

Stable inheritance of transgenes in rice plants transformed by *Agrobacterium tumefaciens*

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Inheritance of foreign genes in rice transformed by *Agrobacterium tumefaciens* has been investigated up to the R₄ progeny. Rice cultivar Tsukinohikari was transformed with *A. tumefaciens* strains LBA4404(pTOK233) and EHA101(pIG121Hm). Cultivar Koshihikari was transformed with LBA4404(pTOK233). pTOK233 is a "super-binary" vector and LBA4404(pTOK233) had a greater transformation efficiency than EHA101(pIG121Hm). The T-DNA of these strains carried a *gus* gene and a hygromycin resistance gene, both of which were driven by the 35S promoter. The *gus* gene contained an intron in the coding region. The selfed progeny of 20 independent plants from each of the three populations of transformants were evaluated for *gus* expression and resistance to hygromycin. The two markers were genetically linked and inherited in a Mendelian fashion. The segregation ratio of 3:1 (positive:negative for the transgenes) was observed in the progeny of 60-75% of the transformants in each of the groups of 20 plants. The R₂ progeny of six plants from each of the groups were scored for GUS expression and hygromycin resistance. Thus, the Mendelian transmission of the two markers was further confirmed and offspring homozygous for the two genes were identified in the R₂ lines. R₃ plants were obtained from the homozygous R₂ offspring, and consequently R₄ plants were produced. The two genes were expressed in the R₃ and R₄ generations and none of the plants in these generations lost the genes. The DNA from selected offspring was analyzed by Southern hybridization and a tight correlation between the phenotype and genotype was demonstrated.

Stable inheritance and expression of foreign genes are of critical importance in application of genetically engineered cereal crops in agriculture, but have not been extensively studied.

Transformants of cereals have been produced mainly via direct uptake of DNA by protoplasts or cells. Mendelian inheritance of transgenes in such transformants was observed on various occasions (Shimamoto et al 1989, Datta et al 1990, Christou et al 1991), but only R_1 progeny (selfed progeny of primary transformant [R_0]) were examined in most of the cases. Non-Mendelian types of segregation patterns of transgenes were also reported. For example, foreign genes (*gus* and *neo*) in three rice plants transformed by the polyethylene glycol (PEG)-mediated method were analyzed up to the R_3 generation (Peng et al 1995), and the progeny of the two plants did not show segregation of the genes in a Mendelian fashion. In another case, *bar* and *gus* genes integrated into a maize plant by particle bombardment were unstable and poorly transmitted to the progeny (Spencer et al 1992).

Non-Mendelian inheritance of transgenes was also reported in dicotyledonous transformants produced by direct transformation methods (Potrykus et al 1985). On the other hand, genes introduced into dicotyledons via *Agrobacterium*-mediated methods appear to be very stable and are usually inherited in Mendelian fashion (De Block et al 1984, Budar et al 1986, Chyi et al 1986, Feldmann and Marks 1987, Muller et al 1987).

We have previously reported the development of an efficient transformation method for japonica rice mediated by *A. tumefaciens*, and observed clear Mendelian transmission of transgenes in the R_1 and R_2 progeny (Hiei et al 1994). In this paper, we present further, large-scale analysis of inheritance, expression and stability of the foreign genes up to the R_4 progeny of the rice plants transformed by *A. tumefaciens*.

Materials and methods

Vectors and transformation

The procedure for transformation of rice and *A. tumefaciens* strains LBA4404(pTOK233) and EHA101(pIG121Hm) were described by Hiei et al (1994). pTOK233 is a "super-binary" vector (Komari 1990, Hiei et al 1994). The T-DNA of these strains carried a β -glucuronidase (*gus*) gene and a hygromycin resistance gene, both of which were driven by the CaMV35S promoter. The *gus* gene contained an intron in the coding region (Fig. 1).

Analysis of progeny

GUS activity in leaves was scored by the procedure described by Hiei et al (1994). Hygromycin resistance of seedlings was assayed by culturing root segments on 2N6 medium (Hiei et al 1994) containing hygromycin (50 mg L^{-1}). Root segments from sensitive seedlings did not show any response and root segments from resistant seedlings produced calli (Fig. 2a).

Southern blot analysis

DNA was extracted from leaf tissue using the procedure described by Komari et al (1989). Ten micrograms of DNA were digested with *Hind*III or *Xba*I and fractionated on a 0.7 % agarose gel by electrophoresis at 1.1 V cm^{-1} for 16 h. Southern hybridiza-

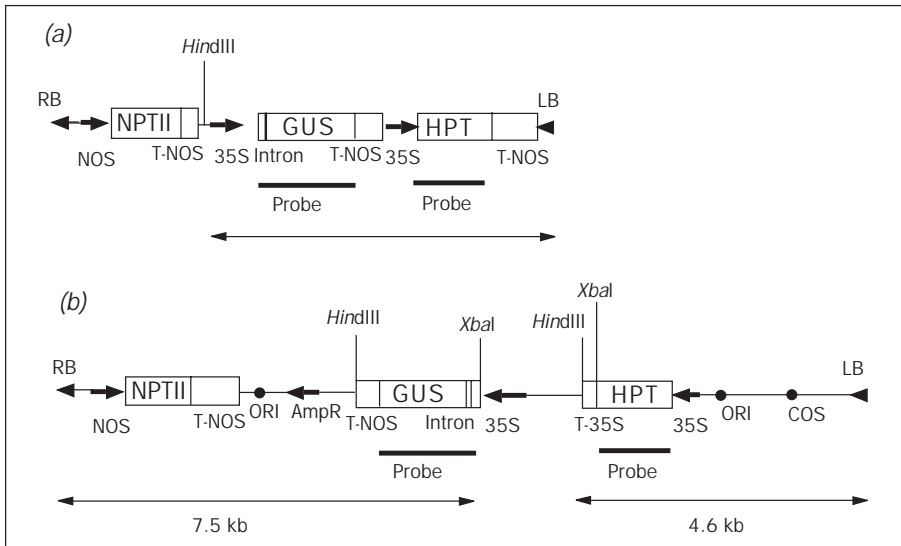


Fig. 1. T-DNA regions of pIG121Hm(a) and pTOK233(b). RB; right border, LB; left border, NPTII; neomycin phosphotransferase, GUS; β -glucuronidase, HPT; hygromycin phosphotransferase, NOS; nopalyn synthase promoter, 35S; 35S promoter, T-NOS; 3' signal of nopalyn synthase, T-35S; 3' signal of 35S RNA, ORI; origin of replication of ColE1, AmpR; ampicillin resistance gene active in *Escherichia coli*.

tion was carried out as described by Sambrook et al (1989). The probe for *hpt* was the 1.1-kb *Bam*HI fragment from pGL2-IG (Hiei et al 1994) and the *Gus* probe was the 1.9-kb *Sal*I-*Sac*I fragment from pGL2-IG.

Results and discussions

Transformation

Japonica rice cv Tsukinohikari was transformed with LBA4404(pTOK233) (population T) and EHA101(pIG121Hm) (population E), and cv Koshihikari was transformed with LBA4404(pTOK233) (population K). Twenty independent, GUS-positive, hygromycin-resistant transgenic plants (R_0) from each of the three populations of transformants were analyzed by Southern hybridization. The copy numbers of integrated genes varied from one to six (Tables 1 and 2), but the majority of the transformants contained one or two copies of the integrated genes. In several plants, the copy numbers of *hpt* and *gus* were different, probably due to rearrangement of DNA upon transformation.

R_1 generation

The selfed progeny were evaluated for resistance to hygromycin and GUS expression. The segregation patterns of offspring from the 20 independent R_0 plants (cv Koshihikari transformed by LBA4404[pTOK233], K lines) are shown in Table 1.

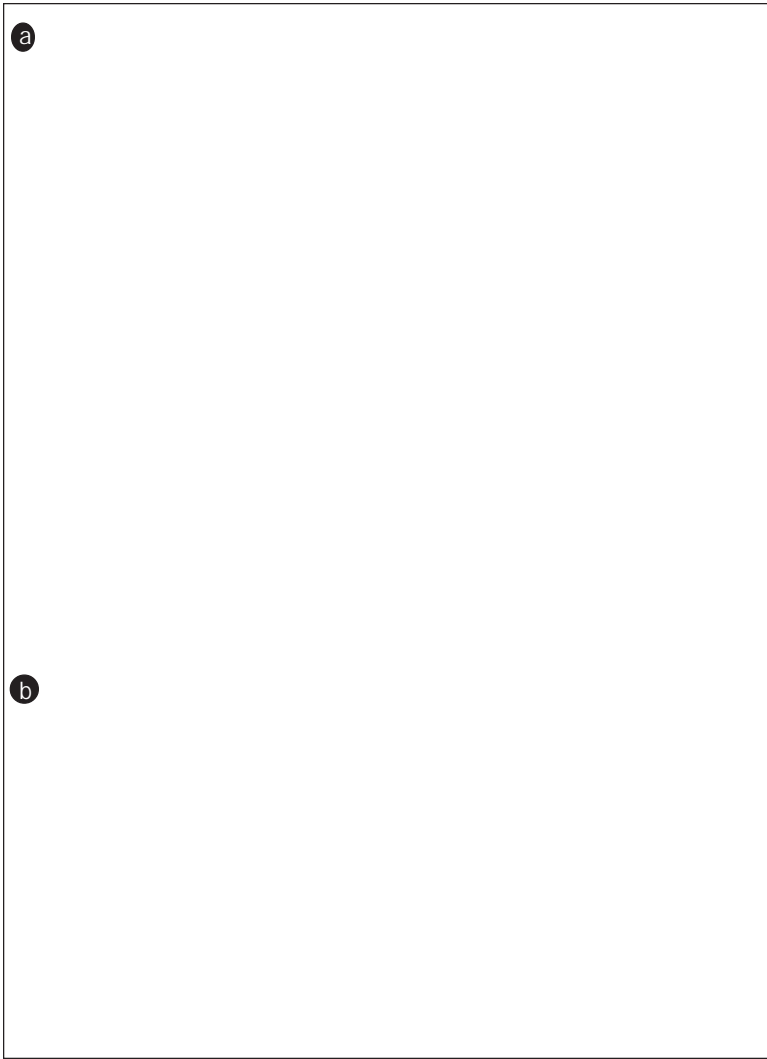


Fig. 2. Test of the R_1 progeny of cv Tsukinohikari transformed by LBA4404(pTOK233) for resistance to hygromycin and for expression of GUS. a) Root segments from R_1 seedlings were plated on selective medium and the photograph was taken 3 wk later. b) R_1 seedlings were cultured in darkness and their leaves were stained with 5-bromo-4-chloro-3-indolyl glucuronidase.

Table 1. Estimation of copy numbers of transgenes in the transformants of cv Koshihikari produced by LBA4404 (pTOK233), and segregation patterns for hygromycin resistance and expression of GUS in the R₁ progeny.

Line no. (R ₀)	Copy number (R ₀)		Number of R ₁ plants		χ^{2c}	
	<i>hpt</i> ^a	<i>gus</i> ^a	GUS+	GUS-	3:1	15:1
			HygR ^b	HygS ^b		
K1	3	4	45	8	2.774	7.075**
K2	1	2	38	12	0.027	26.885**
K3	2	2	31 ^d	8	0.419	13.540**
K4	1	1	29	11	0.133	30.827**
K5	1	2	29	11	0.133	30.827**
K6	3	3	36	12	0.000	28.800**
K7	3	2	31	2	6.313*	0.002
K8	2	2	35	10	0.185	19.593**
K9	2	2	35	2	7.575**	0.045
K10	4	3	71	5	13.754**	0.014
K11	2	2	40	0	13.333	2.667
K12	1	2	32	8	0.533	12.907**
K13	2	2	65	14	2.232	17.743**
K14	3	2	36	2	7.895**	0.063
K15	1	1	52	15	0.244	29.780**
K16	2	3	35	12	0.007	29.823**
K17	2	2	38	1	10.470**	0.904
K18	2	2	65	14	2.232	17.743**
K19	2	1	37	13	0.027	33.285**
K20	1	1	58	15	0.772	25.469**

^a*hpt*: hygromycin resistance gene, *gus*: GUS gene. ^bHygR: hygromycin-resistant, HygS: hygromycin-sensitive. ^c* and **: significant at 5 and 1% level, respectively. ^dExpressing GUS scattered and poor hygromycin-resistant calli.

Table 2. Copy numbers of transgenes in R₀ and number of genetic loci estimated by segregation patterns in the R₁ progeny.

Lines ^a	Lines analyzed (no.)	Transgene ^b	Copy numbers of transgenes			Genetic loci (no.)	
			1	2	3-6	1	2-
E	20	<i>hpt</i>	4	10	6	15	5
		<i>gus</i>	7	8	5	16	4
T	20	<i>hpt</i>	2	8	10	12	8
		<i>gus</i>	4	7	9	13	7
K	20	<i>hpt</i>	6	10	4	14	6
		<i>gus</i>	6	9	5	14	6

^aE: cv Tsukinohikari transformed by EHA101 (pIG121Hm), T: cv Tsukinohikari transformed by LBA4404 (pTOK233), K: cv Koshihikari transformed by LBA4404 (pTOK233). ^b*hpt*: hygromycin resistance gene, *gus*: GUS gene.

GUS expression and hygromycin resistance cosegregated clearly in Mendelian fashion, and 14 of the 20 R₁ lines showed a 3:1 ratio (positive:negative for the transgenes) of segregation.

The leaf segments from most of the GUS-positive R₁ plants uniformly expressed GUS activity, but scattered expression of GUS, which was associated with relatively low level of hygromycin resistance, was observed in line K3. This line still had a segregation ratio of 3:1. Such expression of *gus* and *hpt* was also observed in one of the R₁ lines of Tsukinohikari transformed with EHA101(pIG121Hm).

Analysis of the R₁ progeny of the three populations of transformants is summarized in Table 2. The estimated number of loci was smaller than the copy number of genes measured by Southern blot analysis in some lines. It is likely that more than two copies of genes were integrated close to each other on a chromosome of such plants. The numbers of *gus* and *hpt* loci were different in a few cases, possibly due to rearrangements of T-DNA.

R₂ generation

Fifteen R₁ lines with a segregation ratio of 3:1 and 3 R₁ lines with a segregation ratio of 15:1 were grown to maturity. The R₂ plants from these lines were evaluated.

The R₁ analysis of a 3:1 line, K4, is shown in Table 3. The R₂ progeny from GUS-positive, hygromycin-resistant R₁ plants either had a segregation ratio of 3:1 or

Table 3. Segregation patterns for hygromycin resistance and expression of GUS in the R₂ progeny of transgenic Koshihikari mediated by LBA4404(pTOK233), Line K4.

Line no. (R ₁)	Phenotype of R ₁		Number of R ₂ plants		$\chi^2(3:1)$	Apparent genotype of R ₁ ^c	
	GUS	Hyg	GUS+ ^a HygR ^b	GUS- ^a HygS ^b		<i>gus/gus</i>	<i>hpt/hpt</i>
K4-1	+	R	40	0	0.533	<i>gus/gus</i>	<i>hpt/hpt</i>
K4-2	+	R	32	8		<i>gus/-</i>	<i>hpt/-</i>
K4-3	+	R	40	0		<i>gus/gus</i>	<i>hpt/hpt</i>
K4-4	+	R	40	0	0.533	<i>gus/gus</i>	<i>hpt/hpt</i>
K4-5	+	R	32	8		<i>gus/-</i>	<i>hpt/-</i>
K4-6	+	R	30	10		0.000	<i>gus/-</i>
K4-7	+	R	39	0	0.240	<i>gus/gus</i>	<i>hpt/hpt</i>
K4-8	+	R	36	14		<i>gus/-</i>	<i>hpt/-</i>
K4-9	+	R	41	11		0.410	<i>gus/-</i>
K4-10	+	R	33	16	1.531	<i>gus/-</i>	<i>hpt/-</i>
K4-11	+	R	40	0		<i>gus/gus</i>	<i>hpt/hpt</i>
K4-12	+	R	40	0		<i>gus/gus</i>	<i>hpt/hpt</i>
K4-13	+	R	34	7	1.374	<i>gus/-</i>	<i>hpt/-</i>
K4-14	+	R	47	12	0.684	<i>gus/-</i>	<i>hpt/-</i>
K4-15	+	R	34	6	2.133	<i>gus/-</i>	<i>hpt/-</i>
K4-16	+	R	26	13	1.444	<i>gus/-</i>	<i>hpt/-</i>
K4-17	+	R	29	14	1.310	<i>gus/-</i>	<i>hpt/-</i>
K4-18	+	R	40	0		<i>gus/gus</i>	<i>hpt/hpt</i>
K4-19	-	S	0	40		<i>-/-</i>	<i>-/-</i>
K4-20	-	S	0	40		<i>-/-</i>	<i>-/-</i>

^aGUS+: GUS positive, GUS- and -: GUS negative. ^bHygR: hygromycin-resistant, HygS: hygromycin-sensitive. ^c*Hpt*: hygromycin resistance gene, *gus*: GUS gene.

were all GUS-positive and hygromycin-resistant. The R₂ progeny from GUS-negative, hygromycin-sensitive R₁ plants were all GUS-negative and hygromycin-sensitive. The examination of the other 3:1 lines gave similar results. The scattered expression of GUS and low level of hygromycin resistance in line K3 were also inherited by the R₂ progeny as Mendelian loci.

The R₂ analysis of a 15:1 line, K7, is shown in Table 4. R₂ lines consisting solely of plants positive for the transgenes, 3:1 lines, 15:1 lines, and lines consisting solely of plants negative for the transgenes are expected in a ratio of 7:4:4:1. This expectation was confirmed and examination of the other 15:1 lines gave similar results.

Table 4. Segregation patterns for hygromycin resistance and expression of GUS in the R₂ progeny of transgenic Koshihikari mediated by LBA4404(pTOK233), Line K7.

Line no. (R ₁)	Phenotype of R ₁		Number of R ₂ plants		χ^2 ^c	
	GUS	Hyg	GUS ⁺ ^a HygR ^b	GUS ⁻ ^a HygS ^b	3:1	15:1
K7-1	+	R	39	0	13.000**	2.600
K7-2	+	R	28	9	0.009	20.628**
K7-3	+	R	36	0	12.000**	2.400
K7-4	+	R	35	1	9.481**	0.740
K7-5	+	R	40	0	13.333**	2.667
K7-6	+	R	38	0	12.667**	2.533
K7-7	+	R	38	0	12.667**	2.533
K7-8	+	R	40	0	13.333**	2.667
K7-9	+	R	31	2	6.313*	0.002
K7-10	+	R	38	2	8.533**	0.107
K7-11	+	R	40	0	13.333**	2.667
K7-12	+	R	39	1	10.800**	0.960
K7-13	+	R	42	0	14.000**	2.800
K7-14	+	R	43	0	14.333**	2.867
K7-15	+	R	40	0	13.333**	2.667
K7-16	+	R	30	7	0.730	10.135**
K7-17	+	R	39	0	13.000**	2.600
K7-18	+	R	38	2	8.533**	0.107
K7-19	+	R	39	0	13.000**	2.600
K7-20	+	R	39	9	1.000	12.800**
K7-21	+	R	39	0	13.000**	2.600
K7-22	+	R	39	0	13.000**	2.600
K7-23	+	R	35	14	0.333	41.667
K7-24	+	R	44	17	0.268	48.657**
K7-25	+	R	47	3	9.627**	0.005
K7-26	+	R	39	0	13.000**	2.600
K7-27	+	R	40	0	13.333**	2.667
K7-28	+	R	40	0	13.333**	2.667
K7-29	+	R	45	20	1.155	66.692**
K7-30	+	R	35	18	2.270	69.465**
K7-31	+	R	39	10	0.551	16.763**
K7-32	-	S	0	30	90.000**	450.000**
K7-33	-	S	0	30	90.000**	450.000**

^aGUS+: GUS positive, GUS-: GUS negative. ^bHygR: hygromycin-resistant, HygS: hygromycin-sensitive. ^c* and **: significant at 5 and 1% level, respectively.

R₃ and R₄ generations

R₃ plants were obtained from R₂ plants homozygous for the transgenes, and then R₄ plants were produced. Thus, R₃ and R₄ progeny, which originated from six independent R₀ plants in each of the three populations of the initial transformants were characterized. All of the R₃ and R₄ plants were GUS-positive and hygromycin-resistant. A typical pattern (line E8) of transmission of the transgenes from the R₀ to the R₄ generation is shown in Table 5.

Plants showing scattered expression of GUS and low level of hygromycin resistance were newly identified in two independent R₃ lines, E9 and E10, and these traits were inherited by the R₄ progeny. Expression of the transgenes was normal in the R₁ and R₂ generations. The scattered expression of GUS and low level of hygromycin resistance in line K3 were stably inherited by the homozygous R₄ progeny.

R₃ plants homozygous for a single locus of the transgenes, originating from R₀ plants that contained two loci of the transgenes, were successfully identified in all of the three independent lines tested.

Southern blot analysis of progeny plants

The progeny plants were analyzed by Southern hybridization (parts of data are shown in Figures 3 and 4), and a tight correlation between phenotype and genotype was confirmed. For example, the R₀, R₁, R₂, R₃, and R₄ plants positive for the expression of the transgenes in line E8 showed an identical hybridization pattern (Fig. 3, Table

Table 5. Segregation patterns for hygromycin resistance and expression of GUS up to the R₄ progeny of transgenic Tsukinohikari mediated by EHA101(pIG121Hm), Line E8.

Line no. (generation)	Parent	Phenotype		Number of plants in the progeny		χ^2 (3:1)	Apparent genotype of parent ^c	
		GUS	Hyg	GUS+ ^a HygR ^b	GUS- ^a HygS ^b			
E8	(R ₀)	+	R	45	13	0.207	<i>gus</i> /-	<i>hpt</i> /-
E8-1	(R ₁)	+	R	34	0	0.111	<i>gus</i> / <i>gus</i>	<i>hpt</i> / <i>hpt</i>
E8-4	(R ₁)	+	R	35	13		<i>gus</i> /-	<i>hpt</i> /-
E8-9	(R ₁)	+	R	26	9	0.010	<i>gus</i> /-	<i>hpt</i> /-
E8-10	(R ₁)	+	R	34	0	0.238	<i>gus</i> / <i>gus</i>	<i>hpt</i> / <i>hpt</i>
E8-15	(R ₁)	+	R	25	10		<i>gus</i> /-	<i>hpt</i> /-
E8-16	(R ₁)	+	R	39	17	0.857	<i>gus</i> /-	<i>hpt</i> /-
E8-2	(R ₁)	-	S	0	35		-/-	-/-
E8-6	(R ₁)	-	S	0	35		-/-	-/-
E8-1-1	(R ₂)	+	R	30	0		<i>gus</i> / <i>gus</i>	<i>hpt</i> / <i>hpt</i>
E8-10-5	(R ₂)	+	R	30	0		<i>gus</i> / <i>gus</i>	<i>hpt</i> / <i>hpt</i>
E8-1-1-2	(R ₃)	+	R	30	0		<i>gus</i> / <i>gus</i>	<i>hpt</i> / <i>hpt</i>
E8-1-1-4	(R ₃)	+	R	30	0		<i>gus</i> / <i>gus</i>	<i>hpt</i> / <i>hpt</i>
E8-1-1-5	(R ₃)	+	R	30	0		<i>gus</i> / <i>gus</i>	<i>hpt</i> / <i>hpt</i>

^aGUS+: GUS positive, GUS-: GUS negative. ^bHygR: hygromycin-resistant, HygS: hygromycin-sensitive, ^c*hpt*: hygromycin resistance gene, *Gus*: GUS gene.

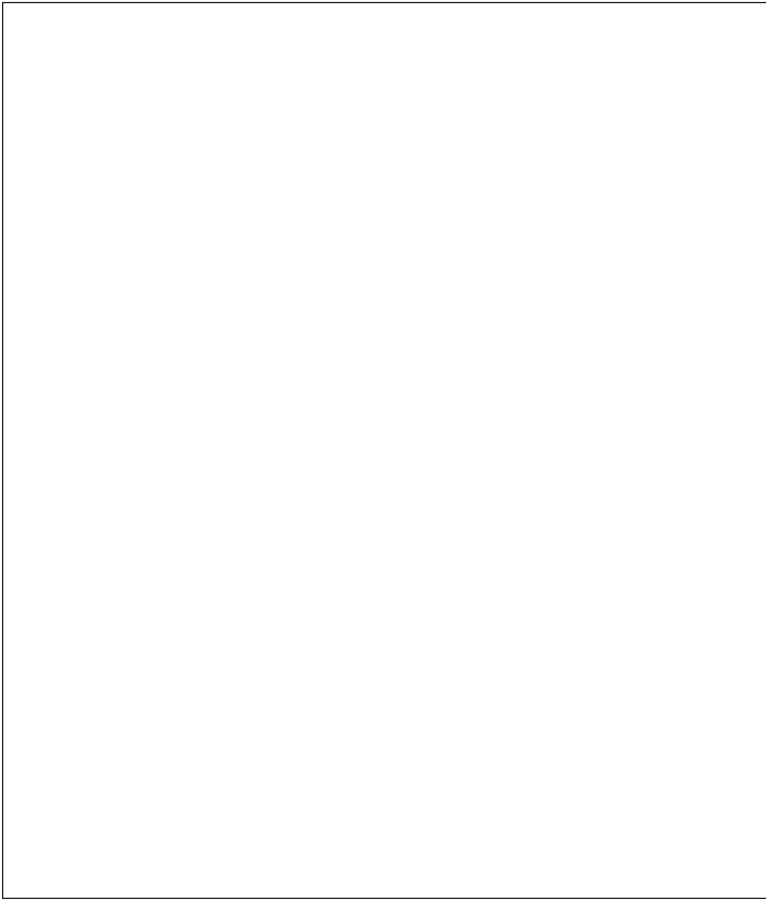


Fig. 3. Southern analysis of the R₁, R₂, R₃, and R₄ progeny of transformant E8. DNAs from R₀ transformant and the progeny were digested with *Hind*III, fractionated by electrophoresis, transferred to a nylon membrane, and allowed to hybridize to the *gus* (a) or the *hpt* (b) probe.

5). Independent transmission of the two copies of the foreign DNA fragments, which corresponded to the two loci of the transgenes, was verified in line K7 (Fig. 4, Table 4).

The plants with scattered expression of GUS and a low level of hygromycin resistance showed hybridization patterns identical to those of their parents, which expressed the transgenes normally.

Conclusion

The present study has demonstrated stable, Mendelian inheritance of two marker genes introduced into rice by *A. tumefaciens*. Progeny of 18 independent transgenic rice

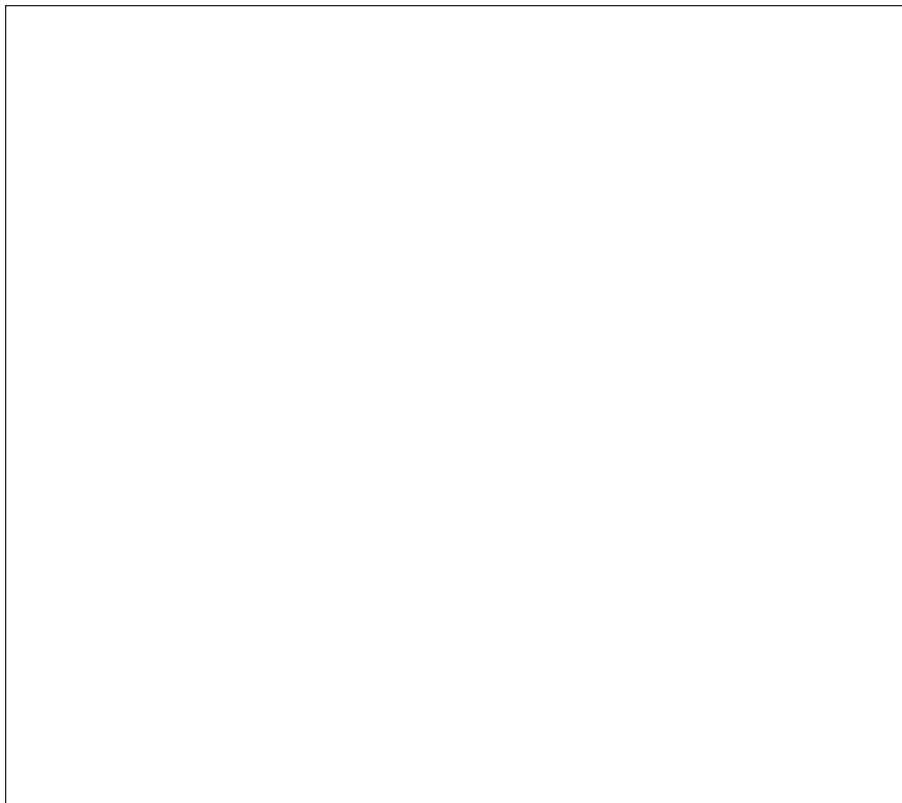


Fig. 4. Southern analysis of the R_1 , R_2 , and R_3 progeny of transformant K7. DNAs from R_0 transformant and the progeny were digested with *Xba*I (a) or *Hind*III (b), fractionated by electrophoresis, transferred to a nylon membrane, and allowed to hybridize to the *gus* (a) or the *hpt* (b) probe.

plants were analyzed up to the R_3 and R_4 generations. All lines showed clear Mendelian transmission of the transgenes, and Southern hybridization confirmed the genetic data. Therefore, the foreign genes integrated into rice in this study appeared to be as stable genetically as those in dicotyledonous transformants produced by *A. tumefaciens*.

Plants showing scattered expression of GUS and a low level of hygromycin resistance emerged in a few lines in the R_1 or R_3 generations. As expression of the foreign genes in their parents was normal and molecular analysis did not detect any flaw, the nature of such expression is not understood. This phenomenon may be related to “gene silencing” observed in various transformation systems.

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Notes

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