

Minimal Transgene cassettes generate transgenic rice plants which accumulate higher levels of insecticidal proteins.

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ABSTRACT

Particle bombardment was used to transform genes encoding the insecticidal proteins snowdrop lectin (*Galanthus nivalis agglutinin*; GNA) and cry1Ac Bt toxin (δ -endotoxin from *Bacillus thuringiensis*) into two 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. Both transformation methods generated similar numbers of independent transformation events. Selected R_0 clonal plant lines and R_1 plants from selected lines of minimal cassette transgenic rice lines were further characterised for presence and expression of transgenes. Co-transformation of the unselected genes (*cry1Ac* and *gna*) with the selectable marker (*hpt*) was at least as efficient for transformation with minimal gene cassettes as with whole plasmid DNA, and higher levels of accumulation of the insecticidal gene products GNA and *cry1Ac* were observed in plants resulting from minimal gene cassette transformation.

Keywords: Particle bombardment, "clean" DNA, *Oryza sativa*, co-transformation, integration.

INTRODUCTION

Two major techniques for rice transformation have been developed and are in routine use; direct DNA transfer (Christou *et al.*, 1991), and *Agrobacterium*-mediated transformation (Hiei *et al.*, 1994). Both methods generally lead to the integration of extraneous sequences (vector backbone) into the host genome along with the desired transgene(s) (Kononov *et al.*, 1997; Tingay *et al.*, 1997). However, integration of backbone sequences may exert undesirable effects in *cis* (Artelt *et al.*, 1991) as well as having a tendency to promote transgene rearrangements. Furthermore, involvement of vector backbone sequences in plasmid-plasmid recombination events has been reported, and recombination hotspots that stimulate illegitimate recombination through the formation of stable secondary structures have been identified (Muller *et al.*, 1999; Kohli *et al.*, 1999). However, despite the routine use of whole plasmids to introduce DNA in direct transfer procedures such as particle

bombardment, the vector backbone serves no purpose. Recently, Fu *et al.* (2000) cotransformed rice by particle bombardment using linear transgene constructs lacking vector backbone sequences (minimal cassettes comprising promoter, open reading frame, terminator) resulting in plants with simple integration patterns, low transgene copy number, and efficient expression.

The present paper extends these results to genes of agronomic interest and describes the production of transgenic rice plants expressing GNA and Bt toxins using linear transgene constructs lacking vector backbone sequences ("clean" DNA). This study also compares integration patterns and expression levels of the transgenes in transgenic plants introduced either as linear DNA fragments or in the whole plasmid form.

MATERIALS AND METHODS

Gene constructs

Minimal transgene expression cassettes isolated from four plasmids were used in the cotransformation experiments.

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- (i) The pUbi-gna plasmid contained the coding sequence of the *gna* gene fused to the constitutive maize ubiquitin-1 promoter (*Ubi-1*); construction of this vector is described elsewhere (Rao *et al.*, 1998).
- (ii) Plasmids pIA2 (Sardana *et al.*, 1996) and pIA2(mod) contained a synthetic *cry1Ac* gene under the control of maize ubiquitin-1 promoter and the nopaline synthase (*nos*) terminator. Plasmid pIA2(mod) differed from pIA2 by virtue of modifications at the C-terminal end of the Cry1Ac coding sequence, but produced a similar protein product, which was indistinguishable from that of pIA2 on Western blots. Transformations carried out using these two plasmids,
- or gene cassettes derived from them, are subsequently considered together, and are referred to as *cry1Ac*.
- (iii) Plasmid 35S-hpt-35S-GusA (Cooley *et al.*, 1995) contained the *gusA* reporter gene and *hpt* selectable marker conferring hygromycin resistance, was controlled by individual copies of the CaMV 35S promoter.
- The constructs are shown in diagrammatic form in fig. 1. Minimal transgene expression cassettes were isolated from the above plasmids by digestion with the restriction enzymes Kpn I and Sac I (*ubi-gna* construct), Hind III (*cry1Ac* construct), Not I and Swa I (modified *cry1Ac* construct), or BamH I (*hpt* gene).

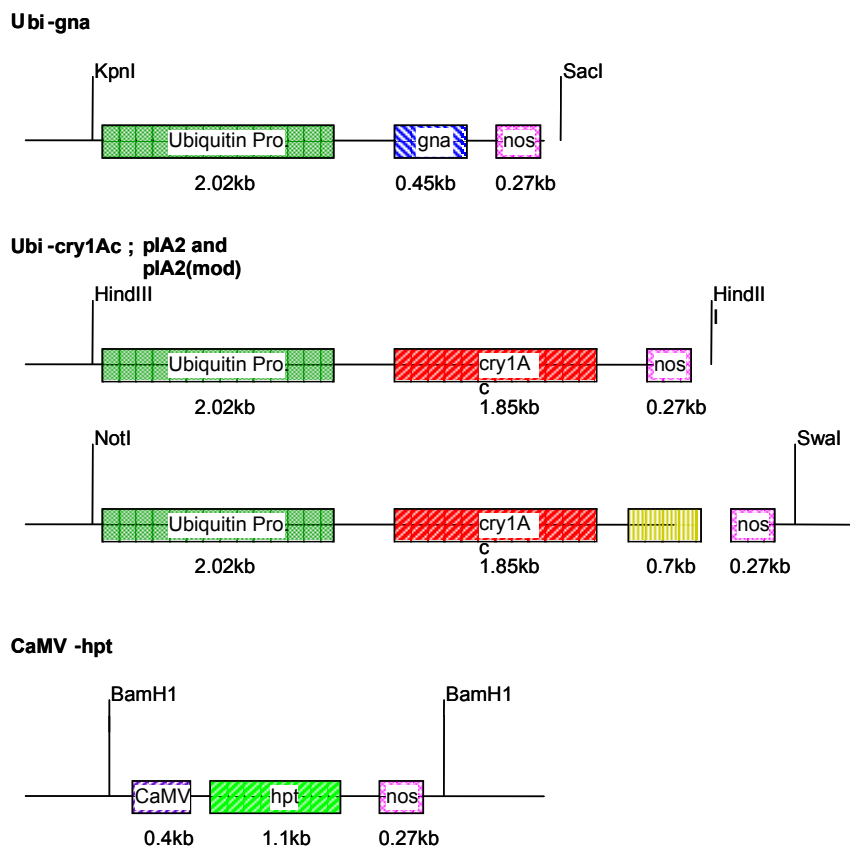


Figure 1: Schematic maps of the gene constructs used in transformation experiments. The enzymes indicated in the maps were used to isolate the minimal cassette for each construct.

Plant material, cotransformation and regeneration

Scutellum-derived embryogenic callus from mature seeds of two elite rice (*Oryza sativa* L.) cultivars, Eyi 105 and Bengal, was used as target tissue for transformation as previously described (Sudhakar *et al.*, 1998a). Two co-transformation experiments were carried out for minimal transgene expression cassettes, and for whole plasmid transformations. A molar ratio of 1:3:3 *hpt* (selectable marker): *gna* (nonselected): *cry1Ac* (nonselected) cassettes, or whole plasmids, was used. The preparation of DNA, co-transformation by particle bombardment, selection and regeneration of transgenic rice plants was carried out as described previously (Sudhakar *et al.*, 1998a). Shoots regenerated from a single callus that had been transformed and subjected to selection were considered to be clonal; this conclusion was verified in several cases by Southern blotting of genomic DNA of regenerated plants. The resulting lines of clonal plants were designated E- if derived from cultivar Eyi 105, and B- if derived from cultivar Bengal.

Polymerase chain reaction (PCR) analysis

Genomic DNA was extracted from young leaf tissue of hygromycin-resistant putatively transgenic rice plants according to the method described by Edwards *et al.* (1991). PCR analysis was carried out using primers specific for the selectable gene *hpt* and unselected genes *gna* and *cry1Ac*. Reaction conditions were as follows: for *hpt* and *gna*, DNA was denatured at 94°C for 3 min followed by 30 amplification cycles (94°C, 50 s; 62°C, 50 s; 72°C, 50 s) and a final extension step at 72°C for 7 min.; for *cry1Ac*, DNA was denatured at 94°C for 5min followed by 35 amplification cycles (94°C, 1 min; 60°C, 1 min; 72°C, 2 min) followed by 7 min final extension at 72°C

Nucleic acid isolation and Southern blot analysis

Total genomic DNA was isolated from 1g samples of fresh leaf material using the Nucleon Phytopure Plant DNA Extraction Kit (Amersham Life Science) according to the manufacturer's instructions. Procedures for enzyme digestion, electrophoresis and Southern blot analyses was carried out as described previously (Magbool, S.B and Christou, P,1999) with some modifications.

Western blot analysis

Samples of young leaf tissue were homogenised in extraction buffer (50mM Tris-HCl, pH9.5 + 1% PMSF), and extracted for 5 hours at 4°C, followed by centrifugation at 12,000g for 10 min at 4°C. Protein concentration in the supernatants was estimated using Bradford Reagent (Bio-Rad). Procedures for Western blot analyses was carried out as described previously (Foissac *et al.*, 2000) with some modifications.

RESULTS AND DISCUSSION

Recovery of transgenic plants and PCR analysis

After particle bombardment, transformed calli were subcultured and regenerated under hygromycin selection, using the *hpt* gene as selectable marker. The proportion of calli which were recovered after selection varied from 14 - 22%, and the efficiency of recovery was similar in the two transformation techniques.

PCR analyses were carried out on selected R₀ transgenic plants to confirm the presence of genes encoding *hpt*, *gna* and *cry1Ac*. We selected a representative population of seven independent transgenic plants, each generated from either "clean DNA" or "whole plasmid" transformations for more in depth analysis. In the minimal gene cassette transformation experiments, analysis of plants from seven independent transformation events showed that five events gave rise to plants containing all 3 genes (*gna*, *cry1Ac* and *hpt*), while two further events gave rise to plants that lacked one or other of the unselected genes (*cry1Ac* or *gna*). Parallel co-transformation experiments carried out using supercoiled whole plasmid DNA showed a similar proportion of events giving rise to plants containing all three genes (4 out of a total of 7 events analysed). As with the minimal gene cassettes, two further transformation events gave rise to plants lacking either one or the other of the unselected genes (*cry1Ac* or *gna*). One transformation event gave rise to plants containing the selected marker (*hpt*) only.

Transgene integration and cointegration analysis

The integration patterns of the genes introduced into transgenic rice plants using co-transformation with minimal gene cassettes or whole plasmids were studied by Southern blot hybridization. DNA from clonal

R₀ plants of each selected independent line was digested with either *SpeI* or *HindIII*. *SpeI* cuts once in the *gna* cassette, whereas *HindIII* cuts either once, or twice (liberating a 4.12 kb fragment containing the minimal expression cassette) in the *cry1Ac* gene constructs. Hybridization was carried out with probes corresponding to the coding sequences of the *cry1Ac* and *gna* genes.

Representative results of the Southern blot analysis of transgenic plants are presented in figure 2. Plants derived from "clean DNA" transformation experiments exhibited a general trend in terms of simpler integration patterns for the transgenes compared to those generated in experiments involving plasmids. However, this was not an absolute behaviour as some plants transformed with fragments has complex integration patterns and some plants transformed with plasmids did exhibit simpler patterns. This is consistent with previous results with marker genes reported by Fu et al. (2000).

In those cases where the restriction enzyme used to digest genomic DNA cut once in the gene construct, the hybridization patterns were variable, reflecting the different sites of transgene integration and different transgene copy numbers. When *HindIII* was used to digest DNA from plants containing the appropriate *cry1Ac* gene construct, the expected 4.12 kb diagnostic fragment (resulting from excision of an intact gene construct) was observed with the *cry1Ac* probe in 1 out of 2 lines transformed with the *cry1Ac* minimal cassette (lane 3, figure 4 with *cry1Ac* probe) and one line transformed with the whole plasmid DNA containing *cry1Ac* (lane 5).



Fig. 2. Small transgenic rice shoots in rooting medium

Although it is common practice for all vector sequences to be removed before microinjecting foreign DNA into animal eggs or embryos (Palmiter and Brinster, 1986), whole plasmid transformation is predominant in plant systems, and is obligatory in *Agrobacterium*-mediated transformation strategies employing cointegrative vectors, that is, those with the *vir* genes linked to the T-DNA. Recently, Fu et al. (2000) reported the successful use of linear transgene constructs lacking vector backbone sequences (minimal cassettes) to transform rice with marker genes by particle bombardment. In these experiments, transformation of rice with minimal cassettes encoding marker genes resulted in predominantly 'simple' integration events (one or two bands on Southern blots), producing transgenic plants with low transgene copy number and a low frequency of transgene rearrangements. The present study has confirmed the utility of the minimal gene cassette technique for rice transformation, showing that bombardment with both minimal cassettes and whole plasmid DNA gives rise to transgenic plant tissue with similar efficiency.

One of the major advantages of particle bombardment as a method for transformation is the ability to transform plants simultaneously with several different constructs. In the present study two different insect-resistance genes (*cry1Ac*, *gna*) plus a marker gene (*hpt*) were co-transformed into rice using either minimal cassettes or whole plasmid DNA. The results showed that cointegration was not affected by the use of minimal gene cassettes.



Fig. 3
BT-GNA
minimal
transgenic
rice plants
giving
seeds
normally

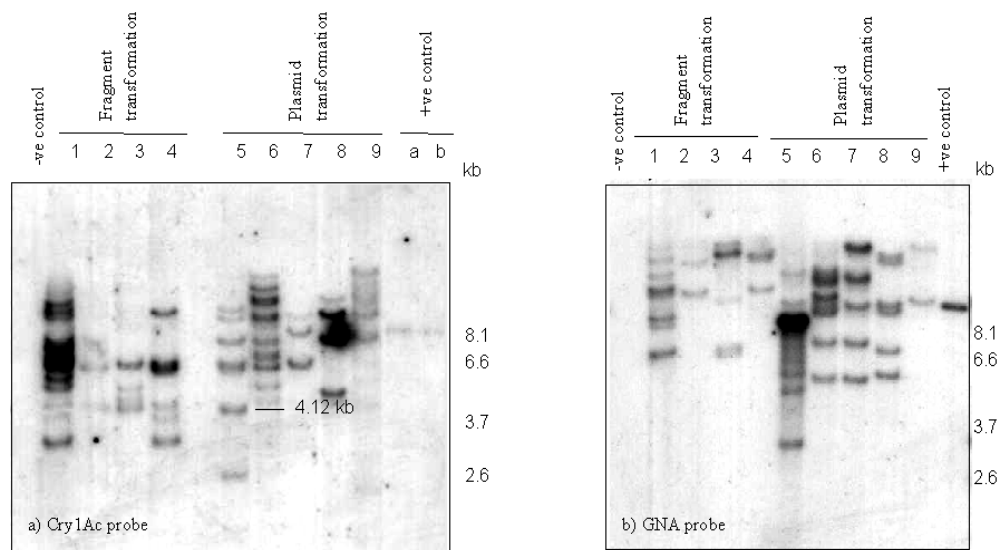


Figure 4. Southern blots of genomic DNA from transgenic plants cotransformed with *cry1Ac*, *gna* and *hpt* genes. a) Lane 1: E-4-7 carrying *gna* + *cry1Ac* genes; lane 2: E-4-3 carrying *cry1Ac* gene; lanes 3, 4: E-6-5, E-6-8 carrying *gna* + *cry1Ac* genes; lanes 5, 6, 7, 8: B-3-2, B-2-2, B-2-7, B-2-10 carrying *gna* + *cry1Ac* genes; lane 9: B-2-8: carrying *cry1Ac* gene. b) Lane 1: E-4-7 carrying *gna* + *cry1Ac* genes; lane 2: E-4-10 carrying *gna* gene; lanes 3, 4: E-6-5, E-6-8 carrying *gna* + *cry1Ac* genes; lanes 5, 6: B-3-2, B-2-2 carrying *gna* + *cry1Ac* genes; lane 7: B-2-5: carrying *gna* gene; lane 8, 9: B-2-7, B-2-10 carrying *gna* + *cry1Ac* genes.

Co-expression analysis

Western blot analysis of proteins extracted from leaves of plants which had been co-transformed either with minimal cassettes or whole plasmids was used to investigate the accumulation of transgene products. Typical blots are shown in fig. 5. GNA in transgenic plants was visualised by probing with anti-GNA antibodies as a polypeptide of approximately 12 kDa, of similar size to GNA produced in snowdrop, suggesting that post-translational processing of this protein occurs similarly in rice as in snowdrop, and that the nature of the transformation method does not affect its processing. The constructs containing *cry1Ac* gave rise to multiple polypeptides when extracts from plants were probed with anti-cry1Ab antibodies. A doublet band at approx. 65 kDa band was similar in size to the major polypeptide present in activated "native" cry1Ac toxin (fig. 5a).

Interestingly, whereas expression of the target transgenes, in terms of accumulation of the protein product, was observed in all the

lines produced by transformation with minimal gene cassettes, 1/5 lines produced by transformation with whole plasmids did not express *cry1Ac*, and 2/5 lines did not express *gna*. Lines B-2-1, containing *cry1Ac*, *gna* and *hpt* genes, and B-2-5, containing only *gna* and *hpt* genes, did not accumulate detectable levels of GNA protein, and line B-2-8, containing *cry1Ac* and *hpt* genes, did not accumulate *cry1Ac* protein (fig 5). Furthermore, all plants produced by transformation with minimal gene cassettes gave higher expression levels of the target transgenes as compared to plants transformed with whole plasmid DNA (fig. 5). The increase in expression level was estimated as up to approx. 2-fold in plants accumulating Bt toxins (fig. 5a), and up to approx. 4-fold in plants accumulating GNA (fig. 5b).

Co-transformation of rice with minimal cassettes resulted in a high frequency of plants expressing the target gene(s). Fu *et al.* (2000) found that approximately 20-30% of rice transformants produced by bombardment with whole plasmid DNA showed low levels of

transgene expression, or complete transgene silencing, which was absent from plants transformed with fragments in our study.

Transgene silencing was not only observed after transformation with minimal gene cassettes in these experiments, but plants co-transformed with minimal cassettes expressed higher levels of the transgene

product. This was particularly true for GNA, where levels of protein accumulated in leaf tissues was increased in the order of 2-4 -fold (Fig 5b). Transformation with minimal gene cassettes thus led to both a higher proportion of plants +ve for the transgene product, and higher expression levels of the product, than when whole plasmid DNA was used.

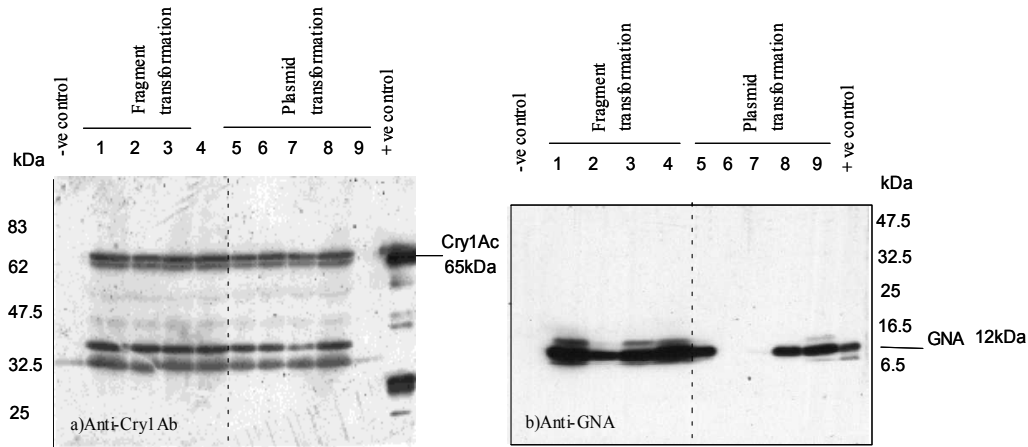


Figure 5: Western blot analysis for a) Cry1Ac and b) GNA in transgenic rice plants a) Lane 1: E-4-7 carrying *gna* + *cry1Ac* genes; lane 2: E-4-3 carrying *cry1Ac* gene; lanes 3, 4: E-6-5, E-6-8 carrying *gna* + *cry1Ac* genes; lanes 5, 6, 7, 8: B-3-2, B-2-2, B-2-7, B-2-10 carrying *gna* + *cry1Ac* genes; lane 9: B-2-8: carrying *cry1Ac* gene; +ve control: cry1Ac protein (400ng), b) Lane 1: E-4-7 carrying *gna* + *cry1Ac* genes; lane 2: E-4-10 carrying *gna* gene; lanes 3, 4: E-6-5, E-6-8 carrying *gna* + *cry1Ac* genes; lanes 5, 6: B-3-2, B-2-2 carrying *gna* + *cry1Ac* genes; lane 7: B-2-5: carrying *gna* gene; lane 8, 9: B-2-7, B-2-10 carrying *gna* + *cry1Ac* genes; +ve control: standard GNA (50 ng) . Equal amounts of protein (20 µg) were loaded in each lane.

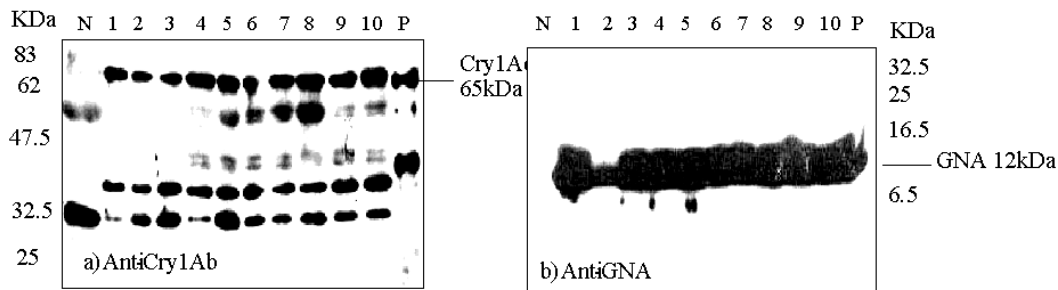


Figure 6: Western blot analysis for a) Cry1Ac and b) GNA in R1minimal cassette transgenic rice plants a) Lane 'N', Negative control; 'P', purified Cry1Ac protein (250 ng); b) Lane 'N', Negative control; 'P', standard GNA (80 ng) ; Lane: 1 - 10: extracts from plants cotransformed with Cry1Ac and GNA. Equal amounts of protein (30µg) were loaded in each lane.

Inheritance of transgenes

Further studies will be required to determine whether the high transgene expression observed for plants transformed with minimal gene cassettes in the present study will be stable? Some experiments have been conducted: R₁ plants from selected lines were analysed for the presence and expression of genes by PCR and western blot analyses. PCR results showed that most of the lines exhibited a ratio consistent with a 3:1 segregation, suggesting integration of the transgene(s) at a single locus. Both *cry1Ac* and *gna* coding sequences were found in 39 out of 52 progenies of a selected minimal cassette transgenic rice line which containing both *cry1Ac* and *gna* genes. The western blot analysis of the same plants detected expression of *cry1Ac* in 35 out of 39 plants determined as positive by PCR and it is very interesting that the GNA protein have been accumulated with very high level in all progeny which contain the *gna* gene. Fu *et al.*

(2000) observed stable and heritable transgene expression in most of the plants they generated using minimal cassettes. .

In conclusion, this is one of the first reports using minimal cassettes for the production of plants expressing insect resistance genes. Transgene expression levels in rice plants produced by this method were higher than those in plants transformed with the whole plasmid DNA.

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SUMMARY IN VIETNAMESE

Chuyển gen kháng sâu hại lúa bằng phương pháp sử dụng “cassette” gen

Súng bắn gen đã được sử dụng để chuyển nạp gen kháng côn trùng, GNA cry1Ac vào lúa. Hai phương pháp chuyển nạp gen đã được đồng thực hiện qua 2 thí nghiệm: (1) sử dụng những plasmid còn nguyên có mang các gen thích hợp; (2) sử dụng các cassette gen tương ứng đó là những đoạn ADN hình thẳng không còn chứa chuỗi vector backbone nữa và những đoạn ADN này được cắt từ những plasmids tương ứng nói trên thông qua việc sử dụng những enzym thích hợp. Những cây chuyển gen độc lập (Ro) và những cây R1 gieo từ hạt của vài cây chuyển gen độc lập chọn lọc đã được phân tích về hiệu quả chuyển gen, sự kết nạp của gen vào nhiễm sắc thể và sự biểu hiện gen. Kết quả cho thấy rằng hiệu quả chuyển gen của cả 2 phương pháp là như nhau, nhưng điều đáng chú ý là sử dụng những đoạn ADN hình thẳng không còn chứa chuỗi vector backbone nữa thì đã cho những cây chuyển nạp gen có mức protein kháng côn trùng (GNA và cry1Ac) cao hơn rất nhiều so với phương pháp cũ là dùng những plasmid còn nguyên vẹn.
