

Introduction of multiple genes into elite rice varieties—evaluation of transgene stability, gene expression, and field performance of herbicide-resistant transgenic plants

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Transgenic rice plants from indica, japonica, and javanica varieties that express several foreign genes were generated via particle gun bombardment of immature embryos. Molecular and genetic characterization of large numbers of these plants (more than 500 independent transgenic plants) provided information on structure, expression, and stability of integrated DNA through multiple generations. Such evaluations were carried out in the greenhouse (five generations) and in the field (three generations). Stability of foreign DNA was found to be dependent on the nature of the promoter and the transgene, and in specific cases, on gene copy number. A general conclusion of the results presented here is that direct DNA transfer utilizing electric discharge particle bombardment for the delivery of foreign DNA into rice tissue results in the recovery of large numbers of independently derived transgenic plants in a variety-independent fashion.

Rice is one of the world's most important food crops. Accordingly, great effort has been directed toward biotechnology to complement conventional breeding methods. Previously, we reported the recovery of transgenic rice plants using direct gene transfer of exogenous DNA into immature zygotic embryos by electric discharge particle acceleration (Christou et al 1991, 1992). The method bypasses traditional variety-dependent, tissue-culture procedures involving protoplast and embryogenic suspension cultures (Zhang and Wu 1988, Shimamoto et al 1989) thus enabling the rapid recovery of desirable transgenic phenotypes in agronomically important cultivars.

A number of advantages make microprojectile bombardment the method of choice for engineering major agronomic crops:

- Stable transformation of a wide range of cell types in organized tissue is possible. The ability to engineer organized and easily regenerable tissue permits introduction of foreign genes into elite germplasm directly. Consequently,

backcrossing is not required to restore the original line as compared with other transformation methods limited by genotype and host specificity. Recovery of transformed R_1 seed is considerably shortened and this saving in time is of paramount importance in commercial programs where timing is vital in bringing products to market.

- Transient gene expression has been demonstrated in almost all tissues for many crops, and this provides an easy method for rapid gene expression studies.
- Stable transformation of recalcitrant species: most of the important agronomic crops, including previously recalcitrant legumes and cereals can be engineered effectively only through bombardment-based technologies. In the case of soybean, *Agrobacterium* host specificity restricts utilization of this technology to specific varieties that are of no commercial importance (Hinchee et al 1988); for cotton, tissue culture limitations only allow engineering of a specific variety (Umbeck et al 1987, Finer and McMullen 1990). Wheat transformation using electroporation of protoplasts resulted in the recovery of infertile plants (He et al 1994). Bombardment-based methodology allowed effective engineering of important species such as soybean (Christou et al 1990), cotton (McCabe and Martinell 1993), maize (Gordon-Kamm et al 1990, Fromm et al 1990), wheat (Vasil et al 1993, Weeks et al 1993), barley (Wan and Lemaux 1994), and others (Vain et al 1996). Breakthroughs have been the consequence of improved transgene engineering (Callis et al 1987, Christensen et al 1992), efficient selection (Gordon-Kamm et al 1990, Fromm et al 1990), screenable markers (Jefferson et al 1987), and reliable tissue culture techniques (Christou et al 1991, Weeks et al 1993).
- Study of basic plant developmental processes: by utilizing chromogenic markers, it is possible to study developmental processes and also better clarify the origin of the germline in regenerated plants (Christou and McCabe 1992).

A number of factors were found to influence successful particle and DNA delivery into regenerable tissues of rice. Parameters examined included condition of the explant prior to bombardment, environmental factors including temperature and humidity, and influence of such parameters on transient activity of the *uidA* marker gene; and depth of particle penetration and degree of tissue damage as a function of accelerating voltage and effect of selective agents. Experiments were carried out to develop a selection system that would eliminate the need to screen all regenerated plants derived from transformation experiments. These studies have been reported elsewhere (Christou et al 1995a,b,c). Selection procedures were optimized to eliminate potential escapes resulting from cross-protection of wild-type cells. This was achieved effectively by incorporating a secondary selection phase (P. Vain et al, pers. commun.).

Until recently, the key barrier in achieving effective transformation of agronomically important species was the DNA delivery method. Particle bombardment has had a tremendous impact on this limitation. The challenge now is shifting to the biology of the explant used in bombardment experiments and also to the integration and subsequent expression of transgenes. It is apparent that conversion frequency of transient to stable transformation events is low. This makes recovery of large numbers

of independently derived transformation events labor-intensive and rather expensive. More attention needs to be paid to the biology of explants prior to and following bombardment. We need to identify how more cells can be induced to become competent for stable DNA uptake and regeneration. Optimization of biological interactions between physical parameters and target tissue needs to be better studied and understood. Not much is known about the fate of DNA from the time particles are introduced into plant cells. Recipient tissue variation and variability due to bombardment conditions complicate the picture even further. Additional issues such as irregular particle size and uniformity as well as improvements in hardware design need also to be addressed. However, recent results from a number of different laboratories are beginning to address some of these problems. Further progress in plant transformation techniques would also depend on developing techniques that allow controlled integration and expression of foreign DNA into the plant genome (Finnegan and McElroy 1994).

Once an efficient transformation system has been developed, it is necessary to assess the fate and function of the exogenous genes in the host organism and its progeny. Of particular interest are the structure and expression of unselected genes, which often encode the desired transgenic phenotype. Goto et al (1993) demonstrated cointegration and coinheritance of selected and unselected exogenous genes transformed on separate plasmids. We also reported cointegration and coinheritance as well as stable coexpression of multiple genes transferred on a single plasmid into rice by electric discharge particle acceleration (Cooley et al 1995).

The transformation method

Ten to fifteen-day-old rice immature seeds from various cultivars (Table 1) were harvested from expanded panicles and sterilized with 2% sodium hypochlorite for 5 min. They were subsequently rinsed repeatedly with sterile distilled water and the glumes were removed under a dissecting microscope. In some cases, immature seeds were resterilized for 10 min as described above. The embryos were then aseptically removed and plated on MS or CC media supplemented with 0.5 or 2.0 mg 2,4-D L⁻¹, respectively, with the adaxial side in contact with the medium. Particle bombardment was carried out as described elsewhere (Christou et al 1991). Explants were bombarded at an accelerating voltage of 14-16 kV. Following bombardment, embryos were plated on fresh medium, and embryogenic calli and plantlets were recovered as described (Hartke and Lorz 1989, Datta et al 1990). Transformed calli and plants were recovered under selective conditions. Explants were plated on hygromycin-containing media (usually 50 mg L⁻¹) at 2 d following bombardment. Once tissue was subjected to selection pressure, this pressure was maintained throughout the proliferation and regeneration phase.

Differential responses of the explants were observed depending on the input plasmid and timing of selection. As expected, nontreated embryos and embryos bombarded with plasmids not conferring resistance to hygromycin or only gold particles (no DNA) died within 48 h of plating on hygromycin-containing media. Explants bombarded with the hygromycin-resistance plasmid proliferated when plated on media

Table 1. Transformation frequencies, genotypes, and fertilities of transgenic plants generated through electric discharge particle bombardment.

Genotype	Explants (no.)	Independently derived transformed plants (no.)	Transformation frequency	Fertility		
				75-100%	50%	Sterile
Gulfmont	650	108	17	94	14	0
Koshihikari	600	93	16	52	41	0
Sasanishiki	300	48	16	29	15	4
CM101	250	42	17	40	2	0
S201	200	36	18	8	28	0
IR26	100	9	9	2	7	0
IR36	350	28	8	22	5	1
IR54	300	67	22	52	15	0
IR72	900	131	15	82	35	14
Cypress	200	23	12	23	0	0
Bengal	200	8	4	8	0	0
Lido	250	15	6	2	8	5
Carnaroli	50	1	2	1	0	0
Thaibonnet	50	3	6	1	0	2
ITA212 ^a	766	20	3	nd	nd	nd
WAB56-104 ^a	627	3	0.5	nd	nd	nd
Lac26 ^a	493	5	1	nd	nd	nd
IDSA6 ^a	408	8	2	nd	nd	nd

^aThese results include optimization experiments.

supplemented with the antibiotic. In parallel experiments in which a plasmid conferring resistance to the herbicide Bialaphos (or glufosinate) was introduced into rice immature embryos, similar results were obtained, i.e., when the bombarded explants were plated on media supplemented with hygromycin, they died, but proliferated when cultured in the presence of Bialaphos.

To monitor and confirm transformation events conveniently, a *uidA* gene (Jefferson et al 1987) was linked to the hygromycin (*hpaIV*) gene. The plasmid containing both genes as well as a third gene (*bar*) was introduced into rice immature embryos and transformation events were monitored using a GUS histochemical assay. The timing of selection was shown to influence the phenotype of transgenic callus lines, and subsequent recovery of transgenic plants. In experiments in which immature embryos were bombarded and plated on hygromycin at various times following bombardment, we observed that a small number of transformed cells on each explant allowed proliferation of wild-type cells by effectively detoxifying the antibiotic. As a result calli from these explants were shown to be chimeric. When selection was delayed for up to 10 d following bombardment, we obtained chimeric calli comprising of transformed and nontransformed cells. Subsequently, when plants regenerated from these cultures, only a small fraction of them was shown to be transformed. In experiments in which selection was applied early, i.e., 48 h following bombardment, we recovered transformed embryogenic calli, as shown by GUS activity. All transgenic plants recovered under the conditions of our experiments were clonal in nature, segregating in a Mendelian fashion in the R₁ generation. The selection procedure was

optimized further by incorporating a secondary selection step to allow complete elimination of wild-type cells, which were cross-protected by transformed cells overexpressing the gene(s) for detoxification of the selectable marker(s). More details on the selection procedure will soon be found in the primary literature (P. Vain et al, pers. commun.).

Table 1 summarizes transformation frequencies, genotypes, and fertilities of transgenic plants generated through these procedures.

Recently, transgenic plants from four West African varieties were recovered. These plants were engineered with the Oryzacystatin-1-delta-86 gene (Urwin et al 1995), which confers resistance to parasitic nematodes. Western blots confirmed expression of detectable levels of the gene in root tissue. Biochemical analysis of these plants will be reported elsewhere (P. Vain et al, pers. commun.).

Transgene integration, levels of expression, and stability

Transgenic plants transformed with a single plasmid containing the selectable gene hygromycin phosphotransferase (*hpaIV*) and one or two unselected genes (*uidA* coding for β -glucuronidase or *bar* coding for phosphinothricin acetyltransferase) were analyzed by Southern blot analyses to determine copy number and cointegration frequencies. Coexpression frequencies for selected and unselected genes were also determined. The physical linkage of transgenes was confirmed by Southern blot patterns and cosegregation of transgenic phenotypes in the R_1 and R_2 generations. In one group of experiments (Cooley et al 1995), Southern blot analysis of primary transformants was used to identify 56 independent transformation events. The analysis was designed to characterize independent events by comparing patterns of bands, which include both the chimeric gene insert and flanking rice genomic DNA. Cointegration of selected and unselected marker genes was determined by probing blots for all chimeric genes (*uidA*, *bar*, and *hpaIV*; Cooley et al 1995). The transforming DNA in each of these transgenic individuals consisted of one of six different plasmid constructs. Southern blot analysis using gene-specific probes indicated that all genes on the transforming DNA were integrated at least once in all independent events with a 100% cointegration frequency of selected and unselected genes on the same plasmid. For most transformants, the complexity of the Southern band patterns was comparable from one gene-specific probe to another, indicating similar copy numbers of the various transferred genes. This suggests that the transforming plasmids generally integrate as a complete unit. Battraw and Hall (1992) also reported a 100% cointegration frequency when using linked genes to produce transgenic rice plants from protoplasts. This cointegration frequency is about 25% higher than other linked-gene transfer methods described for transgenic soybean callus (Christou and Swain 1990) and *Phaseolus vulgaris* (Russell et al 1993).

We examined the inheritance patterns of unselected transgenes in the R_1 progeny of 19 *uidA*- and/or *bar*-expressing transformants. Production of transformants with multiple genetic loci occurred at a very low frequency. Expression of *uidA* and *bar* in transformed plants demonstrated a 3:1 segregation ratio consistent with Mendelian

Table 2. Segregation of transgenes in progeny of primary transformants.

	Transformant	GUS+	BAR+	Total assayed	Expected ratio	χ^2	P
R ₁	495-1	41	51	53	3:1 GUS	0.039	0.80-0.95
					15:1 BAR	0.035	0.80-0.95
	496-1	0	74	101	3:1 BAR	0.040	0.80-0.95
					3:1 GUS	0.512	0.20-0.50
	496-2	31	31	47	3:1 BAR	0.512	0.20-0.50
					3:1 BAR	0.512	0.20-0.50
	496-3	0	41	45	3:1 BAR	1.557	0.20-0.50
					3:1 GUS	0.870	0.20-0.50
	496-4	57	57	86	3:1 BAR	0.870	0.20-0.50
					3:1 BAR	0.870	0.20-0.50
496-5	0	31	46	3:1 BAR	0.355	0.20-0.50	
				1:1 GUS	0.133	0.80-0.95	
517-5	17	17	30	1:1 GUS	0.133	0.80-0.95	
				1:1 BAR	0.133	0.80-0.95	
R ₂	495-1-5	56	56	78	3:1 GUS	0.107	0.50-0.80
					3:1 BAR	0.107	0.50-0.80
	495-1-12	27	44	46	3:1 GUS	1.630	0.20-0.50
					15:1 BAR	0.018	0.50-0.80
	495-1-13	0	33	46	3:1 BAR	0.065	0.50-0.80
					3:1 BAR	0.300	0.50-0.80
	496-3-1	0	72	90	3:1 BAR	0.300	0.50-0.80
					3:1 GUS	0.318	0.50-0.80
496-4-1	48	48	59	3:1 GUS	0.318	0.50-0.80	
				3:1 BAR	0.318	0.50-0.80	

inheritance of a single dominant locus in all but two plants (Table 2). One plant, 517-5, demonstrated a 1:1 segregation ratio for BAR expression. This aberrant segregation can be explained by the passage of the transgene exclusively through one gamete. A second plant, 495-1, produced a 15:1 segregation ratio for BAR expression consistent with the presence of two unlinked genetic loci. However, analysis of GUS expression resulted in a 3:1 ratio in the same group, indicating the presence of only one locus with a functional *uidA* gene. Southern blot analyses using both *bar* and *uidA* probes on multiple R₁ seedlings from 495-1 indicated that *bar* and *uidA* DNA were inherited in all progeny resulting in three distinct band patterns. Two of the patterns had no common bands between them, while the third pattern exhibited a combination of the other two. These patterns represented the progeny with either one or two loci, respectively. Subsequent Southern blot analysis demonstrated that at each locus, there was one full size and one partial plasmid insert with nonplasmid DNA between the two inserts at both loci. At one locus, the partial plasmid copy lacks the *bar* gene, while at the other locus, the partial copy lacks the *uidA* gene. The single *uidA* gene at the latter locus is nonfunctional as determined by lack of GUS staining of progeny possessing only this locus. All three types of progeny were resistant to Basta® with no obvious difference in resistance between R₁ plants that had either or both loci. Southern blot analysis of other independent families demonstrated complete concordance between the presence of enzyme activity and the corresponding genes. Southern blots on progeny resulted in the same banding pattern as the primary regenerant. Segregation analysis from this and other systems indicates that these multiple integration events are genetically linked. In this study, multiple inserts were not generally tandem concatemeric arrays, but had multiple genomic DNA borders

indicated by sequencing. This suggests that the linked multiple inserts are significantly fragmented and/or separated by nonplasmid DNA.

We observed a typical range of 1-10 copies of plasmid DNA per haploid genome. This is consistent with other reported methods of direct gene transfer (Schocher et al 1986, Linn et al 1990, Battraw and Hall 1992, Peng et al 1992, Goto et al 1993). Our experiments have shown that in the vast majority of transformants, Southern blot patterns are conserved in R_0 , R_1 , and R_2 generations and reflect Mendelian inheritance of a single locus. The tightly linked nature of gene fragments associated with multicopy integration events has also been reported elsewhere with both T-DNA integration (De Block and Debrouwer 1991) and direct transfer of linked (Christou et al 1989, Battraw and Hall 1992) and unlinked genes (Kartzke et al 1990, Saul and Potrykus 1990, Rathore et al 1993, Goto et al 1993).

To determine if copy number plays a role in coexpression of transgenes, the number of functional copies of transgenic inserts per individual transformant was estimated (Cooley et al 1995). When the number of *uidA* transgenes exceeded 10, the frequency of GUS expression in hygromycin-resistant individuals decreased sharply from 44 to 13%. In the individuals with *uidA* driven exclusively by 35S, however, all low-copy (1-2 copies) transformants expressed GUS, but none of the individuals with greater than 10 integrated copies were GUS-positive. Conversely, 35S-*bar/hpaIV* plants demonstrated the opposite relationship, with coexpression rate stable or increasing with copy number from 80% for one or two copies to 100% for greater than 10 copies. In parallel experiments in which the *uidA* and *bar* genes were evaluated separately, we found that, in general, levels of expression were reduced with increasing copy number for *uidA*, but not for the *bar* gene. These results suggest that correlation between gene-silencing and multicopy integration events may be gene- or construct-specific.

Our experiments suggest that the key variables affecting gene silencing are inherent to the unselected gene and/or its promoter. All 35S-*uidA* and 35S-*bar* constructs exhibited 66 and 90% coexpression, respectively. Plants transformed with pWRG2426 contained both 35S-*uidA* and 35S-*bar* as unselected transgenes on the same plasmid. However, while more than 90% of these plants expressed BAR, only half of them expressed GUS. These results indicate that GUS expression may be more susceptible to inactivation than BAR expression. The larger *uidA* gene is twice as likely than *bar* to be truncated during integration based on gene size alone. Variation in the levels of expression of transgenes is typical of genetically engineered plants. These variations have been observed utilizing either T-DNA or direct DNA transfer methods and are largely attributed to a number of phenomena including integrative fragmentation or rearrangement (Jongsma et al 1987, Kartzke et al 1990, Battraw and Hall 1992, Peng et al 1992, Rathore et al 1993), position effects (Shirstat et al 1989, Al-Shawl 1990, Allen et al 1993, Assaad et al 1993), and gene silencing (Jorgensen 1991, Hart et al 1992, Allen et al 1993, Assaad et al 1993, Rathore et al 1993). In the present study, these variations are indicated by lack of GUS activity or susceptibility to Basta® in R_0 hygromycin-resistant plants.

Our data indicate a relationship between copy number and gene silencing in plants transformed with *uidA* constructs. Allen et al (1993) also reported an inverse

relationship between copy number and expression of 35S-*uidA* transgenes in transformed tobacco NT1 cell lines. Linn et al (1990) reported reduced gene expression with increased copy number in petunia plants engineered with an unselected 35S-dihydrofoliate reductase (*dhfr*) gene. Shirstat et al (1989) and van der Krol et al (1990) reported no correlation between expression and transgenic copy number of transgenic leguminin and *dhfr* genes, respectively, and Stockhaus et al (1987) reported an increase in gene expression with increased copy number. These apparent discrepancies support our hypothesis that factors affecting gene silencing may be gene- or construct-specific. The sensitivity of the assays used to measure and compare various unselected gene product activities and the level of gene expression required to produce detectable enzyme activity are additional factors that may affect apparent gene silencing. It may be that significantly less protein is required to produce the Basta®-resistant phenotype than is necessary to produce the GUS phenotype. We have shown that choice of promoter is important in attaining detectable levels of GUS expression. Of the tissue-specific promoters used, only the *Arabidopsis ssu* promoter produced GUS expression albeit at a lower frequency (33%; 5 of 15 plants examined). However, it is possible that these weak promoters would be sufficient to produce a positive BAR phenotype.

In experiments in which multiple genes were introduced into rice plants utilizing one construct, we determined that coexpression frequencies of all genes were dependent on promoter nature and orientation and also on the number of genes present on the plasmid. In general, promoter orientation does not seem to be important when two genes are present on the plasmid. However, as the number of genes increases, it becomes crucial (Table 3).

Table 3. The nature and orientation of promoters that influence coexpression frequencies of multiple genes introduced into rice plants utilizing one plasmid.^a

Transgenic plants analyzed (no.)	hmr	gus	bar	x	Plants expressing all genes (no.)
30	+	+			30
30	+	-			30
74	+	+	+		48
45	+	+	-		45
25	+	-	-		15
38	+	+	+	+	8
42	+	+	+	-	20
50	+	+	-	-	30
28	+	-	+	-	16
28	+	-	<i>Arab. ssu</i>	Rice <i>ssu</i>	26

^aPlus and minus signs indicate multiple 35S promoters driving multiple genes in a clockwise or counterclockwise orientation.

Molecular characteristics of field-derived plants

Fifteen lines representing 11 independent transformation events were analyzed. Southern blot analysis of the transgenic lines was used to characterize patterns of integration of the unselected genes (*uidA* and *bar*) carried on the transforming WRG 2426 plasmid DNA (Cooley et al 1995). Analysis was performed on one plant only of a segregating population for each line. Consequently, only general statements regarding the fate of the transgenes can be made as we are not dealing exclusively with homozygous populations for the transgene(s). Northern blot analysis was used to determine the transcriptional state of the *uidA* and *bar* transgenes. Southern blot analysis with gene-specific probes showed that in nine of the plants representing the 15 lines, both the *uidA* and *bar* transgenes were present (Fig. 1). The complexities of band patterns and intensities were similar when the banding patterns within the same line were compared for the *uidA* and *bar* transgenes, indicating that the transforming plasmid was inherited as a complete unit in the nine lines. Comparison of the complexities of banding patterns for the *uidA* and the *bar* transgenes within independent lines showed six different patterns of integration of the *uidA* transgene, whereas at least eight different integration patterns were revealed for the *bar* gene suggesting recombination events. DNA from two lines, GFMT 517-5-R1 and GFMT 517-2-R1, showed hybridization to the *bar* gene probe but not to the *uidA* gene probe. In the remaining four lines (GFMT 526-1 GFMT 517-1-R1, GFMT 517-7-R1, and KOSH 496-3-R2), neither the *bar* nor the *uidA* transgene was detected. Since these plant lineages exhibited resistance in the field following herbicide application and since KOSH 496-3-R1 contains both *bar* and *uidA*, this shows that we are dealing with a mix of transformed and nontransformed plants derived from segregating populations. We are currently developing homozygous lines for the transgenes, which will be used for a more in-depth molecular analysis of transgene structure and function.

A comparison of the Southern and Northern data provide evidence for mechanisms of gene silencing operating at both the transcriptional and posttranscriptional levels. Nucleic acid extracted from the GFMT 517-3-R1 line showed hybridization to the *uidA* gene probe at the DNA level, but no transcript was detected in these plants for the *uidA* transgene at the RNA level. Of the 11 lines in which the *bar* transgene was detected at the DNA level, four lines (KOSH 496-2-R1, KOSH 496-3-R1, KOSH 496-4-R1, and KOSH 496-4-R2) showed no hybridization to the *bar* gene probe at the RNA level. That the silencing effect is seen in plants having anywhere between 1 and 5 hybridizing bands in Southern gel-blot analyses suggests that the transcriptional silencing effect may be independent of the number of integration sites of either the *uidA* or *bar* transgenes. Our data also provide evidence for gene silencing occurring at the posttranscriptional level in some of the plants we analyzed. The expected size of the transcript from the *uidA* gene in plasmid WRG 2426 is 2 kb; such a transcript was observed in RNA only from lines KOSH 495-1-R1, KOSH-495-1-R2, and KOSH-496-2-R1 (Fig. 2). These lines exhibited the highest frequency of GUS expression (three of the nine plants tested, data not shown). GUS activity was rarely detected in any other line. Transcripts of more than 12 kb, detected in RNA from lines KOSH

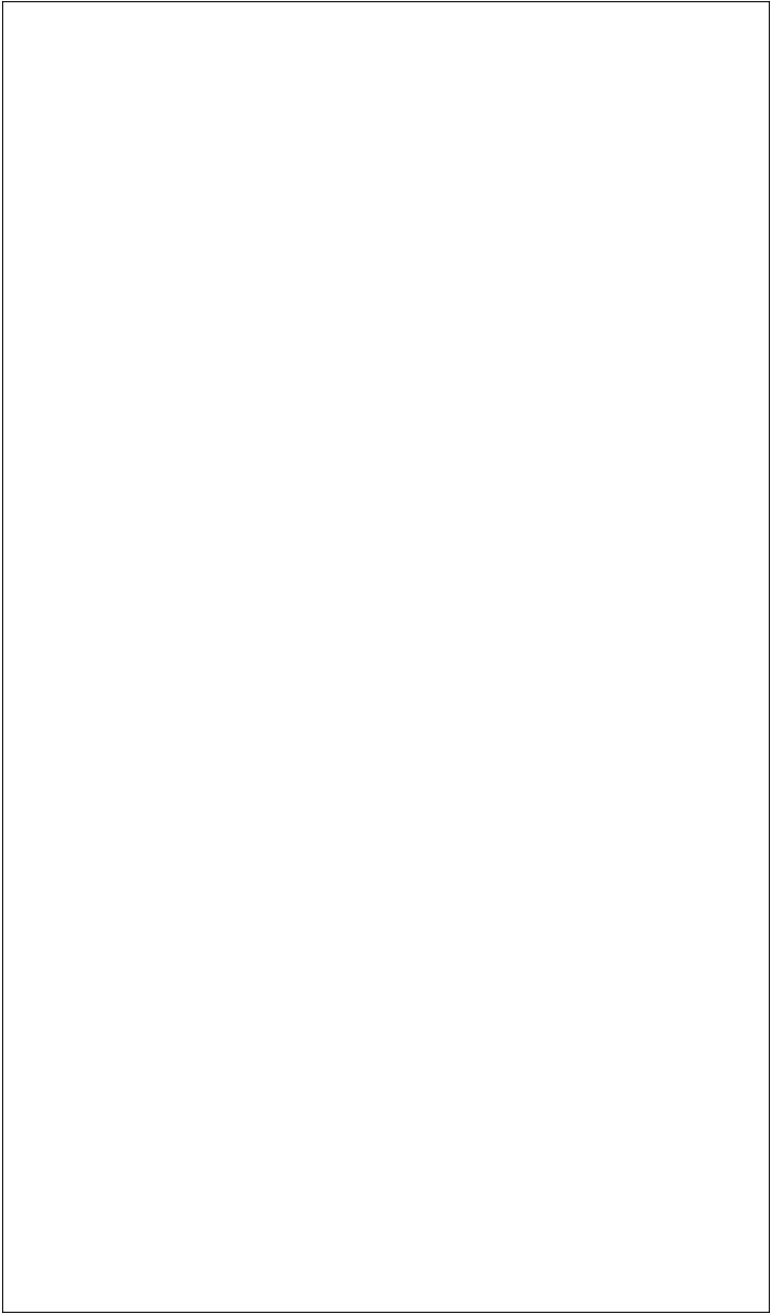


Fig. 1. Southern blots of transgenic lines tested in the field. Genomic DNA was digested with *SacI* (blot A) or *XbaI* (blot B), transferred to nylon membranes and probed with ³²P-labeled *uidA* gene probe DNA (blot A) or similarly labeled *bar* gene DNA (blot B). Blots A and B have identical lane loadings of genomic DNA isolated from the following lines: GFMT 517-2-R1 (lane 1), GFMT 517-5-R1 (2), GFMT 517-3-R1 (3), GFMT 517-1-R1 (4), G 526-1 (5), KOSH 496-3-R2 (6), GFMT 517-7-R1 (7), KOSH 496-4-R1 (8), KOSH 496-4-R2 (9), KOSH 495-1-R2 (10), KOSH 495-1-R1 (11), KOSH 496-3-R1 (12), KOSH 496-1-R2 (13), KOSH 496-1-R1 (14), and KOSH 496-2-R1 (15). Lanes 16, 17, and 18 contain pWRG2426 at 1, 5, and 10 copies, respectively, per genome, digested with either *SacI* (blot A) or *XbaI* (blot B).



Fig. 2. Northern blot analysis of RNA from transgenic rice plants tested in the field. Total RNA (15 µg) was fractionated through 1.5% agarose gels, transferred to nylon membranes, and probed with ³²P-labeled *bar* gene DNA. Lanes 1-6 show RNA from the GFMT lines and lanes 7-15 RNA from the KOSH lines as follows: GFMT 517-1-R1 (lane 1), 517-2-R1 (2), 517-3-R1 (3), 517-5-R1 (4), 517-7-R1 (5), 526-1 (6), KOSH 495-1-R1 (7), 495-1-R2 (8), 496-1-R1 (9), 496-1-R2 (10), 496-2-R1 (11), 496-3-R1 (12), 496-3-R2 (13), 496-4-R1 (14) and 496-4-R2 (15).

496-1-R1, KOSH 496-1-R2, KOSH 496-3-R1, KOSH 496-4-R2, and KOSH 496-4-R1, are likely to be untranslatable since no GUS activity was found in leaf material taken from five independent plants of each of these lines. These experiments are being pursued further to develop a better understanding of these phenomena. Interestingly, those plants from which genomic DNA revealed the greatest number of hybridization bands in Southern blot analyses with the *uidA* gene probe (KOSH 496-1-R1, KOSH 496-1-R2, KOSH 496-3-R1, KOSH 496-4-R1, and KOSH 496-4-R2) also showed the presence of a very large, relatively low abundant transcript of more than 12 kb that hybridized to the *uidA* gene probe in Northern blot analyses. In addition, the lines exhibiting the simplest integration patterns of the *uidA* gene (KOSH 495-1-R1, KOSH-495-1-R2, and KOSH-496-2-R1) showed GUS expression more consistently across different plants of these lines. A large transcript hybridizing to the *bar* gene probe was detected in lines KOSH 496-1-R1 and KOSH 496-1-R2 that similarly demonstrated the more complex integration pattern for the *bar* transgene. These data suggest that there maybe a correlation between complexity of integration sites and reorganization of the input plasmid to produce a large transcription unit, perhaps containing multiple copies of the *uidA* or *bar* messages. How such transcripts are produced remains unclear and will be the subject of future work. We may speculate, however, that it is probably due to complex extrachromosomal preintegration recombination events, which result in the formation of a large transcription unit containing multiple copies of the *gus* gene between a promoter and termination signal. The possibility that the transcripts are polycistronic, being a composite of RNA sequences of *uidA*, *bar*, and possibly an *aphIV*, is not supported by Northern hybridization data.

Agronomic performance of transgenic material after herbicide treatment

All nontransgenic plants at the 3- to 4-leaf stage died within 8 d following application of 1.12 or 2.24 kg glufosinate ha⁻¹ (J.H. Oard et al, pers. commun.). In contrast, transgenic Gulfmont and Koshihikari lines survived glufosinate treatments to produce fertile, normal-looking seeds. These results demonstrate that rice can be genetically engineered for field-level resistance to glufosinate herbicide, which has been shown to be effective in controlling red rice and other noxious weeds. Gulfmont transgenic lines displayed no visible injury from herbicide treatments, but all Koshihikari lines exhibited initial yellowing and some stunting 2-3 d after glufosinate application. The yellowing disappeared 7 d after treatment, however, stunting for some lines was observed at harvest. Significant differences among the Gulfmont- and Koshihikari-derived transgenic lines for grain yield were detected both at the 1.12 and 2.24 kg ha⁻¹ rates. The grain yield of transformed Gulfmont lines varied between 1.8 and 1.7 t ha⁻¹ for 1.12 and 2.24 kg ha⁻¹ herbicide rates, respectively. A similar trend was observed within Koshihikari lines that displayed significant differences in yield, but with greater ranges (2.2 and 4.1 t ha⁻¹) at the two herbicide rates. Under no herbicide conditions, a few transformed Gulfmont and Koshihikari lines yielded below the untransformed parental cultivars, but 80% of the lines yielded equal or better than the controls. Mean

grain yields across herbicide treatments were not statistically significant within Gulfmont-derived lines, which indicates that glufosinate applications did not significantly alter performance of this material. In contrast, grain yields for 33% of the Koshihikari lines were significantly reduced with increasing glufosinate rates. Variation for grain yield among transgenic lines may be explained by variations of BAR expression. As with grain yield, significant differences among transgenic lines for mature plant height were observed for each herbicide rate. A 6- to 7-cm range in height was observed for Gulfmont lines while Koshihikari lines displayed an 11- to 12-cm range within treatments. Mean height values across treatments were not statistically different among Gulfmont lines while a majority (67%) of Koshihikari lines showed significant reductions in plant height with increasing glufosinate rates. Plant height 14 d after spraying of 1.12 kg glufosinate ha⁻¹ showed a slight decrease of 2.4 cm among transgenic lines when compared with the no-herbicide treatment in 1993 experiments. Most Gulfmont and Koshihikari lines (55-67%) displayed no significant differences in days to 50% heading across herbicide treatments. Moreover, heading values were nearly equal or only slightly greater with no-herbicide treatments when compared with the untransformed controls. For those lines where statistical differences in heading were observed, the 2- to 3-d delay in maturity would probably have minimal effect on agronomic performance of the transgenic material.

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Notes

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