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# Insect resistance to *Nilaparvata lugens* and *Cnaphalocrocis medinalis* in transgenic indica rice and the inheritance of *gna+sbti* transgenes

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**Abstract: Molecular genetic analysis and insect bioassay of transgenic indica rice 'Zhuxian B' plants carrying snowdrop lectin gene (*gna*) and soybean trypsin inhibitor gene (*sbti*) were investigated in detail. PCR, 'dot' blot and PCR-Southern blot analysis showed that both transgenes had been incorporated into the rice genome and transmitted up to R3 progeny in most lines tested. Some transgenic lines exhibited Mendelian segregation, but the other showed either 1:1 (positive: negative for the transgenes) or other aberrant segregation patterns. The segregation patterns of *gna* gene crossed between R2 and R3 progeny. In half of transgenic R3 lines, *gna* and *sbti* transgenes co-segregated. Two independent homozygous lines expressing double transgenes were identified in R3 progeny. Southern blot analysis demonstrated that the copy numbers of integrated *gna* and *sbti* transgenes varied from one to ten in different lines. Insect bioassay data showed that most transgenic plants had better resistance to both *Nilaparvata lugens* (Ståhl) and *Cnaphalocrocis medinalis* (Guenée) than wild-type plants. The insect resistance of transgenic lines increased with the increase in transgene positive ratio in most of the transgenic lines. In all, we obtained nine lines of R3 transgenic plants, including one pure line, which had better resistance to both *N lugens* and *C medinalis* than wild-type plants.**


**Keywords:** transgenic rice; insect resistance; *gna* gene; *sbti* gene; inheritance; *Nilapavata lugens*; *Cnaphalocrocis medinalis*

## 1 INTRODUCTION

Among the insect pests of cultivated rice (*Oryza sativa* L), the brown planthopper (*Nilaparvata lugens* Ståhl) and the rice leaf-folder (*Cnaphalocrocis medinalis* Guenée) are two of the most damaging pests in terms of crop losses in the last 20–30 years.[1] In order to develop a new strategy for pest control as an alternative to the extensive use of chemical pesticides, great efforts have been made in rice genetic engineering.[2] Although many papers have been published on successful gene transfer,[3,4] studies on the fate of transgenes in the progeny of primary transformants have not been extensively investigated. The mechanism of transgene integration into host plants by direct DNA transfer procedures is also not well understood. Ideally, all transgenic rice plants are expected to carry single and complete copies of the primary transgene. However, the reality is that transgenes always insert into host genomic DNA randomly, often jumbled and rearranged together with large fragments of vector backbone, which can disrupt the expression of endogenous plant genes. Another most important matter is whether transgenic plants can effectively resist target insects long-term; there are cases where transgenic plants have successfully killed target pests for only one or two generations, and some insect populations have evolved resistance to transgenic plants. Take the most widely used, *Bacillus thuringiensis* (Bt) transgenic plants, as an example. So far, only one insect species has evolved significant levels of resistance in the field, but laboratory selection experiments have shown the high potential

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of other species to evolve resistance against Bt.[5] Hence, the successful commercialization of genetically engineered rice depends critically not only on the stable inheritance of transgenes, but also the on stable resistance of transgenic plants to target pests over several generations.

However, to our knowledge, there are only a few researches that have investigated both the molecular genetic inheritance on interest genes in transgenic plants and the resistance capability of transgenic lines for several generations. Thus, in order to develop new transgenic varieties successfully, the insect resistance and the transmission mechanism of transgenes from parents to progenies need to be well studied. In the present study, we examined the molecular and genetic properties of transgenic indica rice 'Zhuxian B' plants transformed by a selectable marker gene, *hpt*, and two useful genes, *gna* and *sbti*, on separate plasmids, via particle bombardment. Insect bioassay of transgenic plants was also investigated in detail.

## 2 MATERIALS AND METHODS

### 2.1 Plant materials

*GNA* + *SBTi*-expressing rice plants were selfed R1, R2 and R3 progeny of transgenic indica rice (*Oryza sativa* L cv Zhuxian B) plants genetically transformed with three plasmids (pIP860, pIP801 and p35H) containing *gna*, *sbti* and *hpt* genes at a molar ratio of 2:2:1 by the biolistic-mediated method.[6] pIP860 and pIP801 contained *gna* and *sbti* genes, respectively, and were provided by Professor Ray Wu (Department of Molecular Biology and Genetics, Cornell University, NY, USA).[6] p35H contained the selectable marker gene hygromycin phosphotransferase (*hpt*) and was provided by Dr Fauquet (The Scripps Research Institute, CA, USA).

### 2.2 DNA isolation and PCR analysis of transgenic plants

Genomic DNA was isolated from the fresh leaf tissue of transgenic R2 and R3 plants by using the cetyltrimethylammonium bromide DNA extraction method.[7] To obtain the coding region of the *gna* gene, forward primer (5′-GCT AAG GCA AGC TCC TCA TTT-3′) and reverse primer (5′-TCA CAA GCT TTA TCT TTC CAG C-3′) were designed to amplify the entire 460-bp coding region. The PCR amplification was carried out in 20 μl of reaction mixture using a PCR kit (TakaRa). The samples were denatured initially at 94 °C for 3 min, followed by 35 cycles of 1 min denaturation at 94 °C, 1 min of primer annealing at 58 °C and 1.5 min of synthesis at 72 °C, with a final extension step of 72 °C for 5 min.

### 2.3 'Dot', Southern blot, PCR-Southern hybridization

Aliquots (10 μg) of genomic DNA with (for Southern blot) or without (for 'dot' blot) restriction endonuclease digestion were loaded onto and fractionated in 0.8% agarose gels. The 'dot' blot technique differed slightly from the conventional dot blot in that the genomic DNA purification procedure with agarose gels was used; otherwise the procedure was as in the conventional method. For PCR-Southern blot, PCR reaction was carried out as described in Section 2.2, and PCR products were subsequently separated in 2% agarose gels. All three kinds of gel were then denatured, neutralized and blotted onto Hybond-$N^+$ nylon membranes following the method described by Sambrook *et al*.[8] DNA was fixed to the membrane by baking in a vacuum oven at 80 °C for 2 h. Gene-specific probes were generated using the following digests: a *Hind*III-*Sac*I digest of pIP860 to isolate the 450-bp *gna* fragment, and a *Hind*III-*BamH*I digest of pIP801 to release the 500-bp *sbti* fragment. Probe labeling, pre-hybridization and hybridization were carried out according to the hybridization kit instructions supplied by TakaRa Biotechnology Co Ltd, China. After hybridization, the membranes were sealed with a plastic sheet and exposed to X-ray film.

### 2.4 Insect bioassay

The insecticidal activities of the transgenic rice plants against *N lugens* and *C medinalis* were assayed using methods described in the literature.[9] For the *N lugens* bioassay, five to six second- to third-instar nymphs were introduced onto each four- to five-leaf stage plant. Percentage seedling mortality was assessed visually and compared with susceptible TN1 rice plants (≥95% death), and the data were converted into resistance grade according to the revised IRRI damage-grading standard. For the *C medinalis* bioassay, 40–50 pairs of recently emerged moths were introduced into each container at the rice tillering stage. At the same time, honeydew was provided for extra nutrition of adults, and the damage severity was monitored after two weeks. Damage-grading was determined and converted into resistance grade according to the revised IRRI damage-grading standard.

## 3 RESULTS AND DISCUSSION

### 3.1 Inheritance and integration of the *gna* and *sbti* genes in transgenic rice

The inheritance of foreign genes has been studied in different transgenic rice varieties. Typically, a single foreign gene inserted in the host genome often leads to the expected 3:1 segregation ratio in the selfed population.[10,11] Three plasmids were cotransformed into indica rice using particle bombardment by Maqbool *et al*,[12] and a 3:1 segregation ratio for all four transgenes was observed in R1 plants with a few exceptions. Chen *et al*[13] also co-transformed 14 different genes into japonica rice, and all transgenes showed a stable integration pattern through three generations. However, in several instances, the foreign gene displayed complicated segregation profiles instead of classical Mendelian segregation.[14–16]

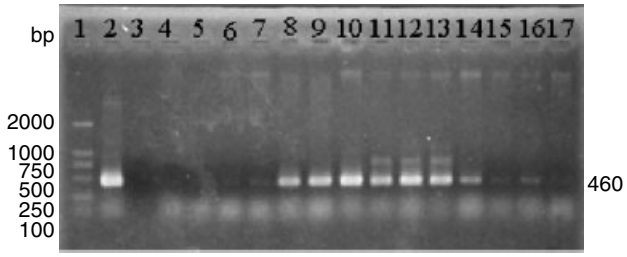


**Figure 1.** PCR analysis of R2 progeny genomic DNA to monitor the presence of the *gna* coding region. Amplification products visualized by ethidium bromide staining on 2% agarose gel. Line 1: DNA Marker DL 2 000; line 2: plasmid pIP860; line 3: control genomic DNA from non-transformed 'Zhuxian B'; lanes: 4–17 genomic DNA of transgenic plants.

In the present experiment, the selfed R1 progeny was selected using 30 mg litre$^{-1}$ hygromycin. The inheritance of both *gna* and *sbti* genes was then studied in selfed R2 and R3 progenies using PCR, 'dot' blot and PCR-Southern blot analysis. One typical PCR analysis profile of *gna* transgene in R2 progeny is presented in Fig 1. In the figure, 11 out of 14 transgenic plants had the expected PCR products with identical sizes of 460 bp and difference band intensity, whereas three of them did not yield PCR products. Integrating the results of the three analysis methods, we found that, in the selfed R2 population, 45.5% of total 363 transgenic rice plants contained *gna* gene. Valued by $\chi^2$ test, it was found that the segregation patterns of *gna* gene were variable, and each line showed a different segregation profile.

In a total of 60 transgenic lines, 20.0% of $GNA^+/GNA^+$ and 21.7% of $GNA^-/GNA^-$ transgenic lines were obtained. Approximately 21.7% of the transgenic lines demonstrated a one-locus Mendelian 3:1 inheritance in the selfed population. Only 1.7% of the lines displayed a 15:1 two-locus Mendelian segregation pattern; 11.7% of the lines showed 1:1 segregation and 23.3% of the lines displayed other aberrant segregation ratios (positive number was less than negative number). It was noted that most of transgenic lines obeyed the Mendelian law, but some transgenic lines displayed aberrant segregation. The aberrant segregation may be due to transgene rearrangement or loss, recessive lethal or homozygous deletion, male gamete lethal or gene escape, so that the positive plants were smaller than expected. In order to further screen out the homozygous lines and find the transgene inheritance fashion in R3 progeny, part of the offspring of positive R2 plants, a total of 27 transgenic R3 lines, were subjected to 'dot' blot, PCR and PCR-Southern blot analysis. The typical profile of *gna* 'dot' blot is displayed in Fig 2. The *gna* probe hybridized to genomic DNA in some transgenic plants, indicating that the transgenes were integrated into the plant genome, and gene segregation also occurred in R3 progeny. Combining the data of PCR and 'dot' blot analysis, we found that the integration frequency of *gna* gene in a total of 340 transgenic plants was 59.4%. Valued by $\chi^2$ test, 37.0% lines demonstrated Mendelian 3:1 inheritance, 11.3% displayed 15:1 segregation, 3.7% showed 1:1, and 25.9% displayed other aberrant segregation ratios. Also, 18.5% of $GNA^+/GNA^+$ and 3.7% of $GNA^-/GNA^-$ transgenic lines were obtained. To further validate the data, PCR-Southern blot analysis was carried out (Fig 3). Comparing Fig 2 with Fig 3 indicates that consistent results were obtained from the two analysis methods, ie in Fig 3, the same positive results were obtained as the selected positive plants in Fig 2.

From the above two generations molecular analysis, we found that the integration frequency of *gna* transgene in R3 was higher than that in R2 progeny. Moreover, the percentages of 3:1 and 15:1 segregation were also much higher in R3 than in R2. This indicated that the *gna* gene was successfully integrated into in R0 plants, and then steadily transmitted up to R3 progeny. In this study, it is also very interesting to find that the segregation patterns of *gna* gene crossed between R2 and R3 progeny, as summarized in Table 1. In R3, there existed distorted segregation as well as the expected 3:1 segregation in the offspring of R2 with 3:1 pattern. In contrast, in R3, there also existed 3:1 ratio from the progeny of distorted segregation in R2 lines. Scott *et al*[16] considered that the segregation distortion was due to deleterious or lethal mutations

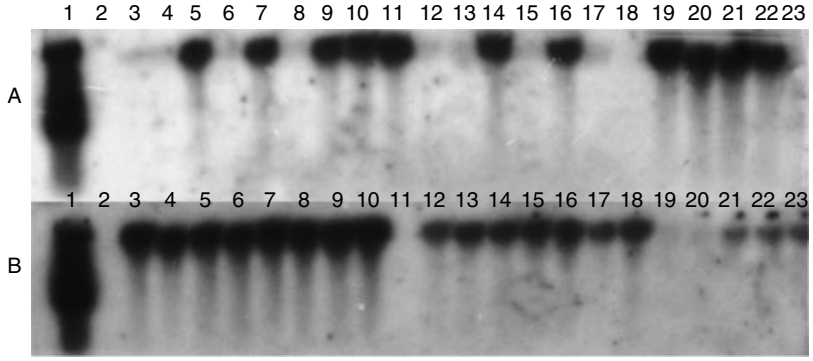


**Figure 2.** 'Dot' blot analysis of R3 progeny genomic DNA to monitor the presence of the *gna* coding region. 'Dot' blot was a little different from conventional dot blot: the genomic DNA purification procedure with agarose gels was used in this method, the rest of the procedure being the same as in the conventional method. Lines A1, B1: plasmid pIP860; lines A2–A17, B2–B11: control genomic DNA from non-transformed 'Zhuxian B'; the remaining lanes: genomic DNA of transgenic plants.

**Table 1.** Comparison of *gna* gene inherence patterns of transgenic R2 with R3 progeny[a]

| Line | Positive:negative (R2 Progeny) | Positive:negative (R3 Progeny) | Line | Positive:negative (R2 Progeny) | Positive:negative (R3 Progeny) |
|---|---|---|---|---|---|
| HZ1-1 | 3:1 | Pure line | HZ7-3 | 1:1 | 15:1 |
| HZ2-2 | 3:1 | < | HZ8-1 | Pure line | Pure line |
| HZ2-3 | 15:1 | Pure line | HZ8-2 | < | < |
| HZ4-1 | 3:1 | Pure line | HZ9-1 | < | 3:1 |
| HZ5-1 | < | 3:1 | HZ10-2 | 3:1 | 3:1 |
| HZ6-1 | 3:1 | 1:1 | HZ11-1 | 1:1 | 3:1 |
| HZ6-2 | Pure line | Pure line | HZ11-3 | 3:1 | < |
| HZ7-1 | 3:1 | < | HZ12-2 | < | < |

[a] 'Positive' means *gna* positive transgenic plant number; 'Negative' means *gna* negative transgenic plant number; Pure line means $GNA^+$ : $GNA^+$; < means that *gna* positive plant number was less than *gna* negative plant number.

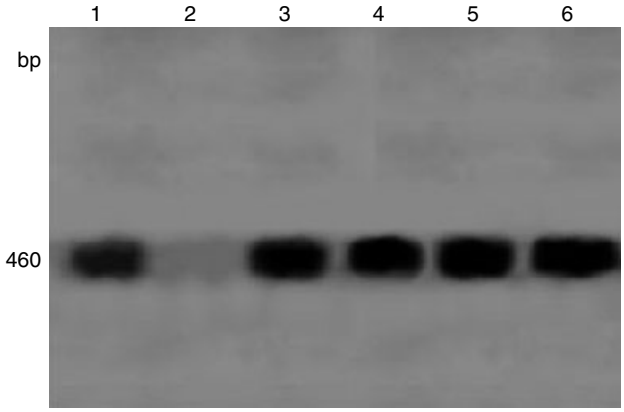


**Figure 3.** PCR-Southern blot analysis of *gna* gene in transgenic R3 progeny plants. PCR reaction was carried out as conventional PCR section, and PCR products were subsequently separated in 2% agarose gels. Then the gel was denatured, neutralised and blotted onto Hybond-$N^+$ nylon membranes. Lane 1: plasmid pIP860; lane 2: control genomic DNA from nontransformed 'Zhuxian B'; lanes 3–6: genomic DNA of transgenic plants.

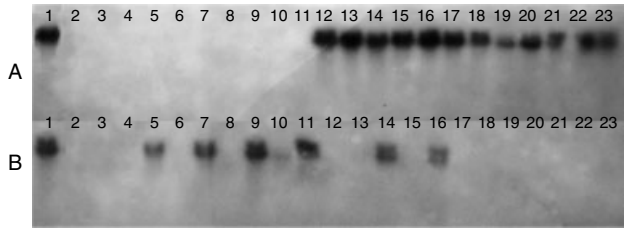


**Figure 4.** 'Dot' blot analysis (see Fig 2) of R3 progeny genomic DNA to monitor the presence of the *sbti* coding region. Lines A1, B1: plasmid pIP860; lines A2–A11, B2: control genomic DNA from non-transformed 'Zhuxian B'; the remaining lanes: genomic DNA of transgenic plants.

occurring during the insertion event. However, other reports concluded that the aberrant ratio resulted from suppression of female or male gamete, or from some environmental factor.[17] While suppression of female or male gamete is not equal to gene deletion or lethal mutations, it may be reactivated in subsequent selfed progeny. We could conclude that, in the present work, *gna* gene crossing resulted from suppression of female or male gamete other than gene deletion or lethal mutations in transgenic plants. Thus, in lines HZ5-1, HZ9-1, HZ11-1, etc, *gna* gene might be suppressed in R2 progeny, and reactivated in R3 progeny, while in lines HZ2-2, HZ7-1, HZ11-3, etc, the *gna* gene was active in R2, and female or male gamete suppression occurred in R3. However, in lines HZ8-2 and HZ12-2, the female or male gamete was suppressed in transgenic *gna* gene plants for two generations, and in lines HZ6-2, HZ10-2 and HZ8-1, the *gna* gene was active all the time in R2 and R3 progenies. A similar phenomenon was also observed in transgenic rice over several generations by Peng *et al*.[18]

Inherence analysis of *sbti* gene also revealed that the integration frequency was 43.8% of a total of 333 transgenic plants in R3 progeny; 23.0% of total 26 transgenic lines demonstrated Mendelian 3:1 inheritance, 3.8% showed 15:1 segregation, 15.4% showed 1:1, and 26.9% displayed other aberrant segregation ratios. Moreover, 11.5% of $SBTi^+/SBTi^+$ and 19.2% of $SBTi^-/SBTi^-$ transgenic lines were obtained. One typical *sbti* gene 'dot' blot analysis is illustrated in Fig 4.

Some researchers have reported that particle bombardment generated a higher frequency of transgenic plants with multiple transgenes integrated at a single locus. If the transgenes were inserted in a single locus, then all transgenes would co-segregate.[13,19] In this experiment, *gna* and *sbti* transgenes had almost the same segregation pattern in 13 out of 26 transgenic lines in R3 progeny. These 13 lines include five 3:1 lines, two pure positive lines, one pure negative line, two 15:1 lines and three aberrant segregation lines. That is to say, in only some transgenic lines were *gna* and *sbti* transgenes integrated into the same locus, but in other transgenic lines, the two transgenes might be present in different locations, resulting in a recombination among transgenes. Further research is required to understand the reasons for this.

Ideally, all transgenic plants would carry a neat, single copy of the primary transgene within a well-characterized expression structure integrated at a precisely defined locus. However, in most cases, there exist multiple copies of transgenes, which are often jumbled and rearranged together with large fragments of vector backbone.[20,21] In this study, Southern blot analyses were used to confirm further the inheritance

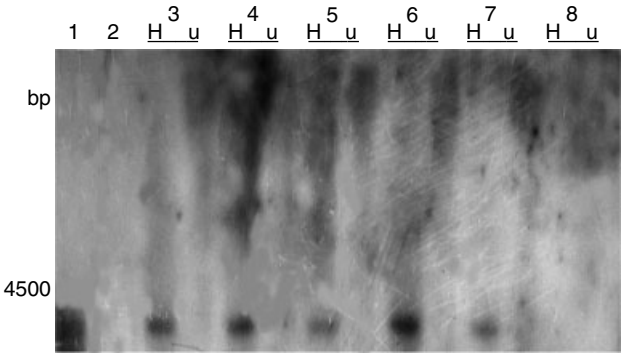


**Figure 5.** Southern blot analysis of *gna* gene in transgenic R2 progeny plants. Plant genomic DNA was digested with *Hind*III and hybridized with the *gna* probe. A single 4.5-kb band was observed in lanes 1–7 when *Hind*III was used to digest genomic DNA and two of them do not display any band. Lane 1: plasmid pIP860; lane 2: control genomic DNA from non-transformed 'Zhuxian B'; lanes 3–8: genomic DNA from six transgenic plants of HZ4-1 line; H: *Hind*III -treated samples; U: non-digested samples.

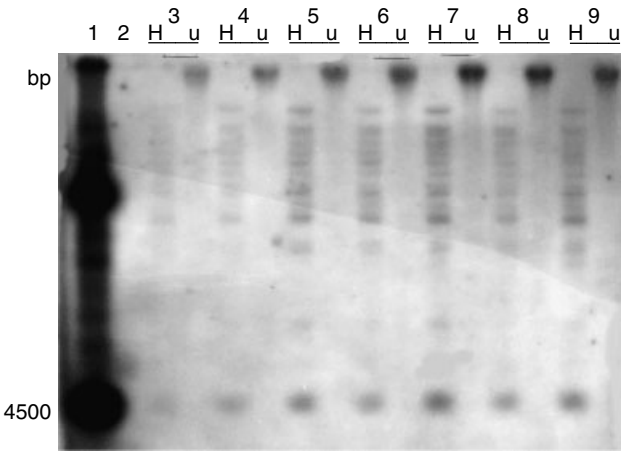


**Figure 6.** Southern blot analysis of *gna* gene in transgenic R3 progeny plants. Plant genomic DNA was digested with *Hind*III and hybridized with the *gna* probe. All transgenic plants carried approximately the same number of copies of the *gna* transgene, and the copy number was about ten. Lane 1: plasmid pIP860; lane 2: control genomic DNA from non-transformed 'Zhuxian B'; lanes 3–9: genomic DNA from seven transgenic plants of HZ6-1 line; H: *Hind*III-treated samples; U: non-digested samples.

fashion of transgenes and evaluate the number of transgene copies integrated into the rice genome. It was found that the *gna* and *sbti* probes from pIP860 and pIP801 hybridized to high-molecular-weight DNA in undigested DNA samples from the transgenic plant lines, indicating that the transgenes were integrated into the plant genome, and the data also supported the conclusion that a large part of transgenic lines obeyed a Mendelian law. In addition, it revealed a set of unique and complex hybridization bands for each of the two transgenes when certain restriction endonuclease was used to digest transgenic rice genomic DNA. The copy number of transgene was estimated based on the number of bands in the figure of Southern blots, and the number of integrated copies fell within the range from one to ten in different lines. As seen from Fig 5, in the line HZ4-1 single 4.5-kb bands were observed in lanes 1–7 when *Hind*III was used to digest genomic DNA. Two of them did not display any band with the size expected from the corresponding plasmid DNA fragments. From Fig 6 we found that, after hybridization with *gna* probe, all R3 progeny transgenic plants of HZ6-1 line contained additional truncated copies besides the expected *gna* band, and the *gna* transgene copy number was approximately ten.

### 3.2 Insect bioassays

The lectin from snowdrop (GNA) is toxic to a number of important insect pests including Homoptera, Coleoptera and Lepidoptera due to its antifeedant properties when incorporated into artificial diet. Transgenic rice expressing GNA did indeed show enhanced resistance to *N lugens* in bioassay and feeding tests.[22] Recent studies have demonstrated that transgenic rice expressing GNA also conferred enhanced resistance to the green leafhopper (*Nephotettix virescens* Dist) besides *N lugens*.[23] Soybean trypsin inhibitor (SBTI) was highly effective against the proteolytic activity of insect gut extract and was inhibitory to insect growth when present in artificial diet. There is also a report that transgenic rice plants expressing SBTI are effectively resistant to *C medinalis*.[24]

In this study, parts of transgenic rice R2 and R3 plants which were transformed with *gna* and *sbti* transgenes and studied extensively by molecular analysis were also infested with *N lugens* and *C medinalis*. The transgenic plants used for the insect bioassay were based on both molecular genetic analysis and insect bioassay of their parents. That is to say, when their parents' molecular genetic analyses were positive and insect bioassays showed a certain grade of insect-resistant capability, then they were used to study insect resistance in the subsequent progeny.

In bioassays using *N lugens*, as expected, more transgenic plants survived than wild-type plants, and most transgenic lines expressing *gna* gene demonstrated an increase in resistance to *N lugens* larvae. We also found that, in most of the transgenic lines, resistance capability increased as *gna* gene positive ratio increased, and was displayed as a normal distribution. That is, most transgenic plants showed moderate resistance capability, while a small number part exhibited high resistance capability. As we can see from Table 2, 60% of transgenic plants in line HZ8-1 of R3 progeny had high resistance capability (grade 0), and molecular genetic analysis showed that line HZ8-1 was a *gna* gene pure line (Table 1). The two results were in accord with each other. That is to say, the high resistance capability of transgenic rice plants is mainly due to the resistance function of GNA. In line HZ4-1, 80% transgenic plants had a better resistance capability than control plants in R2 progeny. Inheritance analysis revealed that *gna* gene obeyed 3:1segregation (Table 1) and only one single copy was observed (Fig 5). This result also further

**Table 2.** Bioassay of R2 and R3 progeny of transgenic rice plants against brown plant hopper[a]

| | Mean resistance grade | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | | 1 | | 3 | | 5 | | 7 | | 9 | |
| Line | R2 | R3 | R2 | R3 | R2 | R3 | R2 | R3 | R2 | R3 | R2 | R3 |
| HZ2-3 | | | | | 20 | | 20 | | 40 | 50 | 20 | 50 |
| HZ4-1 | | | | | | | 20 | | 60 | 100 | 20 | |
| HZ5-1 | | | | | | | | | | 100 | 100 | |
| HZ6-1 | | | | | | | | 33.3 | | 33.3 | | 33.3 |
| HZ6-2 | | | | | | | | | | | | |
| HZ7-4 | | | | | | | | 100 | | | | |
| HZ8-1 | | 60 | | | | | | | | 20 | | 20 |
| HZ8-2 | | | | | | | | | 100 | 100 | | |
| HZ10-1 | | | | | | | | | | 100 | | |
| HZ10-2 | | | | | | | 75 | | | | 25 | |
| HZ11-1 | | | | | | | 40 | | 40 | | 20 | |
| HZ12-2 | | | | | | | | | 75 | | 25 | |
| TN1 (S CK) | | | | | | | | | | | 100 | 100 |
| ZhuxianB (C CK) | | | | | | | | | | | 100 | 100 |

[a] R2 and R3 mean R2 progeny and R3 progeny, respectively; the numbers are the percentage of transgenic plants in different resistance grade in the same line.

**Table 3.** Bioassay of R2 and R3 progeny of transgenic rice plants against rice leaf-folder[a]

| | Mean resistance grade | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | | 1 | | 3 | | 5 | | 7 | | 9 | |
| Line | R2 | R3 | R2 | R3 | R2 | R3 | R2 | R3 | R2 | R3 | R2 | R3 |
| HZ2-3 | | | 0.8 | | 5.4 | 12.5 | 34.9 | 25 | 36.4 | 37.5 | 22.5 | 25 |
| HZ4-1 | 1.4 | | 0.7 | | 9.9 | 12.5 | 43.3 | 25 | 35.5 | 50 | 9.2 | 12.5 |
| HZ5-1 | | | 4.8 | | 12.9 | | 33.9 | | 43.6 | | 4.8 | |
| HZ6-1 | | | | | | 50 | | | | 50 | | |
| HZ6-2 | | | | | | 12.5 | | 62.5 | | 12.5 | | 12.5 |
| HZ7-4 | | | | | | 25 | | 50 | | 12.5 | | 12.5 |
| HZ8-1 | | | | | | | | 50 | | 50 | | |
| HZ8-2 | | | | | 8.7 | | 32.6 | | 28.3 | 40 | 30.4 | 60 |
| HZ10-1 | | | | | | | | | | | | |
| HZ10-2 | | | | | 6.4 | | 24.4 | 25 | 33.3 | 50 | 35.9 | 25 |
| HZ11-1 | | | | | 5.4 | | 38 | 60 | 31.5 | 20 | 54.3 | 20 |
| HZ12-2 | | | 7.9 | | | | 31.6 | | 26.3 | | 34.2 | |
| TN1 (S.CK) | | | | | | | | | | | 100 | 100 |
| ZhuxianB (C.CK) | | | | | | | | | | | 100 | 100 |

[a] R2 and R3 mean R2 progeny and R3 progeny, respectively; the numbers are the percentage of transgenic plants in different resistance grade in the same line.

confirms the above viewpoint. In line HZ5-1, the *N lugens* resistance was grade 9 in R2 progeny but grade 7 in R3; that is, R3 plants had better resistance than R2 plants to *N lugens* in line HZ5-1. The reason may be that in line HZ5-1 the suppression of female or male gamete in transgenic *gna* gene plant took place in R2, and then some factor led to gene reactivation in selfed R3 progeny. This viewpoint can also be confirmed by the results of molecular genetic analysis. As we can see from Table 1, in line HZ5-1, the positive number of *gna* genes was less than the negative number in R2 progeny, which was in conflict with expected data. However, in R3 progeny, the transgenic line displayed a one-locus Mendelian segregation fashion.

Transgenic plants were also infested with *C medinalis*, and the results are summarized in Table 3. It was found that control plants were highly susceptible and exhibited no resistance to *C medinalis*, but most of the transgenic lines displayed a better insecticidal effect on *C medinalis*. As expected, the resistance capability of all tested transgenic lines showed a normal distribution and some transgenic plants exhibited high resistance capability. Combining molecular analysis with insect bioassay, we found that the resistance capability increased with increase in positive ratio of *sbti* gene in most transgenic lines. In line HZ4-1, the resistance grade of some plants to *C medinalis* was up to zero (highly resistant) in R2 progeny, and approximately

90% of plants had a better resistance capability than second generation control plants. Inheritance analysis showed that s*bti* gene in this line obeyed 3:1segregation in R3. However, it is worth pointing out that, in HZ8-2 of R3 progeny, the positive ratio of *sbti* gene was much higher than that in other lines, such as HZ2-3, but the insect resistance capability was much lower than that of other lines. This is interpreted as being due to some plants containing multiple copies of *sbti* transgene being insect-sensitive due to some environmental factor or other reason. The result may be similar to the study of Kim *et al*.[25] From the above insect bioassays, we obtained nine lines of R3 transgenic plants, including one pure line, which had better resistance to both *N lugens* and *C medinalis* than wild-type plants.

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