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MINIMAL GENE CASSETTE TRANSGENIC RICE PLANTS EXPRESSING GNA AND BT TOXINS RESISTANCE TO BROWN PLANTHOPPER AND RICE STEM BORER.

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ABSTRACT

Biolistic transformation was used to introduce genes encoding the insecticidal proteins snowdrop lectin (Galanthus nivalis agglutinin; GNA) and cry1Ac Bt toxin (δ-endotoxin from Bacillus thuringiensis) into elite rice (Oryza sativa) cultivars. Plant transformation was carried out in parallel experiments simultaneously by using either whole plasmids containing suitable gene constructs, or the corresponding minimal gene cassettes which were linear DNA fragments lacking vector backbone sequences excised from the plasmids. Insect bioassays with major pests of rice showed that transgenic rice plants expressing GNA were enhanced the levels of resistance to brown planthopper (Nilaparvata lugens), and plants expressing cry1Ac were protected against attack by striped stem borer (Chilo suppressalis). Expression of both transgenes gave protection against both pests, irrespective as to whether the plants were co-transformed with supercoiled whole plasmid DNA, or minimal cassettes. Comparison of results obtained in the present study show that plants transformed with minimal gene cassettes gave slightly higher levels of resistance to the target pest (possibly due to higher expression levels for the transgene product) when compared to plants cotransformed with whole plasmid DNA.

Keywords: Biolistic transformation, "Clean" DNA, Crop pests, Minimal gene cassette, *Oryza sativa*.

INTRODUCTION

Enhanced levels of resistance to insect pests is an economically important agronomic trait which is being engineered into crops via recombinant DNA technology (Gatehouse and Gatehouse 1999). The crystal protein genes (*cry* genes) from *Bacillus thuringiensis* (Bt) encode insecticidal δ-endotoxins and are primary candidates for exploitation in the development of crops resistant to insect pests. Such genetically modified crops have been commercially available since 1995 following the introduction of Bt-potato (expressing *cry 3A*), and thereafter by the commercialisation of Bt-cotton (*cry 1Ac*) and Bt-maize (*cry 1Ab, cry1Ac, cry9C*); these crops exhibit enhanced levels of resistance to coleopteran (*cry 3A*) and lepidopteran pests, respectively (reviewed in Carozzi and Koziel 1997). Rice resistant to lepidopteran insect pests has also been produced by transformation with Bt *cry* genes (Bennett *et al.* 1997). Field trials with rice expressing Bt toxin have shown a high level of protection against yellow stem borer and leaf folder (Tu *et al.* 2000)

In order to develop crops with enhanced levels of resistance to sap-sucking insects (Homoptera), other strategies are being actively pursued. The mannose specific lectin from snowdrop (*Galanthus nivalis*), GNA, is toxic towards a number of insect pests of different orders, including Homoptera. GNA inhibits development and reduces fecundity in aphids when fed in artificial diet (Sauvion *et al.* 1996; Down *et al.* 1996), and when expressed in transgenic potato plants (Gatehouse *et al.* 1997, Down *et al.* 1996). This lectin also reduces nymph survival of the two most important homopteran pests of rice, the brown planthopper *Nilaparvata lugens*

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(BPH) and the green leafhopper *Nephotettix virescens* (GLH), when fed in artificial diet (Powell *et al.* 1993). Expression of GNA in transgenic rice (Sudhakar *et al.* 1998) was shown to confer significantly enhanced levels of resistance to BPH (Rao *et al*. 1998; Tinjuangjun *et al*. 2000), and to GLH (Foissac *et al*. 2000).

Fu *et al*. (2000) cotransformed rice by particle bombardment using linear transgene constructs lacking vector backbone sequences resulting in plants with simple integration patterns, low transgene copy number, and efficient expression. We have extended these results to genes of agronomic interest and described the production of transgenic rice plants expressing GNA and Bt toxins using linear transgene constructs lacking vector backbone sequences ("clean" DNA). In that study we also compared the integration patterns and expression levels of the transgenes in transgenic plants introduced either as fragments or in plasmid form (Loc *et al.* 2001).

In the present paper, we compare the consequences of using minimal cassettes in terms of levels of resistance to two major pests of rice, brown planthopper *Nilaparvata lugens* and striped stem borer *Chilo suppressali*s, with that of plants transformed with whole plasmids in parallel experiments.

MATERIALS AND METHODS

Gene constructs, plant material, cotrans-formation and regeneration, polymerase chain reaction (PCR) analysis, nucleic acid isolation and Southern/Northern blot analysis and Western blot analysis are described in OMonRice 9 (Loc *et al*, 2001). In this study we have used those minimal gene cassette transgenic rice plants expressing GNA and Bt toxins for the insect bioassays with major pests of rice crop. The bioassay have been conducted as following:

Rice Brown Planthopper:

Rice brown planthopper (BPH, *Nilaparvata lugens*), obtained originally from Rhone-Poulenc Agriculture Ltd (Essex, UK), was maintained on two- to three-month-old rice plants (susceptible variety Taichung Native 1) under controlled environmental conditions (70-80% relative humidity, 25 ± 2°C, 16-h photoperiod). Insect stock cultures were held under MAFF licence PHL 51A/3438. Insects were released onto 30-day-old (ca. 60 cm high) R_0 rice plants (10 neonates per plant), individually confined within insect-proof fine-mesh nylon cages to prevent migration of insects between plants. Six replicates were set up for each transgenic line and the non-transformed controls. Insect survival (number of live insects as a proportion of the number of insects initially released) was monitored 6 h after release onto the plants (day 0), after 1 day, and thereafter every three days throughout the 19-day trial period. Data analysis was carried out using the Statview software package (Version 5.0; SAS Institute Inc., Cary, North Carolina, U.S.A.) and statistical differences between treatments were assessed pairwise using the unpaired t-test and its nonparametric equivalent, the Mann-Whitney U-test.

Rice Stem Borer:

Egg masses of striped stem borer (*Chilo suppressalis)* were obtained from International Rice Research Institute, the Philippines. The egg masses were maintained under controlled environmental conditions (70-80% relative humidity, 25 ± 2°C, 16-h photoperiod).

Bioassays were carried out on rice stem sections from primary transformant R_0 rice plants and non-transformed parental controls. A single stem section, 7 cm long with at least one node, was taken from each plant. Sections were placed on moist filter paper in petri dishes and infested with neonate stem borer larvae (<5 h old) by placing five at each end of the cut sections to facilitate entry into the stem (10 neonates/replicate). Eight replicates were set up for each line. The petri dishes were then sealed with parafilm and left for 5 days in a controlled growth chamber under the conditions specified above. After the trial period, stem sections were dissected under a binocular microscope and insect survival, development and weight were recorded. Statistical analysis of insect data was performed with Statview software as above.

Analysis of variance (ANOVA) was used to test for significant differences between treatments. A rejection limit of P>0.05 was used.

RESULTS AND DISCUSSION

Bioassays were carried out with two insect species on plants derived from transformation with both whole plasmids and minimal gene cassettes. Where linear fragment constructs were used, the non-transformed parental line was Eyi 105; transgenic lines were designated E-4-10 (accumulating GNA), E-4-3 (*cry1Ac*), E-4-7 and E-6-5 (GNA + *cry1Ac*). Where whole plasmids were used for transformation, the control parental line was Bengal, with transgenic lines designated B-2-2 (accumulating *cry1Ac*), B-3-2 and B-2-10 (GNA + *cry1Ac*). Trials were carried out using clonally replicated R_0 transgenic plants derived from independent transgenic lines. No differences in susceptibility to attack by both insect species used between the two parental lines were observed.

Assays with brown planthopper (*Nilaparvata lugens*)

N. lugens survival on plants was monitored over a period of 19 days, corresponding to the interval required for complete insect development. Survival on plants of both parental cultivars was over 90% during this period.

As expected, the transgenic rice lines expressing *gna* which had been produced by transformation with whole plasmids were significantly resistant to this homopteran pest. By day 4 insect survival on lines B-2-10 and B-3-2 was significantly reduced ($p<0.05$) as compared to that on non-transformed control plants. This trend was maintained throughout, with reduced survival being highly significant by the end of the trial ($p<0.0001$). At this stage *N. lugens* survival had been reduced by 43% and 38%, respectively, for lines B-2-10 and B-3-2 relative to performance on control plants (Fig 1a). However, there was no significant difference between insect survival on line B-2-2 (expressing *cry1Ac* only) and on the control plants, at any stage during the trial period.

a) Plants transformed with minimal gene cassettes b) Plants transformed with whole plasmids

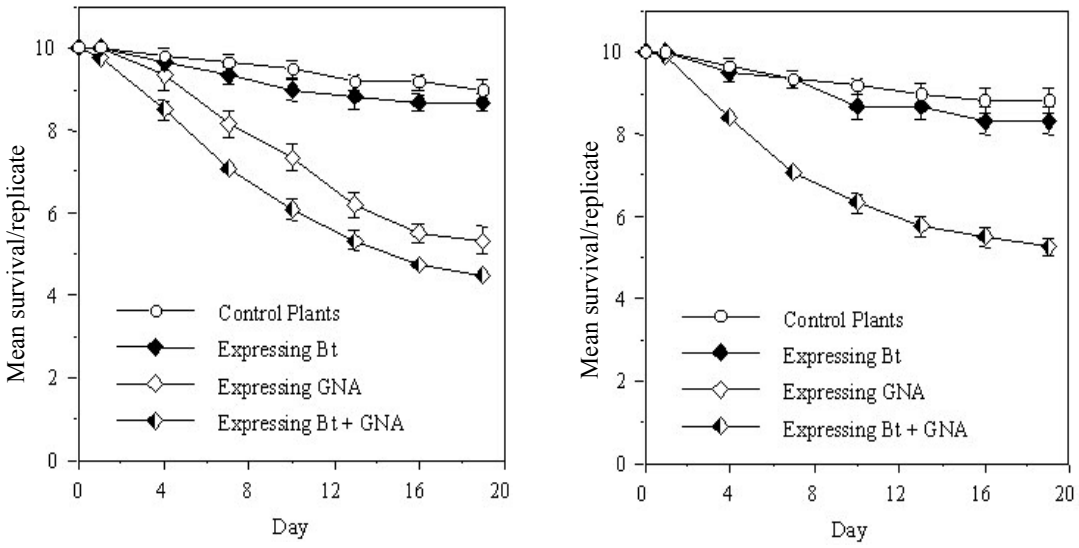

Figure 1. Bioassay of transgenic rice plants against brown planthopper (*Nilaparvata lugens*). Assays were carried out with first instar nymphs of BPH; 10 nymphs per plant were set up for 6 replicates of each transgenic plant line (whole plasmid transformation; *cry1Ac* = B-2-2, GNA + *cry1Ac* = B-2-10, B-3-2; minimal cassette transformation; GNA = E-4-10, *cry1Ac* = E-4-3, GNA + *cry1Ac* = E-4-7, E-6-5). Plants of the parental cultivars were used as controls. Graphs show mean survival per plant (replicate) vs time. Points show mean+SE. Graph legends show protein(s) expressed by each line tested.

Similar results were obtained with plants transformed with minimal gene cassettes. By day 4, survival of *N. lugens* on 2 GNA-expressing lines, E-4-7 and E-6-5, was significantly reduced ($p<0.05$) compared to that on non-transformed control plants; by day 7, survival on the other GNA-expressing line, E-4-10, was also significantly reduced ($p<0.05$) compared to that on non-transformed control plants. This trend was maintained throughout the remainder of the trial, and by the end of the trial the difference in survival between all the GNA-expressing lines and the control was highly significant ($p <0.0001$). At this stage, insect survival had been reduced by 52%, 48% and 41% for lines E-4-7, E-6-5 and E-4-10, respectively, relative to the control parental line (Fig.1b). Furthermore, differences in *N. lugens* survival between lines E-4-10 and E-4-7 were also significant throughout the trial, with line E-4-7 exhibiting higher levels of resistance. As in trial, on transgenic plants which did not accumulate GNA (line E-4-3; *cry1Ac* only accumulated) insect survival was not significantly different to that on non-transformed control plants at any stage during the trial.

Expression of GNA in all transgenic lines of rice were shown to confer significantly ($p<0.0001$) enhanced levels of resistance to brown planthopper, irrespective as to whether the plants were co-transformed with supercoiled whole plasmid DNA, or minimal cassettes where extraneous backbone sequences had been removed. These results for enhanced resistance to brown planthopper in transgenic rice containing GNA, with survival decreased by 38-52% over insect development are in broad agreement with those previously published for other transgenic rice varieties expressing *gna*, where constitutive expression was shown to reduce BPH survival by 41% (Rao *et al.*, 1998) and 32% (Tinjuangjun *et al.* 2000). It is interesting to note that whilst *cry1Ac*, in keeping with all other Bt endotoxins screened to date, had no effect on BPH survival, lines expressing the *cry1Ac* toxin together with GNA gave small, but significantly ($p < 0.05$) higher levels of protection against this insect pest as compared to expression of GNA alone. The reason for this observation is not clear, although GNA may be facilitating uptake of the endotoxin.

Comparison of results obtained in the present study show that the reduction in brown planthopper survival was slightly greater (approx. 10%) on plants cotransformed with minimal cassettes, compared to the corresponding plants cotransformed with whole plasmid DNA (Fig 1a, b). The improved performance of plants containing minimal cassettes may be a consequence of the significantly higher levels of GNA expression obtained for these lines.

Assays with striped stem borer (*Chilo supressalis*)

Rice stem borer bioassays were carried out using stem sections of non-transformed parental control plants and plants from transgenic rice lines carrying different transgenes and transformed with either minimal cassettes or whole plasmid DNA. The feeding habit of this insect meant that detached stem sections had to be used for these assays, which could only be carried out for a limited time (5 days), during which period the neonate larvae developed to 2nd instar on control stem sections, with survivals of 91% and 94% on plants of cultivars Eyi105 and Bengal, respectively. Both insect survival and growth could be monitored at the end of the assay. Lines used were as described for BPH assays.

All plants expressing Bt toxins showed a high level of resistance to this lepidopteran pest. Survival of stem borer larvae on plants containing *cry1Ac* protein but not GNA was reduced to 29% (line E-4-3) and 28% (line B-2-2), for plants transformed with the minimal cassette and whole plasmid, respectively (Fig 2); both reductions were highly significant ($p<0.0001$) when compared to survival on the parental cultivar controls. Plants containing both GNA and *cry1Ac* performed comparably to plants containing *cry1Ac* only in this assay, with insect survival being reduced to 17% and 42% (lines E-4-7 and E-6-5, respectively) and 25% and 49% (lines B-2-10 and B-3-2, respectively) for plants transformed with the

minimal cassette and whole plasmid, respectively. Data for these lines is combined pairwise in fig. 2. In all cases, the reduction in survival on plants expressing the two insecticidal proteins was highly significant ($p<0.0001$) as compared to performance on the respective control plants. Survival of stem borer larvae on plants containing GNA but no Bt toxin (line E-4-10) was slightly less than the parental control (86% vs. 91%), but this slight reduction was not statistically significant (Figure 2a).

Effects of transgene expression on the growth and development of rice stemborer larvae were also monitored. Development of surviving larvae was significantly reduced on all plants expressing *cry1Ac*, with no larvae developing beyond the 1st instar as compared to almost all insects on the controls reaching 2nd instar. Expression of *cry1Ac* alone resulted in a decrease in mean weight of surviving larvae of 62% (line E-4-3; transformation with minimal gene cassette) and 60% (line B-2-2; transformation with whole plasmid DNA); these reductions were highly significant ($p<0.0001$). On plants containing GNA + cry1Ac, mean larval weight was decreased by 71% and 48% (lines E-4-7 and E-6-5, respectively) and 65% and 46% (lines B-2-10 and B-3-2, respectively) relative to controls (significant at $p<0.0001$), for plants cotransformed with minimal cassettes and whole plasmid DNA, respectively. Data for these lines is again combined pairwise in fig. 3. In the lines that performed best in these assays, E-4-7 and B-2-10, no damage to stem sections was observed. As with stem borer survival, plants cotransformed with minimal gene cassettes appeared to perform marginally better, in that larval weight was lower compared to those cotransformed with whole plasmids, although differences did not reach statistically significant levels at $p < 0.05$.

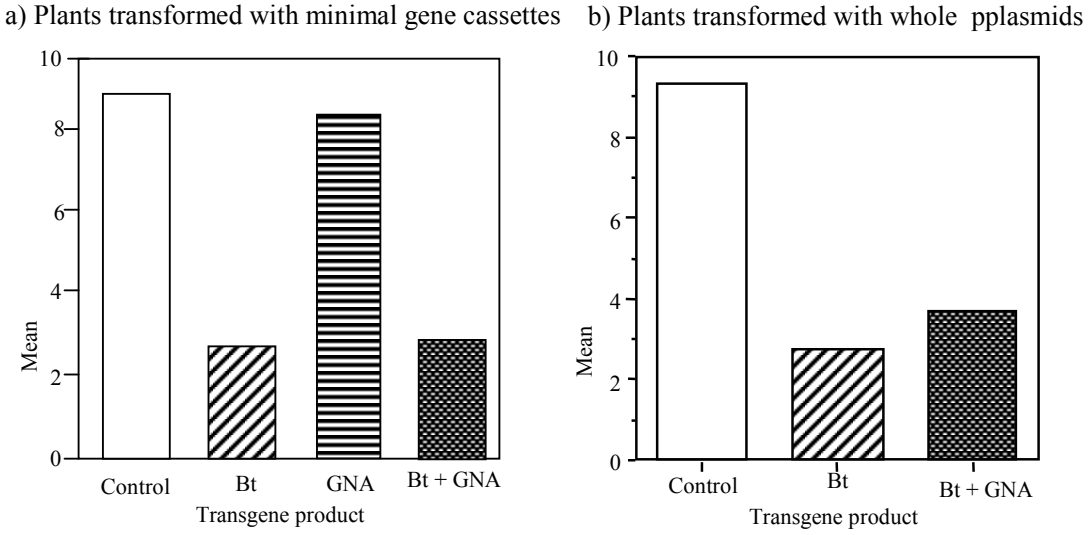

Figure 2. Survival of striped stem borer (*Chilo suppressalis)* larvae feeding on stem sections from transgenic rice plants and (parental) controls. Transgenic plant lines used were as given in fig. 1. Plants of the parental cultivars were used as controls. Graph legends show protein(s) expressed by each line tested. Graphs showing mean insect survival (+SE) after 5 days (initial inoculum of 10 neonate larvae). Each value represents the mean of eight replicate bioassays.

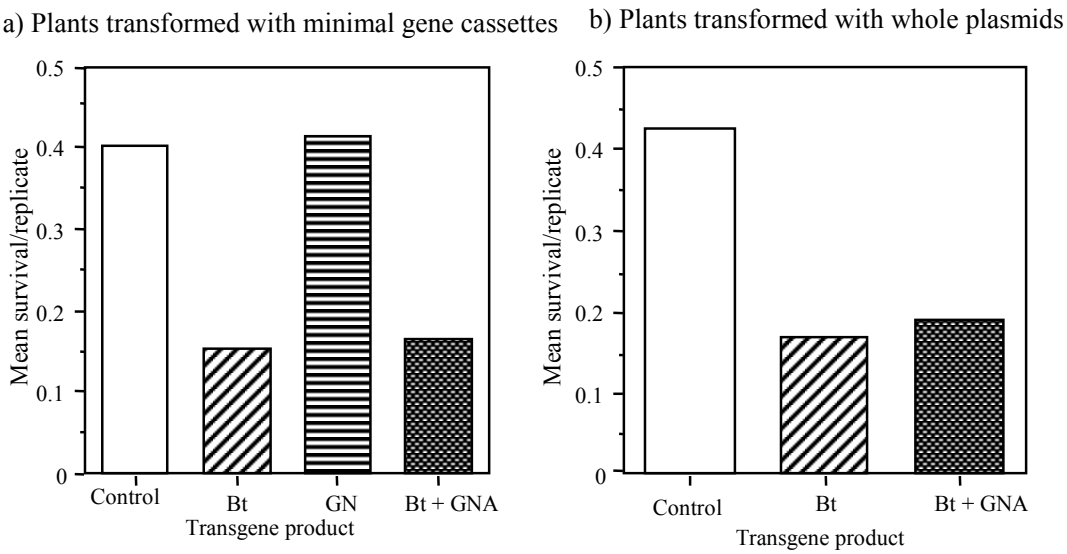

Figure 3. Growth of striped stem borer (*Chilo suppressalis)* larvae feeding on stem sections from transgenic rice plants and (parental) controls. Transgenic plant lines used were as given in fig. 4. Plants of the parental cultivars were used as controls. Graph legends show protein(s) expressed by each line tested. Graphs showing mean larval weight for surviving larvae (+SE; mg) after 5 days. Each value represents the mean of eight replicate bioassays.

In contrast to its effects on the homopteran pest brown planthopper, accumulation of GNA alone has no significant insecticidal effect on larvae of striped stem borer (fig. 2a, 3a). The insecticidal effects of GNA on lepidopteran larvae are generally limited, although previous bioassays in which the protein was fed in diet, or produced in transgenic plants, have shown significant effects on both survival and development. The present results suggest that striped stem borer is not susceptible to this monocot lectin, possibly due to exposure to similar proteins in rice. In agreement with the apparent lack of toxicity of GNA, plants containing both GNA and *cry1Ac* toxin performed no better in the bioassay than those containing *cry1Ac* toxin alone. However, differences between lines accumulating cry1Ac were observed; among lines expressing *gna* and *cry1Ac*, plants of line E-4-7 caused a reduction in larval survival of 83%, as compared to plants of line E-6-5, where the reduction in survival was 58% (significant difference at $p < 0.001$). Possibly this is due to higher levels of accumulation of the *cry1Ac* gene product. Trends for reduction in larval weight were similar to those seen for survival. As for resistance to brown planthopper, plants transformed with minimal gene cassettes exhibited slightly higher levels of resistance to the target pest (possibly due to higher expression levels for the transgene product) when compared to plants cotransformed with whole plasmid DNA, although once again the differences were not individually statistically significant at the $p < 0.05$ level.

The results obtained for stem borer mortality are comparable to previous reports in the literature. Fujimoto *et al.* (1993) reported mortality levels of up to 50% for striped stem borer on transgenic rice plants expressing a codon-optimised *cry1Ab* gene under the control of the CaMV 35S promoter. Wunn *et al*. (1996) reported mortality for this pest species up to 100% on plants expressing a truncated *cry1Ab* gene under the control of the CaMV 35S promoter. High mortality levels for stem borer larvae on transgenic rice were reported by Ghareyazie et al (1997) who

demonstrated that expression of this truncated *cry1Ab* gene, but driven by the maize C_4 PEP carboxylase promoter (which directs transgene expression to leaves), offered significant levels of protection. However, in that study the recovery of insects from control plants was much lower than in the present study (>90%), and thus direct comparisons in terms of insect survival cannot be made. Synthetic *cry1Ab* and *cry1Ac* genes gave 100% mortality of striped stem borer larvae in stem section assays when expressed in transgenic rice (Cheng et al., 1998; Shu et al., 2000).

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SUMMARY IN VIETNAMESE

Sử dụng "CASSETTE" gen tạo ra những cây lúa chuyển nạp gen biểu hiện độc tố GNA và BT, kháng rầy nâu và sâu đục thân hại lúa

Súng bắn gen đã được sử dụng để chuyển nạp gen GNA và *cry1Ac* vào lúa để điều khiển tính kháng côn trùng. Hai phương pháp chuyển nạp gen đã được đồng thực hiện thông qua 2 thí nghiệm: một là sử dụng những plasmid còn nguyên có mang các gen thich hợp; hai là sử dụng các "cassette" gen tương ứng đó là những đọan DNA hình thẳng không còn chứa chuỗi vector backbone nữa và những đoạn DNA này được cắt từ những plasmids tương ứng nói trên thông qua việc sử dụng những enzyme thích hợp. Những xét nghiệm sinh học đối với một số côn trùng chính hại lúa cho thấy rằng: những cây chuyển nạp gen biểu hiện GNA đã được tăng cường tính kháng đối với rầy nâu (*Nilaparvata lugens)*, những cây biểu hiện gen *cry1Ac* kháng sâu đục thân sọc nâu hại lúa (*Chilo suppressalis)* và những cây biểu hiện cả 2 gen thì đã tăng tính kháng đối với cả 2 loài sâu hại nói trên. So sánh kết quả đã đạt được trong nghiên cứu này cho thấy rằng những cây lúa chuyển nạp với "cassette" gen tỏ ra có mức kháng rầy nâu và sâu đục thân tương đối cao hơn so với những cây chuyển nạp với những plasmid còn nguyên vẹn.
