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Full Length Research Paper

CRY 1AB trangenic cowpea obtained by nodal electroporation

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Electroporation-mediated genetic transformation was used to introduce *Cry 1 Ab* insecticidal gene into cowpea. Nodal buds were electroporated *in planta* with a plasmid carrying the *Cry 1Ab* and antibiotic resistance *npt II* genes driven by a 35S CaMV promoter. T_1 seeds derived from electroporated branches were selected *in vitro* on a medium containing geneticin. PCR and Southern blot analyses confirmed the stable integration of the *Cry 1Ab* gene into genome of transgenic T_1 cowpea plants. Copy numbers of the gene in cowpea were estimated to be between one to three per genome. However transgene integration occurred at high molecular weights and corresponded to the hybridisation bands obtained for plasmid control. When tested for resistance to *Maruca vitrata,* T_1 plant progenies reduced larval survival to as low as 11% three days after infestation (DAI). T_2 progenies of *Cry 1Ab* transgenic lines also positively hybridised with *npt II* probes when subjected to southern analyses. T_3 progenies significantly reduced larval survival and larval weight and inhibited *M. vitrata* feeding on cowpea leaves.

Key words: Cowpea transformation, ***in planta***, nodal bud electroporation, ***Maruca vitrata***, genetic engineering, ***Bacillus thuringiensis.***

INTRODUCTION

Cowpea (***Vigna unguiculata*** (L.) Walp.) is consumed by approximately two hundred million people in sub-Saharan Africa and is a source of income for millions of low resource farmers (NGICA, 2002). It is the most economically important indigenous African grain legume (Langyintuo, 2005). However, due to heavy insect damage at various stages in its life cycle, actual yield on the field is less than 10% of its yield potential under optimal conditions (Murdock, 1992). ***Maruca vitrata***, one of its most devastating post-flowering pests can sometimes cause up to 80% yield losses (Sharma, 1998; Jackai and Adalla, 1997).

Chemical insecticides are expensive and pose serious hazards to both the user and the ecosystem. Conventional breeding has failed in producing pest resistant genotypes of cowpea due to the lack of resistance genes among the cultivated cowpea and because of cross incompatibility between cultivated and wild ***Vigna*** species that possess some inherent resistance (Jackai et al**.**, 1996; Sharma, 1998; Fatokun et al**.**, 1997). Despite several resentments against the use of GM crops, many plant species have been transformed with insect resistance genes (Dunell, 2000). Recent investigations have indicated that there are no direct effects of Bt plants on natural enemies of insect pests and other components of the ecosystem (Romeis et al**.**, 2006). Therefore develop-ing transgenic plants that express insecticidal proteins provide new hope for the control of ***Maruca*** pod borer (MPB) (Machuka et al**.**, 1999, 2002). Bioassays have shown that ***Bacillus thuringiensis*** (Bt) genes have potential for controlling a number of insect pests of cowpea and have demonstrated that MPB in particular is susceptible to several different forms of Bt crystal toxins when these are fed in its diet (Murdock, 1992; Jackai and Raulston, 1988; Jackai et al., 1997). Also African maize and cotton varieties expressing ***Cry 1Ab*** insecticidal proteins for the control of other lepidopteran insect pests have been documented (Berg and Wyk, 2007; Gouse et al., 2004).

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Although cowpea transformation by both ***Agrobacterium*** and direct gene transfer methods has been reported (Chaudhury et al., 2007; Popelka et al., 2006; Ikea et al., 2003; Lingaraj et al., 2000; Machuka et al., 2002), no common method has emerged for routine transformation of cowpea. As a result, cowpea transformation using insecticidal genes has not been reported. Cowpea had been very recalcitrant to ***in vitro*** manipulations and regeneration (Fillipone, 1990; Christou, 1992; Walden and Wingender, 1995; Monti et al., 1997). In some instances transgenic calli and chimeric plantlets were obtained (Garcia et al., 1986; 1987; Penza et al., 1992; Akella and Lurquin, 1993; Muthukumar et al., 1995, 1996; Sijen, 1996) but no transgenic plants regenerated. Moreover, when transformation technique depends on ***in vitro*** manipulations alone, fluctuations in gene expression and gene silencing may be observed (Charity et al., 2005). ***In planta*** techniques however, either with ***Agrobacterium*** or electroporation of intact meristems, hold the promise of much higher frequencies and more simple means of regenerating transformants (Atkins et al., 1997). In order to bye pass the hurdle of ***in vitro*** regeneration, therefore, Chowrira et al. (1995, 1996, 1998) attempted the first ***in vivo*** cowpea transformation approach by nodal bud electroporation. Since growth and development occur in the meristems, nodal meristems are good targets for transformation. Their results provide evidence of transgene integration into T_1 progenies. It also showed the integration and expression of the pea enation virus resistance gene in ***Pisum sativum*** progenies. In this work, we modified the technique by employing ***in vitro*** antibiotic selection of progeny seeds obtained by ***in planta*** electrotransformation and have demonstrated for the first time the integration of ***Cry 1Ab*** gene in cowpea.

MATERIALS AND METHODS

Plant material and nodal bud electroporation

TVu 201, Ife Brown, IT90K-277-2, IT90K-288 and IT90K-391 cowpea cultivars were selected at the International institute of Tropical Agriculture (IITA) and used for this study. Twenty-five three week old plants of each variety were prepared and their nodal buds were electroporated as described by Chowrira et al. (1995). The most terminal buds were exposed by decapitating the apical portions of the cowpea plant and removing stipules and adjacent petioles. They were microinjected with 2 µl of plasmid DNA/spermine/MS salts containing 350 µg DNA per ml solution prior to electroporation with two pulses of 80 - 200 volts at 5 s interval. All other buds were excised. T_1 seeds were obtained from new shoots produced by the surviving buds.

Plasmid DNA

The plasmid ptrval was used in this study. It contains the ***B. thuringiensis*** (Bt) gene coding for the production of the Cry 1Ab protoxin as well as the neomycin phosphotransferase II. This enzyme confers resistance to the antibiotic kanamycin (Bevan et al., 1983) as well as other aminoglycosides including geneticin and neomycin. Both genes are controlled by the CaMV 35S promoter.

In vitro selection for neomycin phosphotransferase II gene expression in T_1 seeds

T_1 seeds were collected and evaluated for expression of the ***npt*** II gene by culturing their embryos on 10 mg/l geneticin according to Obembe et al. (2005). Seeds were sterilized in 0.5% (w/v) calcium hypochlorite solution containing two drops of Tween 20 for about 24 h. They were rinsed with three changes of sterile distilled water. The seeds were split longitudinally and embryos were excised under asceptic conditions and cultured on full strength MS medium containing 10 mg/l geneticin, 0.05% naphthalene acetic acid, 3.0 % sucrose and 0.8% agar (pH 5.8). Culture conditions were kept constant at 25 ± 4°C under a 16 h light and 8 h dark photoperiod. Plantlets retaining green leaves after 2 - 3 weeks were selected and transferred to antibiotic-free MS medium for at least 4 weeks before been transferred to peat inside PHYTACON™ bowls for acclimatization.

Polymerase chain reaction (PCR)

Total genomic DNA was isolated from leaf tissue of transformed and control plants. The leaves were freeze-dried and ground in a mortar and pestle. DNA was extracted using the Dellaporta et al. (1983) method. Plants that survived screening on geneticin were subjected to PCR analysis. Two 24-mer primes, which are homologous to the ***Cry 1Ab*** gene (5′ – GCA TAG TTA AGC CAG CCC CGA CAC – 3′) and (5′ – TAG CTC CTT CGG TCC TCC GAT CGT – 3′) were used to detect Bt gene sequences introduced into the plant genome. PCR amplification of DNA were performed in 25 µl reaction volumes containing 2 µg of total plant DNA, 5.0 µl of each primer, 10 mM of dNTP (promega), 2.5 µl of 10 x PCR buffer, 2.0 µl of 25 mM $MgCl_2$. 2.0 µl of 5% Tween 20, and 10 units Taq DNA polymerase (promega) and made up with 2.5 µl of sterile distilled water. The reaction was overlaid with mineral oil and cycled in a Perkin Elmer-Cetus DNA cycler model 480 though the following temperature profile: 2 min at 94°C, 1 min at 94°C, 1 min at 60°C and 2 min at 72°C. The final incubation at 72°C was extended for another 10 min to allow better termination of the polymerase reaction. The PCR was completed in 40 cycles and products were electrophoresed in 1.4% agarose gel and visualized under UV by ethidium bromide staining.

Southern blot analysis

Southern blotting and hybridization were performed as described by Southern (1975), Sambrook et al. (1989). Genomic DNA (10 – 20 µg) of DNA was digested with Eco RV (Integrated DNA technologies, U.K.) separated on 0.8% agarose gel, and blotted on nylon membrane (Hybrid M Amersham). This nylon was baked at 80°C for 2 h to immobilize transferred DNA. Probes were prepared by digesting the plasmid DNA with Apa I enzyme (Figure 1) and labelled by non-radioactive process using Biotin following the manufacturers manual (Bioprime Labelling Kit, Biolabs, England).

Insect larval culture and bioassay of transformed plants against *Maruca vitrata*

M. vitrata larvae were cultured on artificial diets and produced for

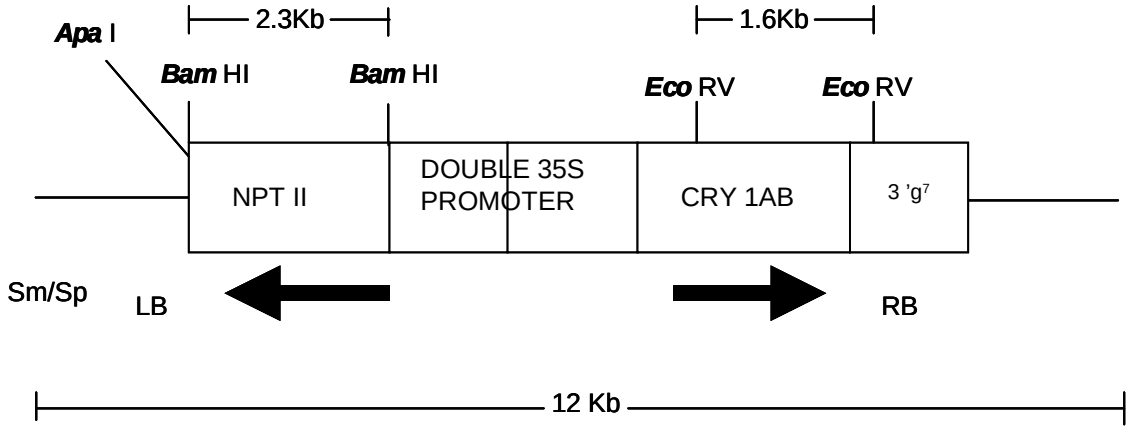


Figure 1. Schematic map of the plasmid ptrval carrying ***Cry 1Ab*** and ***nptII*** genes. Restriction sites for enzymes used in southern blot analyses are shown.

bioassays according to (Jackai and Raulston, 1988). Transgenic T_1 plants surviving geneticin selection and ***Cry1Ab*** positive by PCR and southern analyses were assayed for resistance to ***M. vitrata*** using a detached leaf bioassay method (Machuka et al*.,* 1999; Oghiake et al*.,* 1993). With further antibiotic selection and molecular analyses, T_2 and T_3 progenies were generated, but only T_3 plants were evaluated for insecticidal effects.

Three leaflets from test plants and non-transformed controls were each infested with three, third instar ***M. vitrata***, larvae (i.e. 8 – 10 days old) inside a Petri dish with moist paper towel. Larval survival at 1, 2 and 3 days after infestation (DAI) and feeding deterrence score (FDS) were determined for T_1 plants while in addition larval weight was measured at 1, 2, and 3 DAI for T_3 plants. The FDS is a visual assessment based on a score of 0 – 3 to indicate the extent of inhibition of larval feeding on the diet A score of 0 indicates no feeding deterrence, that is, larvae are alive and feeding well. A score of 3 indicates high mortality with little or no survival and little or no feeding. Scores of 1 and 2 indicate low and moderate feeding deterrence, respectively. Larval weight difference was estimated from weight taken on first and third days of the experiment. Analysis of variance (ANOVA) test was done on larval weight, FDS, and larval survival percentages and significantly different means were separated by Duncan's multiple range test (SAS Institute, 1989).

In vivo selection for *npt* II gene in T_2 progeny

T_2 progeny obtained by selfing was germinated in the green house and selected for expression of the marker gene encoding ***npt*** II by painting the two surfaces of each primary leaf with 1.0% kanamycin sulfate (sigma) according to Zapata et al*.* (1999). This was achieved by submerging the freshly emerging leaves on 1 - 2 weeks old plants in the kanamycin solution. The choice of this dosage was informed by an unpublished killing curve data obtained in our laboratory for cowpea leaves.

Molecular analysis of T_2 progenies

Plants derived from kanamycin screening were subjected to southern analysis by digesting their DNA with ***Eco*** RV. These were probed with ***Bam*** HI fragment (2.3Kb) containing the ***npt*** II cassette of the ***Bt*** plasmid (Figure 1).

RESULTS AND DISCUSSION

Electroporation and geneticin selection of T_1 plants

Forty five plants survived when 75 were electroporated. Four hundred and sixty six T_1 seeds were produced and screened for ***nptII*** gene expression on 10 mg/l geneticin from which 138 geneticin-resistant plants were obtained. This antibiotic concentration was chosen based on our earlier investigation (Obembe et al*.,* 2005), showing that clear distinction can be made between non-transformed zygotic embryos growing on 10 mg/l geneticin and non-transformed controls growing on selection-free medium. Our data showed that after 17 days on selection (Figure 2), shoots of putative Bt transgenic plantlets were still growing vigorously and their leaves remained fresh and green while non-transformed controls showed high sensitivity to the antibiotic as revealed by necrotic and bleached roots and failure to produce leaves. This confirms the earlier findings of Garcia et al*.* (1986), Perkins et al*.* (1987) and Fillipone (1990). When they transformed cowpea leaf discs, mature embryos and cotyledonary nodes by cocultivation with ***Agrobacterium***, viable and stably transformed calli were obtained by selecting explants on 50 mg/l geneticin but no transgenic whole plants were regenerated. Find et al*.* (2005) demonstrated the efficiency of geneticin as a selective agent for transgenic ***Abies normanniana*** plants. After five weeks there was no growth recorded for any non-transformed genotype on 15 mg/l geneticin. Grace et al*.* (2005) also obtained independent ***Cry 1Ac*** transgenic

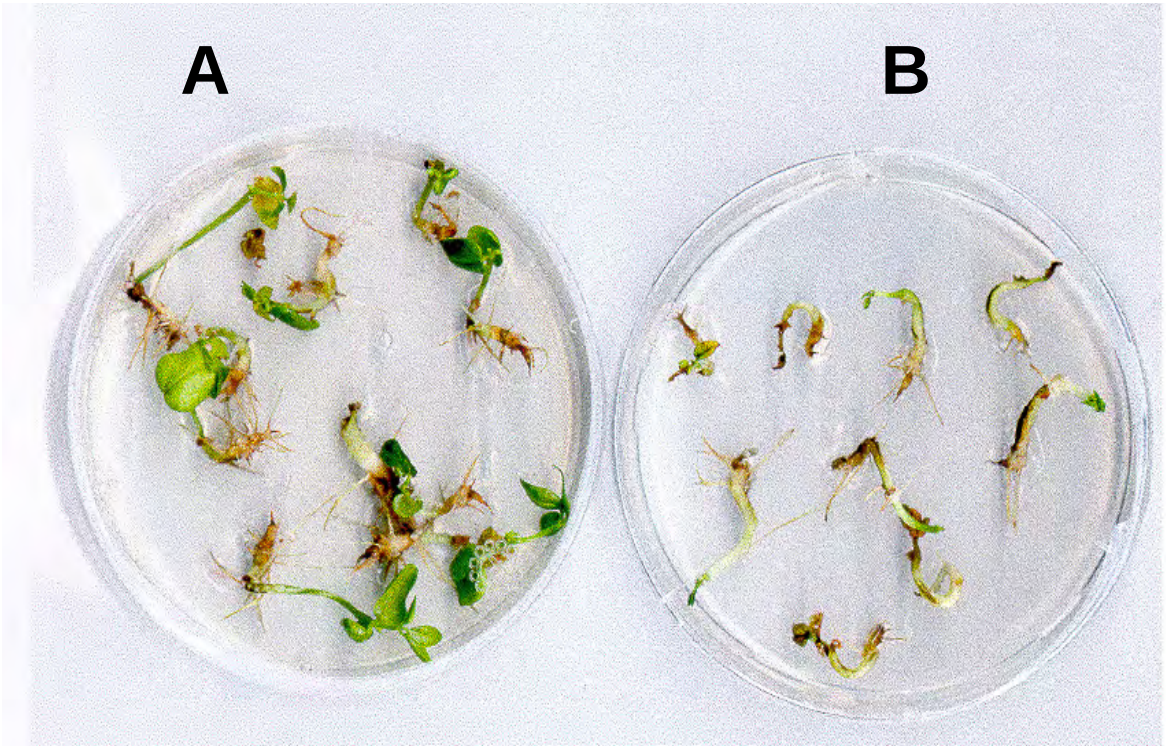


Figure 2. Evaluation of **A**: T_1 seed embryos of ***Cry 1 Ab*** transformed cowpea on 10 mg/L geneticin **B**: T_1 seed embryos of non-transformed cowpea on 10 mg/L geneticin for ***npt II*** gene expression after 17 days of selection.

Pinus radiata lines by selecting embryogenic tissues on geneticin. ***Picea glauca*** was similarly selected and geneticin was found to effectively inhibit growth of non-transformed zygotic embryos at 70 mg/l. Various other transformed plants have been selected on geneticin including suspension cultures (Ritala et al***.,*** 1993), shoot cultures (Zhang et al***.,*** 2000), protoplasts (Asano and Ugaki, 1994) and embroygenic tissues (Walter et al***.,*** 1998).

It is usual of cowpea to produce low number of seeds especially in pots and this makes genetic analyses difficult as with other large-seeded legumes (Chowrira et al***.***, 1998). However, from T_1 progenies recovered, the ratio of geneticin resistant to geneticin sensitive plants was non-mendelian (Table 1). This indicates that the T_0 plants were chimeric, compromising both transformed and non-transformed cells, with the latter accounting for a higher proportion of non-transformed progeny in the T_1 than expected. Chimeric transformation has been documented in other legumes such as pea (Bean et al***.,*** 1997), peanuts (Cheng et al***.,*** 1997) and ***Phaseolus vulgaris*** (Aragao et al***.,*** 1996). Chowrira et al***.*** (1998) asserted that T_0 branches developing from electroporated pea buds were highly chimeric and this explains why only few T_1 plants derived from such parents carry the transgene. Similarly, Dominiguez et al***.*** (2004) obtained chimeric citrus plants at unexpectedly high frequencies after ***Agrobacterium***-mediated transformation and attributed this to inefficient selection. Wu et al***.*** (2002) also explained that non-Mendelian ratios might be due to complicated and random integration of foreign genes in the host genome. However, chimeras do not prevent effective germline transformation and the production of transgenic sexual progenies has been demonstrated in soybean (Christou and McCabe, 1992). Inheritance of ***Cry 1Ab*** gene in cowpea progenies in this study as illustrated by molecular analyses confirms germline transformation through nodal bud electroporation.

Molecular analyses of T_1 and their effect on *M. vitrata* larvae

TVu 201 plants were analysed further. Presence of ***Cry1Ab*** coding sequence in 18 of these T_1 plants was identified by PCR analysis using primers that amplified an internal 750 base pair ***Cry1Ab*** fragment. A fragment with the expected size was detected in all of the 18 samples examined (Figure 3). No fragments were detected in untransformed control plants. This suggests that the ***Cry 1Ab*** gene was inherited in T_1 progeny.

Genomic DNA samples from 14 of these PCR-positive

Table 1. T_1 progeny resistance to geneticin and effect on ***Maruca vitrata*** larval survival.

T_o Plant	Number of T_1 progeny screened	Number resistant (R)	Number susceptible (S)	Ratio R:S	Larval survival % 3 DAI
E2	15	5	10	1:2	91.14
E3	24	0	24	-	-
E5	17	7	10	1:1.4	82.22
E7	20	4	16	1:4	83.35
E8	25	4	21	1:5.3	96.30
E12	10	0	10	-	-
E15	21	10	11	1:1	90.15
E18	32	11	21	1:2	94.40
E25	26	13	13	1:1	67.78
CNTL	10	0	10	-	100

DAI = days after infestation with larvae.

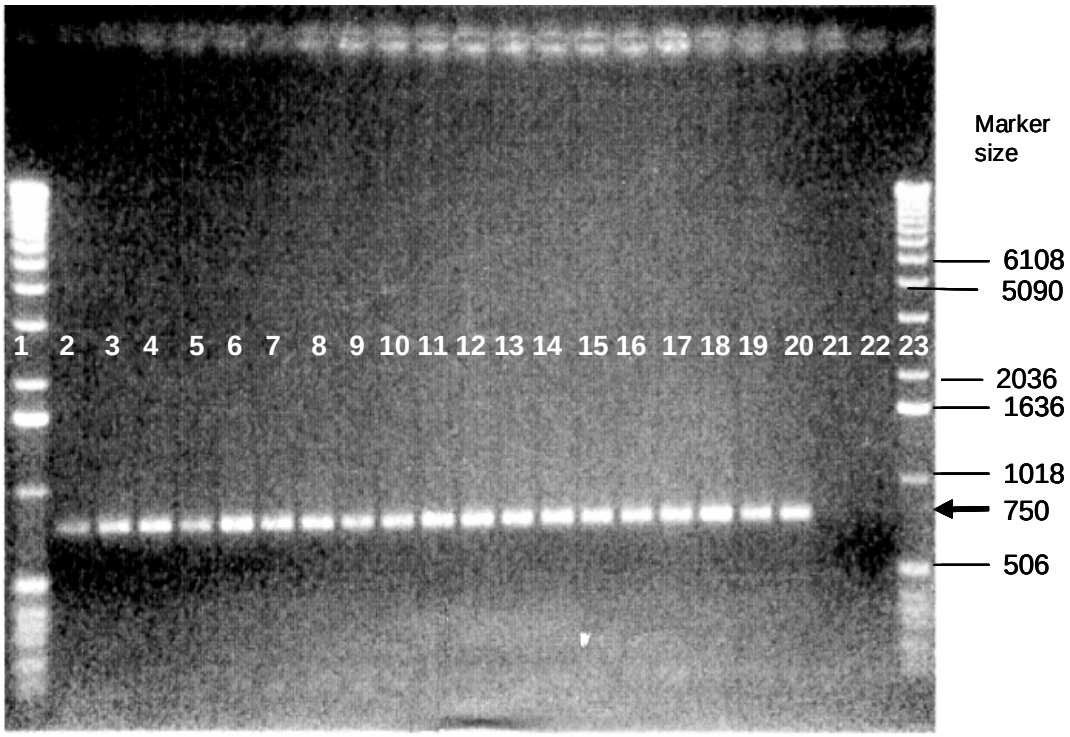


Figure 3. Agarose gel electrophoresis of PCR amplified DNA fragments of ***Cry1 Ab*** gene from T_1 progeny of plants electroporated with Bt plasmid. Lanes 1 and 23: 1Kb Ladder DNA marker; Lane 2: Bt plasmid control; Lanes 3-20: Genomic DNA from T_1 plants; Lanes 21-22: Untransformed cowpea DNA and water controls.

plants were subjected to southern hybridization analysis (Figure 4a). Restriction enzyme ***Kpn*** I linearized the 12 kb ptrval.seq plasmid carrying the ***Cry1Ab*** gene (Figure 1) and this was used as probe. The probe hybridised to ***Eco*** RV-digested genomic DNA from transformed plants. A hybridization band corresponding to the plasmid size was detected in 12 of these plants while none was observed for the non-transformed plant and blank DNA controls (Figure 4a). With nodal bud electroporation-mediated transformation Chowrira and co-workers (1996, 1998)

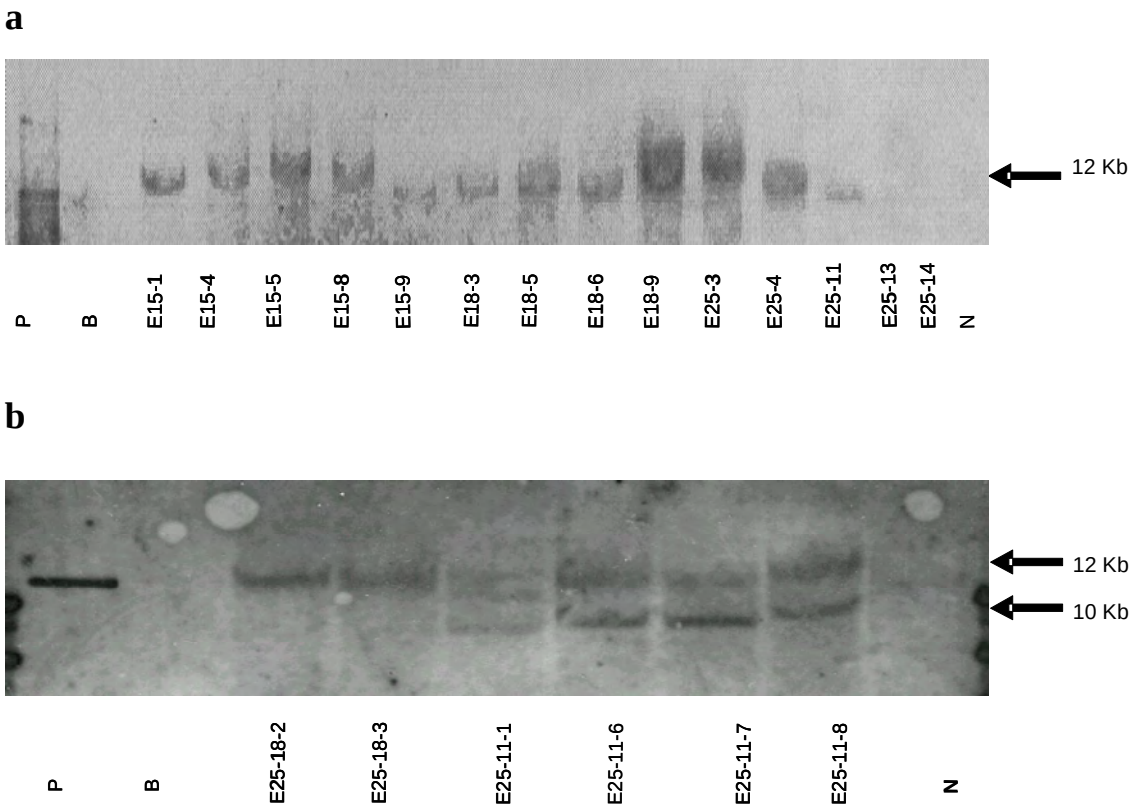


Figure 4. a: Southern hybridization of T_1 progenies from ***Cry 1Ab*** electroporated plants probed with linearized Bt plasmid Lanes 3-16: Genomic DNA from T_1 progeny; B: No DNA control (Blank); P: Plasmid control; N: Untransformed cowpea DNA control. **b**: Southern hybridization of T_2 progenies ***Cry 1 Ab*** electroporated plants probed with ***npt II*** fragment of plasmid. Lanes 1 - 6: Genomic DNA from T_2 plants; Lane 7: Untransformed cowpea DNA control; B: No DNA control; P: Plasmid control.

demonstrated the integration of GUS and pea enation virus resistance genes into cowpea and pea (***Pisum sativum***) respectively at T_1.

Copy numbers of the ***Cry1Ab*** gene in the plants shown in Figure 4a were estimated to be one to several per genome from the visual comparison of the band intensities (Register et al*.,* 1997). Datta et al*.* (1998) similarly observed the integration of one to several copies of ***Cry 1Ab*** transgene in rice following microprojectile bombardment. They noticed that uncut genomic DNA from transgenic plants showed different banding patterns and ***Cry 1Ab*** gene integrated at high molecular weights. ***Cry 1Ab*** transgene occurred at very high molecular weight in this work despite the fact that the genomic DNA was well restricted with ***Eco*** RV. These 12 transgenic plants were derived from 3 different T_0 parents, that is from three independent transformation events (Figure 4a). They are thus expected to produce three distinct patterns only. We observed that there were more than three integration sites within the host genome. However, according to Chowrira et al*.* (1998), T_1 progeny plants arising from a given T_0 parent are not really identical due to the chimeric nature of these primary transformants produced by nodal electroporation, therefore more than three integration sites could be expected. They were also inserted at slightly different sites within the host genome. Plants of lanes 16 and 17 showed no hybridisation with the probe even though they were positive with PCR analysis.

Transgenic plants expressing insecticidal crystal proteins (ICP) are powerful tools in an integrated pest management program (Sharma et al*.*, 2004). In attempt to identify reliable insect resistance gene that can be deployed in cowpea transformation against ***M. vitrata,*** effective bioassay methods have been developed (Monti et al*.,* 1997). Bioassays carried out at the Purdue University in collaboration with Auburn University had demonstrated that of all the several Bt crystal toxins available, Cry 1Ab had the most detrimental effect on the cowpea pod borer (Jackai et al*.*, 1997). This was achieved through bioassay using artificial diets. Also, Oghiake et al*.* (1993) noticed that though first instar larvae may not feed well on cowpea foliage; older larvae are able to do better. This informs the choice of the third

Table 2. Survival and feeding deterrence of ***Maruca vitrata*** larvae fed on T_1 progeny transformed with ***Cry 1Ab*** gene.

T_1Plant	Larval survival 3DAI (%)	Feeding deterrence
E15-1	100a	1.33abc
E15-4	88.9a	1.0abc
E15-5	88.9a	0.67abc
E15-8	88.9a	1.0abc
E15-9	100a	1.0abc
E18-3	88.9a	2.33abc
E18-5	100a	1.33abc
E18-6	100a	2.67ab
E18-9	77.8ab	1.33abc
E25-3	22.2b	0c
E25-4	88.9a	3.0 a
E25-11	77.8a	0c
Control	100a	1.67abc

Numbers followed by the same alphabets are not significantly different at p≤0.05 using Duncan's multiple range test. DAI = days after infestation.

instar larvae for bioassays carried out in this study. Starving the larvae for more than 1 h before infestation and reducing the amount of moisture and water supply on the paper towel induced better feeding of the insect larvae on the leaves. This was necessary since MPB naturally has more preference for pods. Similar moisture quantity (200 µl) was used by Machuka et al*.* (1999) in control diets for MPB larvae (i.e. without lectin solution). When leaves of 12 transgenic plants were infested with ***M. vitrata*** larvae, eight of them reduced larval survival within 3 days after infestation (DAI) to between 11.1 and 77.8% (Table 2). The most severe effect on survival of larvae and the highest level of feeding inhibition were observed amongst T_1 progeny of transgenic line E25.

Kanamycin leaf painting of T_2 progeny

Two hundred and forty-three T_2 seedlings obtained from 11 T_1 transformants were evaluated by kanamycin leaf painting. Forty-seven plants were kanamycin resistant. Zapata et al*.* (1999) confirmed the integration of the GUS gene in two successive generations derived from T_0 regenerants after previous selection by painting 2% kanamycin on the leaves. This approach was used to select T_2 progenies ***in vivo*** to avoid losing plants to contamination as with ***in vitro*** selection. Moreover, it appears that kanamycin selection is more reliable after initial selection with geneticin as Garcia et al. (1986) obtained fully transformed cowpea calli using a similar method.

Molecular analysis of T_2 progeny

Four plants derived from line E25-11 (Table 3) were collected for southern analysis. They were digested with ***Eco*** RV and probed with ***Bam*** HI fragment containing the ***npt*** II gene of the Bt plasmid. Two copies of the ***npt*** II gene were integrated into their genome. The copy number of ***npt*** II in these plants is the same as that observed for ***Cry 1Ab*** gene in the parent line E25-11 (Figure 4a). Plasmid positive control hybridization band corresponded to the transgenic plants DNA (Lane P, Figure 4b) while the no DNA and non-transformed cowpea controls had no bands. This proves the stable integration of the ***npt*** II gene into the cowpea genome at T_2. Southern blot analysis for ***Cry 1Ab*** gene was not performed on these plants but ten T_3 progenies obtained from one of them, i.e. from line E25-11-8, were subjected to insect bioassay to cross check the stability of the ***Cry 1Ab*** gene and its effect on the survival of ***M. vitrata***.

Insecticidal effect of T_3 progeny on *M. vitrata* larvae

Leaves of 10 T_2 plants when assayed resulted in a significant decrease (P = 0.05) in percentage survival of insect larvae as compared to non-transformed cowpea (Table 4). After 3 days no insect survived on 9 of them while all the larvae survived on the control plant leaves of the same age. Significant differences between control and test plants became noticeable and clear on the third day of the experiment.

Weight gain is usually an index of growth and development in insect larvae. The weight gain 3 DAI by larvae on non-transformed controls was significantly higher than weight gain by those on all transformed lines. Line E25-11-8-5, where none of the larvae were killed still resulted in larvae with significantly lower weight (6.37 mg) than those on negative control plants (9.8 mg) suggesting that some insecticidal effect was still produced. Percentage larval survival was significantly lower (p=0.05) on the other nine lines tested than on controls (Table 4). On 6 out of 10 transformed lines, total weight difference was negative i.e. the larvae weighed less at the end of the experiment than at the beginning. A similar trend was obtained for feeding deference scores taken 3 DAI. Insects fed voraciously on control leaves as also in the E25-11-8-5. In 6 out of 10 transformed lines, above 50% feeding inhibition was noticed. Their leaves were well protected from insect feeding damage (Figure 5).

Machuka et al*.* (1999) noted that although older (i.e 3^{rd} – 6^{th}) instars of ***M. vitrata*** were expected to consume more food than 1^{st} and 2^{nd} instars, yet they consumed less than optimal of the artificial diets supplemented with various concentrations of lectin solutions as to affect subsequent stages negatively. It can be concluded that those lines that produced significantly higher feeding

Table 3. Segregation of the ***npt***II gene in T_2 progeny based on resistance to Kanamycin leaf painting.

T_1 Plant	No T_2 seeds produced	No of T_2 plants painted with kanamycin	Resistant progeny (R)	Sensitive progeny (S)	R:S ratio
E15-1	13	13	2	11	1:5.5
E15-4	23	23	2	21	1:10.5
E15-5	16	16	3	13	1:4.3
E15-8	26	26	7	19	1:2.7
E15-9	25	25	4	21	1:5.3
E18-5	9	9	2	7	1:3.5
E18-6	34	34	6	28	1:4.7
E18-9	16	16	4	12	1:3
E25-3	43	43	8	35	1:4.4
E25-4	16	16	1	15	1:15
E25-11	22	22	8	14	1:1.8

Table 4. Survival and larval weight difference of ***Maruca vitrata*** larvae fed on T_3 progeny transformed with ***Cry 1Ab*** gene.

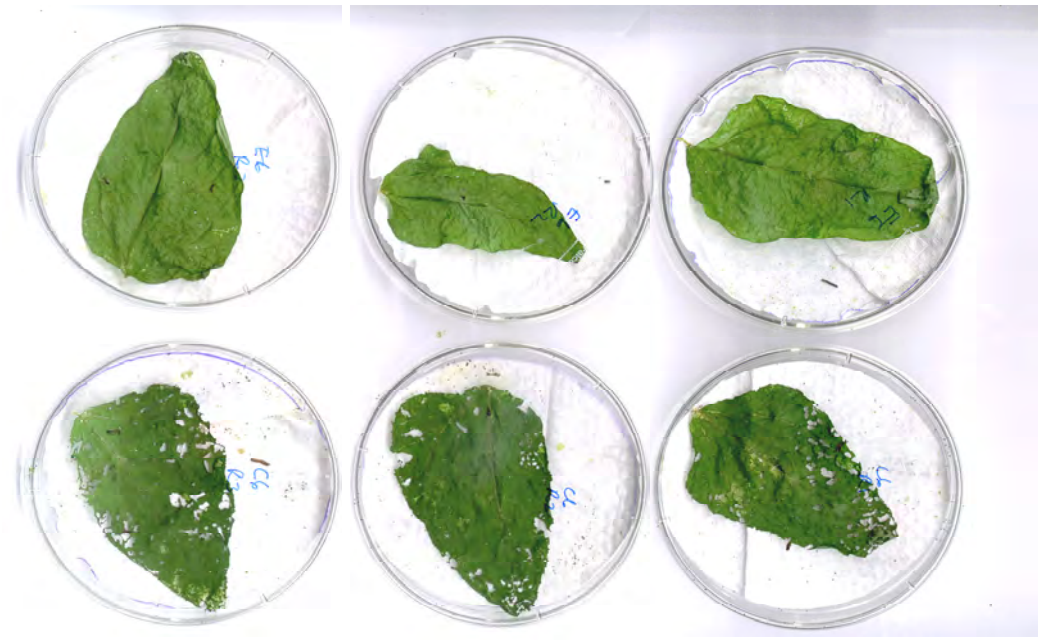
	%Survival			Weight difference (mg)
T_3 Plant	1DAI	2DAI	3DAI	3DAI
E25-11-8-1	100.0a	88.9ab	0e	-3.23e
E25-11-8-2	77.8b	77.8ab	66.7bc	1.73 c
E25-11-8-3	100.0a	100.0a	88.9ab	4.87 b
E25-11-8-4	88.9ab	88.9ab	44.43cd	0.33 cd
E25-11-8-5	100.0a	100.0a	100.0a	6.37d
E25-11-8-6	100.0a	88.9 ab	22.2de	-3.93e
E25-11-8-7	100.0a	88.9 ab	33.3d	-1.23de
E25-11-8-8	88.9ab	88.9 ab	0e	-3.83e
E25-11-8-9	88.9ab	66.7 b	0e	-2.33de
E25-11-8-10	100.0a	100.0a	22.2de	-1.13de
Control	100.0a	100.0a	100.0a	9.8 a

Numbers followed by the same alphabets are not significantly different at p≤0.05 using Duncan's multiple range test. DAI = days after infestation.

deterrence scores can effectively control this insect pest by antibiosis. According to Sharma (1998), antibiosis appears to be the most common mechanism of host plant resistance to MPB as demonstrated in wild cowpea variety Tvu 946. Reduction in larval survival was due to nutritional and antibiotic factors. Machuka et al*.* (1999) noted that MPB larvae do not survive for long without feeding.

The ability of ***B. thuringiensis*** crystal protein genes to reduce the crop damage level, growth and survival of economically important insect pests and their larvae has been documented for many crops. Corn earworm (***Helicoverpa zea***) infestation resulted in less than 3% defoliation on ***Cry 1AC*** transgenic soybean compared with more other 40% leaf damage on susceptible cultivars (Stewart et al*.,* 1996). The synthetic version of ***Cry 1Ab3*** gene used by Jin et al*.* (2000) provided effective control of the diamondback moth (***Plutella xylostella***) in transgenic cabbage. There was 100% larval mortality after three days of infestation in this study. Similarly, four transgenic lines of rice expressing ***Cry 1B*** gene were protected against second instar stripped stem

a



b

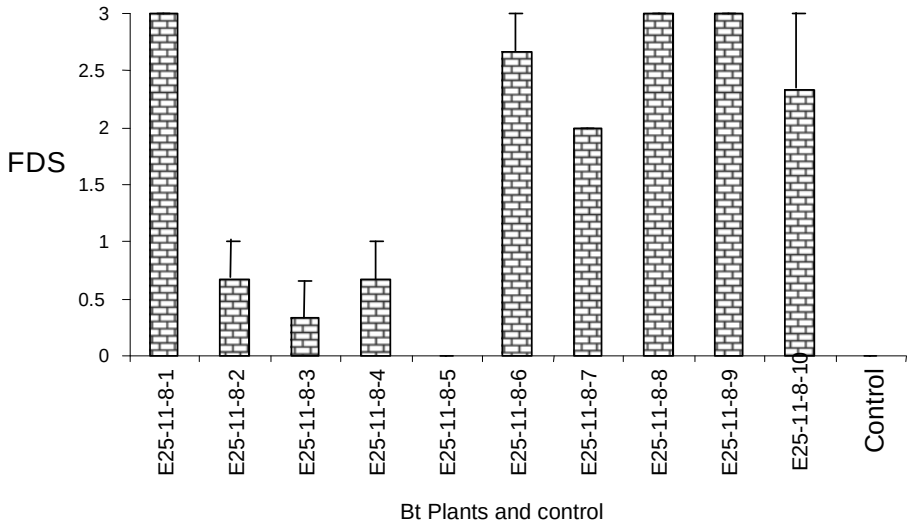


Figure 5. a: ***Maruca vitrata*** bioassay of detached leaves from T_3 ***Cry 1Ab*** transgenic cowpea. **b**: Histogram of ***Maruca vitrata*** feeding deterrence on T_3 ***Cry 1Ab*** transgenic cowpea.

borer (***Chilo suppressalis*** Walker) larvae in whole plant feeding assays, while the larvae exhibited 90 – 100% mortality 7 DAI (Breitler et al**.,** 2000). Similar trends were observed in this study as feeding damage, larval survival percentage and larval weight reduced drastically within three DAI among the resistant transgenic lines. Less feeding led to weight loss and lower survival rates. In most cases, ***Maruca*** larvae fed on control plants in the study gained weight while those on the transgenic lines lost weight. This probably indicates that the insects starved to death. At the same time, larvae must have ingested enough toxin to result in death as indicated by the minute feeding lesions on the transformed cowpea leaves (Figure 5). Berg and Wyk (2007) reported that the

drastic reduction in weight of ***Sesamia calamistis*** larvae fed on Bt transformed maize indicated an immediate toxic effect by the Cry 1Ab proteins. Similarly Walker et al*.* (2004) tested antibiosis resistance of ***Cry 1AC*** transgenic soybean to tobacco bud worm, ***Heliothis virescens*** (a lepidopteran pest) using detached leaf bioassays. Insect resistance was evaluated based on larval weight gain on detached leaves. All dead larvae eventually dried to a hard brown or black scale as similarly observed when Taylor (1968) used ***B. thuringiensis*** var. ***thuringiensis*** bacteria as biocontrol agent against Maruca pod borer.

Conclusion

This study ascertains that ***Cry 1Ab*** gene was integrated into cowpea genome at T_1 as confirmed by PCR and southern analyses and this resulted in antibiosis against MPB larvae and protection of cowpea from damage. The fact that T_3 plants with MPB resistant phenotypes were obtained suggests that the transgene is stably inherited in cowpea. Although the use of ***in planta*** electroporation for cowpea transformation has not become widespread, it has been shown to be a simple means of generating transgenic cowpea.

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