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Expression and inheritance of multiple transgenes in rice plants

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The ability to control integration, inheritance, and expression of multiple transgenes is a prerequisite for manipulating biosynthetic pathways and complex agronomic characteristics in plants. One hundred and twenty-five independent transgenic rice plants were regenerated after cobombarding embryogenic tissues with a mixture of 14 different pUC-based plasmids. Eighty-five percent of the R0 plants contained more than two, and 17% more than nine, of the target genes. Plants containing multiple transgenes displayed normal morphologies and 63% set viable seed. Multigene cotransformation efficiency was correlated with the ratio in which the plasmids were mixed with respect to the selectable marker. All target genes had an equal chance of integration, indicating that the nature of the coding region had no effect on the efficiency of integration. Three plant lines containing 11, 10, and 9 transgenes, respectively, were analyzed for patterns of integration and inheritance until the R3 generation. Integration of multiple transgenes occurred at either one or two genetic loci, with inheritance conforming to a 3:1 Mendelian ratio. Coexpression of four marker genes was investigated until the R2 generation.

Keywords: agricultural biotechnology, metabolic engineering, particle bombardment

The introduction and expression of foreign genes in plants by genetic transformation is now routine for many species[1]. In most cases, one or a few genes are transferred to the plant genome along with a selectable marker that facilitates selection and recovery of transgenic tissues. Genetic transformation with a single target gene has been used for the production of transgenic crop plants expressing herbicide tolerance[2,3] as well as resistance to fungal[4], viral[5,6], and bacterial[7,8] diseases and insect pests[9]. In addition, improved agronomic characteristics have been achieved by manipulating metabolic pathways through overexpression of a specific gene or the use of antisense sequences[10,11]. As most agronomic characteristics are polygenic in nature, plant genetic engineering will require manipulation of complex metabolic or regulatory pathways involving multiple genes or gene complexes. Redirecting complex biosynthetic pathways and modifying polygenic agronomic traits requires the integration of multiple transgenes into the plant genome, while ensuring their stable inheritance and expression in succeeding generations.

Integrating multiple genes by repetitive insertion of single coding sequences is impractical due to the time and effort required for recovery of the transgenic tissues and the necessity of using a different selectable marker for each new transformation event. Transfer of multiple genes via *Agrobacterium*-mediated transformation, although possible[12], is technically demanding and becomes increasingly problematic as the number of genes and the size of the tDNA increases. Cobombardment is a simple process in which genes carried on separate plasmids are mixed prior to transfer by particle bombardment. In this manner, numerous genes can be transferred simultaneously using a single selectable marker. Rice plants can be genetically transformed with two genes by cobombardment in which the coding sequences are inherited in a Mendelian manner to the R1 generation[8,13,14]. Genetic transformation with 12 different plasmids was demonstrated in soybean by cobombardment of a single "cocktail" of gene constructs into embryogenic suspension tissues[15]. However, in this case, no information was provided on the integration, coexpression, or inheritance of the transgenes in regenerated plants.

We have capitalized on an efficient transformation system available for *Japonica* rice[16] to investigate the number of transgenes that can be integrated by the rice plant. We performed cobombardment with 14 different genetic constructs resulting in the production of fertile transgenic plants. As many as 13 genes can be inserted into the rice genome by this method, and in many cases these are cointegrated at a single genetic locus. The expression of the four marker genes is stable and predictable across sexual generations.

Results

Effect of plasmid ratios on cotransformation efficiency. Embryogenic callus and embryogenic suspension-derived cell aggregates of the *Japonica* rice variety Taipei 309 were used as target tissues. To ensure that each transgenic plant was derived from an independent transformation event, only one plant was recovered from each hygromycin B resistant (hyg^r) callus or suspension-derived cell aggregate.

Parameters for efficient cobombardment of multiple transgenes in rice were established by bombarding DNA mixtures with different ratios of plasmids carrying hygromycin phosphotransferase gene (*hph*, conferring resistance to hygromycin B) and β-glucuronidase (GUS) gene (*uidA*), into embryogenic tissues. The molar ratio at which the two plasmids were cobombarded influenced both the total number of transgenic plants regenerated and the frequency of R0 plants expressing both genes (Table 1). As the molar amount of the *uidA* plasmid was increased relative to the plasmid containing the *hph* gene, fewer transgenic plant lines were recovered, but the efficiency of coexpression increased. A 1:1 (*hph:uidA*) ratio resulted in the recovery of 29 independently obtained transgenic R0 plants, 38% of which showed a positive GUS staining reaction; cobombardment at a 1:12 ratio produced 20 plants, 85% coexpressed both genes.

Cobombardment with multiple genes. Embryogenic rice tissues were initially transformed with four different coding sequences. The three coat protein genes *CP1, CP2,* and *CP3* from the rice tungro spherical virus (RTSV), were cloned into separate pUC18/19 vectors and cobombarded with the *hph* gene. Cobombardment took place at a molar ratio of 1:2:2:2 (*hph:CP1:CP2:CP3*). 89% of the 90 independent hyg^r R0 plant lines recovered contained more than one gene and 55% carried all four transgenes, as determined by PCR. The frequency of insertion of each gene was similar, varying from 74% for the *CP1* coding region, to 79% and 67% for *CP2* and *CP3*, respectively (data not shown).

To determine how many transgenes could be integrated simultaneously into the rice genome, 14 plasmids—including the *hph*; *uidA*; phosphinotricine acetyl transferase (*bar*); firefly luciferase (*luc*); green fluorescent protein (*GFP*) genes; and nine plasmids containing different coding sequences from RTSV, the rice yellow mottle virus (RYMV), the rice tungro bacilliform virus (RTBV)—were cobombarded into embryogenic rice tissues. All coding sequences were cloned into pUC-based vectors. As no significant difference was observed between the cotransformation efficiencies for plasmid ratios of 1:7 and 1:12 in the previous experiment (Table 1), the genetic constructs were cobombarded at a 1:9 molar ratio, representing approximately a 1:0.7 molar ratio between the selectable marker gene and each target gene. Twelve plates of embryogenic rice material were bombarded, leading to the regeneration of 140 independent R0 hyg^r plant lines, recovered 8–9 weeks after gene transfer.

PCR analysis of R0 plants. One hundred and twenty-five R0 plant lines were randomly selected for analysis from the 140 regenerated lines. PCR assays were performed with primers specific for each of the coding sequences, except that of the *bar* gene. Efficient PCR analysis for the latter was problematic in our hands, and gene expression was confirmed by resistance to the herbicide BASTA[17].

One hundred twenty-five R0 plants were found to contain 1–13 of the cobombarded coding sequences (Fig. 1A). No escapes were detected and all plants contained at least the *hph* selectable marker gene. Presence of only the *hph* gene was relatively rare, such that 94% of the plants tested were found to contain two or more coding sequences. The percentage of plants transformed with multiple genes formed a skewed, normal distribution with 75% of the R0 plants containing between two and eight differ-

Table 1. Effect of plasmid DNA ratios on efficiency of coexpression of the *hph* and *uidA* genes.

Molar DNA ratios *hph:uidA*	Number of hyg^r lines regenerated	Number of hyg^r lines/100 bombarded calli±SD	% of hyg^r lines expressing GUS±SD
1:1	29	32.2±5.1^a	37.7±7.8^a
1:4	26	28.9±1.9^a	61.6±5.7^b
1:7	20	22.2±6.9^{a,b}	80.4±2.8^c
1:12	20	22.2±1.9^b	85.9±14.3^c

Data are from three petri dish replicates each containing 30 callus explants. $^{a-c}$Significant differences at the 5% level using Student's *t*-test . Plasmid pILTAB310 carries the *hph* gene under the control of the CaMV35S promoter. The *uidA* gene was contained within pRNB114 and driven by the RTBV (the rice tungro bacilliform virus) promoter. hyg^r: hygromycin B resistance.

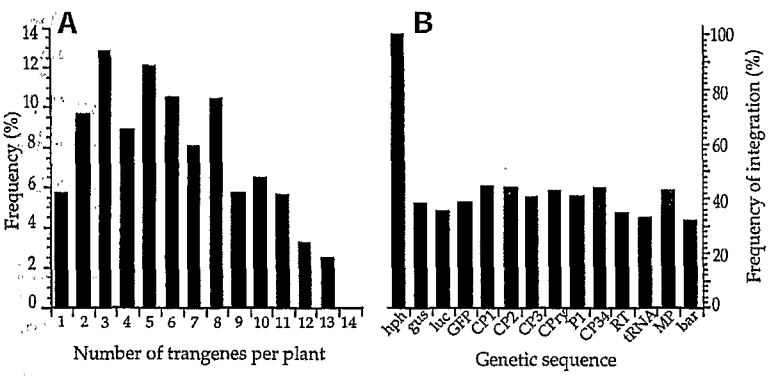

Figure 1. Identification of transgenes in R0 rice plants by PCR. (A) Frequency of transgenic plants containing 1–14 genes. (B) Insertion frequency for each transgene in rice plants. Presence of the *bar* gene was assessed by resistance to the herbicide BASTA.

Table 2. Profile of R0 plant lines containing 13 and 3 transgenes as determined by PCR analysis.

Number of transgenes	Transgenic lines	Transgenes detected													
		hph	*uidA*	*luc*	*GFP*	*CP1*	*CP2*	*CP3*	*CPry*	*P1*	*CP34*	*RT*	*tRNA*	*MP*	*bar*
13	C35	+	+	+	-	+	+	+	+	+	+	+	+	+	+
	C58	+	+	+	+	+	+	+	+	+	+	+	+	+	-
	C82	+	+	+	+	+	+	+	+	+	+	-	+	+	+
3	C9	+	-	-	-	-	-	+	-	-	-	-	+	-	-
	C13	+	-	+	-	+	-	-	-	-	-	-	-	-	-
	C56	+	-	-	-	-	-	+	-	-	-	-	-	+	-
	C59	+	+	-	-	-	-	-	-	-	-	-	-	+	-
	C102	+	-	-	-	-	-	+	-	-	+	-	-	-	-
	C107	+	-	+	-	-	-	-	+	-	-	-	-	-	-
	C133	+	-	-	+	-	-	-	-	-	-	-	-	+	-
	C154	+	-	-	-	-	-	-	-	-	+	-	-	-	+
	S29	+	-	-	-	-	-	+	-	-	-	-	-	-	+
	S39	+	-	+	+	-	-	-	-	-	-	-	-	-	-
	S73	+	-	-	-	-	-	+	-	-	-	-	-	-	+
	S92	+	-	-	-	-	-	-	+	+	-	-	-	-	-
	S97	+	+	-	-	-	-	+	-	-	-	-	-	-	-
	S112	+	-	-	-	+	-	-	-	-	-	-	+	-	-
	S140	+	-	-	-	+	-	-	-	-	+	-	-	-	-
	S142	+	+	-	-	-	-	-	-	-	+	-	-	-	-
	S152	+	+	-	-	-	-	-	-	-	+	-	-	-	-

hph: hygromycin phosphotransferase gene, *uidA*: β-glucuronidase gene, *luc*: firefly luciferase gene, *GFP*: green fluorescent protein gene, *CP1*: RTSV (the rice tungro spherical virus) CP1 coding sequence, *CP2*: RTSV-CP2, *CP3*: RTSV-CP3, *CPry*: RYMV (the rice yellow mottle virus) CP, *P1*: RYMV-P1, *CP34*: RTBV (the rice tungro bacilliform virus) CP, *RT*: RTBV reverse transcriptase, tRNA: DNA replication initiation site on RTBV, *MP*: RTBV putative movement protein. *bar*: phosphinotricine acetyl transferase gene, PCR not performed, insertion assessed by application of BASTA.

ent genes. Although no plants were recovered that contained all 14 genes, 22 R0 plants (17.6%) contained 10 or more genes, and three plants contained 13 different target genes. The independent nature of each R0 plant line was confirmed by its transgene profile. Information from the R0 plants containing 13 and 3 transgenes (Table 2) shows that 19 out of the 20 plant lines contained a unique set from the 14 plasmids included in the cobombardment mixture.

Data from PCR of the R0 plants were analyzed to determine the frequency of transformation for each individual plasmid (Fig. 1B). Other than the *hph* gene, for which positive selection was applied, no significant differences were seen in the frequency of incorporation for the remaining 13 coding sequences. Each gene had an equal chance of insertion, and all were introduced at a frequency of 33% to 44% (mean, 39.4 ± 4.0%).

Growth and fertility of R0 plants. Hyg^R R0 plants were grown to maturity in the greenhouse. All plants, except one, had normal morphology and growth habit compared with nontransgenic controls. Although all three plants containing 13 coding sequences were sterile, four, six, and two fertile lines were recovered containing 10, 11, and 12 transgenes, respectively. Overall, 63.5% of the plants set viable seed (Fig. 2A). No correlation was observed between sterility and the number of coding sequences integrated, nor was the overall frequency of sterility higher than for R0 transgenic *Japonica* plants produced from previous experiments under laboratory and greenhouse conditions[16].

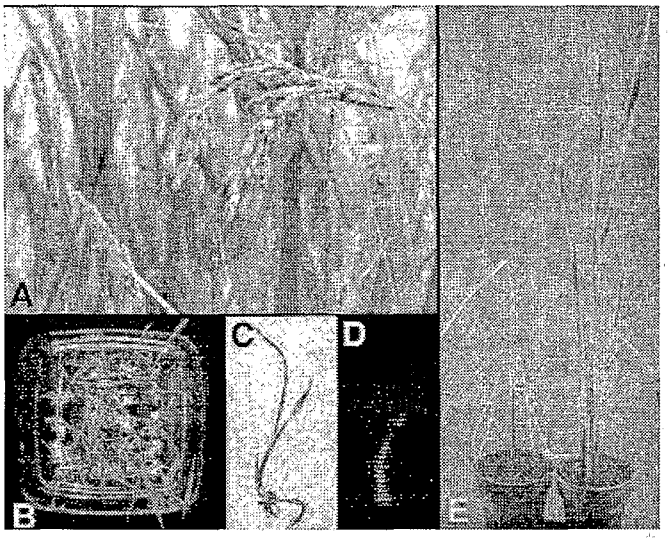

Figure 2. (A) Fertile rice plants containing 12 transgenes in the greenhouse. (B) R2 plantlets of C27-19 on medium containing 50 mg/L hygromycin B. (C) GUS activity in a C27-19 plantlet. (D) Luciferase activity in a C27-19 plantlet. (E) A C27-19 plant (right) and a nontransgenic control plant (left) 7 days after the application of the herbicide BASTA.

Segregation of integrated DNA in the R1 generation. Segregation of the introduced genetic material was investigated by germinating seeds from two R0 lines on 1/2 Murashige and Skoog (MS) medium and performing PCR analysis on 20 R1 seedlings. Lines C27 and C69 were chosen for study as they contained 10 and 9 different coding sequences in the R0 plants, respectively. PCR confirmed that in both these plant lines all the transgenes exhibited a 3:1 Mendelian segregation ratio in the R1 generation (Table 3).

Line S88 was also studied for segregation of its 11 transgenes. In this case the R1 seedlings were screened for expression of the *uidA* and *hph* genes. Sixty-seven from a total of 92 R1 seedlings tested were GUS positive and hyg^r, representing a 3:1 ratio for coexpression of the two genes. Twenty of the 67 *uidA*- and *hph*-expressing plantlets were analyzed further, and all were subsequently confirmed by PCR to contain both the *hph* and *uidA* coding sequences, in addition to eight of the nine remaining transgenes present in the R0 generation (Table 3). The ninth, *RT* (RTBV reverse transcriptase), was detected in only 13 of the 20 R1 plantlets analyzed. This indicated that, in the case of C27 and C69 R1 plants, all the introduced genes had cointegrated at a single genetic locus, while in S88, gene integration would appear to have occurred at two separate loci. We presume, therefore, that the *RT* coding sequence inserted at one site and the 10 remaining transgenes at a second locus, or in close proximity (genetically linked).

Southern blot analysis of inherited transgenes. Southern blot analysis was performed to estimate transgene copy number and to confirm the stability of integration of the genetic sequences over several plant generations. DNA hybridization was performed on C27, C69, and S88 for seven of the transgenes common to these lines. One plant from each line was analyzed by Southern blotting at the R0, R1, and R2 generations for the *hph*, *luc*, and *CP3* genes (Fig. 3), and for the *CP1*, *CP2*, *RT*, and *uid*A genes in the R1, R2, and R3 generations, respectively.

All transgenes showed a stable pattern of integration through the three generations. The three plant lines produced different integration patterns, confirming that they resulted from different transformation events, while stable integration of the transgenes was confirmed by the identical banding patterns across the generations studied. At least one intact gene cassette was identified for each gene, except for the *CP3* coding sequence in S88 and the *luc* gene in C69. Gene copy numbers between one and four were estimated for all seven of the transgenes from the three lines analyzed.

Expression of marker genes in R0, R1, and R2 transgenic plants. R0, R1, and R2 plants from C69, C27, and S88 were investigated for coexpression of the *hph*, *uidA*, *bar*, and *luc* genes (Table 4; Fig. 2B–E). Coexpression of these genes in the transgenic plants conformed to the pattern of the PCR and Southern blot analysis, and were as predicted by a 3:1 Mendelian segregation ratio (Tables 3 and 4; Fig. 3).

Table 3. Segregation of coding sequences in R1 transgenic rice plants.

Plant lines tested	Number of progeny containing coding sequence as confirmed by PCR Coding sequences *hph*	*uidA*	*luc*	*GFP*	*CP1*	*CP2*	*CP3*	*CPry*	*P1*	*CP34*	*RT*	*tRNA*	*MP*	*bar*
C69 R0	+	+	+	–	–	+	+	–	+	–	+	–	+	+
R1	14+	14+	14+	0+	0+	14+	14+	0+	14+	0+	14+	0+	14+	14+
	6–	6–	6–	20–	20–	6–	6–	20–	6–	20–	6–	20–	6–	6–
C27 R0	+	+	+	–	–	+	+	+	+	–	+	–	+	+
R1	14+	14+	14+	0+	0+	14+	14+	14+	14+	0+	14+	0+	14+	14+
	6–	6–	6–	20–	20–	6–	6–	6–	6–	20–	6–	20–	6–	6–
S88 R0	+	+	+	–	+	+	+	-	+	+	+	+	+	+
R1	20+	20+	20+	0+	20+	20+	20+	0+	20+	20+	13+	20+	20+	20+
	0–	0–	0–	20–	0–	0–	0–	20–	0–	0–	7–	0–	0–	0–

Abbreviations as in Table 2.

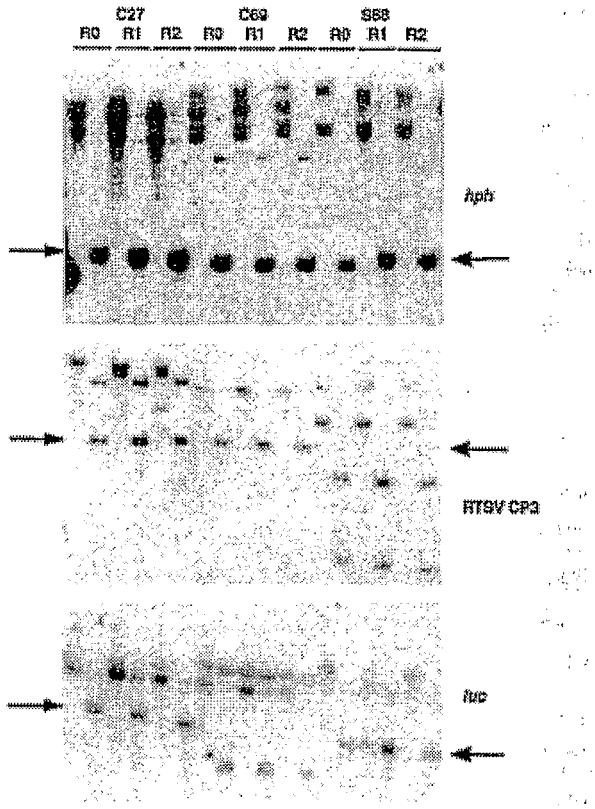

Figure 3. Southern blot hybridization of the *hph*, RTSV-CP3, and *luc* genes from R0, R1, and R2 plants of C27, C69, and S88. Two restriction enzyme digestions were performed on each DNA; in the first lane of each sample, a single cutting enzyme was used and in the second a double digestion, with one or two enzymes, was performed to liberate the whole cassette (SphI and SstI for *hph*, EcoRV and EcoRV+SstI for RTSV-CP3, and HindIII and AflI+HindIII for *luc*). Arrows indicate the expected size of each cassette.

Table 4. Expression of the *hph, uidA, bar, luc* genes in successive generations of transgenic rice plants.

Plant lines tested	Number of plants expressing marker gene *hph*	*uidA*	*bar*	*luc*	Ratio	X^2
S88 R0	+	+	-	+		
R1	16+/4-	16+/4-	-	16+/4-	3:1	0.267*
R2 S88/20	15+/5-	15+/5-	-	15+/5-	3:1	
C27 R0	+	+	+	+		
R1	14+/6-	14+/6-	14+/6-	13+/7-	3:1	0.267*
R2 C27/1	17+/3-	17+/3-	17+/3-	17+/3-	3:1	1.067
C27/19	16+/4-	16+/4-	16+/4-	16+/4-	3:1	0.267*
C69 R0	+	+	+	+		
R1	14+/6-	14+/6-	14+/6-	14+/6-	3:1	0.267*
R2 C69/1	20+/0-	20+/0-	20+/0-	20+/0-	1:0	
C69/4	20+/0-	20+/0-	20+/0-	20+/0-	1:0	
C69/6	16+/4-	16+/4-	16+/4-	16+/4-	3:1	0.267*
C69/15	20+/0-	20+/0-	20+/0-	20+/0-	1:0	
C69/18	20+/0-	20+/0-	20+/0-	20+/0-	1:0	

*X^2 tests indicate good agreement with segregation ratios of 3:1.

In the case of C69, four out of 14 R2 plants tested were homozygous for all four of the marker genes. Presence, but not expression, of the *luc* gene was observed in one of the C27 R1 plants and in some R2 plants (data not shown). As expression of this gene was confirmed in the R0 generation, gene silencing occurred in this line. This was the only example of gene silencing observed in the plants investigated. In C69 plants, *luc* expression, although still detectable, was 25 to 30 times lower than in C27 and S88, and may be related to the loss of part of this gene cassette, as shown by the Southern blot (Fig. 3).

Discussion

Mixing 14 different plasmid DNAs prior to particle bombardment and regeneration of R0 rice plants, resulted in plants that contained multiple transgenes, grew normally, and were fertile. Among the transgenic plants studied, analysis of segregation patterns showed integration of the multiple target genes to have occurred at one or a few loci, resulting in stable and predictable patterns of inheritance and expression through three sexual generations.

The efficiency of multiple-gene cointegration might be improved further by manipulating the selectable marker:target gene ratio. This variable has not been investigated in previous reports, and most often the plasmid ratios have been fixed at between 1:1 and 1:3 (refs. 13–15). In this study, increasing the amount of target gene from a 1:1 to a 1:12 ratio with respect to the selectable marker, more than doubled the percentage of R0 plants expressing both transgenes (Table 1). Cotransformation with 14 genes effectively took place at a 1:0.7 ratio with respect to each of the individual genes. This resulted in an average cotransformation efficiency of 39.4% (Fig. 1); a value very similar to the 1:1 ratio recorded in the *hph/uidA* experiment. Efficiencies greater than 39% might be achieved for multigene insertion by increasing the amount of target gene DNA in relation to the selectable marker. Furthermore, the skewed normal distribution curve of multiple gene insertion (Fig. 1A) indicates that the maximum number of insertable genes has probably not been reached, and that plants containing greater than 13 genes could be recovered if more plants were to be regenerated.

Coexpression of the *hph, uidA, luc,* and *bar* marker genes was stable over three generations (Table 4) and correlated with the presence of the respective genes. Gene silencing was only observed in the case of the *luc* gene in some of the C27 plants, and was therefore not a widespread phenomenon despite the large number of homologous sequences used. Integration and the behavior of multiple transgenes inserted by cobombardment was therefore similar to that for a single gene, and exhibited the patterns of stable and predictable inheritance necessary for incorporation into crop breeding programs.

Integration of many genes at one or a few loci could not have taken place by chance alone; however, the mechanisms governing this phenomenon are not known. The coding sequences may have been inserted individually at the same locus, or the plasmid DNAs may have become joined, either by ligation or recombination, prior to the integration event. Plasmids with high levels of sequence homology (70–90%, excepting pDO432 and pHX4), were intentionally used in this study. The fact that all the genes had an equal chance of being integrated indicates that the nature of the coding sequence did not influence efficiency of the integration process.

Southern blot analysis indicated that in most cases gene copy numbers ranged from one to four for each of the transgenes investigated (Fig. 3). Estimates can be made, therefore, that segments of DNA between 78 and 300 kb in length were integrated. An upper limit for the total amount of foreign DNA, which can be inserted into the rice genome is not known, but incorporation of such large segments is compatible with direct genetic transformation of DNA fragments contained in bacterial artificial chromosome (BAC) vectors, which are generally between 100 and 200 kb in length[18].

While *Japonica* rice was chosen as the model plant for these studies, we would predict that other plant species will be amenable to cotransformation with multiple genes.

Experimental protocol

Plant material. *Oryza sativa* L. *Japonica* cv. Taipei 309 was used. Both

embryogenic callus and embryogenic suspension cell aggregates derived from mature seeds were used as target tissues as described[16,19,20]. Embryogenic callus was subjected to particle bombardment 2 months after initiation; cell suspension cultures were bombarded 6 weeks after transfer to liquid medium. Osmotic treatment, selection, and regeneration of transformed plants were carried out as reported[8,16,19]. To ensure that each plant resulted from an independent transformation event, only one plant was recovered from each bombarded callus or cell aggregate.

Plasmids and particle bombardment. Plasmid pHX4 containing the *hph* gene (J. Finer, Ohio State University, Columbus, OH) was cobombarded with plasmids pAHC27 containing the *uidA* gene (P. Quail, Center of Gene Expression, Albany, CA); pDO432 carrying the *luc* gene[21]; pAHC20 containing the *bar* gene (P. Quail); pRNB135 with *RT*; and pILTAB326, 219, 220, 221, 269, 306, 209, 285, and 343 containing the gene for green fluorescent protein, *CP1* (RTSV-CP1), *CP2* (RTSV-CP2), *CP3* (RTSV-CP3), *CPry* (RYMV-CP), *P1* (RYMV-P1), *CP34* (RTBV-CP), tRNA (DNA replication initiation site on RTBV), and *MP* (RTBV putative movement protein), respectively. All the genes or coding sequences were cloned into separate pUC-based vectors, under the control of Ubiquitin promoter, except for the *hph* and *luc* genes, which were driven by the CaMV 35S promoter. The 14 plasmids varied from 5.5–7.2 kb in size, and the homology (common sequence) across all the plasmids ranged from 70–90% except pHX4 (45%) and pDO432 (41%). The plasmid DNAs were mixed at a 1:9 molar ratio (*hph*:total target DNA) such that 0.52 µg of pHX4 was mixed in an Eppendorf tube with equimolar amounts of the remaining 13 plasmids to make a total of 5 µg DNA. In the *hph/uidA* experiment, plasmid DNAs were mixed at 1:1, 1:4, 1:7 and 1:12 molar ratios prior to bombardment. Embryogenic tissues (80–100 pieces) were arranged to form a 2-cm-diameter circle in the center of a petri dish, and each sample bombarded twice at 1100 psi and 27 in. of Hg vacuum[16].

Analysis of transgenic plants. DNA was extracted and purified according to Dellaporta et al.'s procedure[22]. Presence of all transgenes except *bar* was verified by PCR. The primer sequences for PCR were as follows: *hph*, forward sequence (F) 5′-CGTCTGTCGAGAAGTTTC-3′, reverse sequence (R) 5′-TACTTCTACACAGCCATC-3′; *uidA*, F 5′-GGTGGGAAAGCGCGTTACAA-3′, R 5′-GTTTACGCGTTGCTTCCGCCA-3′; *luc*, F 5′-GAGGATGGAACCGCTGGAGAGCAA-3′, R 5′-CTTCTTGGCCTTTATGAGGATCTC-3′; GFP, F 5′-CACTGGAGTTGTCCCAATTCTTG-3′, R 5′-CGGCCGCGACTAGTTCATCCATGC-3′; *CP1*, F 5′-GCTGGCGAAACGGTCAT-3′, R Nos (*Nos* terminator reverse sequence, 5′-GTAACATAGATGACACCGCG-3′); *CP2*, F 5′-GAGCGGGGGTTTTGAAGA-3′, R Nos; *CP3*, F 5′-GGATTTTGGAAGAAGCCT-3′, R Nos; *CPry*, F 5′-AAGATGGCCAGGAAGGGC-3′, R Nos; *P1*, F 5′-GCATCGTGTATGACACGGTT-3′, R Nos; *CP34*, F 5′-TCCCATAGAAGCCTCAA-3′, R 5′-GGATCTACAGAATGCTAAGGATGC-3′; *RT*, F 5′-TACGACTCTGATACCTAACAACAA-3′, R Nos; tRNA, F 5′-GAATCAGAAAGAAAGCATACC-3′, R Nos; *MP*, F 5′-AGAACTATAACCCAAGATTCAACC-3′, R 5′-TAAGGATTCTCCTCCTTGTAAATT-3′. PCR conditions were those recommended by the Taq DNA polymerase manufacturer (Life Technologies, Gaithersburg, MD). Resistance to the herbicide BASTA was used to detect insertion and expression of the *bar* gene.

Southern blot analysis. Appropriate enzymes were used to liberate the entire cassette (promoter::target gene::terminator) to confirm integrity of the transgenes, and an enzyme that cut once within the plasmid was used to estimate transgene copy number. DNAs were electrophoresed on 0.8% agarose gels, transferred onto Hybond N or N$^+$ membranes (Amersham, Arlington Heights, IL) and hybridization and washing carried out as described by Sambrook et al.[23] DNA probes, approximately 1 kb in size, consisted of a restricted fragment or a purified PCR amplified product from each target gene. Probes were randomly radiolabeled with nucleotides (Amersham) using the Prime It II kit (Stratagene, La Jolla, CA). After washing, the membranes were placed in a cassette on Kodak XAR-5 film with intensifying screen at -80 °C to visualize restriction patterns.

Expression of transgenes. Analysis of transgene expression was carried out using in vitro grown plants. For the R0 generation, tissues were taken from regenerated plants. For R1 and R2 material, sterile seeds were placed on 1/2 MS medium that contains half strength MS basal salts[24] supplemented with 10 g/L sucrose and 2.5 g/L Phytagel, and analysis was carried out on leaf tissues 2 weeks after germination. For *hph* analysis R0 plantlets were assessed after 14 days' growth on 1/2 MS medium containing 50 mg/L hygromycin B. R1 and R2 seeds were germinated on 1/2 MS medium over a 1 week period, after which seedlings were transferred to 1/2 MS medium containing 50 mg/L hygromycin B and left for 7 days. Plantlets that grew normally were scored as positive for expression of the *hph* gene[16]. For *uidA* analysis, GUS activity was determined by application of 5-bromo-4-chloro-3-indolyl-beta-D-glucuronide acid (X-Gluc) (Biosynth AG, Naperville, IL) and assessed according to Jefferson et al.[25] For *luc* analysis, luciferase activity was assessed as described[21] except that 1 mM dithiothreitol was dissolved in water instead of buffer. Measurement of luciferase activity was performed with a luminometer (Turner TD-20e) 10 s after addition of the substrates (Analytical Luminescence Lab, Ann Arbor, MI). For *bar* analysis commercial herbicide (BASTA) containing 60 mg/ml of ammonium glufosinate, the ammonium salt of phosphinothricin (PPT), was used to test for gene expression. Three leaves were scored with a marker pen approximately 5 cm below the tip (to facilitate penetration of the cuticle) and dipped into 750 ppm BASTA to a depth of 10 cm below the tip for 5 s. Plants were incubated in growth-chamber at 32 °C by day, 22 °C by night, and at 95% humidity, and were scored for resistance 4 days after herbicide application. Resistant plants showed no response, while the marked region in susceptible types became necrotic.

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