Transgenic rice homozygous lines expressing GNA showed enhanced resistance to rice brown planthopper

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Abstract **Mature seed-derived calli from two elite Chinese japonica rice (Oryza sativa L.) cultivars Eyi 105 and Ewan 5 were co-transformed with two plasmids, pWRG1515 and pRSSGNA1, containing the selectable marker hygromycin phosphotransferase gene (hpt), the reporter β-glucuronidase gene (gusA) and the snowdrop (Galanthus nivalis) lectin gene (gna) via particle bombardment. 61 independent transgenic rice plants were regenerated from 329 bombarded calli. 79% transgenic plants contained all the three genes, revealed by PCR/Southern blot analysis. Western blot analysis revealed that 36 out of 48 gna-containing transgenic plants expressed GNA (75%) at various levels with the highest expression being approximately 0.5% of total soluble protein. Genetic analysis confirmed Mendelian segregation of transgenes in progeny. From the R2 generations whose R1 parent plants showing 3:1 Mendelian segregation patterns, we identified five independent homozygous lines containing and expressing all the three transgenes. Insect bioassay and feeding tests showed that these homozygous lines had significant inhibition to rice brown planthopper (Nilaparvata lugens, BPH) by decreasing BPH survival and overall fecundity, retarding BPH development and declining BPH feeding. These BPH-resistant lines have been incorporated into rice insect resistance breeding program. This is the first report that homozygous transgenic rice lines expressing GNA, developed by genetic transformation and through genetic analysis-based selection, conferred enhanced resistance to BPH, one of the most damaging insect pests in rice.**

Keywords: ***Oryza sativa*** **L.,** ***gna*****, transgenic rice, bioassay, feeding test.**

Rice is one of the most important food crops in the world, providing the principal source of calories for nearly one third of the population. However, rice yields can be severely compromised by pests and diseases, and this problem is exacerbated because rice is grown predominantly in the developing countries where financial and technological resources are limited[1]. Therefore, there has been an immense effort to improve rice yields using conventional breeding and, more recently, genetic engineering[2]. The latter strategy provides an opportunity to introduce novel genes into the rice gene pool, particularly genes conferring pest or disease resistance that can be transmitted as Mendelian traits.

Genetic engineering has been used successfully to generate insect- and disease-resistant rice plants. Transgenic rice plants expressing *Bacillus thuringiensis* (*Bt*) crystal protein genes have been tested in bioassays, and show strong resistance to a number of important insect pests that feed by chewing[3]. Similarly, rice plants expressing the *Xa21* gene provide resistance against rice bacterial blight disease[4]. This indicates that genetic transformation is one of the potent approaches for rice improvement.

Brown planthopper (*Nilaparvata lugens*, BPH) is one of the most damaging insect pests causing significant yield losses of rice through the direct damage to rice plant by the sucking of phloem sap and acting as vectors for several economically important viruses such as stunt virus and tungro virus[5]. Recent studies show that some plant lectins are toxic to BPH in artificial diet assay[6, 7], among which the lectin (GNA) from snowdrop (*Galanthus nivalis*) is the most toxic. GNA's toxicity to BPH and non-toxic to mammals[8] makes it a potential candidate to be used in genetic engineering of rice for the control of BPH.

The *gna* gene was previously reported to be introduced into rice[9, 10] and the transgenic rice plants showed enhanced resistance to BPH in bioassay and feeding tests[11]. However, until now, there is no report on either the production of transgenic rice homozygous lines containing the *gna* gene or the BPH bioassay and feed tests of the *gna*-containing homozygous transgenic rice lines, which is crucial for the use of such lines in rice insect resistance breeding program. Here we report on the production of homozygous transgenic rice lines containing the *gna* gene via particle bombardment-mediated co-transformation combined with genetic analysis-based selection and the bioassay and feeding tests of the homozygous lines.

1 Materials and methods

(ⅰ) Plasmid constructs. Two plasmids, pWRG1515 and pRSSGNA1, kindly provided by Dr. Paul Christou (John Innes Centre, Norwich, UK), were used in co-transformation experiments. pWRG1515 contained the *gusA* gene and the hygromycin-resistance gene *(hpt*), both under the control of the CaMV 35S promoter[12]. pRSSGNA1 contained the *gna* gene driven by the Rss1 promoter[13], which was demonstrated to be phloem specific[9]. The *gna* gene from snowdrop (*Galanthus nivalis*) encodes for *Galanthus nivalis* agglutinin (GNA). GNA is known to exhibit significant insecticidal activity towards BPH when incorporated into artificial diets at concentrations as low as 4 μmol/L[6, 7].

(ii) Transformation, selection and plant regenera-

tion. Mature seed-derived calli (14—21 d-old) of two elite Chinese japonica rice cultivars Eyi 105 and Ewan 5 were bombarded with pWRG1515 and pRSSGNA1 at a molar ratio of 1∶3 using an electric discharge gun as described before[9]. The transformation, selection and regeneration of hygromycin (30 mg/L)-resistant callus and plants were essentially the same as described by Sudhakar et al.[9]. The regenerated plantlets approximately 1.0—2.0 cm high were transferred to rooting medium containing the same selection agents. Plants of 8—12 cm in height and with vigorously-growing roots were transferred to soil and grown to maturity.

(iii) PCR, Western blot, Southern blot and GUS histochemical analysis. Plant DNA used in PCR analysis was extracted from young leaves using a protocol described by Edwards et al.[14]. All the putative transgenic plants growing vigorously on hygromycin-containing rooting medium were analyzed by PCR for the presence of the *hpt*, *gusA* and *gna* genes. Three pairs of specific PCR primers, HPT-1 and HPT-2, GUS-1 and GUS-2, GNA-1 and GNA-2, were used to analyze the presence of the *hpt*, *gusA* and *gna* genes respectively in the transgenic rice plants and progenies. The amplified band sizes were 1270, 1540 and 477 bp for the *hpt*, *gusA* and *gna* genes respectively. The PCR primer sequences and PCR amplification were essentially the same as described before[10].

All the 48 *gna*-PCR positive plants were analyzed for GNA expression by Western blot using the protocol essentially as described before[9]. Protein aliquots (30 μg per lane), extracted from young leaves, were loaded and fractionated on a 15% SDS-polyacrylamide gel and blotted to a nitrocellulose membrane. The membrane was first probed with polyclonal rabbit anti-GNA antiserum, then treated with alkaline phosphatase-conjugated goat anti-rabbit and finally incubated in the Western Blue® stabilized substrate for alkaline phosphatase (Promega) until bands developed. Levels of GNA protein were measured by scanning densitometry using purified GNA protein (30 ng) as a reference.

21 independent transgenic rice plants expressing GNA were subjected to Southern blot analysis following the manufacture's instructions (Boehringer Mannheim). A 10-μg aliquot of plant DNA per sample, digested with *Sac* Ⅰ which cuts only once in pRSSGNA1, was used and digoxigenin (DIG)-dUTP labeled *gna* fragment, generated by PCR using the primer set (GNA-1 and GNA-2), was used as the probe in Southern hybridization.

All hygromycin-resistant plants were analyzed for GUS activity using the histochemical assay described by Jefferson et al.[15].

(iv) Genetic analysis and selection of transgenic rice pure lines. 21 independent R_0 primary transgenic plants expressing GNA were grown to maturity. R_1 seeds were harvested and R_1 progenies from 10 independent R_0 plants (plant Nos. W1, W2, W3, W4, Y7, Y8, Y9, Y10, Y15 and Y16; W: Ewan 5; Y: Eyi 105), which had GNA expression of over 0.1% of total soluble protein, were analyzed by PCR for the segregation patterns. The seeds from independent R_1 plants derived from primary transformant plants (Nos. W1, W2, Y7, Y8, Y16) showing 3:1 Mendelian segregation ratios and expressing GNA of over 0.3% were harvested separately. Up to 50 R_2 seeds harvested from each R_1 plant were germinated on hygromycin-containing rooting medium. If one of the seeds tested failed to germinate on hygromycin-containing rooting medium, the whole batch of the tested R_2 seeds derived from the same R_1 plant was abandoned. The screening for the germination of R_2 seeds derived from another R_1 plant on hygromycin-containing rooting medium was performed repeatedly until all the tested R_2 seeds harvested from one R_1 plant were found to germinate plants. The germinated seedlings were then analyzed by PCR, Western blot and GUS staining to identify homozygous lines.

(v) BPH bioassay and feeding tests. Seed-germinated plants (20—30 cm high, in three-leaf stage) of the 5 transgenic homozygous lines (Nos. W1, W2, Y7, Y8 and Y16) were challenged by BPH (biotype Ⅰ) and investigated for their effects on BPH survival, fecundity, development and feeding behavior using a protocol essentially the same as described by Rao et al.[11]. Each plant was confined to an insect-proof fine-mesh nylon cage. For BPH survival assay, 10 first-instar BPH nymphs were introduced into each plant and the insect survival was measured at 2-d intervals for 22 d. For BPH development assay, 25 first-instar BPH nymphs were released onto each plant and the survived BPH nymphs and adults were monitored after 13 d. For BPH fecundity test, the adult BPH was allowed to mate at random and total nymphs produced from the eggs laid were counted after 40 d. For BPH feeding test, honeydew secretion by BPH was measured. 25 first-instar BPH nymphs (pre-starved for 5 h) were released onto each plant in feeding chamber and honeydew was collected onto a filter paper placed at the bottom of the chamber over a 24-h period. The filter papers were removed and air-dried, and sprayed with 0.1% ninhydrin solution in acetone. The area of the honeydew spots, which was made visible by the development of honeydew deposits at 100℃ for 5 min, was measured.

2 Results

(i) Characterization of R_0 plants. After bombardment, callus was transferred to selection medium containing 30 mg/L hygromycin for 2 weeks. Continuous selection of the proliferating tissues on hygromycin-containing medium for additional 2 weeks resulted in the development of vigorously-growing hygromycin-resistant callus. When such callus was transferred to hygromycin-containing regeneration medium, plants were regenerated and roots developed when transferred to hygromy-

cin-containing rooting medium. All plants that survived and developed roots on hygromycin-containing rooting medium were shown to be transgenic by molecular analysis, without escape.

In total, 61 independent transgenic plants were regenerated from 329 bombarded calli, accounting for a transformation frequency of 18.5%. GUS assay revealed that 83% of the plants proliferated on hygromycin-containing medium had GUS expression at various levels. All the plants that were GUS-negative in the histochemical assays but proliferated on hygromycin-containing rooting medium were found to contain the *hpt* gene and the *gusA* gene by PCR (figures not shown). Based on the population we analyzed, the co-integration frequencies of genes (*hpt* and *gusA*) in the co-integrate vector were 100%.

All the transgenic plants were analyzed for the presence of the *gna* gene by PCR. 48 out of 61 independent transgenic plants contained the *gna* gene (figure not shown). Thus, co-integration frequency of the gene (the *gna* gene) in the non-selected plasmid and genes (*hpt* or *gusA*) in the selected plasmid was 79%. Western blot analysis revealed that 36 out of 48 *gna*-containing transgenic plants (75%) expressed GNA at various levels, with the highest GNA expression being approximately 0.5% of total soluble protein (fig. 1).

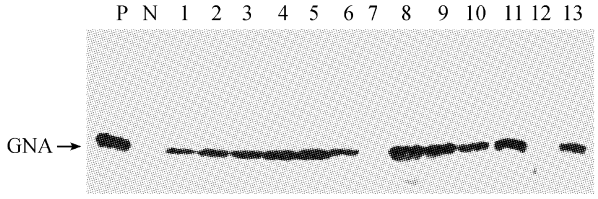

Fig. 1. Representative Western blot analysis for GNA expression in transgenic rice plants. Plant protein was extracted from young leaves and 30 μg was loaded in each lane. P, 30 ng purified GNA protein (positive control, 12 ku); N, an untransformed plant (negative control); lanes 1—5, transgenic Ewan 5 plants; lanes 6—13, transgenic Eyi 105 plants.

Southern blot analysis revealed the unique and complex hybridization patterns among the analyzed 21 plants expressing GNA (fig. 2). Thus, Southern blot analysis further confirmed that these GNA-expressing plants were indeed independent transformation events.

(ii) Analysis of transgenic R_1 progeny. R_1 progenies of 10 independently derived primary transformants (plant Nos. W1, W2, W3, W4, Y7, Y8, Y9, Y10, Y15 and Y16) with the GNA expression of over 0.1% of total soluble protein were analyzed by PCR for the segregation patterns of the introduced genes. Results showed that all the three transgenes in 8 lines (Nos. W1, W2, W4, Y7, Y8, Y9, Y10, Y16) were inherited together at a segregation ratio of 3∶1, indicating the integration of all the transgenes into a single genetic locus of rice genome (table 1). Transgenes in lines W3 and Y15 showed an aberrant segregation pattern.

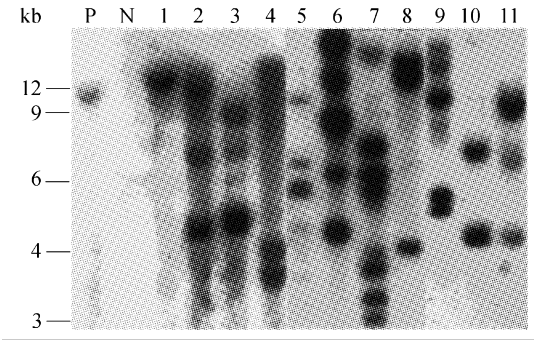

Fig. 2. Representative Southern blot analysis of transgenic rice plants. Plant genomic DNA (10 μg) and plasmid DNA were digested with *Sac* Ⅰ, separated on 0.8% agarose gel and hybridized with the DIG-labeled *gna* gene probe. P, Positive control (pRSSGNA1); N, an untransformed plant; lanes 1—11, transgenic plants corresponding to plant Nos. W1, W2, W3, W4, Y7, Y8, Y9, Y10, Y12, Y15 and Y16 respectively.

Table 1 Genetic analysis of R_1 progeny of transgenic rice lines[a)]

Transformants	*gusA*+	*hpt*+	*gna*+	No. of plants analyzed	Segregation ratio	χ^2	*P*
W1	26	26	26	35	3∶1 *hpt*	0.010	0.922
					3∶1 *gusA*	0.010	0.922
					3∶1 *gna*	0.010	0.922
W2	36	36	36	47	3∶1 *hpt*	0.064	0.801
					3∶1 *gusA*	0.064	0.801
					3∶1 *gna*	0.064	0.801
W3	11	11	11	41	– *hpt*	–	–
					– *gusA*	–	–
					– *gna*	–	–
W4	30	30	30	42	3∶1 *hpt*	0.286	0.593
					3∶1 *gusA*	0.286	0.593
					3∶1 *gna*	0.286	0.593
Y7	31	31	31	46	3∶1 *hpt*	1.420	0.233
					3∶1 *gusA*	1.420	0.233
					3∶1 *gna*	1.420	0.233
Y8	34	34	34	43	3∶1 *hpt*	0.380	0.538
					3∶1 *gusA*	0.380	0.538
					3∶1 *gna*	0.380	0.538
Y9	40	40	40	47	3∶1 *hpt*	2.560	0.110
					3∶1 *gusA*	2.560	0.110
					3∶1 *gna*	2.560	0.110
Y10	23	23	23	35	3∶1 *hpt*	1.610	0.205
					3∶1 *gusA*	1.610	0.205
					3∶1 *gna*	1.610	0.205
Y15	8	8	8	45	– *hpt*	–	–
					– *gusA*	–	–
					– *gna*	–	–
Y16	26	26	26	38	3∶1 *hpt*	0.877	0.349
					3∶1 *gusA*	0.877	0.349
					3∶1 *gna*	0.877	0.349

a) Plants were analyzed by PCR for the presence of *hpt*, *gusA* and *gna* genes. –, aberrant ratios.

(iii) Analysis of transgenic R_2 progeny and the selec-

tion of transgenic rice pure lines. Seeds of independent R_1 plants derived from the 5 primary transgenic plants (Nos. W1, W2, Y7, Y8 and Y16) showing 3∶1 Mendelian segregation ratios and expressing GNA of over 0.3% of total soluble protein were harvested separately. Up to 50 R_2 seeds harvested from each R_1 plant were germinated on hygromycin-containing rooting medium. By using the method described in this note, we obtained homozygous lines derived from the 5 independent primary transformants (Nos. W1, W2, Y7, Y8 and Y16). We were able to identify one homozygous line by randomly screening 5—10 R_2 lines derived from independent R_1 parent plants. PCR analysis confirmed their homozygous status. All the R_2 plants from the homozygous lines expressed all the three transgenes (*hpt, gusA* and *gna*), indicated by their vigorous growth on hygromycin-containing rooting medium, by GUS staining and by Western blot (figures not shown).

(iv) BPH bioassay and feeding tests. The 5 transgenic rice homozygous lines (Nos. W1, W2, Y7, Y8 and Y16) were tested for their effects on BPH survival, fecundity, development and feeding behavior. For BPH survival bioassay, 10 first-instar BPH nymphs were introduced into each plant and the insect survival was measured at 2-d intervals for 22 d. Results showed that BPH survival declined from 10 insects per plant (initial inoculum) to 6.2 and 6 insects per plant over a 22-d assay period on the control Eyi 105 and Ewan 5 plants respectively. However, BPH survival significantly declined, constantly through the assay period, from 10 insects per plant (initial inoculum) to an average of 1.7, 2.0, 1.7, 1.8 and 1.4 insects per plant on day 22 on the lines Nos. Y7, Y8, Y16, W1 and W2 respectively, compared to that on the control plants with the differences significant at $P<0.05$ after day 2 (fig. 3(a)). The BPH survival was decreased by 73%, 68%, 73%, 70% and 77% on lines Nos. Y7, Y8, Y16, W1 and W2 respectively, compared to the controls.

BPH development assay revealed that BPH survival on the transgenic lines Nos. Y7, Y8, Y16, W1 and W2 was significantly reduced from initial inoculum of 25 first-instar BPH nymphs/plant to 12.0, 9.7, 9.7, 11.9 and 10.8 insects/plant respectively, compared to 13.6 and 14.6 insects/plant on the control Eyi 105 and Ewan 5 plants respectively. The development of insects was also significantly retarded on all the transgenic lines, with only 2.6 (22% of survivors), 2.0 (21% of survivors), 1.4 (14% of

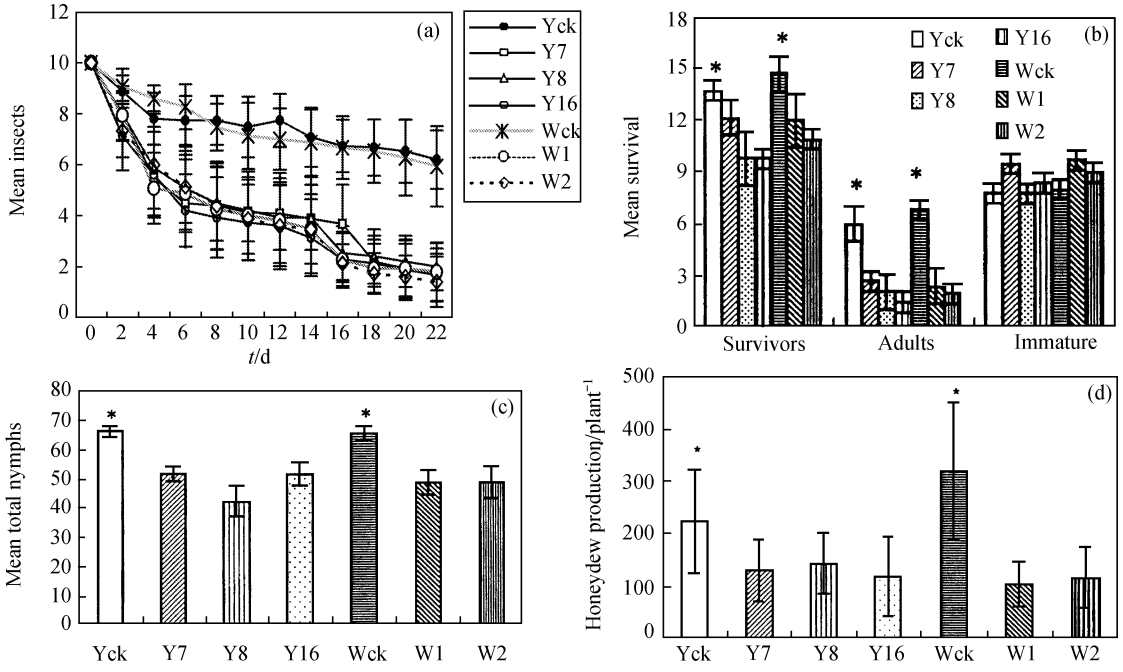

Fig. 3. BPH bioassay and feeding tests of transgenic rice homozygous lines (Nos. W1, W2, Y7, Y8 and Y16). Points and bars indicate means and SE (standard error). (a) BPH survival on the transgenic lines. 10 first-instar BPH nymphs were introduced into each plant on day 0 and the insect survival was measured at 2-d intervals for 22-d period. Differences between transgenic lines and the controls are significant at $P < 0.05$ after day 2. (b) BPH development assay. 25 first-instar BPH nymphs were released onto each plant on day 0 and the survived BPH nymphs and adults were monitored after 13 d; * significant at $P < 0.05$ for BPH survivors and adults compared to the transgenic lines. (c) BPH fecundity assay. The adult BPH was allowed to mate at random and total nymphs produced from the eggs laid were counted after 40 d; * significant at $P < 0.05$ compared to the transgenic lines. (d) BPH feeding test. Honeydew excretion by BPH nymphs feeding on transgenic lines and the controls was measured over a 24-h period; ∗ significant at $P<0.05$ compared to the transgenic lines.

survivors), 2.3 (19% of survivors) and 1.9 (18% of survivors) insects/plant reaching adulthood after 13 d on the transgenic lines Nos. Y7, Y8, Y16, W1 and W2 respectively, compared to 5.9 (43% of survivors) and 6.7 insects (46% of survivors) per plant on the control Eyi 105 and Ewan 5 plants respectively (P<0.05) (fig. 3(b)).

In agreement with the above results, fecundity test over a 40-d period also showed that the number of BPH nymphs produced on transgenic lines Nos. Y7, Y8, Y16, W1 and W2 was significantly reduced by approximately 22%, 37%, 22%, 26% and 26% respectively (P<0.05), compared to the controls (fig. 3(c)).

BPH feeding assay also revealed that transgenic lines had significant deterrent effects on BPH feeding. Honeydew production of first-instar BPH nymphs feeding on transgenic lines Nos. Y7, Y8, Y16, W1 and W2 over a 24-h period was significantly decreased by 42%, 36%, 47%, 68% and 64% respectively (P<0.05), compared to the controls (fig. 3(d)).

3 Discussion

Particle bombardment has been successfully used to generate transgenic lines of different crop plants[16]. It is still the method of choice for the production of transgenic plants, especially for monocot species due to its variety-independent, simple and efficient although substantial progress has been made recently on the development of *Agrobacterium*-mediated transformation systems for monocot species[17]. Its additional advantage is its ability to generate transgenic plants with multiple genes by co-transformation, which has been demonstrated clearly before[10, 18]. In one report, rice was co-transformed with 14 different plasmids and up to 13 of the 14 plasmids were simultaneously introduced into the rice genome[18]. In the present study, we again demonstrate that multiple genes can be simultaneously introduced and integrated into rice genome by co-transformation using bombardment. All of the transgenes were stably integrated into rice genome and most of them were inherited in Mendelian manner as one genetic locus (3:1 segregation ratios), which was consistent with previous reports[10, 19], although aberrant segregation was also observed in two cases.

In the present study, one hundred percent of co-integration frequency of linked genes (*gusA* and *hpt*) in transgenic rice plants was achieved, in consistence with the figures reported by Cooley et al.[19] and Kohli et al.[20]. 79% transgenic plants contained the gene (the *gna* gene) in the non-selected plasmid and genes (*hpt* or *gusA*) in the selected plasmid. Similar co-integration frequencies of genes in the non-selected and selected plasmids in transgenic plants were also reported by Tang et al.[10] and Cooley et al.[19]. This confirms that particle bombardment allows high frequency co-transformation using separate plasmids, making the use of co-integrate vector unnecessary, and is the method of choice for introducing multiple agronomically useful genes into plants.

In an earlier report using BPH bioassays based on the artificial diet feeding system, GNA showed significant deterrent effect on BPH survival when delivered at levels above 4 μmol/L (approximately 0.08%), and decreased BPH survival by 50% at concentrations as low as 6 μmol/L (approximately 0.12%) although the insect fecundity could not be measured using this system[7]. In another report, BPH bioassays and feeding studies showed that transgenic rice R_1 plants expressing GNA decreased BPH survival and overall fecundity, retarded insect development and had deterrent effect on BPH feeding[11]. In the present study, through genetic analysis-based selection, we successfully identified 5 homozygous lines from R_2 progenies derived from R_1 parent lines showing 3:1 Mendelian segregation ratios. These lines contained and expressed all the three transgenes (*gusA*, *hpt* and *gna*), with the GNA expression of over 0.3%. BPH bioassay and feeding test results showed that these transgenic pure lines significantly decreased BPH survival, declined BPH fecundity, retarded BPH development and had deterrent effects on BPH feeding, compared with the results presented by Rao et al.[11]. These homozygous lines have been incorporated into rice insect resistance breeding program.

In this study, we have demonstrated that the homozygous transgenic rice lines containing multiple genes including an agromonically important gene can be obtained via particle bombardment-mediated co-transformation and through genetic analysis-based selection. This further demonstrates that transformation technology is an important supplement to conventional breeding for improving multiple traits in crop plants.

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