

# *Agrobacterium*-mediated transformation of an elite cultivar of Texas rice

J.J. Dong, W.M. Teng, W.G. Buchholz, and T.C. Hall

While useful advances have been made using biolistic and other naked DNA uptake methods for rice transformation, high copy numbers of rearranged sequences are common and lack of stable expression due to silencing or other causes is proving to be a major problem. Attempts to transform rice and other monocots using *Agrobacterium tumefaciens*-based vectors have typically yielded results that were promising, but inconclusive. Recently, an *Agrobacterium*-based system starting from scutellar tissue has been described in the literature. Using only minor modifications of this system, we were able to repeat their transformation of callus derived from the cultivar Koshihikari. We have also extended the work to include Gulfmont, a javonica variety widely used for commercial cultivation in Texas, and Taipei 309, a japonica line that has been widely used for transformation by direct DNA transfer. Transformation using the *Agrobacterium* approach occurred at a reasonably high frequency and vigorous, phenotypically normal plants were recovered. Only one or two unrearranged copies of the T-DNA were present at a single locus; no silencing of the selectable marker or the gene of interest (*gus*) was observed and the introduced genes were transmitted in a normal Mendelian fashion.

Transformation of rice (*Oryza sativa*) and other agronomically important graminaceous monocot crops has frequently been reported using naked DNA uptake stimulated by polyethylene glycol or electroporation (Shimamoto et al 1989; Battra and Hall 1990, 1992; Datta et al 1990; Li et al 1990; Davey et al 1991) or by biolistics (Christou et al 1991). Unfortunately, DNA introduced by these direct transfer methods is often incorporated into the genome as multiple fragmented or rearranged copies. Transformation events resulting in a single-copy, unrearranged sequence containing the gene of interest are rare. Presumably as a result of these complex integration events, the expression pattern of the gene of interest is often aberrant (Xu et al 1995) and/or unstable in R<sub>0</sub> and progeny plants. Furthermore, data obtained from promoter studies

are difficult to interpret due to the presence of multiple copies of rearranged promoter fragments, uncertainty in the number of functional copies, and potential competition for *trans*-acting factors.

In contrast to direct DNA uptake methods, *Agrobacterium*-mediated transformation usually results in a discrete, unrearranged segment of DNA being inserted in the recipient genome at a fairly low copy number. Though successful transformation of rice by *Agrobacterium* has been reported several times (Raineri et al 1990, Chan et al 1992), the data have been equivocal and direct confirmation has been lacking. Potrykus (1990) has provided a critical review describing several ways in which false positive results may have been obtained. In contrast to earlier reports, the data presented by Hiei et al (1994) of *Agrobacterium*-mediated rice transformation appeared persuasive. Since the impact of this technology would be so great, we felt it was very important that the results be independently confirmed. Therefore, we obtained the bacterial strains and plasmids used by Hiei et al and attempted to reproduce their results.

Here, we describe experiments in which we successfully reproduced the earlier observation that japonica lines of rice could be stably transformed using the pTOK233 vector (Hiei et al 1994). Using relatively minor modifications of the described procedure, we were able to extend their results to transformation of two additional rice cultivars.

## Results

### Derivation and transformation of scutellar calli

In the original report by Hiei et al (1994), four Japonica cultivars of rice were used, one of which was Koshihikari. In the studies reported here, we used the cultivars Koshihikari, Taipei 309, and Gulfmont, an elite japonica line used commercially in Texas.

Twelve- to fifteen-day-old immature rice embryos were isolated from greenhouse-grown plants of each cultivar, and placed scutellum side up on MS medium containing 2 mg L<sup>-1</sup> of 2,4-D (MS2). After 7-10 d, callus began to develop from the scutellum. This was removed and placed on fresh MS2 medium for 3-4 wk.

The scutellum-derived calli were transformed essentially as described by Hiei et al (1994) by cocultivation with either of two disarmed *Agrobacterium tumefaciens* strains LBA 4404 (Hoekema et al 1983) or EHA 101 (Hood et al 1986) harboring binary vectors (pTOK233) or (pIG121hm), respectively. *Agrobacterium* binary vector plasmids pTOK233 and pIG121hm have similar complements of genes encoded in their T-DNA regions; importantly, both encode an intron-containing  $\beta$ -glucuronidase (*gus*) gene and a hygromycin phosphotransferase (*hpt*) gene, each driven by the CaMV35S promoter. The calli were cocultivated for 10 min in AAM solution (Hiei et al 1994) containing around 5 x 10<sup>9</sup> *Agrobacteria* ml<sup>-1</sup>, after which they were placed on MS medium supplemented with 2 mg L<sup>-1</sup> of 2,4-D and 150 EM acetosyringone, pH 5.2. After 3 d, *Agrobacteria* that were overgrowing the calli were washed away using cefotaxime solution (250 mg L<sup>-1</sup>) before transferring the calli to MS medium containing 2 mg L<sup>-1</sup> of 2,4-D and 250 mg L<sup>-1</sup> of cefotaxime. If no bacteria were

evident after 1 wk, the calli were ready for selection on hygromycin; if bacteria were present, a second round of cefotaxime treatment was performed and the calli were reevaluated 1 wk later.

### **Selection of resistant calli and plant regeneration**

Cocultivated calli were placed on selective medium (MS2 supplemented with 50 mg L<sup>-1</sup> of hygromycin B). After 3-4 wk, resistant calli were visible (Fig. 1A). Continuous selection on hygromycin-containing medium resulted in the appearance of proliferating, apparently resistant, calli from all three cultivars used. To confirm transformation, segments of the calli were subjected to histochemical staining for GUS activity (Jefferson et al 1987) and many regions of intense blue were observed (Fig. 1B-D). Calli derived from Gulfmont (Fig. 1B) and Taipei 309 (Fig. 1D) appeared to be embryogenic, and transfer to regeneration medium (MSD4) resulted in the development of plantlets after 3-4 wk (Fig. 1E). Thus far, we have not been successful in regenerating plants from the Koshihikari calli (Fig. 1C).

The plantlets were first transferred to magenta boxes and subsequently transferred to soil and grown to maturity in a confined greenhouse. Tissue samples (Fig. 1F-H) confirmed that GUS expression was retained in the mature vegetative tissues. Intense expression of GUS was readily seen in roots (Fig. 1F), but initial experiments with leaves resulted in staining only at cut surfaces (Fig. 1G). We determined that this resulted from limited penetration of the X-gluc substrate, rather than from a wounding phenomenon, by solubilization of leaf membranes by brief (3 min) immersion in diethyl ether. Mature leaves treated in this way stained uniformly blue (Fig. 1H). The primary transformants were all vigorous and fertile.

### **DNA analysis of transgenic rice plants**

A summary of experiments conducted using the three rice cultivars in combination with two strains of *Agrobacterium* is presented in Table 1. Thus far, 96 plants expressing *gus* as well as the selectable marker have been obtained. Of these, 29 were Gulfmont. Because of our special interest in this elite Texas cultivar, we have undertaken initial molecular analyses on these plants. Genomic DNA blot analyses of the Gulfmont transformants have shown that four independent lines were obtained in one experiment (Fig. 2) and three independent lines in two other, entirely separate, experiments (Table 1). Further analyses (not shown) of the four independent lines from experiment 1 revealed that two contained single-copy inserts of the T-DNA and two contained two inserts. No rearrangement of the transgene occurred and probing of plant genomic DNA with region failed to detect any bacterial DNA outside the T-DNA region.

### **Analysis of progeny plants**

Vigorous, phenotypically normal and fertile plants were obtained (Fig. 1I) in about 3 mo after transfer from regeneration medium (Fig. 1E). Seeds from these plants showed deep blue GUS activity in less than 2 h after exposure to the X-gluc substrate (Fig. 1J). Histochemical staining of 7-d-old seedlings showed high levels of GUS activity in

**Table 1. *Agrobacterium*-mediated transformation of rice.**

Experiment	Number of calli				Number of plants		
	Cultivar Strain (plasmid)	Initial calli	After 1st selection	After 2nd selection	Total	GUS+	Independent transformants
Gulfmont	1	70	23	4	16	14	4
LBA4404 (pTOK233)	2	77	16	3	8	7	3
	3	71	27	3	9	8	3
Gulfmont	1	70	10	0	0	0	0
EHA101 (pIG121hm)	2	78	4	0	0	0	0
	3	74	7	0	0	0	0
Taipei 309	1	80	21	6	17	12	nd
LBA4404 (pTOK233)	2	51	17	5	21	15	nd
Taipei 309	1	75	30	11	27	21	nd
EHA101 (pIG121hm)	2	59	23	9	28	20	nd
Koshihikari	1	45	8	4	5	0	0
LBA4404 (pTOK233)	2	32	11	1	2	0	0
	3	53	10	5	7	0	0
Koshihikari	1	48	6	2	5	0	0
EHA101 (pIG121hm)	2	50	12	3	9	0	0
	3	25	3	1	4	0	0

**Table 2. Segregation of Gulfmont R<sub>1</sub> progeny.<sup>a</sup>**

Line	Hygromycin			GUS		
	Resistant	Sensitive	Ratio	Positive	Negative	Ratio
W1	149	50	3.0 : 1	72	21	3.4 : 1
W10	31	9	3.4 : 1	35	11	3.2 : 1
W17	28	9	3.1 : 1	25	8	3.1 : 1
W21	48	15	3.2 : 1	45	14	3.2 : 1

<sup>a</sup>Segregation tests of progeny of four independently transformed rice lines. Sterilized and dehusked seeds were germinated on hormone-free medium, with or without 50 mg L<sup>-1</sup> of hygromycin B. Germination rates on hormone-free medium without hygromycin were more than 90% (data not shown). Segregation ratios were close to 3:1 for hygromycin resistant : sensitive or GUS positive:negative. These data indicate that the transgenes were inserted at a single locus.

root tissues. As was the case for the leaves of the primary transformants, treatment of the leaves was required to allow penetration of the substrate. However, three cycles of freeze-thaw treatment (liquid nitrogen/room temperature) proved to be more effective for the young seedling leaves (Fig. 1L) than was immersion in diethyl ether.

Germination tests were conducted on progeny from each of the primary Gulfmont transformants of experiment 1 (Table 1). As anticipated for a single-locus insertion, segregation ratios close to 3:1 (hygromycin resistant:sensitive) were obtained for all four lines (Table 2). In a parallel experiment, seedlings were germinated and stained for GUS activity. In accord with the segregation of the selectable marker, we obtained 3:1 ratios for GUS positive:negative seedlings (Table 2). We have established that progeny of each of these four Gulfmont lines retained the DNA restriction fragment profile of the parental, primary transformant (data not shown).

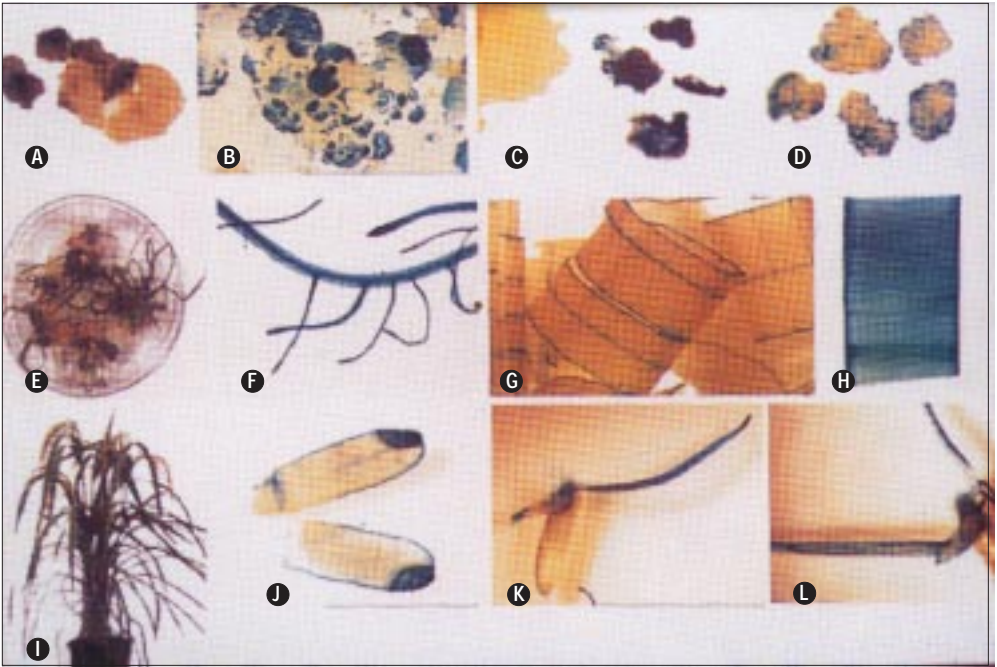


Fig. 1. Production of fertile transgenic rice plants by *Agrobacterium*-mediated transformation of scutellum-derived calli. A) Gulfmont calli on hygromycin selection ( $50 \text{ mg L}^{-1}$ ) following cocultivation. B-D) GUS expression in hygromycin-resistant calli from B) Gulfmont, C) Koshihikari, D) Taipei 309; bar = 1 mm. (E) Gulfmont plants after 3-4 wk on regeneration medium. F-H) GUS expression in tissues of a Gulfmont primary transformant. Roots show uniform blue staining (F), but mature leaf segments (G) only show staining at cut edges and wound sites unless penetration of substrate is assisted, e.g., by treatment with ether (H). I) Fertile plant. J) Longitudinal section of a seed showing intense staining of embryonic and maternal (testa) tissues but little staining in the endosperm, even after wounding. K-L) The roots of progeny seedlings stain readily, but penetration of leaf tissues by the X-Gluc substrate needs assistance, e.g., by several freeze-thaw cycles (L).

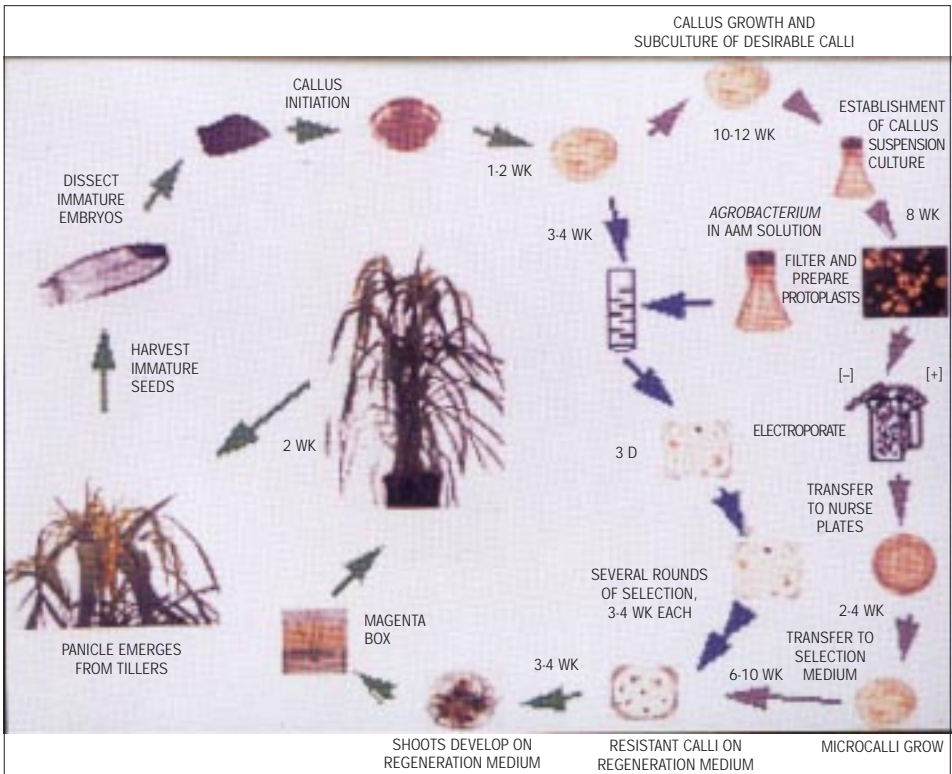
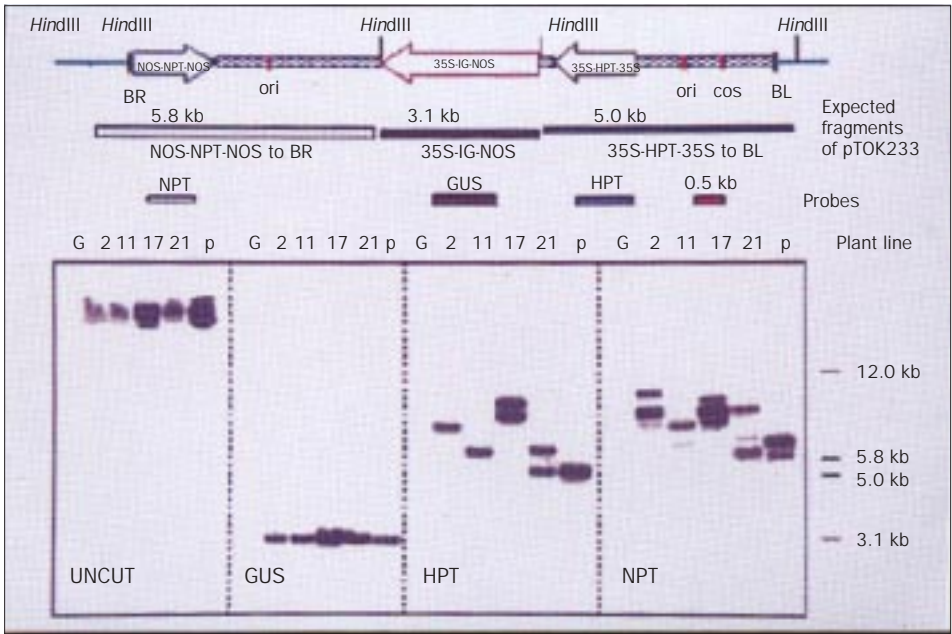


Fig. 2. Genomic DNA blot analysis of transgenic Gulfmont. The upper panel shows a diagram of the T-DNA of pTOK233, the probes used in the analysis and expected fragments following digestion with *Hind*III. The scale bar (red) indicates 0.5 kb. Genomic DNA from Gulfmont (G), four different independent transformants of Gulfmont (2, 11, 17, 21), and pTOK233 (p) was digested to completion with *Hind*III, fractionated by electrophoresis in an agarose gel (0.7%), blotted by standard pressure blot procedures, and probed with <sup>32</sup>P-labeled fragments of GUS or NPT or HPT. The Southern blots in the lower panel show the lack of signal from wild type Gulfmont probed for GUS or NPT or HPT, and four independent transformants: lines 2 and 11 appear to have a single complete insertion and another partial insertion; lines 17 and 21 have two inserted copies of T-DNA. The blot of uncut genomic DNA was probed using the <sup>32</sup>P-labeled GUS fragment.

Fig.3. Procedures for *Agrobacterium*-mediated and electroporation-mediated transformation and regeneration of rice. Arrows indicating steps common to both systems are shown in green; those specific for the *Agrobacterium* approach are in blue and those for electroporation approach in gray. Immature embryos were dissected 10-12 d after pollination and placed on callus induction medium. After 4-6 wk of growth, with one subculture, the scutellum-derived calli were inoculated with *Agrobacterium* and cocultivated in the presence of acetosyringone. After washing with cefotaxime solution, the calli were put onto selection medium with cefotaxime and subcultured at least three times at 3 to 4-wk intervals. Actively growing calli were transferred to regeneration medium. Regenerated plants, confirmed to be transformed by molecular analysis (Fig.2) were transferred to magenta boxes and then to soil in the greenhouse. As shown, it takes about 6 mo from initiation of callus to obtain mature seeds; in contrast, the electroporation approach requires at least 12 mo.

## Discussion

The results presented here represent the confirmation of a report of *Agrobacterium*-mediated transformation of rice, and extend the initial observations by showing production of fertile, stably transformed plants from the japonica cultivar Taipei 309 and the javanica cultivar Gulfmont. Cotransformation of *hpt* and *gus* was essentially 100%, although this has yet to be rigorously established. Importantly, only a single, or two copies of the introduced genes were present and they segregated as expected for a single-locus insertion.

The plants obtained by the present procedure were phenotypically normal and much more vigorous than plants we have previously obtained using electroporation of protoplasts (Battraw and Hall 1990, 1992). In contrast to the protoplast system, which takes 1 yr to obtain fertile plants, we obtained progeny seed in 5-6 mo (Fig. 3). This is similar to the time taken for plants derived from biolistic transformation (Christou et al 1991), but the low copy number of inserted genes is a notable advantage. Additionally, in *Agrobacterium*-mediated transformation, only the DNA sequence between the T-DNA borders is transferred, unrearranged, to the recipient genome. In contrast, bombardment often results in the delivery of multiple, ill-defined plasmid fragments along with the desired genes.

As shown in Fig. 1C, we obtained calli from the cultivar Koshihikari that were transformed, and stained deep blue with X-gluc. However, thus far, we have not been successful in regenerating transformed plants from this cultivar. Hiei et al (1994) also noted that, of the cultivars examined, Koshihikari was relatively recalcitrant to regeneration.

Although both *Agrobacterium* strains used were competent for transformation of Taipei 309, we were not successful in transforming Gulfmont with EHA101(pIG121hm) (Table 1). From these findings, it is evident that strain and cultivar compatibilities exist. However, it seems likely that modifications in culture conditions for both the plant and bacterial strains could yield successful results.

*Agrobacterium*-mediated transformation of many agronomically important dicot species has provided a sound approach for the development of commercial crops having new traits. The proven ability to use a similar approach for transformation of rice, one of the most important monocot crops in the world, promises to be revolutionary in the application of biotechnological advances to agriculture.

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## Notes

*Authors' address:* Institute of Developmental and Molecular Biology and Department of Biology Texas A&M University, College Station, TX 77843-3155, USA.

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