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Publication Date
1999

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Planta (1999) 208: 552–563

Planta
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Particle-bombardment-mediated co-transformation of elite Chinese rice cultivars with genes conferring resistance to bacterial blight and sap-sucking insect pests

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Received: 12 October 1998 / Accepted: 29 December 1998

Abstract. Transgenic rice plants were generated using particle bombardment to simultaneously introduce the rice *Xa21* gene effective against bacterial blight and the *Galanthus nivalis* agglutinin (snowdrop lectin; *gna*) gene effective against sap-sucking insect pests, specifically the brown plant hopper. Using three plasmids, we co-transformed 5- to 10-d-old, mature seed-derived rice (*Oryza sativa* L.) callus of two elite Chinese rice cultivars, Eyi 105 and Ewan 5. The plasmids carried a total of four genes. The *gna* and *Xa21* genes were carried on separate plasmids. The selectable marker hygromycin phosphotransferase (*hpt*) and the reporter gene β-glucuronidase (*gusA*) were linked on the same, co-integrate vector. We recovered over 160 independently derived transgenic rice plants. Over 70% of the transgenic plants carried all four genes, as confirmed by polymerase chain reaction and/or Southern blot analysis. Furthermore, 70% of transgenic plants carrying all four genes also co-expressed all four genes, as confirmed by growth on selection media (*hpt*), GUS histochemical assays (*gusA*), western blotting (*gna*) and reverse transcriptase-polymerase chain reaction (*Xa21*) analysis. The co-expression efficiency reported for the four transgenes in our study is the highest ever found in any transgenic plant population generated through co-transformation. The linked genes (*hpt* and *gusA*) co-integrated with a frequency of near 100%, and we observed a co-integration frequency greater than 70% for the genes carried on separate plasmids. We observed no preferential integration of any particular gene(s). Genetic analysis confirmed Mendelian segregation of the transgenes in subsequent generations. We report, for the first time, generation and analysis of transgenic rice lines carrying genes effective against more than one taxa of pathogen or pest, substantiating that particle bombardment represents an effective way to introduce unlinked complex multiple traits into plants.

Key words: Bacterial blight (*Xa21* gene) – Lectin (*gna* gene) – *Oryza* (co-transformation) – Resistance genes (*gna*, *Xa21*)

Abbreviations: *gna* (GNA) = gene (protein) for *Galanthus nivalis* agglutinin; *gusA* (GUS) = gene (protein) for β-glucuronidase; *hpt* (HPT) = gene (protein) for hygromycin phosphotransferase; *Xa21* (XA21) = gene (protein) for resistance to bacterial blight, RT-PCR = reverse transcriptase-polymerase chain reaction

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Introduction

Rice is one of the world's most important food crops. Substantial effort has been directed towards improving rice production using both conventional breeding and genetic engineering techniques. Although a decade ago rice was considered one of the most recalcitrant crops in terms of genetic manipulation, it has recently emerged as the model cereal (Izawa and Shimamoto 1996) for the study of plant genomics (Dean and Schmidt 1995; Nagamura et al. 1997; Sasaki 1998), plant pathology (Valent 1990; Ronald 1997), gene regulation (Sheu et al. 1994; Zhao et al. 1994; Kyozuka et al. 1998) gene expression (Chan et al. 1994; Tsuchiya et al. 1994; Kohli et al. 1996, 1999a; Kumpatla et al. 1997; Kumpatla and Hall 1998) metabolic pathway manipulation (Burkhardt et al. 1997; Nakamura et al. 1997) and the inheritance, organization, rearrangement and fate of transgenes (Hiei et al. 1994; Cooley et al. 1995; Kohli et al. 1998, 1999b). This revolution was due, in part, to progress in the establishment of efficient rice transformation systems (for review, see Christou 1997).

A number of workers have now reported stable transgene inheritance and expression. However, only a few have carried out such analysis on multiple transgenes of agronomic importance. Goto et al. (1993)

studied the co-integration of two unlinked marker genes in transgenic rice plants, and their co-inheritance up to the R3 generation. They reported a high frequency of co-integration, although some plants carried only the selectable marker. Cooley et al. (1995) showed that selectable and non-selectable genes linked on the same vector co-integrated at a frequency of 100% in transgenic rice. Recently Wakita et al. (1998) reported co-integration, co-expression and co-inheritance of a selectable marker gene and a fatty acid desaturase gene. Chen et al. (1998) demonstrated the power of particle bombardment by co-transforming rice plants with 13 unlinked genes. They have also reported the co-expression and co-inheritance of transgenes; however, their analysis was limited to four marker genes in just three of the many lines they generated. Hadi et al. (1996) bombarded soybean callus with 12 unlinked genes on separate plasmids and showed that the majority of transgenic clones carried all 12 genes. However, no expression studies were reported.

Our long-term aims are to improve the quality of rice using an approach in which multiple disease- and insect-resistance traits can be introduced into the same lines. Transgenic lines carrying genes effective against more than one taxa of pathogen or pest have not been reported.

In the present study, we describe the use of particle bombardment to co-transform elite Chinese rice cultivars with four genes carried on three plasmids. Two of these genes on the same plasmid are markers regularly used in rice transformation (*hpt* and *gusA*). The other two, each on a separate plasmid, are effective against two separate taxa of pathogens or pests. The *Xa21* gene provides resistance against bacterial blight (Wang et al. 1998; Zhang et al. 1998) and the *gna* gene provides resistance against sap-sucking insects such as the brown plant hopper (Sudhakar et al. 1998b; Rao et al. 1998). We present data on co-integration frequencies and the relationship between co-integration, co-expression and co-inheritance of these agronomically important multiple transgenes in the rice plants we analyzed.

Materials and methods

Plasmid constructs. Three plasmids were used in the co-transformation experiments. pJIMB15 contained the *gusA* reporter gene and the *hpt* selectable marker (conferring hygromycin resistance) on the same plasmid, each under the control of the cauliflower mosaic virus (CaMV) 35S promoter. Plasmid pRSSGNA1 contained the *gna* gene driven by the rice sucrose synthase promoter (Wang et al. 1992; Sudhakar et al. 1998b). A genomic subclone of the rice *Xa21* gene, driven by the maize ubiquitin 1 promoter was carried on pAHXA21. It was constructed as follows: the *Kpn*I fragment of plasmid pB822, containing *Xa21* (Song et al. 1995), was inserted into the *Kpn*I restriction site of pAHC17 (Christensen and Quail 1996). The resulting intermediate vector was partially digested with *Eco*RV to remove the noncoding sequence upstream of the *Xa21* coding region, thus generating pAHXA21. The three plasmids are shown in Fig. 1.

Transformation, selection and regeneration procedure. The target material for particle bombardment was mature, seed-derived callus (5- to 10-d-old) from two elite Chinese rice (*Oryza sativa* L.) cultivars, Eyi 105 and Ewan 5. The calli were bombarded with 0.75-μm gold particles coated with the three plasmids at a molar ratio of 1 (pJIMB15):3 (pRSSGNA1):3 (pAHXA21). A total of 10.5 μg DNA (1.5:4.5:4.5) was used to coat 5-mg gold particles (2.1 μg DNA per mg gold). Transformation and selection of hygromycin-resistant callus was carried out essentially as described by Sudhakar et al. (1998a). After two rounds of selection on medium supplemented with 30 mg l^{-1} hygromycin, resistant callus was transferred to 20 ml MSKN (MS basal medium, from Sigma, supplemented with 2.0 mg l^{-1} kinetin, 0.5 mg l^{-1} naphthalene-1-acetic acid and 30 g l^{-1} maltose) medium solidified with 0.25% phytagel and containing 30 mg l^{-1} hygromycin. After incubation for 1 week in the dark at 28 °C, the proliferating callus was transferred to fresh medium and maintained under light with a 16-h photoperiod for plant regeneration (photosynthetic photon flux of 55 μmol m^{-2} s^{-1}, cool-white fluorescent tubes). Two to three weeks later, regenerated

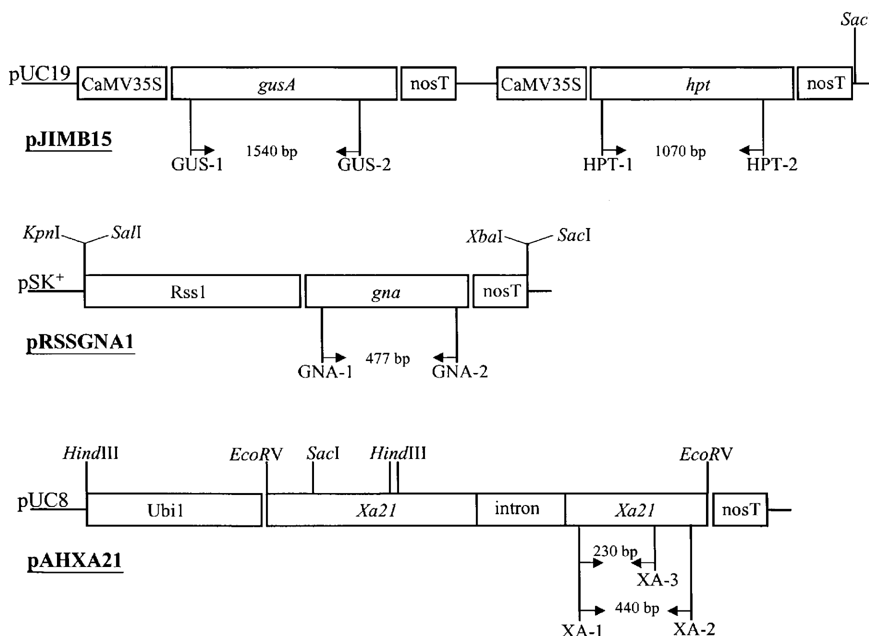

Fig. 1. Schematic representation of the three plasmids pJIMB15, pRSSGNA1 and pAHXA21 used in rice transformation. The relevant restriction sites and the position of primers used in genomic PCR, RT-PCR and to generate Southern blot probes is indicated

plantlets approximately 1–2 cm high were transferred to rooting medium (MS basal medium solidified with 0.25% phytagel and supplemented with 30 g l^{-1} sucrose, 30 mg l^{-1} hygromycin). One to two weeks later, plants with vigorously growing roots were transferred to soil.

Polymerase chain reaction (PCR) analysis. Plant DNA was extracted from young leaves essentially as described by Edwards et al. (1991). All the putative transgenic (hygromycin resistant) plants were analysed by PCR for the presence of the *gna* and *Xa21* transgenes. Plants shown by histochemical analysis to be GUS-negative (Jefferson et al. 1987) were analyzed by PCR for the presence of the *gusA* and *hpt* markers. Four pairs of specific primers were used to detect the *gna*, *Xa21*, *gusA* and *hpt* genes: for the *gna* gene, forward primer (GNA-1) 5′-ATGGCTAAGGCAAGTCTCCTC-3′ and reverse primer (GNA-2) 5′-TCATTACTTTGCCGTCACAAG-3′; for the *Xa21* gene, forward primer (XA-1) 5′-GTGCTGGAAATAGTAACCGGG-3′ and reverse primer (XA-2) 5′-AATCATCGCAAGACCGGCAA-3′; for the *gusA* gene, forward primer (GUS-1) 5′-CCATACCTGTTCACCGACGA-3′ and reverse primer (GUS-2) 5′-GGAATTGATCAGCGTTGGTG-3′; and for the *hpt* gene, forward primer (HPT-1) 5′-ACTCACCGCGACGTCTGTCG-3′ and reverse primer (HPT–2) 5′-GATCTCCAATCTGCGGGATC-3′. Primer positions and PCR product sizes are indicated in Fig. 1. The PCR reactions were carried out in a total volume of 25 µl, comprising 50 ng of rice genomic DNA, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 µM each dNTP, 1.25 units *Taq* DNA polymerase and 125 pmol of each primer. For PCR analysis of the *gna*, *gusA* and *hpt* genes, DNA was denatured at 94 °C for 3 min followed by 30 amplification cycles (94 °C for 50 s, 62 °C for 50 s, 72 °C for 50 s) and 7 min at 72 °C. For PCR analysis of the *Xa21* gene, DNA was denatured at 94 °C for 3 min followed by 30 amplification cycles (94 °C for 1 min, 52 °C for 1 min and 72 °C for 1 min) and 10 min at 72 °C.

Southern blot analysis. Total genomic DNA was isolated from 1 g fresh weight leaf material using the Nucleon Phytopure Plant DNA Extraction Kit according to the manufacturer's instructions (Amersham Life Sciences). A 10-µg aliquot of DNA was digested overnight at 37 °C with appropriate restriction enzyme. The digested DNA was fractionated in a 0.8% agarose gel, transferred to a positively charged nylon membrane (Boehringer Mannheim) and hybridized to digoxigenin (DIG)-dUTP labelled probes according to the manufacturer's instructions (Boehringer Mannheim). Probes were synthesized using the PCR DIG Probe Synthesis Kit (Boehringer Mannheim) and purified prior to use with the QIAquick Gel Extraction Kit (QIAGEN). Three of the primer sets used for PCR analysis (GNA-1 and GNA-2, GUS-1 and GUS-2, HPT-1 and HPT-2) were also used to generate DIG-labelled probes for Southern blot analysis. For the *Xa21* gene, we used the XA-1 forward primer in conjunction with a new reverse primer XA-3 to generate a 230-bp probe. Detection was achieved using the DIG Luminescent Detection Kit (Boehringer Mannheim) and the hybridization signals were visualized by exposure to Fuji X-ray film at 37 °C for 40 min. Filters were probed first with the *gna* probe, stripped for re-hybridization by washing twice for 15 min in 0.2 N NaOH, 0.1% (w/v) SDS at 37 °C, and then probed with the *Xa21* probe. The filters were then stripped and reprobed with the *gusA* probe, and finally with the *hpt* probe.

Reverse transcriptase (RT)-PCR analysis. Twenty-two independently derived R0 transgenic plants, confirmed to contain the *Xa21* transgene, were analyzed by RT-PCR to characterize expression of *Xa21* at the RNA level. Total plant RNA was extracted using Trizol reagent according to the manufacturer's instructions (GIBCO Life Technologies). The RT-PCR was performed using the Access RT-PCR System (Promega) in a total volume of 50 µl, containing 200 pg template RNA, 10 µl AMV/*Tfl* 5x reaction buffer, 1 µl dNTP Mix (10 mM each dNTP), 1 µl AMV reverse transcriptase (5 U μl^{-1}), 1 µl *Tfl* DNA polymerase (5 U μl^{-1}), 2 µl 25 mM $MgSO_4$ and 50 pmol of each primers (XA-1 and XA-2, as described above). The RT-PCR reaction conditions and cycling parameters were as follows: 48 °C for 45 min, 94 °C for 2 min, followed by 40 amplification cycles (94 °C for 30 s, 52 °C for 1 min, 68 °C for 2 min) and 7 min at 68 °C.

β-Glucuronidase histochemical assay. All hygromycin-resistant plants were analyzed for GUS activity using the histochemical assay described by Jefferson et al. (1987).

Western blot analysis. All hygromycin-resistant plants were analyzed for GNA expression by western blot. Protein was extracted from young leaves as described by Koziel et al. (1993). Protein concentration was determined using the standard Bradford assay (Bradford, 1976). Protein aliquots (20 µg per lane) were loaded and fractionated on a 15% SDS-polyacrylamide gel (Laemmli, 1970) before transfer to a nitrocellulose membrane. The membrane was incubated with a polyclonal rabbit anti-GNA primary antiserum and subsequently with an alkaline-phosphatase-conjugated goat anti-rabbit second antibody (Promega). The membrane was incubated in the Western Blue stabilized substrate for alkaline phosphatase until bands developed. Levels of GNA protein were measured by scanning densitometry using purified control GNA protein (100 ng) as a reference.

Genetic analysis. Seventeen independently derived R0 transgenic plants were grown to maturity. R1 grain was harvested and progeny were grown and analyzed by PCR. Some of the R1 progeny showing 3:1 transgene segregation were also analyzed by Southern blot, GUS assays, and western blots to compare gene integration and gene expression patterns with those of the parental plants.

Results

Selection and plant regeneration. Following transformation by particle bombardment, we transferred callus to selection medium containing hygromycin. Continuous selection of the proliferating tissues for two weeks resulted in the appearance of vigorously growing embryogenic callus. This was transferred to regeneration medium, and then to rooting medium, containing hygromycin. Plants were successfully regenerated, and developed roots when transferred to the rooting medium. We recovered over 160 independent transgenic rice lines. We determined the overall transformation efficiency by calculating the number of independently derived plants regenerated on hygromycin-containing medium as a proportion of the total number of calli subjected to particle bombardment (Table 1). We achieved a transformation frequency of 16% for Eyi 105 and 18% for Ewan 5. We used approx. 20 calli per bombardment. For the two cultivars Eyi 105 and Ewan 5, 22 and 27 plates were bombarded, respectively. On average, we recovered 3.5 and 3.6 transgenic plants per bombardment, for Eyi 105 and Ewan 5 respectively.

Characterization of R0 plants: integration of transgenes. We showed all surviving plants to be transgenic by molecular analysis. Continuous selection of callus up to rooting of the plantlets on medium containing hygromycin ensured there were no escapes (Christou et al. 1991; Valdez et al. 1998; Sudhakar et al. 1998a; Vain et al. 1998) and confirmed the presence and activity of

Table 1. Efficiency of particle-bombardment-mediated transformation of rice. Hm^R, hygromycin resistant. GUS^+, GUS-positive by histochemical assay

Rice cultivar	No. of callus pieces bombarded (A)	No. of independent Hm^R plants produced (B)	No. of Hm^R and GUS^+ plants produced	Transformation frequency (%) (B/A)
Eyi 105	444	72	54	16
Ewan 5	542	96	65	18

the *hpt* gene. Initially, histochemical GUS assays were carried out on all 160 R0 plants. Polymerase chain reaction analysis was then performed for the *hpt* and the *gusA* genes on plants that did not show GUS activity. Taken together, these results confirmed that all plants carried both genes except for Eyi 105 plant No. 4, which appeared to contain the *hpt* gene but not the *gusA* gene. Since the *hpt* and *gusA* genes were initially linked on the same transforming plasmid, the status of this plant was further addressed by Southern blot analysis to confirm the PCR results (see below). The co-integration frequencies of the linked *hpt* and *gusA* genes were 99% for Eyi 105 and 100% for Ewan 5.

We analyzed all of the independently derived transgenic plants by PCR for the presence of the *gna* and *Xa21* transgenes. We found that 55 out of 72 (76%) Eyi 105, and 79 out of 97 (81%) Ewan 5 transgenic plants contained the *gna* gene, while 62 out of 72 (86%) Eyi 105, and 77 out of 97 (79%) Ewan 5 transgenic plants contained the *Xa21* gene. The frequency at which genes from the non-selected plasmid (*gna* or *Xa21*) and the selected plasmid (*hpt-gusA*) co-integrated was thus over 75%. We observed no preferential integration of any gene.

Transgenic plants could be divided into four categories based on co-transformation patterns: (a) those containing the selectable (*hpt*) and screenable (*gusA*) markers alone; (b) those containing the two marker genes and the *gna* gene; (c) those containing the two marker genes and the *Xa21* gene and (d) those containing all four genes, indicating co-transformation by all three plasmids. Most of the transgenic plants (71%) belonged to category (d), showing that co-transformation events involving all three plasmids were very efficient, even though selection was carried out for a marker (*hpt*) present on only one of the plasmids. This demonstrates that transgenic plants containing multiple transgenes can be obtained at relatively high frequency using co-transformation, making the use of large co-integrate vectors unnecessary.

We used Southern blot analysis to characterize the transgenic status of these plants in detail, and to estimate the transgene copy number. We used restriction enzyme *Sac*I, which recognizes a single site in each of the three transforming plasmids. This site is not present within the PCR products used as probes. We were able to obtain rough estimates of copy numbers according to the number and intensity of hybridizing bands on Southern blots. We analyzed 20 independently derived R0 transgenic plants by Southern blot for the *gna*, *Xa21*, *gusA* and *hpt* genes (Figs. 2a–d, respectively). Unique and complex hybridization patterns were revealed, indicating that these plants indeed originated from independent integration events. As suggested by initial PCR data, the Southern blot analysis also confirmed that in plant No. 4 the *hpt* gene had integrated but the *gusA* gene had not.

Copy numbers of different genes varied among the plants and ranged from low (1–3 copies) or moderate (4–6 copies) to high (more than 6 copies). Most of the hybridization bands were between 3.0 and 12.0 kbp in length. Interestingly, although the number of copies of the *gna* gene varied substantially between independent R0 transgenic plants, less variation was seen for the linked *gusA* and *hpt* genes. Most of the transgenic plants carried one to three copies of the *gusA* and *hpt* genes, with only two plants carrying more than three copies of the *gusA* gene (plant Nos. 1 and 2) and one plant carrying more than three copies of the *hpt* gene (plant No. 8). This indicated a probable bias resulting from the use of lower molar amount of plasmid containing the *hpt* and *gusA* genes in the gold preparation.

Southern blot analysis of the *Xa21* gene after digestion of genomic DNA with *Sac*I also revealed different hybridization patterns among independently derived transgenic plants (Fig. 2). However, the presence of hybridization bands for the endogenous *Xa21* gene family did not provide clear results for the *Xa21* transgene. We therefore digested genomic DNA from 17 plants with *Hin*dIII. This releases a diagnostic 4.8-kbp fragment. Results of Southern hybridization performed are shown in Fig. 3. We obtained the 4.8-kbp fragment in 7 lines. A number of additional smaller and larger transgene-specific bands were obtained indicating truncations or loss of *Hin*dIII site most likely due to rearrangements of the plasmid before or during integration.

Characterization of R0 plants: expression of integrated genes. Expression of the *hpt* selectable marker was confirmed by the recovery of transgenic lines grown on selective media. β-Glucuronidase histochemical assay was used to confirm expression of the *gusA* gene. The results showed that 75% of Eyi 105 and 67% of Ewan 5 plants proliferating on hygromycin-supplemented medium also showed GUS activity (Table 1). Although PCRs showed that nearly all the transgenic lines of both cultivars contained the *gusA* gene, we could not detect GUS activity using the histochemical assay in about 30% of the plants. These plants did not show GUS activity at any of the three different stages tested – in rooting medium, after 15 d in soil, and after 40 d in soil. This indicated that the lack of *gusA* expression was due either to the phenomenon of transgene silencing setting

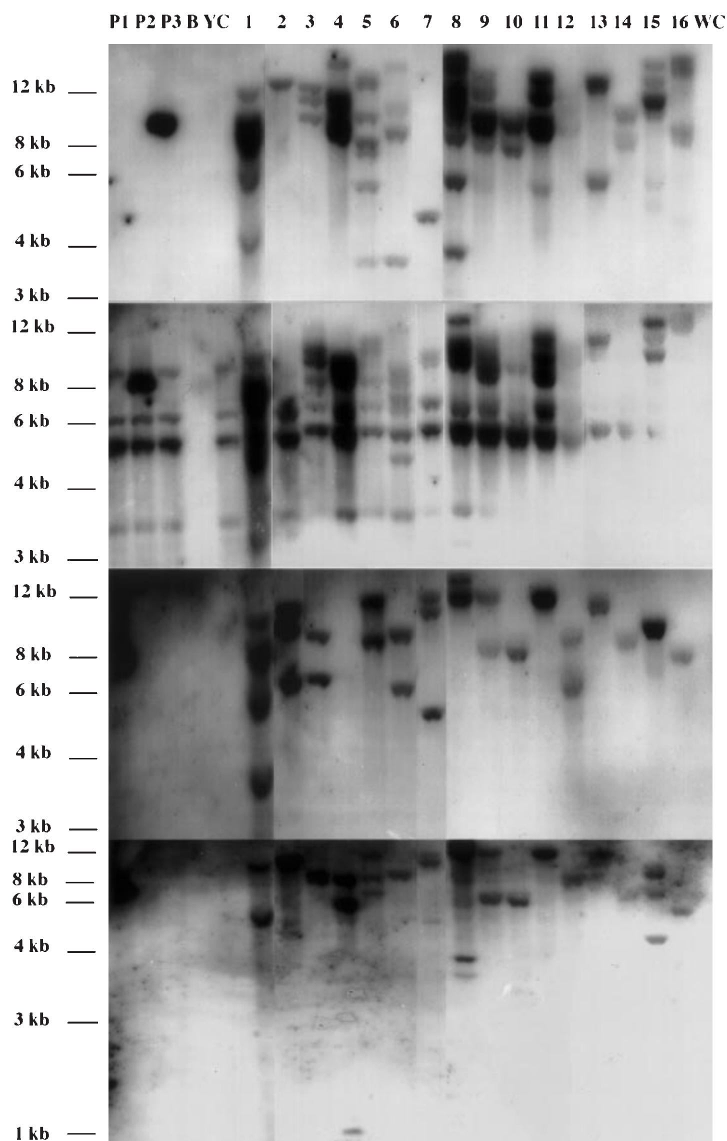

Fig. 2a–d. Representative Southern blot analysis of R0 transgenic rice plants. Plant genomic DNA and plasmid DNA were digested with *Sac*I, separated in a 0.8% agarose gel and hybridized to a DIG-labeled *gna* (**a**), *Xa21* (**b**), *gusA* (**c**) or *hpt* (**d**) probe. *P1*, pJIMB15 plus untransformed plant DNA; *P2*, pAHXA21 plus untransformed plant DNA; *P3*, pRSSGNA1 plus untransformed plant DNA; *B*, blank lane; *YC*, untransformed Eyi 105 plant; *lanes 1–16*, transgenic plants corresponding to plant Nos. 1–16; *lane 17*, untransformed Ewan 5 plant

in very early during development, or to the absence of an intact copy of the gene cassette.

We tested all independently derived transgenic plants for *gna* gene expression by western blot analysis. Figure 4 shows the results from a representative population of plants tested for the expression of the *gna* gene. The numbers of plants shown to be positive for the presence of *gna* by PCR were 55 and 79 for Eyi 105 and Ewan 5, respectively. We obtained 51 and 66 GNA western-positive plants for Eyi 105 and Ewan 5, respectively. Therefore nearly 93% of Eyi 105 plants and 84% of Ewan 5 plants expressed the *gna* gene. However, the levels of GNA among individual plants varied from 0.01% to 0.5% total cellular protein.

We investigated *Xa21* gene expression at the RNA level by RT-PCR analysis (Fig. 5). We found eight out of 11 (73%) Eyi 105 and seven out of 11 (64%) Ewan 5 transgenic plants synthesized the *Xa21* RNA.

We found no relationship between transgene copy number and expression. For example, plant No.7 had a low *gna* copy number (one or two copies) and at the level of sensitivity provided by western analysis, did not express GNA protein. Plant No. 1 carried approximately five copies of *gna*, but the level of GNA expression was high (about 0.35% total soluble protein). However, plant No. 8, which carried over ten copies of the gene also expressed GNA at moderate levels (approximately 0.2% total soluble protein). We observed similar evidence of dosage-independent expression for the *gusA* gene. Plant No. 13 carried two copies of *gusA* but GUS could not be detected, while plant Nos. 1 and 2 carried more than four copies and showed GUS activity. The lack of GUS activity in plant 13 could be due to the absence of an intact gene cassette, so the comparison between plant No. 13 and plant Nos. 1 and 2 might not be valid. However, our contention that high transgene copy number does not necessarily lead to a concomitant decrease in expression was borne out by the higher GUS activities in plant Nos. 1 and 2 in comparison to other plants

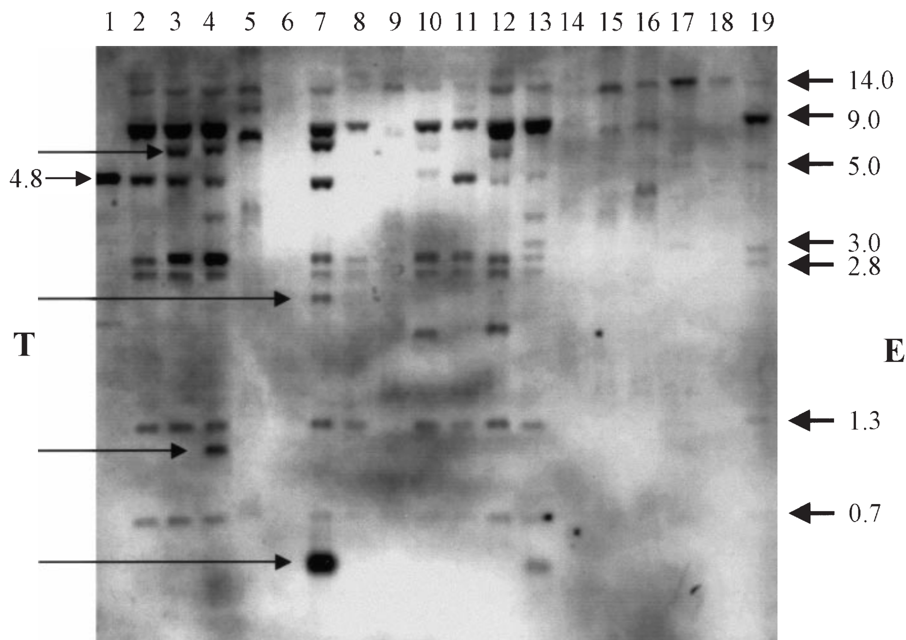

Fig. 3. Southern blot analysis of selected plants for *Xa21* gene after digestion of genomic DNA with restriction enzyme *Hin*dIII which generates a 4.8-kbp fragment specific for part of the transgene cassette. Numbers on the sides represent the molecular sizes of the endogenous (*E*) and the transgenic (*T*) fragments in kbp. *Arrows on the left* indicate some of the transgene-specific fragments. *Arrows on the right* indicate the endogenous bands. *Lane 1* contains the plasmid pAH-XA21 digested with *Hin*dIII. *Lanes 2–18* represent transgenic plant Nos. 1 to 17. Lane 18 contains untransformed Ewan 5 DNA

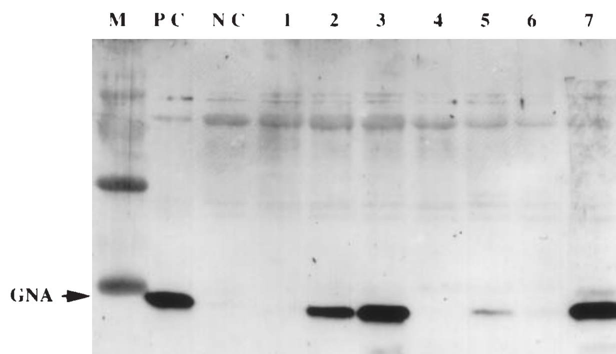

Fig. 4. Representative western blot analysis for GNA expression in R0 transgenic rice plants. Protein was extracted from young leaves and 20 μg was loaded in each lane. M, protein marker; PC, 100 ng purified GNA protein (positive control, 12 kDa); NC; untransformed plant (negative control); *lanes 1–7*, transgenic plant Nos. 7, 8, 9, 10, 11, 12 and 1 respectively. Plant Nos. 7 and 10 are negative for *gna* expression, plant Nos. 11 and 12 are weak expressers, plant No. 8 is a moderate expresser whereas plant Nos. 9 and 1 are high expressers

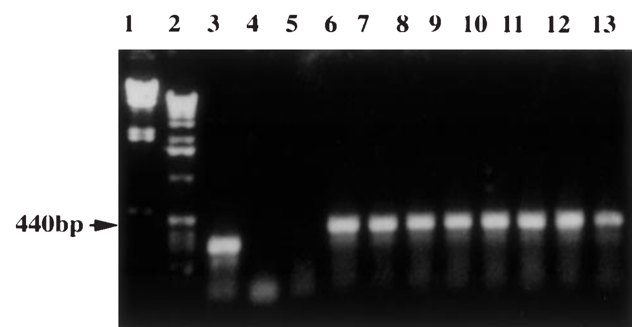

Fig. 5. Representative RT-PCR analysis for *Xa21* transcription in R0 transgenic rice plants. *Lane 1*, size markers (*Hin*dIII-digested λ DNA); *lane 2*, size markers (1-kbp DNA ladder); *lane 3*, positive control from Access RT-PCR System, producing a 323-bp band; *lane 4*, water (negative control); *lane 5*, untransformed plant (negative control); *lanes 6–13*, transgenic plants Nos. 1, 7, 8, 9, 10, 11, 12, and 13 respectively. The *arrow* indicates the expected RT-PCR product of the *Xa21* mRNA (440 bp)

containing fewer *gusA* copies. Expression levels and estimates of transgene copy numbers are summarised in Table 2.

Of the 20 independently derived transgenic plants analyzed by Southern blot hybridization, 14 carried all 4 genes, indicating a co-integration frequency of 70%. This figure (arising from a representative population) is similar to that obtained after PCR and histochemical GUS analysis of the entire population of transgenic lines for all four transgenes. Of the 14 plants containing the 4 transgenes, 10 plants also expressed all 4 genes. The co-expression frequency of the four genes was therefore approximately 70%, and this figure is also close to that generated by testing all PCR-positive plants for the expression of *gusA* and *gna*.

Characterization of R1 plants: co-integration and co-segregation of transgenes. R1 progeny of five independently derived Eyi 105 primary transformants (plant Nos. 2, 3, 5, 17 and 18) and four Ewan 5 primary transformants (plant Nos. 11, 12, 13 and 20) were analyzed by PCR for segregation patterns (Table 3). Our results showed that in five lines (from primary transformants 2, 3, 11, 12 and 13) all four genes co-segregated at a ratio of 3:1, indicating that all four genes had integrated at a single genetic locus. One line (derived from plant No. 17) showed a 15:1 segregation ratio for all four genes, suggesting two independent integration events each involving all four genes. Another line (derived from plant No. 18) showed 15:1 segregation ratios for the *gna* and *Xa21* genes, but 3:1 ratios for the

Table 2. Estimated copy number and expression level of transgenes in transgenic rice plants. Plants Nos. 1–6 and 17–18: Eyi 105. Plant Nos. 7–16 and 19–20: Ewan 5

Transformant No.	Transgene copy number[a]				Expression			
	gna	*gusA*	*hpt*	*Xa21*[b]	GNA % of total protein	GUS[c]	HPT[d]	XA21
1	M	M	L	+	≥0.3	+ + +	+	+
2	L	M	L	–	≥0.3	+ + +	+	–
3	L	L	L	+	≥0.3	+	+	+
4	M	0	L	+	≥0.1	–	+	+
5	M	L	L	+	≥0.3	+	+	+
6	M	L	L	+	≤0.1	+ +	+	+
7	L	L	L	+	0	+	+	+
8	H	L	H	+	≥0.1	+	+	+
9	M	L	L	+	≥0.3	+ + +	+	–
10	L	L	L	–	≤0.1	+	+	–
11	M	L	L	+	≥0.3	+ +	+	+
12	L	L	L	+	≥0.3	+ + +	+	+
13	L	L	L	+	≥0.3	–	+	+
14	L	L	L	–	≥0.3	+	+	–
15	H	L	L	+	≥0.1	–	+	–
16	M	L	L	+	≥0.3	+ +	+	+
17	L	L	L	–	≥0.3	+ +	+	–
18	H	L	L	+	≥0.3	+ +	+	+
19	M	L	L	–	≤0.1	+	+	–
20	H	L	L	+	≥0.3	+	+	+

[a]Estimates of transgene copy number presented as L (low: 1–3), M (moderate: 4–6) and H (high: > 6)
[b]Copy number not presented due to presence of endogenous *Xa21* hybridization bands
[c]Transgenic plants with strong (+ + +), moderate (+ +), low (+) or no (–) GUS expression
[d]Hygromycin resistance confirmed by germination selection of R0 and germination of R1 plants

gusA and *hpt* genes. This suggested that the linked *gusA* and *hpt* genes had integrated into one locus, but the *gna* and *Xa21* genes carried on separate plasmids had co-integrated at two unlinked loci. Another two lines (derived from plant Nos. 5 and 20) showed a 3:1 segregation ratio for the *Xa21* gene but an aberrant segregation ratio of 1:1 for the other three transgenes. Southern blot analysis of some of the R1 plants showing typical Mendelian segregation patterns revealed identical hybridization band patterns to the parental R0 plants (data not shown). For each of the nine lines, 27–40 R1 plants were also tested for expression patterns. Co-expression, as detected by germinating the grain on hygromycin-supplemented medium (*hpt*), histochemical GUS assay (*gusA*), western blot (*gna*), and RT-PCR (*Xa21*), was observed among these R1 plants in accordance with the co-expression patterns of R0 plants. All 28 R1 progeny plants derived from R0 plant No. 13 (in which the *gusA* gene was not expressed), were also GUS-negative. In each of the other eight lines, none of the genes expressed in the R0 generation exhibited silencing in the R1 generation. These data indicate stable integration, co-segregation and co-expression of the foreign genes. Additionally, the segregation analysis reveals co-integration of genes from different plasmids at a frequency of nearly 100% because in all the lines, at least two of the three plasmids integrated – there were no lines transformed with the *hpt*-*gusA* construct alone. Co-integration of all four genes at one (lines 2, 3, 11, 12, and 13) or two loci (line 17) was, on average, limited to 70% of the lines.

Discussion

Particle bombardment has been successfully used to obtain transgenic lines of different crop plants (Christou 1995). Substantial progress has been made in recent years with the development of *Agrobacterium*-mediated transformation systems for monocot species (Komari et al. 1998). This has led to the recovery of transgenic rice (Hiei et al. 1994; Cheng et al. 1998) maize (Ishida et al. 1996) barley (Tingay et al. 1997) and wheat (Cheng et al. 1997). However, particle bombardment is still the method of choice for the production of transgenic plants, particularly for monocot species, because it is variety-independent (at least in rice), simple and efficient. A further advantage is its ability to produce transgenic plants with multiple genes by co-transformation. This has been demonstrated clearly by Hadi et al. (1996) and Chen et al. (1998) who successfully co-transformed soybean and rice with 12 and 14 different plasmids, respectively. Hadi and colleagues found the majority of transgenic soybean clones had integrated all 12 plasmids, while Chen and colleagues showed that up to 13 of the 14 plasmids could be simultaneously introduced into the rice genome.

We have used co-transformation by particle bombardment to deliver two agronomically useful genes (*gna* and *Xa21*) in addition to the selectable (*hpt*) and screenable (*gusA*) markers, into rice. These genes confer resistance against sap-sucking insects such as the brown plant hopper, and bacterial blight respectively. There have been previous reports of rice transformation with

Table 3. Genetic analysis of R1 progeny of selected transgenic rice lines

Transformant No.	Total No. of plants analyzed	Number of PCR-positive plants				Segregation ratio	χ^2	*P*
		gna	*Xa21*	*hpt*	*gusA*			
2	39	30	–	30	30	3:1 *gna*	0.077	0.782
						3:1 *gusA*	0.077	0.782
						3:1 *hpt*	0.077	0.782
3	28	20	20	20	20	3:1 *gna*	0.191	0.663
						3:1 *Xa21*	0.191	0.663
						3:1 *gusA*	0.191	0.663
						3:1 *hpt*	0.191	0.663
5	34	15	22	nd[a]	nd[a]	1:1 *gna*	2.941	0.086
						3:1 *Xa21*	1.922	0.166
11	40	29	29	29	29	3:1 *gna*	0.133	0.751
						3:1 *Xa21*	0.133	0.751
						3:1 *gusA*	0.133	0.751
						3:1 *hpt*	0.133	0.751
12	35	26	26	26	26	3:1 *gna*	0.067	0.796
						3:1 *Xa21*	0.067	0.796
						3:1 *gusA*	0.067	0.796
						3:1 *hpt*	0.067	0.796
13	38	28	28	28	28	3:1 *gna*	0.035	0.851
						3:1 *Xa21*	0.035	0.851
						3:1 *gusA*	0.035	0.851
						3:1 *hpt*	0.035	0.851
17	34	30	–	30	30	15:1 *gna*	1.765	0.184
						15:1 *gusA*	1.765	0.184
						15:1 *hpt*	1.765	0.184
18	27	25	25	21	21	15:1 *gna*	0.062	0.804
						15:1 *Xa21*	0.062	0.804
						3:1 *gusA*	0.111	0.739
						3:1 *hpt*	0.111	0.739
20	33	15	24	12	12	1:1 *gna*	0.273	0.398
						3:1 *Xa21*	0.091	0.763
						1:1 *gusA*	2.455	0.117
						1:1 *hpt*	2.455	0.117

[a]nd, not determined

the *Xa21* gene (Song et al. 1995; Tu et al. 1998; Zhang et al. 1998). These plants were resistant to the bacterial-blight-causing pathogen *Xanthomonas oryzae* (Wang et al. 1996; Zhang et al. 1998). Similarly Rao et al. (1998) and Sudhakar et al. (1998b) generated transgenic rice lines expressing the *gna* gene. Expression of GNA at levels of up to 2.0% total soluble protein was reported in some of the transgenic plants by Rao et al. (1998). Transgenic rice plants expressing GNA caused decreased survival and fecundity in the brown plant hopper, which causes 'hopper burn', and is an important virus vector.

The rationale behind our approach was to generate elite Chinese rice cultivars carrying resistance to two unrelated yet vitally important classes of pathogen or pest, a phenotype of immense agronomic value. Cultivars Eyi 105 and Ewan 5 have been widely grown in central China, the Hubei region. Transformation of these cultivars is the first important step in the extended process of generating germplasm for breeding rice varieties simultaneously resistant to bacterial blight and the brown plant hopper. We also discuss co-integration, co-expression and co-segregation of the four genes, *Xa21*, *gna*, *hpt* and *gusA* in the transgenic lines we generated.

We used particle bombardment to deliver four genes carried on three plasmids. We obtained near 100% co-integration of the two genes (*hpt* and *gusA*) carried on the same plasmid. Of the 160 transgenic lines generated, only one line (Eyi 105 line No. 4) carried one (*hpt*) and not the other (*gusA*) gene. This indicated that genomic co-integration of genes linked on a single construct occurs at very high efficiency. A 100% co-integration frequency of two genes carried on one plasmid has been reported previously in transgenic rice plants (Battraw and Hall 1992; Cooley et al. 1995). A 75% co-integration frequency has also been reported in transgenic soybean callus (Christou and Swain 1990) and *Phaseolus vulgaris* (Russell et al. 1993). Kohli et al. (1998) investigated 16 transgenic rice lines transformed with co-integrate constructs containing 2 or 3 genes. They also verified the presence of all co-introduced genes in 100% of cases. In some of their lines transformed with the two-gene co-integrate construct, single copies of each gene integrated at adjacent sites were shown to belong to separate plasmid molecules. This interesting observation provided new insights to the mechanisms of foreign DNA integration in plant genomes.

A 100% co-integration frequency of linked multiple genes indicates that transformation using co-integrate vectors is an efficient strategy. However, the delivery of multiple genes using a single plasmid is likely to become progressively more difficult, both with respect to cloning and the delivery procedure itself, as the number of genes increases. The size of such multiple-gene plasmids may

determine the amount of transgene rearrangement, as foreign DNA is known to undergo rearrangement before or during integration (Stam et al. 1997; Kohli et al. 1998, 1999b). Although plants have been transformed with very large constructs of up to 150 kbp (Hamilton et al. 1996), the optimal size range needed to maintain a high ratio of intact to rearranged/truncated transgenic sequences is not known. Particle-bombardment-mediated co-transformation with multiple genes carried on separate plasmids was shown to be an effective strategy to deliver exogenous DNA (Hadi et al. 1996; Chen et al. 1998; Wakita et al. 1998). We delivered the two agronomically useful genes *Xa21* and *gna* on separate plasmids through co-transformation with a third plasmid carrying the marker genes. We obtained a co-integration efficiency of nearly 70%. This result is consistent with figures reported by Wakita et al. (1998) and Schocher et al. (1986).

Co-transformation efficiency is strictly defined as the expression of multiple genes, and has been shown to range from 20 to 40% in previous reports (Schocher et al. 1986; Peng et al. 1995; Li et al. 1993; Rathore et al. 1993). Wakita et al. (1998) reported 28% co-transformation efficiency in rice for the bialaphos resistance gene (*bar*) and the tobacco fatty acid desaturase gene (*NtFAD*). Chen et al. (1998) investigated the effect of plasmid ratios on co-transformation efficiency. Using the genes *hpt* and *gusA*, they concluded that the molar ratio at which the two plasmids carrying these genes were co-bombarded influenced both the total number of transgenic plants regenerated and the frequency of R0 plants expressing both genes. Varying the *hpt*:*gusA* molar ratio from 1:1 to 1:12 in three steps, they discovered that transformation efficiency (number of *hpt* resistant calli per 100 bombarded calli) decreased by nearly 10%, but the co-transformation efficiency (both genes expressing) rose from 38% (for 1:1) to 85% (for 1:12). They did not find much difference in co-transformation efficiency between the ratios 1:7 and 1:12, and used the former ratio to deliver 14 different plasmids into a model japonica variety of rice. This resulted in an average co-integration efficiency of 40% with respect to each of the individual genes. Though no R0 plant containing all 14 genes was recovered, nearly 85% of the R0 plants contained more than two genes. However, the highest co-integration efficiency of 13% was achieved for three genes. Co-transformation efficiencies could not be determined as only three independent transgenic lines were investigated, and only for the expression of 4 marker genes from the 14 genes available. In comparison, using a molar ratio of 1:3:3 for the three plasmids, we obtained a co-integration and co-transformation efficiency of nearly 70% (all four genes expressing). We have thus obtained comparable co-integration efficiency but much higher co-transformation efficiency than ever reported for two or more transgenes in rice. Moreover, our study relates to the expression of two agronomically useful genes in addition to marker genes.

The presence of transgenes (detected by PCR or Southern blot) and the absence of expression (shown by RT-PCR, western blot or enzyme activity) may indicate the integration of truncated or rearranged transgenic sequences. Kohli et al. (1999b) characterized plasmid-plasmid recombinant junctions in transgenic rice lines demonstrating frequent rearrangements. The Southern blot shown in Figure 3 represents 17 transgenic lines, one positive control (plasmid DNA, lane 1) and one negative control (genomic DNA from a wild-type plant, lane 19). Many of the plants carry the specific 4.8-kbp band corresponding to the linearized plasmid in lane 1. The presence of the 4.8-kbp band serves as a diagnostic marker and does not guarantee expression of *Xa21*. However, only three lines (5, 13 and 17) that did not contain the specific band also did not produce an *Xa21* RT-PCR product. The presence of the expected RT-PCR product in spite of the absence of the diagnostic band indicates rearrangement of the exogenous DNA. Additionally, other non-endogenous transgene-specific bands of different sizes (T) also reflect transgene rearrangements causing either truncation (smaller fragments), or the abolition of the *Hin*dIII site (larger fragments).

There are several bands of varying intensity (E) in the negative control lane, representing the presence of an *Xa* multigene family, with paralogous copies varying in their complementarity to the probe. The strongest endogenous band (9 kbp, lane 19) probably represents the gene with closest resemblance to the probe. The endogenous bands are also seen in many of the transgenic plants. The absence of some of the endogenous bands in certain lines may indicate preferential binding of the probe first to the homologous transgenic *Xa21* sequence and then sequentially to endogenous fragments of decreasing complementarity. This would result in a weak signal for some of the endogenous fragments, which may be undetectable under the Southern hybridization conditions used. It is also likely, however, that the absence of endogenous bands reflects the signal saturation in areas of the filter with a low signal-to-noise ratio. The 5-kbp endogenous band could not be detected separately in lines carrying the transgene-specific 4.8-kbp band (lines 2, 3, 4, 8, 12 and 13). Interestingly, the 9-kbp endogenous band is more intense in most of the transgenic lines. Similarly the 3-kbp band is more intense in lanes 3 and 4. Higher intensity of these bands cannot be attributed to difference in the amount of DNA loaded in each lane because the 14-kbp endogenous band is of nearly the similar intensity in all transgenic lines. A possible explanation is that transgenic *Hin*dIII fragments co-migrate with the endogenous band of similar size. However, it would be unlikely to obtain rearranged transgenic fragments of similar size in a number of lines. Alternatively, transgene integration in the vicinity of a homologous endogenous sequence may result in higher intensity of the 9-kbp band. This argument can be supported by the fact that it is the 9-kbp endogenous band that seems to have the strongest complementarity to the probe, which therefore attracts the transgenic fragments, leading to a possible 'homology-dependent integration'.

The plant in lane No. 7 (Fig. 3) contained the 4.8-kbp fragment yet the RT-PCR product could not be detected.

Lack of transgene expression may in this case be due to the phenomenon of gene silencing or loss of structural integrity of the cassette. For example, about 30% of transgenic plants carrying the *gusA* gene did not express GUS at detectable levels. We tested these R0 plants at three different stages of development (see *Results*) but did not observe GUS activity. It is possible that in some of these plants, gene silencing set in very early during development. Kohli et al. (1996) reported transgenic rice lines that expressed the *gusA* gene in callus and early leaf tissue of the R0 plant but became progressively silenced. However, we believe that in most of the plants, GUS non-expression was more likely to result from rearranged transgenic sequences.

The unique Southern hybridization bands among individual transgenic plants confirmed their independent origins. In the present study, we noted that the copy number of the non-selected (*gna*) gene was variable, ranging from 1 to 11 among independently derived transgenic plants. However, there was much less variation in the copy number of the selectable marker *hpt*. Most of the plants had low copy numbers (1–3) of the *gusA* and *hpt* genes. We believe that lower molar ratio of the plasmid carrying the *hpt* and the *gusA* gene contributed to this result.

There have been several studies concerning the relationship between transgene copy number and expression. The results have been inconclusive, with some groups showing no correlation (Shirsat et al. 1989; Van der Krol et al. 1990; Cooley et al. 1995; Hadi et al. 1996; Vain et al. 1998) some showing inverse correlation (Linn et al. 1990; Allen et al. 1993) and one report claiming positive correlation (Stockaus et al. 1987). In our study, we confirmed no correlation between transgene copy number and expression. Table 2 shows that, on the one hand, low *gusA* copy number could result in expression levels ranging from low or moderate to high. On the other hand, low, moderate or high copy numbers of *gna* exhibit comparable high expression levels (≥30.3% total cellular protein). Stoger et al. (1999) and Kohli et al. (1999a) have also made such observations.

We also noted that while the transgenic plants generated unique multiple hybridization bands on Southern blots and varied with respect to their transgene copy numbers, segregation analysis in the R1 generation generally showed transgene inheritance as a single Mendelian trait (3:1 segregation ratio). These results indicate that multiple copies of the various transforming plasmids preferentially integrate at a single locus, so that they function as, and are inherited as, a single genetic unit. Other workers have also reported the insertion of multiple genes or fragments thereof into a single locus (Hiei et al. 1994; Register et al. 1994; Cooley et al. 1995; Kumpatla et al. 1997; Kohli et al. 1998).

This appears to be a species-independent phenomenon, which is not affected by the method of gene delivery. However, as evident from Table 3, we obtained some lines with more than one transgenic locus. Co-inheritance and co-expression of the transgenes in the segregating population indicated the stability of transgene integration and expression in our lines. We observed no gene silencing in the R1 generation if the corresponding genes were expressed in the R0 generation.

In conclusion, we have generated over 160 independent transgenic rice plants by co-transformation using particle bombardment. Over 70% of our transgenic plants contained four different genes, originating from three co-transforming plasmids, and 70% of these plants co-expressed all four genes. The transgenes in most of the plants were inherited as Mendelian traits. This demonstrates particle bombardment is an effective way to deliver multiple foreign genes into the plant genome. Our studies, including not only markers but also genes of agronomic interest, represent a transition from model systems to applied approaches in plant biotechnology. We have studied two genes of agronomic interest, expressed in the same line at a much higher levels than ever reported before. This is also the first time that rice has been engineered with genes conferring resistance to two separate taxa of pests and pathogens.

A Rockefeller Foundation Rice Biotechnology Career Fellowship to K.T. is acknowledged. We thank Ms. Julie Smith for her help with glasshouse work, Dr. Duraialagaraja Sudhakar and Mr. Xiangdong Fu for helpful discussion, and Dr. Richard Twyman for help with the preparation of the manuscript. This research was funded by the Rockefeller Foundation, USA (Grant no. RF97001 #594) and China National High-Tech A863@ program (Grant No. 101-01-01-01). The JIC is supported by a grant-in-aid by the Biotechnology and Biological Sciences Research Council.

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