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Development of transgenic sorghum for insect resistance against the spotted stem borer (*Chilo partellus*)

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Abstract Transgenic sorghum plants expressing a synthetic *cry1Ac* gene from *Bacillus thuringiensis* (*Bt*) under the control of a wound-inducible promoter from the maize protease inhibitor gene (*mpiC1*) were produced via particle bombardment of shoot apices. Plants were regenerated from the transformed shoot apices via direct somatic embryogenesis with an intermittent three-step selection strategy using the herbicide Basta. Molecular characterisation based on polymerase chain reaction and Southern blot analysis revealed multiple insertions of the *cry1Ac* gene in five plants from three independent transformation events. Inheritance and expression of the *Bt* gene was confirmed in T_1 plants. Enzyme-linked immunosorbant assay indicated that Cry1Ac protein accumulated at levels of 1–8 ng per gram of fresh tissue in leaves that were mechanically wounded. Transgenic sorghum plants were evaluated for resistance against the spotted stem borer (*Chilo partellus* Swinhoe) in insect bioassays, which indicated partial resistance to damage by the neonate larvae of the spotted stem borer. Reduction in leaf damage 5 days after infestation was up to 60%; larval mortality was 40%, with the surviving larvae showing a 36% reduction in weight over those fed on control plants. Despite the low levels of expression of Bt δ-endotoxin under the control of the wound-inducible promoter, the transgenic plants showed partial tolerance against first instar larvae of the spotted stem borer.

Keywords *Chilo partellus* · *cry1Ac* · Insect resistance · *Sorghum bicolor* · Wound-inducible promoter

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Introduction

Sorghum [*Sorghum bicolor* (L.) Moench] is the fifth most important cereal crop, feeding millions of people in arid and semi-arid zones of the world. It is also an important source of animal feed and fodder, especially in dry areas as it is generally grown as a rain-fed crop. Insect pests are the major constraints on its production and productivity, of which stem borers [mainly *Chilo partellus* (Swinhoe)] are most important worldwide (Sharma 1993). Until recently, genetic improvement of sorghum for superior agronomic performance, quality, and insect resistance traits was carried out through traditional plant breeding methods (Emani et al. 2002). Resistance levels to insect pests in cultivated sorghums are not absolute, but resistant wild relatives cannot be successfully used due to reproductive barriers. Biological limitations, such as sexual incompatibility and narrow genetic variability, mean that increases in grain yield and productivity cannot be sustained (Able et al. 2001).

Molecular biology and genetic engineering tools can facilitate harnessing and deployment of naturally available insecticidal proteins in crops in a safe and sustainable manner (Ranjekar et al. 2003). Various insecticidal crystal proteins

(ICPs), or δ-endotoxins from *Bacillus thuringiensis* (*Bt*) have been found to be very effective against lepidopteran insects (Hofte and Whiteley 1989). Crops expressing *Bt* genes have shown significant savings in terms of cost, time, labour, and reduction in damage to the environment compared to the chemical insecticides that are conventionally used to control insects (Peferoen 1997). The successful transfer of *Bt* genes into cereal crops, and high levels of resistance against stem borers, have been reported in rice and maize (Koziel et al. 1993; Hill et al. 1995; Brietler et al. 2001; Khanna and Raina 2002). Since sorghum has tended to be the most recalcitrant crop for tissue culture, regeneration, and genetic transformation (Emani et al. 2002), the application of transgenic approaches to genetic improvement of sorghum has lagged compared to other cereal crops (Zhong et al. 1998). To date, there is no transgenic sorghum under commercial cultivation (Seetharama et al. 2003).

Much of the work on sorghum transformation by earlier investigators focussed on the optimisation of parameters for tissue culture and gene transfer methods, assessing the strength of promoters, and identification of efficient selectable marker and reporter genes (Zhong et al. 1998; Able et al. 2001; Harshavardhan et al. 2002; Jeoung et al. 2002; Tadesse et al. 2003). There is only one report dealing with transformation of sorghum with an agronomically important gene, rice chitinase, that confers resistance against stalk rot (Zhu et al. 1998; Krishnaveni et al. 2000). Selection of a promoter that drives transgene expression to detectable and desired levels is the key to success for genetic transformation of sorghum for the trait of interest. Use of wound-inducible promoters to direct expression of genes encoding insecticidal proteins in transgenic crops, both to save energy and delay the occurrence of resistance in the target insect population, is critical (De Maagd et al. 1999). However, the efficiency of wound-inducible promoters over constitutive promoters has not been established in sorghum.

In maize, expression of the *mpi* (maize protease inhibitor) gene coding for a protease inhibitor is induced in response to mechanical wounding and insect feeding (Cordero et al. 1994; Tamayo et al. 2000). We report the successful production of transgenic sorghum via microprojectile bombardment, and expression of the *cry1Ac* gene under the control of the regulatory region of the *mpi* gene, the *mpiC1* −689/+197 promoter. Further, *cry1Ac* expression and efficacy against the sorghum spotted stem borer were evaluated.

Materials and methods

Plant material

Sorghum genotype BT×623, a popular seed parent used for the production of hybrids worldwide was used in the present study. This popular maintainer line (B-line), developed at Texas A&M University (College Station, Tex.), is used for gene mapping, sorghum genomics, and also for breeding. Shoot apices of 7-day-old seedlings were excised from hypocotyls under a dissection microscope (Fig. 2a). A cut was made at the base of the apex below the attachment of the largest expanded leaf separating the membranous sheath of the primordial leaf, leaving parts of the unexpanded primordial leaves intact (Harshavardhan et al. 2002).

Plasmids used for co-bombardment

Co-bombardment of the shoot apices was carried out with plasmids pJS108 and p*mpiC1cry1Ac* (Fig. 1a, b). Plasmid pJS108 (kindly provided by R. Wu, Cornell University, Ithaca, N.Y.) carried the *bar* gene as a selectable marker under the control of the cauliflower mosaic virus 35S (CaMV35S) promoter along with the *uid*A gene encoding β-glucuronidase (GUS) under the control of the rice actin (*act1*) promoter. Plasmid p*mpiC1cry1Ac* carried a synthetic *cry1Ac* gene under the control of the wound-inducible *mpi* gene regulatory region −689/+197 (C1) (Sardana et al. 1996; Brietler et al. 2001)

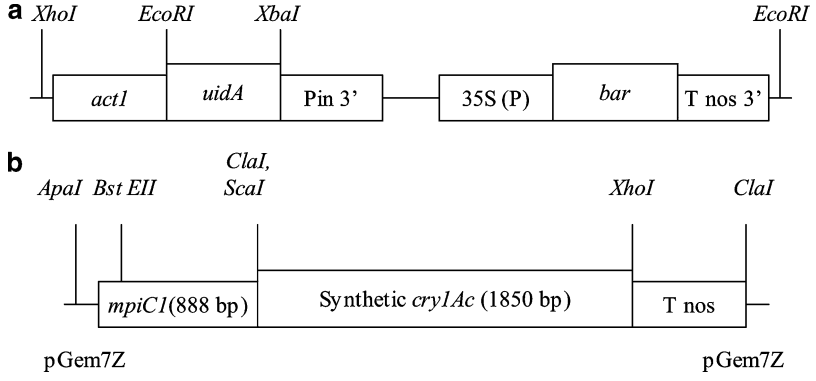

Fig. 1a, b Schematic representation of gene constructs used for the co-bombardment of sorghum shoot apices. **a** Partial map of pJS108. *Act1* Promoter of rice actin (*act1*) gene, *uidA* β-glucuronidase (GUS) coding sequence, 35S *CaMV35S* promoter, *bar* phosphinothricin acetyl transferase coding sequence, *Pin* and *Nos* terminator sequences. **b** Partial map of *PmpiC1cry1Ac*. *mpiC1* Maize protease inhibitor (*mpi*) regulatory region (C1), *cry1Ac* synthetic *Bacillus thuringiensis* (*Bt*) gene, *T nos* nopaline synthetase termination sequence, pGem7Z the entire gene construct is cloned into multiple cloning site of pGem7Z plasmid vector

Particle bombardment

Shoot apices from 7-day-old seedlings (Fig. 2a) were placed horizontally on somatic embryo stimulation medium (SESM) comprising the basal medium of Murashige and Skoog 1962 (MS) fortified with 5 μ*M* thidiazuron (TDZ), 4.0 mg/l benzylaminopurine (BAP), and 0.1 mg/l naphthalene acetic acid (NAA), and incubated overnight under light (16 h day-length) at 26°C and 45% relative humidity (RH). Before bombardment, explants were placed on an osmotic medium containing MS with 0.4 *M* mannitol and 0.4 *M* sorbitol for 4 h. A home-made particle inflow gun (PIG) based on the principle of Finer et al. (1992) was used for gene transfer. Genetic transformation of the explants was carried out using tungsten particles (1 μm) coated with the plasmids at a ratio of 1:1 (5 μl each) under a helium gas pressure of 12 kg/cm^2 and partial vacuum (600 mm Hg). The bombarded explants were then left on the osmotic medium overnight. The next day, a few explants were randomly selected to assay for

Fig. 2a–k Tissue culture, regeneration, and bioassays of sorghum plants carrying the *mpiC1cry1Ac* gene. **a** Shoot tips isolated from 7-day-old germinated seedlings after bombardment, on somatic embryo induction medium (SEIM). **b** Transient GUS expression in freshly bombarded shoot apices. **c** Selection of somatic embryos in somatic embryo germination medium (SEGM). Explants exhibiting browning and necrosis during selection on 1 mg/ml Basta were eliminated. **d** Enlarged somatic embryo in 1 mg/ml Basta selection agent with germinating somatic embryos. **e** Multiple somatic embryos surviving after selection on 2 mg/ml Basta. **f** Enlarged view of shoot clump showing multiple somatic embryos. **g** Explants in shoot elongation medium (SEM). **h** Elongated shoots with well-developed roots ready for transfer to Jiffy cups. **I** Cluster of plantlets with roots and shoots. **j** T_0 transgenic plants growing in the containment greenhouse. **k** Leaf disc bioassay. Grades of resistance (leaf feeding) in transgenic plants (*upper row*) and control (*lower row*) against neonate larva of *C. partellus*. *C* Leaf disc from untransformed control

transient GUS expression according to Jefferson (1987), and the remainder were used in plant regeneration.

Plant regeneration and selection strategy

The bombarded explants were sub-cultured on SESM, following which the enlarged primordial leaves were trimmed while retaining the bulged shoot apex. These explants were further sub-cultured for 2 weeks on somatic embryo induction medium [SEIM; MS supplemented with 4 mg/l BAP and 0.5 mg/l 2,4-dichlorophenoxy acetic acid (2,4-D)]. Within 2 weeks on SEIM, the meristems enlarge resulting in the formation of multiple shoots. After 2 weeks on SEIM, the meristematic masses containing multiple buds were dissected into two or three pieces and sub-cultured on somatic embryo germination medium (SEGM; MS +4 mg/l BAP, and 0.1 mg/l NAA), where the multiple bud initials develop auxiliary plantlets. The explants containing shoot bud initials were maintained on this medium for 2 weeks prior to the transfer of young plantlets to shoot elongation medium [SEM; MS +1 mg/l BAP and 0.5 mg/l indole butyric acid (IBA)] and sub-cultured every 2 weeks. Thus the regeneration of bombarded explants was carried out by direct somatic embryogenesis over a period of 16 weeks. At 4 weeks from the time of bombardment, a three-step selection strategy was imposed using 1 mg/l Basta in SEGM followed by 2, 2.5 mg/l Basta in SEM with intermittent subculturing after every selection step. After 1 month of culturing on SEM, the plantlets were transferred to hormone-free MS until they set 2–3 roots. The rooted plantlets were removed from the Magenta box, rinsed thoroughly with sterile water, and transferred to jiffy cups containing autoclaved vermiculite mixture. Plants were hardened for 7 days in the culture room at 26±2°C and 45% RH with a photoperiod of 16 h. The surviving plants were finally transferred to 25-cm diameter pots containing sterilised soil and grown to maturity in a P2 greenhouse containment facility.

Characterisation of transgenic plants

PCR and RT-PCR

Genomic DNA was isolated from lyophilised leaf samples of transformed and control plants by the CTAB method (Sambrook et al. 1989). PCR amplification of a 506 bp DNA fragment of the *cry1Ac* gene was carried out in an MJ 200 Thermocycler using specific primers 5′-CTCCTTGTCCTTGACACAG-3′ and 5′-GTTGTACCAACGAACAGCG-3′ annealing at position 128 on the non-coding strand, and 624 on the coding strand, respectively. The PCR reaction mixture contained 100 ng genomic DNA in a final volume of 25 µl containing 1× PCR buffer, 10 m*M* dNTPs, 10 pmol of each primer, and *Taq* DNA Polymerase (2.5 U). Amplification was carried out by denaturation at 94°C for 1 min, annealing at 58.5°C for 1 min, and extension at 72°C for 1.5 min for 40 cycles. A pre-denaturation step of 2 min and a final elongation step of 5 min were included. In case of the *uid*A (1.8 kb) gene, a 1.2 kb fragment was amplified using the forward primer 5′-GGTGGGAAAGCGCGTTACAAG-3′ and reverse primer 5′-GTTTACGCGTTGCTTCCGCCA-3′. Denaturation was carried out at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1.5 min for 40 cycles.

RT-PCR analysis was carried out with total RNA (0.5 µg) obtained from wounded leaves of 3-week-old T_1 plants. First strand cDNA was synthesised using Moloney murine leukemia virus (MMLV) reverse transcriptase and an oligo dT primer. Reaction mixture (RT) consisted of 1.0 µl 2.0 µ*M* oligo dT primer (dT_{15} from Gene Hunter, Brookline, Mass.), 100 U MMLV reverse transcriptase, 2.0 µl 1 m*M* dNTPs, 1.0 µl 100 m*M* DTT, and 2 µl 5× first strand buffer [125 m*M* Tris (pH 8.3), 7.5 m*M* $MgCl_2$, and 188 m*M* KCl]. Total RNA was heat denatured by incubating at 65°C for 5 min, followed by the addition of RT mix to the samples, and incubated at 37°C for 120 min. The cDNA-RNA hybrid was separated by heating the tubes at 75°C for 5 min., and the products were stored at −20°C until further use. PCR was carried out for each gene as described above separately. Samples of PCR products were resolved on 1.2% agarose gels.

Southern blot analysis

Southern blot analysis to detect the presence of the *cry1Ac* gene in the putative transgenics was carried out according to standard protocol (Sambrook et al. 1989). Genomic DNA (25 µg) of putative transgenics and control plants was digested with the restriction endonuclease *Bst*EII and separated on an 0.8% agarose gel in TBE buffer. DNA was blotted onto a Hybond N^+ nylon membrane according to the manufacturer's instructions (Amersham, Piscataway, N.J.). The probe was prepared by digesting the plasmid carrying *cry1Ac* with the restriction enzymes *Cla*I and *Xho*I to release a DNA fragment of 1.87 kb. The probe fragment was separated on an agarose gel, purified, and radio-labeled with (α-^{32}P) dATP using the random primer DNA labelling kit (NEBlot Kit, New England Biolabs, Beverly, Mass.). Prehybridisation and hybridisation were carried out in a buffer containing 6× HSB, 0.5% SDS, 5× Denhardt solution, and 25% 5 µg ml^{-1} sonicated and denatured salmon sperm DNA (Sambrook et al. 1989). Blots were pre-hybridised for 6 h at 65°C in 30 ml buffer. The prehybridisation solution was then removed and replaced with 10 ml buffer containing denatured radioactive probe at 1×10^6 cpm per millilitre. Hybridisation was then performed overnight (16 h) at 65°C. Hybridized DNA was visualised on Hyperfilm (Kodak) after exposure of the blot for 4 days.

Enzyme-linked immunosorbant assay

A double-antibody sandwich enzyme-linked immunosorbant assay (ELISA) was used to detect the presence of the Cry1Ac protein expressed in the leaves of transgenic plants. The test was conducted using the Patho-Screen

Kit for Bt-Cry1Ab/Ac protein (Peroxidase label, cat. no. PSP 06200, Agdia, Elkhart, Ind.). Three-week-old leaves of T_1 plants were mechanically wounded at close intervals (3 mm distance) with scissors and samples were collected after 6 and 12 h intervals for analysis. Although colour changes in the ELISA plate were not detected with the naked eye, OD values registered by the ELISA detector were used to calculate the amount of Cry1Ac protein accumulation.

Insect bioassay

Leaf disc assay

Ten healthy neonate larvae of spotted stem borer (*C. partellus*) were carefully released onto leaf discs (2.5 cm diameter) placed on a moist filter paper in a small cup (25 ml capacity). A moist filter paper was placed inside the lid of the plastic cup to keep the leaf discs in a turgid condition. Leaf discs from non-transformed plants were used as controls. Each treatment was replicated three times in a randomised block design. These cups were stored at 27±1°C and data were recorded when the extent of damage to the leaf discs in the controls was >80%. The leaf discs were rated visually after 5 days for insect feeding (damage rating) on a scale of 1–9 (1=<10% leaf disc area consumed, 2=11–20%, 3=21–30%, 4=31–40%, and so on, with 9=>80% leaf area consumed). The number of surviving larvae was also determined and their weights were recorded after 4 h.

$$\text{Larval mortality (\%)} = \left[\frac{(\text{mortality on transgenic plants} - \text{mortality on non-transgenic plants})}{\text{mortality on non-transgenic plants}}\right] \times 100$$

Shoot assay

Shoots (10 cm long) of transgenic and non-transgenic plants (3 weeks old) were cut with scissors and placed on solidified agar in a 15 cm ×6 cm plastic jar. Ten, freshly hatched, neonate larvae of spotted stem borer were then carefully released onto the leaf whorls of plants in each bottle. Each experiment was replicated three times. The plastic jars were covered with a lid and kept at 27±1°C. On the 8th day of the experiment, the surviving larvae were carefully examined by dissecting the shoots; data on larval survival and larval weight were recorded. Genstat 6 software (Lawes Agricultural Trust, Rothamsted, UK) was used for statistical analysis of data obtained from bioassay experiments.

Results

Transformation of sorghum

Shoot apices of sorghum BT×623 genotype were co-transformed with a selectable marker (*bar*) and an insect resistance (*cry1Ac*) gene using plasmids pJS108 and p*mpiCIcry1Ac*, respectively (Fig. 1). A total of 200 shoot apices (Fig. 2a) were co-bombarded, of which 157 were subjected to first round selection on Basta-containing (1 mg/l) SEGM. About 10% of the shoot apices were damaged due to the helium gas pressure at the time of bombardment, and some Petri-plates were eliminated because of contamination. A few tiny, green, healthy Basta-resistant shoot bud clumps (Fig. 2c–e) emerged from the necrotic tissues at the end of first round of selection, while 46% of explants developed acute necrosis. The remaining 33.5% of shoot clumps was transferred to SEM containing 2 or 2.5 mg/l Basta, during which period most of the bombarded and non-bombarded (control) shoot clumps turned brown and became necrotic. By the end of second and third rounds of selection on herbicide-containing medium (Fig. 2g) only a few clumps originating from 12 explants remained. The shoot clumps from untransformed explants, however, did not survive by the end of the second round of selection. Healthy, transformed shoot clumps, which withstood 6 weeks of continuous selection, were regenerated into shoots that were rooted and maintained in the greenhouse to maturity (Fig. 2h–j). A total of 48 plants were regenerated successfully from the surviving 12 explants, among which 45 survived and set fertile seed.

Transient GUS expression in bombarded explants

Shoot apices bombarded with the *uid*A gene under the control of rice *act1* promoter were stained with X-Gluc solution 24 h after bombardment. A maximum of 27 blue foci per explant was observed (Fig. 2b). On average, one in every ten shoot tips bombarded showed at least one GUS spot. However, most of these GUS spots were seen on the primordial leaves and very few were observed near the base of the unexpanded primordial leaf, which is the region from which multiple shoots emerge. GUS expression was not observed in the leaves of greenhouse grown T_0 plants, or in 2-week-old T_1 generation plants.

Molecular characterisation

PCR amplification of T_0 transformants was carried out to detect the presence of transgenes in the putative transgenic plants. When tested for the presence of the *uid*A gene, 6 of the 45 established plants showed the expected 1.2 kb fragment (Fig. 3a, Table 1); 5 of these 6 plants also showed the expected 506 bp fragment that corresponds to the *cry1Ac* gene (Fig. 3b, Table 1). As expected, no amplification was observed when genomic DNA from untransformed

Table 1 Summary of molecular characterisation of T_0 generation transgenic sorghum plants

Transformation events[a]	Number of plants regenerated	PCR analysis		Southern analysis (*cry1Ac*)	T_0 plant number
		*uid*A	*cry1Ac* + *uid*A		
I	15	1	1	1	81
II	13	4	3	3	106, 109, 125
III	17	1	1	1	52

[a]From a total of 200 shoot apices bombarded five plants obtained from three independent explants were confirmed as transgenic. The transformation frequency was 1.5%

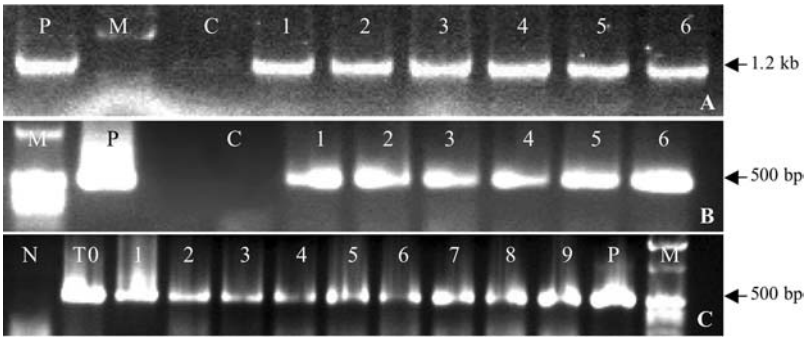

Fig. 3a–c PCR analysis for *uid*A and *cry1Ac* transgenes in T_0 and T_1 generation transgenic plants. **a** PCR amplification of *uid*A gene, showing the 1.2 kb GUS fragment in T_0 plants (lanes *1–6*). **b** PCR amplification of a 506 bp fragment of the *cry1Ac* gene in T_0 putative transformants (lanes *1–6*). **c** PCR analysis of T_1 generation plants for the *cry1Ac* gene (lanes *1–9*). Lanes: *P* Positive controls (plasmids), *M* molecular size markers, *C*/*N* control sorghum plants

control plants was used as template. A summary of the transformation experiments and molecular characterisation of regenerated plants is presented in Table 1.

To determine the copy number and integration pattern of the transgenes, Southern blot hybridisation analysis was performed. DNA extracted from the leaves of T_0 plants was digested with *Bst*EII, which cuts once in the upstream region at 150 bp from the 5′ region of the *mpiC1* promoter DNA sequence. Digested genomic DNA was resolved, blotted, and probed with the radiolabelled *cry1Ac* DNA fragment, revealing the integration pattern and number of copies of the *cry1Ac* gene inserted in the host genome. Of the 45 plants analysed, DNA from 5 plants showed hybridisation with the 1.87 kb *cry1Ac* probe (Fig. 4). As expected, transgenic plants that gave a positive PCR signal for both *uid*A and *cry1Ac* were also positive in Southern hybridisation for *cry1Ac*. Based on these observations, the five T_0 plants originated from three different transformation events, giving a transformation efficiency of 1.5%. Thus, the T_0 plants T_0-81 (lane 1) and T_0-52 (lane 7) showed two and three hybridisation bands, respectively, indicating that they originated from two independent transformation events (I and III), while the T_0 plants T_0-106, T_0-109, and T_0-125 shared a similar hybridisation banding pattern (lanes 3, 4, and 5), confirming their origin from a single

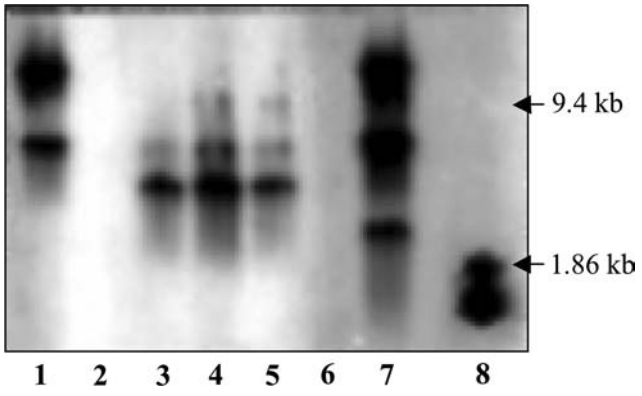

Fig. 4 Southern blot analysis of T_0 putative transformants. Genomic DNA was restricted with *Bst* EII and hybridised with a radiolabelled 1.87 kb *cry1Ac* fragment of plasmid DNA (lane *8*). Lanes: *1*, *3–5*, *7* Transformed plants T_0-52, T_0-106, T_0-109, T_0-112, and T_0-81, respectively; *2* negative control, *6* putative transgenic event that was negative

Table 2 Inheritance of the transgenic *cry1Ac* gene in the progeny of five primary transgenic events based on chi-square test of the *cry1Ac* gene (506 bp) fragment amplified by PCR

T_0 parent	Number of T_1 plants tested	Number of *cry1Ac* PCR positive plants	Number of *cry1Ac* PCR negative plants	Expected ratio	3:1 segregation[a] χ^2
81	20	16	4	3:1	0.27
106	20	14	6	3:1	0.27
125	20	12	8	3:1	2.4
109	20	17	3	3:1	1.07
52	20	13	7	3:1	1.07

[a]Tabulated χ^2 value at 5% probability for 1 degree of freedom is 3.841. The calculated χ^2 value is less than the χ^2 table value. The progeny plants showed a Mendelian segregation ratio (3:1)

Table 3 Summary of insect bioassay (leaf disc) with neonate larvae of sorghum stem borer. Values shown are the average of three replications. *LSD* Least squares difference

Transformation event	T_0 parent line	T_0 progeny line	Larval mortality (%)	Reduction in larval weight (%)[a]	Reduction in leaf feeding (%)[a]
I	81	1	45	26	58
		2	58	47	73
		3	40	36	60
		6	58	33	66
		7	49	30	54
II	106	4	17	19	−3
		6	22	22	12
		7	17	7	8
	109	3	8	6	16
		6	17	21	4
		8	12	22	8
		10	22	−11	−3
	125	2	31	−14	27
		3	26	17	25
		4	22	19	1
		7	17	14	20
III	52	3	54	60	61
		5	63	21	54
		6	45	39	62
		9	22	14	27
	F probability		<0.001	0.007	<0.001
	Standard error of means		8.93	11.44	7.60
	LSD at 5% level		25.58	32.76	21.75

[a]Reduction in land weight / leaf damage in relation to that on the non-transgenic plants

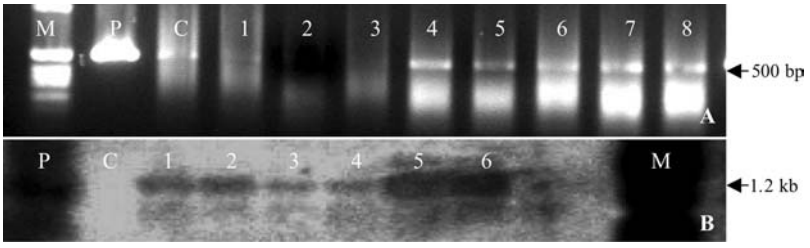

Fig. 5a, b RT-PCR analysis of *cry1Ac* and *uidA* genes in T_1 generation plants. **a** RT-PCR amplification of *cry1Ac* fragment in wounded leaf samples of T_1 generation plants, showing the 506 bp *cry1Ac* fragments in the samples tested (lanes *1*, *4–8*) compared to the positive control (lane *P*). No amplification of *cry1Ac* was observed in plants analysed in lanes *2* and *3*. *C* Control plants, *M* molecular size markers. **b** RT-PCR analysis of the *uid*A gene in T_1 plants, showing the 1.2 kb *uidA* fragments in the samples tested (lanes *1–6*) compared to the positive control (*P* =plasmid). *C* Control plants, *M* molecular markers

transformation event (II). This was further supported by the data recorded during plant regeneration, which indicated that plants T_0-81 and T_0-52 regenerated from two independently transformed explants and plants T_0-106, T_0-109, and T_0-125 regenerated from a single transformed explant. Variations in the intensity of hybridisation bands were also observed. The five T_0 plants harbouring the *cry1Ac* transgene were analysed further. All of them were fertile and set normal seed.

Transgene inheritance and wound-inducible Cry1Ac accumulation

PCR and RT-PCR analyses were carried out to determine transgene expression in the progeny of Southern-positive T_0 plants. The expected 506 bp amplification band corresponding to the *cry1Ac* gene was detected (Fig. 5a). Equally, the 1.2 kb fragment corresponding to the *uid*A gene was also observed in these lines (Fig. 5b). The progeny of the selected transgenic events show inheritance of the transgene in a Mendelian manner (Table 2). These results confirmed that the *cry1Ac* gene was efficiently inherited and transcribed in T_0 progeny plants.

ELISA tests were carried out to determine the amount of Cry1Ac protein expressed in mechanically wounded leaves of T_1 transgenic plants. Cry protein was not detected 6 h after wounding of transgenic leaves, but after 12 h it was detected at low levels. Based on ELISA values two plants showed accumulation of δ-endotoxin to 1 and 8 ng per gram of fresh leaf tissue, respectively.

Insect bioassay

To assess the protection afforded by transgenic expression of *cry1Ac* in sorghum plants, 3-week-old control and transgenic plants grown in the greenhouse were artificially infested with *C. partellus* larvae under controlled laboratory conditions. Leaf disc and young shoots of T_1 plants of transgenic lines T_0-52, T_0-81, T_0-106, T_0-109, and T_0-112 were fed to the neonate larvae of the spotted stem borer. The bioassays (leaf disc and shoot assay) were carried out on 50 T_1 plants (10 from each selfed T_0 parent line).

Leaf disc bioassay

The effects of *cry1Ac* transgene expression on the development of stem borer larvae were recorded 5 days after infestation. Larvae that fed on the leaf discs from transgenic plants had varying levels of effect on their weight, damage rates, and mortalities. The results of the leaf disc bioassay are summarised in Table 3. Larvae feeding on non-transgenic control plants consumed more than 80% of the leaf disc material, compared to <50% leaf disc feeding with some transgenic lines. Plants T_1-81-1, T_1-81-2, T_1-81-3, T_1-81-6, and T_1-81-7, along with T_1-52-3, T_1-52-5, and T_1-52-6 (except 52-9), showed larval mortality rates of 40% and a significant reduction in larval weight (36% less than the non-transformed control). The transgenic expression of *Bt* protein also reduced larval leaf feeding by 60% in some of the above lines. Progeny of T_0-81 and T_0-52 showed a better level of resistance to first instar neonate larvae of *C. partellus* compared to that observed for progeny of T_0-106, T_0-109, and T_0-125. However, the selected 2 generation lines did not show resistance to the neonate larvae (data not shown).

Shoot bioassay

Shoot bioassays were carried with the progeny of T_0-81 and T_0-52 plants that showed a high level of resistance compared to other progeny lines in the leaf disc bioassay. Observations recorded 5 days after release of neonate larvae indicated that larval mortality was <25% in all lines tested (data not shown). The reduction in weight of the surviving larvae was not significantly different from that of the larvae reared on non-transgenic control plants. Most of the surviving larvae were quite active and were tunnelling inside the transgenic shoots.

Discussion

In the present study, transgenic sorghum plants expressing a synthetic *Bt cry1Ac* gene under the control of a wound-inducible promoter from a maize protease inhibitor gene (*mpi*) were produced via particle bombardment. The microprojectile method of DNA transfer used in the present study has been used routinely to transform recalcitrant crop species such as rice (Alam et al. 1998) and sorghum (Zhong et al. 1998; Tadesse et al. 2003; Able et al. 2004). The selection pressure imposed to screen out non-transformed explants was not effective since many escapes regenerated and were grown to maturity. PCR and Southern analysis confirmed that 5 out of 45 established plants carried the *cry1Ac* gene. Multiple copies of the p*mpiC1cry1Ac* transgene were integrated into the genome of sorghum plants, as is usually observed in transgenic plants produced by particle bombardment.

Transgenic sorghum plants expressing the *cry1Ac* gene under the control of the *mpi* promoter were evaluated in insect bioassays using the neonate larvae of sorghum spotted stem borer. The results indicated that wound-inducible expression of *cry1Ac* in transgenic sorghum had a negative effect on growth of *C. partellus* larvae. To date, there are no other published reports on transgenic sorghum developed for insect resistance. However, wound-inducible expression of the *cry1Ac* gene in transgenic sorghum plants did not confer complete protection against first instar neonate larvae of *C. partellus*. Leaf disc bioassays showed that transgenic plants were only partially tolerant to damage by first instar larvae. Reduction in leaf damage (60%), larval mortality (40%), and larval weight (36%) was recorded in comparison to larvae fed on non-transformed control plants at 5 days after infestation. Shoot bioassay results indicated that larval mortality was less than 25%, and no significant reduction in the surviving larval weight was observed as most of the surviving larvae were quite active and made tunnels inside whorls of the young shoots. First instar larvae are known to be the most susceptible stage and should be exposed to the *Bt* toxins as soon as they begin to feed on the plant (Koziel et al. 1993). However, the neonate larvae of *C. partellus* were partially susceptible to these transgenic sorghum plants.

In the present study, a detailed analysis on the level of expression of the *cry* gene in different tissues of transgenic sorghum plants was not carried out. However, lower levels of *Bt* transgene expression in shoot tissues of sorghum plants might explain the differences in the effects observed on larval weight reduction in the two bioassays. Similar observations have been reported by Husnain et al. (2002), who showed that the *cry1Ab* gene under the control of the phosphoenol pyruvate carboxylase (PEPC) promoter expressed more Bt δ-endotoxin in the leaves than in stems and panicles of transgenic rice plants.

Investigations comparing the efficiencies of promoters used to drive expression of transgenes in sorghum were based on expression of reporter- or herbicide-resistance-genes. In the present study, only two of the T_1 transgenic sorghum plants had detectable Cry protein levels in the leaves (1 and 8 ng per gram of leaf tissue). Brietler et al. (2001) 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). In this context, it is interesting to note that the level of Cry1Ac accumulation in transgenic sorghum lines is lower

than that reported for transgenic plants expressing different *Bt* genes in other crops. Datta et al. (1998) reported that, in transgenic rice plants, 10–200 ng Cry1Ab protein per gram of leaf tissue is capable of conferring 100% larval mortality on the yellow stem borer in cut-stem bioassays. Nayak et al. (1997) reported that transgenic rice plants expressing *cry1Ac* from the maize *ubiquitin-1* promoter expressed the Bt crystal protein in a range of 58–240 ng per milligram of total soluble protein. Bioassays with these sorghum transgenics showed that a few plants carrying p*mpiC1cry1Ac* exhibited partial tolerance to *C. partellus* first instar larvae, while the remaining transgenics were susceptible. The low levels of Bt proteins produced were far below the lethal dose required to give complete protection against neonate larvae of *C. partellus*.

The phenomenon of transgene silencing appears to be a major obstacle in the transformation of sorghum (Emani et al. 2002). Although silencing of the *cry1Ac* transgene was not observed in the present investigation, a very low level of *cry1Ac* transgene expression was observed in a few plants, while most show no expression as evidenced by ELISA tests. However, PCR and RT-PCR analysis of T_1 transgenic plants carrying the *cry1Ac* gene revealed the presence of *cry1Ac* mRNA transcripts in leaf samples. Similarly, while *uid*A gene expression was observed in RT-PCR analysis with RNA isolated from leaves, GUS expression could not be visualised.

It is known that mRNA transcripts of synthetic *cry1Ac* genes degrade and disappear at a rate similar to that of GUS mRNA transcripts, since their half-lives are almost the same (De Rocher et al. 1998). These latter authors also identified lower levels of synthetic *cry1Ac* mRNA accumulation in maize than in Arabidopsis and tobacco, and recorded highest levels of transcripts when the synthetic *cry1Ac* sequence is modified and synthesised according to the codon bias typical of maize genes. These studies on synthetic *Bt* genes indicated that some of these expression-enhancing and mRNA-stabilizing sequences are differentially recognised in different crops. Hill-Ambroz and Weeks (2001) noted that, in comparison to gene expression in wheat, the *ubi1* promoter directs very low levels of transgene expression in sorghum. Hagio et al. (1991) observed the accumulation of aberrant transgene RNA transcripts in sorghum, which was attributed to in vivo degradation of these transcripts. It has been demonstrated that the construction of synthetic *Bt* genes with highly modified nucleotide sequences can result in increased levels of mRNA accumulation, leading to high expression in plants as the design criteria for the synthetic genes often includes sequence changes targeted at potential mRNA instability elements (De Rocher et al. 1998).

Methylation-based transgene silencing of *uid*A and *bar* genes in transgenic sorghum was reported by Emani et al. (2002). More recently, using PIG, Able et al. (2004) obtained a 70% increase in GUS expression in sorghum callus when tobacco RB7 MAR (matrix attachment regions) flanked the *uid*A construct. Based on the above discussion, it would be advisable to carry out investigations on comparison of efficiencies of different promoters not only for the marker genes but also for the trait of interest. Further, it would be beneficial if regulatory regions from within the sorghum genome are isolated and used for transformation, or if transgenes were flanked on either side by nuclear MARs such as the sorghum *Adh* MAR sequence to enhance the expression of the target gene (Able et al. 2004). Our investigations on the efficiency of the constitutive promoter *ubiquitin1* in enhancing expression levels of synthetic *Bt* genes in transgenic sorghum are in progress.

To summarise, our results demonstrate that the *mpiC1* promoter from maize is functional in sorghum and drives expression of the *cry1Ac* gene at low levels, which in turn can confer partial protection against neonate larvae of the spotted stem borer. To the best of our knowledge, this is the first report of the study of a transgene expressed under the control of a wound-inducible promoter in sorghum. Information on the mechanism of transgene integration into the host genome and understanding of the molecular evolution of the genome is indispensable for improving genetic transformation technology for recalcitrant plant species (Morikawa et al. 2002). Future research on these areas would certainly help in combatting the problems associated with the genetic transformation of sorghum.

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References

Able JA, Rathus C, Godwin ID (2001) The investigation of optimal bombardment parameters for transient and stable transgene expression in sorghum. In Vitro Cell Dev Biol Plant 37:341–348

Able JA, Rathus C, Carroll BJ, Godwin ID (2004) Enhancing transgene expression levels in sorghum: current status and future goals. In: Seeetharama N, Godwin ID (eds) Sorghum tissue culture and transformation. Oxford, New York, pp 85–96

Alam MF, Datta K, Abrigo E, Vasquez A, Senadhira D, Datta SK (1998) Production of transgenic deep water rice plants expressing a synthetic *Bacillus thuringiensis cry1A*(*b*) gene with enhanced resistance to yellow stem borer. Plant Sci 135:25–30

Brietler JC, Cordero MJ, Royer M, Meynard D, San Segundo B, Guiderdoni E (2001) The −689/+197 region of the maize protease inhibitor gene directs high level, wound-inducible expression of the *cry1B* gene which protects transgenic rice plants from stem borer attack. Mol Breed 7:259–274

Cordero MJ, Raventós D, San Segundo B (1994) Expression of a maize proteinase inhibitor is induced in response to wounding and fungal infection: systemic wound-response of a monocot gene. Plant J 6:141–150

Datta K, Vasquez A, Tu J, Torrizo L, Alam MF, Oliva N, Abrigo E, Khush GS, Datta SK (1998) Constitutive and tissue-specific differential expression of *cry1A*(*b*) gene in transgenic rice plants conferring resistance to rice insect pests. Theor Appl Genet 97:20–30

De Maagd RA, Bosch S, Stiekema W (1999) *Bacillus thuringiensis* toxin-mediated insect resistance in plants. Trends Plant Sci 4:9–13

De Rocher EJ, Vargo-Gogola TC, Diehn SH, Green PJ (1998) Direct evidence for rapid degradation of *Bacillus thuringiensis* toxin mRNA as a cause of poor expression in plants. Plant Physiol 117:1445–1461

Emani C, Sunilkumar G, Rathore KS (2002) Transgene silencing and reactivation in sorghum. Plant Sci 162:181–192

Finer JJ, Vain P, Jones MW, Mc Mullen MD (1992) Development of the particle inflow gun for DNA delivery to plant cells. Plant Cell Rep 11:323–328

Hagio T, Blowers AD, Earle ED (1991) Stable transformation of sorghum cell cultures after bombardment with DNA-coated microprojectiles. Plant Cell Rep 10:260–264

Harshavardhan D, Rani TS, Ugalanathan K, Seetharama N (2002) An improved protocol for regeneration of *Sorghum bicolor* from isolated shoot apices. Plant Biotechnol 19:163–171

Hill-Ambroz KL, Weeks JT (2001) Comparision of constitutive promoters for sorghum transformation. Cereal Res Commun 29:17–24

Hill M, Launis K, Bowman C, Mc Pherson K, Dawson J, Watkins J, Koziel M, Wright MS (1995) Biolistic introduction of a synthetic *Bt* gene into elite maize. Euphytica 85:119–123

Hofte H, Whiteley HR (1989) Insecticidal crystal proteins of *Bacillus thuringiensis*. Microbiol Rev 53:242–255

Husnain T, Asad J, Maqbool SB, Datta SK, Riazuddin S (2002) Variability in expression of insecticidal *Cry1Ab* gene in Indica Basmati rice. Euphytica 128:121–128

Jefferson RA (1987) Assaying chimeric genes in plants: the GUS gene fusion system. Plant Mol Biol Rep 5:387–405

Jeoung MJ, Krishnaveni S, Muthukrishnan S, Trick HN, Liang GH (2002) Optimization of sorghum transformation parameters using genes for green fluorescent protein and β-glucuronidase as visual markers. Hereditas 137:20–28

Khanna HK, Raina SK (2002) Elite Indica transgenic rice plants expressing modified *Cry1Ac* endotoxin of *Bacillus thuringiensis* show enhanced resistance to yellow stem borer (*Scirpophaga incertulas*). Transgenic Res 11:411–423

Koziel MG, Beland GL, Bowman C, Carozzi NB, Crenshaw R, Crossland L, Dawson J, Desai N, Hill M, Kadwell S, Launis K, Lewis K, Maddox D, McPherson K, Meghji MR, Merlin E, Rhodes R, Warren GW, Wright M, Evola S (1993) Field performance of elite transgenic maize plants expressing an insecticidal protein derived from *Bacillus thuringiensis*. Biotechnology 11:194–200

Krishnaveni S, Jeoung JM, Muthukrishnan S, Liang GH (2000) Transgenic sorghum plants constitutively expressing a rice chitinase gene show improved resistance to stalk rot. J Genet Breed 55:151–158

Morikawa H, Sakamoto A, Hokazono H, Irifune K, Takahashmi M (2002) Mechanism of transgene integration into a host genome by particle bombardment. Plant Biotechnol 19:219–228

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco tissue cultures. Plant Physiol 15:473–497

Nayak P, Basu D, Das S, Basu A, Ghosh D, Ramakrishnan NA, Ghosh M, Sen SK (1997) Transgenic elite Indica rice plants expressing Cry1Ac δ-endotoxin of *Bacillus thuringiensis* are resistant against yellow stem borer (*Scirpophaga incertulas*). Proc Natl Acad Sci USA 94:2111–2116

Peferoen M (1997) Progress and prospects for field use of *Bt* genes in crops. Trends Biotechnol 15:173–177

Ranjekar PK, Patankar A, Gupta V, Bhatnagar R, Bentur J, Kumar PA (2003) Genetic engineering of crop plants for insect resistance. Curr Sci 84:321–329

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning, a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Sardana R, Dukiandjiev S, Giband M, Cheng XY, Cowan K, Sauder C, Altosaar I (1996) Construction and rapid testing of synthetic and modified toxin gene sequences CryIA (b&c) by expression in maize endosperme culture. Plant Cell Rep 15:677–681

Seetharama N, Mythili PK, Rani TS, Harshavardhan D, Ranjani A, Sharma HC (2003) Tissue culture and alien gene transfer in sorghum. In: Jaiwal PK, Singh R (eds) Plant genetic engineering: improvement of food crops, vol 2. Sci Tech Publishing, New Dehlli, India, pp 235–265

Sharma HC (1993) Host plant resistance to insects in sorghum and its role in integrated pest management. Crop Prot 12:11–34

Tadesse Y, Sagi L, Swennen R, Jacobs M (2003) Optimization of transformation conditions and production of transgenic sorghum (*Sorghum bicolor*) via microparticle bombardment. Plant Cell Tissue Organ Cult 75:1–18

Tamayo MC, Rufat M, Bravo JM, San Segundo B (2000) Accumulation of a maize protease inhibitor in response to wounding and insect feeding, and characterization of its activity toward digestive proteinases of *Spodoptera littoralis* larvae. Planta 211:62–71

Zhong H, Wang W, Sticklen M (1998) In vitro morphogenesis of *Sorghum bicolor* (L.) Moench: efficient plant regeneration from shoot apices. J Plant Physiol 153:719–726

Zhu H, Muthukrishnan S, Krishnaveni S, Wilde G, Jeoung JM, Liang GH (1998) Biolistic transformation of sorghum using a rice chitinase gene. J Genet Breed 52:243–252