Agrobacterium-Mediated Transformation of Élite Indica and Japonica Rice Cultivars

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Abstract

A rapid, efficient, routine system has been established for *Agrobacterium tumefaciens*-mediated production of hundreds of fertile transgenic plants from commercially important rice cultivars, including an indica cultivar, Pusa Basmati 1. Calli induced from embryos of mature rice seeds were cocultivated with *A. tumefaciens* strain LBA4404 carrying the plasmid pTOK233, then exposed to hygromycin selection followed by an efficient regeneration system. Based on the total number of calli co-cultivated, the transformation frequencies of independent transgenic rice plants including cultivars Pusa Basmati 1, E-yi 105, E-wan 5 and Zhong-shu-wan-geng, were 13.5, 13.0, 9.1, and 9.3%, respectively. T1 seeds were harvested within 7–8 mo of initiation of mature embryo cultures. Data from Southern hybridization analysis proved that foreign genes on T-DNA were stably integrated into the rice genome at low copy/site numbers. Mendelian inheritance of the transgenes was confirmed in T1 progeny.

Index Entries: *A. tumefaciens*; indica rice; japonica rice; *O.sativa*; transgenic plants.

1. Introduction

Transgenic rice (*Oryza sativa* L.) plants have previously been produced chiefly by use of two direct DNA delivery approaches, namely protoplast uptake of foreign DNA *(1,2)*, and DNA delivery into intact cells or tissues by biolistic bombardment *(3,4)*. However, transgenic plants generated via these systems frequently have multiple copies of transgenes integrated in the genome. This situation is often associated with unpredictable gene silencing, sterility *(5,6)*, and non-Mendelian inheritance of the transgenes *(7)*. It is for this reason that numerous attempts have been made to use *Agrobacterium* to transform rice *(8–10)*, but only in 1993 was it reported that *A. tumefaciens* could be used to produce transgenic japonica rice plants at a low frequency *(11)*. In 1994, Hiei et al. *(12)* reported efficient transformation of three japonica rice varieties using *A. tumefaciens* LBA4404 (pTOK233). In 1996, Rashid et al. *(13)* and Aldemita and Hodges *(14)* reported efficient *Agrobacterium*-mediated transformation of both japonica and indica varieties.

This technological development needs to be applied quickly to commercially important indica and japonica rice cultivars, so as to equip such established cultivars with novel traits without the need for time-consuming backcrosses. In this study, we chose the elite indica cultivar, Pusa Basmati 1, and the japonica cultivars, E-yi 105, E-wan 5, and Zhong-shu-wan-geng (ZSWG), as targets for *Agrobacterium*-mediated transformation. Pusa Basmati 1 is a popular aromatic dwarf indica rice variety cultivated in the North Western states of India, commercially important as a high-priced export. E-yi 105, E-wan 5, and ZSWG are grown on more than 3,300,000, 600,000, and 600,000 hectares, respectively, of southern China (D-C. An, personal communication).

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2. Materials and Methods

2.1. Plant Materials, Bacterial Strains, and Plasmids

De-husked mature seeds of Pusa Basmati 1, E-yi 105, E-wan 5, and ZSWG were sterilized in 70% ethanol for 2–5 min and then transferred into 40% (v/v) "Domestos" (Lever Bros., Runcorn, UK) bleach solution for 30 min with shaking. The seeds were rinsed several times with sterile water. Seeds of Pusa Basmati 1 were plated on callus induction medium–N6 medium *(15)* supplemented with 2.5 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 30 g/L maltose or sucrose, 300 mg/L casein hydrolysate, 300 mg/L yeast extract, and 2.5 mg/L Gelrite, pH 5.8 (N6I)–at 28°C in the dark. Seeds of the three japonica cultivars were cultured on LS basal medium *(16)* with the same supplements (LSI medium) and the same culture conditions.

The *A. tumefaciens* strain LBA4404 harbouring the super-binary vector pTOK233 *(12)* was used. The bacteria were grown in liquid AB medium *(17)* containing hygromycin (50 mg/L, Sigma, Dorset, UK), and kanamycin (50 mg/L, Sigma) at 28°C for 3–5 d. For inoculation of calli, the bacteria were suspended at a density of $1–3 \times 10^9$ cells/mL.

2.2. Production of Fertile Transgenic Rice Plants

Three weeks after initiation, embryogenic calli of the four rice cultivars were immersed in *A. tumefaciens* suspension at room temperature for 10–15 min. Infected calli of the three japonica cultivars were cultured on cocultivation medium–LSI medium supplemented with acetosyringone ([AS]; Aldrich, Dorset, UK) at various concentrations (0, 50, 100, 200 μ*M*), without yeast extract, at pH 5.2. After 5–6 d cocultivation, calli were plated on selection medium–LSI supplemented with hygromycin (50 mg/L) and cefotaxime (250 mg/L; Claforan, Roussel Laboratories, UK), pH 5.8 (LSS medium)–and incubated at 28°C in the dark for 3 wk. Infected callus of Pusa Basmati 1 was cultured under the same conditions but on N6 medium. Hygromycin-resistant (hyg^R) calli of the four cultivars were subcultured for two to three cycles onto fresh LSS medium every 3 wk. The hyg^R calli produced somatic embryos from which green buds/shoots developed on regeneration medium–LSS medium containing 3.0 mg/L 6-benzylaminopurine (BAP) instead of 2,4-D and 6.25 g/L Gelrite, without yeast extract, at pH 5.8 (LSR medium)–at 25°C in constant light (3.0 W/m^2) for 3–4 wk. Green buds/shoots were transferred onto development medium (LSR medium with Gelrite concentration reduced to 2.5 g/L) for further shoot development, then to rooting medium–half strength LS medium containing hygromycin (50 mg/L) without plant growth regulators ($^1/_2$ LSO medium)—where they developed vigorous root systems. Robust transgenic rice plants and seeds of T_0 plants were potted into a 3:1:1 (v:v:v) mixture of Levington M3 compost (Levington Horticulture, Ipswich, UK), John Innes No. 3 compost (Croxden Horticulture Products, Norwich, UK), and standard Perlite (Silvaperl Products, Lincoln, UK). These plants were grown in a controlled environment cabinet (Fitron, Sanyo Gallenkamp PLC, Leicester, UK) at 80–90% humidity, with 10 h photoperiod of 25 W/m^2 at 28°C with night temperatures of 24°C, where they matured, flowered, and set seeds.

2.3. Histochemical Assay for β-Glucuronidase (GUS) Activity

GUS activity was assayed histochemically by a procedure based on that described by Jefferson *(18)*. Calli or plant explants were incubated at 55°C in staining solution containing 20% methanol overnight (10–16 h). The stained materials were fixed in 70% ethanol.

2.4. Southern Blot Analysis

Genomic DNA was isolated from leaves of rice plants. *Hin*dIII digested DNA, separated by agarose gel electrophoresis (10 μg/lane), was transferred to Hybon-N (Amersham Life Sciences, Little Chalfont, UK) and hybridized at 65°C in hybridization solution with [α-^{32}P]dCTP probes. The *hpt*, *gus*A probes were prepared from the plasmid pWRG1515 *(19)* and the *npt*II probe from pCaMVNEO *(20)* using a Rediprime kit (Amersham) in all experiments. Membranes were washed

twice at room temperature in 2X SSPE/0.1% sodium dodecyl sulfate (SDS) for 10 min and at 65°C in 1 and 0.1X SSPE/0.1% SDS for 15 min each time and then autoradiographed using Amersham Hyperfilm.

2.5. Germination Tests of T_1 Seeds

Seeds of the T_0 generation were dehusked, sterilized, and plated onto LS medium containing hygromycin 50 mg/L without plant growth regulators under the same conditions used for callus induction. Segregation of the *hpt* gene in seedlings of T_1 plants was recorded after 10 d in the medium.

3. Results

3.1. Plant Regeneration

Embryogenic callus, observed as being compact, yellowish, and granular, was formed from the scutellar tissue of mature seeds of the four rice cultivars within 2–3 wk of culture initiation on N6I or LSI medium. The number of embryogenic calli produced from Pusa Basmati 1 was two- to fourfold lower than that from the japonica cultivars. On induction medium, which contained maltose as a carbon source, 80–95% of the seeds produced embryogenic calli, while less than 50% of the seeds produced such calli on media containing sucrose. Transfer of these embryogenic cultures onto LSR medium led to the formation of somatic embryos which gave rise to plantlets at a high frequency (50–70%). Gelrite at 6.25 g/L in the regeneration medium increased the regeneration frequency by about two- to fourfold in comparison with the frequency at a Gelrite concentration of 2.5 g/L. Fertile plants were obtained from the rooted plantlets after 3–4 mo in controlled environment cabinets.

3.2. Cocultivation of Rice Calli with A. tumefaciens

Embryogenic calli from each of the four cultivars were selected for cocultivation. The expression of the *gus*A gene was monitored in the infected calli for 1–8 d following bacterial inoculation. GUS activity was detectable from the first day of cocultivation; the number of blue GUS foci increased with the time of cocultivation and reached a maximum at d 5 (60–110/9 mm^2 callus). Therefore, the infected calli at d 5 were transferred onto selection medium. The blue GUS foci were observed only in rapidly growing areas of the calli but not in slow-growing areas. Calli of Pusa Basmati 1 gave fewer blue GUS foci than those of the three japonica cultivars (**Table 1**). Without AS treatment, only 10–15 blue GUS foci were detected per 9 mm^2 callus, whilst with AS at 100 μ*M* blue GUS foci, on average, increased by six- to sevenfold, and a maximum number of 400 blue foci were observed (**Fig. 1A,B**). AS at 200 μ*M* did not further increase the number of GUS foci.

3.3. Production of Primary Transgenic (T_0) Plants

Hyg^R calli were obtained after 3-wk selection (**Fig. 1C**). The GUS assay revealed that only 30–40% of the calli were stained uniformly blue, while the remaining 60–70% were presumably chimeric (**Fig. 1D**). When the calli were selected for another 3–6 wk, 95% were uniformly blue after the GUS assay (**Fig.1E**). The formation of green hyg^R buds/shoots from somatic embryos (**Fig. 1F**) of the four rice cultivars occurred within 3 wk of transferring hyg^R calli onto LSR medium. The embryoid shoot primordia and the emerging primary shoots and roots showed uniform *gus*A gene expression (**Fig. 1G**). Healthy plantlets with extensive root systems were established on $^1/_2$ LS0 medium containing hygromycin at 50 mg/L after another 2–3 wk (**Fig. 1H**). GUS activity was observed in the leaves, roots, immature embryos, and endosperms of the primary transgenic plants (**Fig. 1I,J,L,M**), but no GUS activity was detected in the corresponding parts of non-transformed plants. Most of these T_0 plants (95%) grew to phenotypically normal maturity (**Fig. 1K**).

3.4. Transformation Frequency

In **Table 1**, the frequency of transient and stable *gus*A expression in embryogenic calli from the four cultivars has been recorded. High levels of transient gene expression (87–100%) were observed in all four cultivars. However, the number of hyg^R calli produced on the selection medium was significantly lower than the number of GUS

Table 1
Efficiencies of transformation of élite rice cultivars by *A. tumefaciens* LBA4404 (pTOK233)

Rice cultivars	No. of expts.	Transient gene expression: % of GUS+ calli/total calli tested[a]	Transient gene expression: Mean no. of GUS foci (±SD)/9 mm^2 callus[b]	Stable transformation: No. of co-cutivated calli (A)	Stable transformation: No. of hyg^R calli (B)	Stable transformation: No. of hyg^R plantlets (C)	Stable transformation: No. of GUS+ plantlets (D)	Frequencies, % (B/A)	(D/B)	(C/A)	(D/A)
E-yi 105	1	98.6	58.2 ± 27.8	840	391	122	116	46.5	29.7	14.5	13.8
	2	99.3	62.6 ± 12.9	800	401	142	140	50.1	34.9	17.8	17.5
	3	100.0	96.9 ± 36.4	800	398	102	98	49.8	24.6	12.8	12.3
	4	97.8	62.4 ± 28.5	619	361	70	68	58.3	19.1	11.3	11.1
	5	92.6	56.0 ± 30.3	800	342	86	80	42.8	23.4	10.8	10.0
Total		97.7	67.2 ± 15.1	3859	1893	522	503	49.1	26.6	13.5	13.0
E-wan 5	1	86.7	54.4 ± 17.3	950	536	87	87	56.4	16.2	9.2	9.2
	2	93.3	38.3 ± 20.5	600	227	55	53	37.8	23.3	9.2	8.8
Total		90.0	46.4 ± 8.1	1550	763	142	141	49.2	18.5	9.2	9.1
ZSWG	1	100.0	49.7 ± 24.1	446	118	55	52	26.5	44.1	12.3	11.7
	2	96.1	36.1 ± 28.4	600	254	48	45	42.3	17.7	8.0	7.5
Total		98.1	42.9 ± 6.8	1046	372	103	97	35.6	26.1	9.8	9.3
Pusa	1	100.0	13.3 ± 3.9	595	152	83	82	25.5	53.9	13.9	13.8
Basmati 1	2	98.7	13.9 ± 2.8	402	82	56	53	20.4	61.6	13.9	13.2
Total		99.4	13.6 ± 0.3	997	234	139	135	23.5	57.7	13.9	13.5

[a]More than 100 pieces of callus were tested in each experiment.
[b]Data were collected from 30 randomly selected calli 4 d after cocultivation.

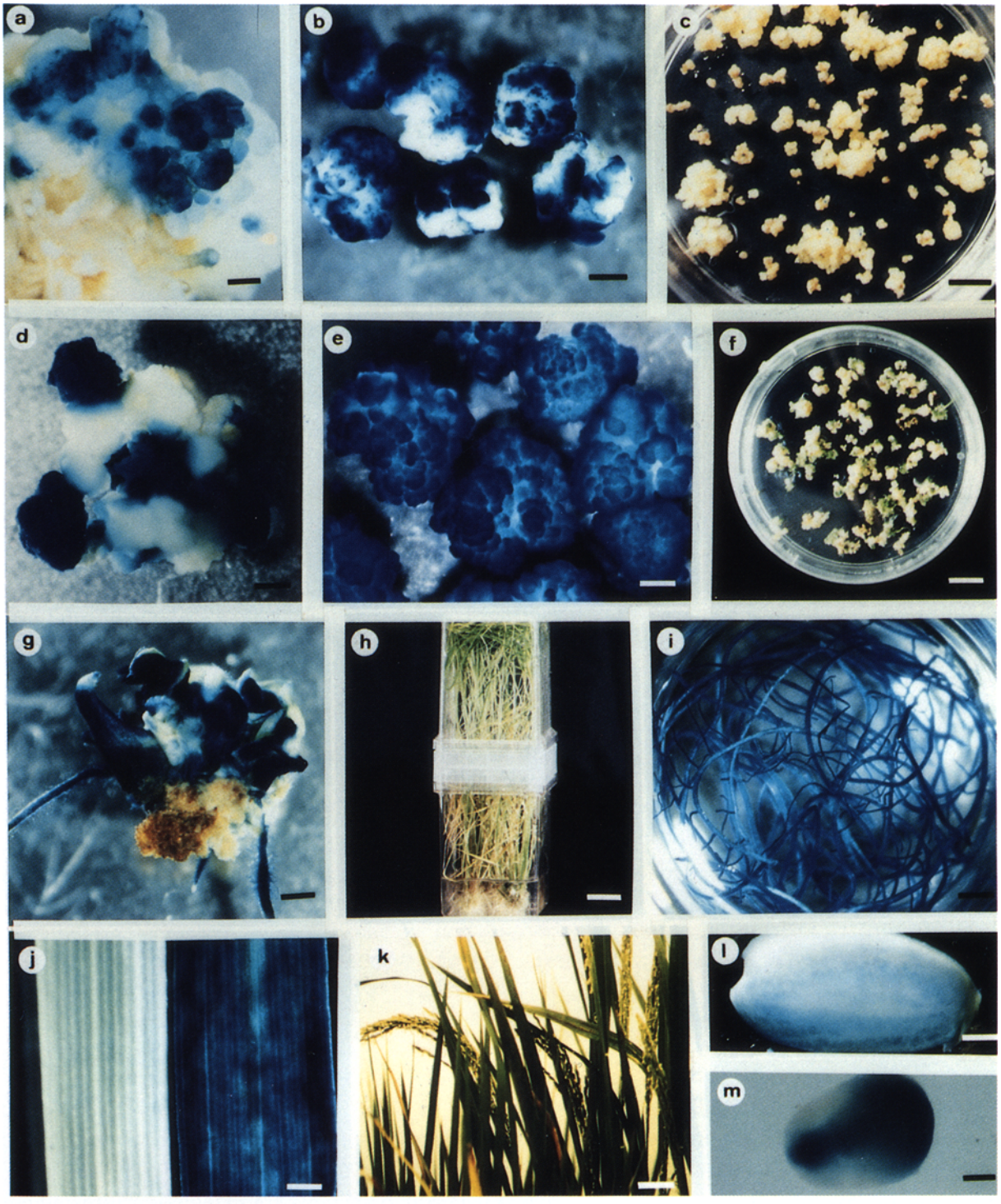

Fig 1. Production of transgenic rice plants by *A. tumefaciens*. **(a,b)** Callus derived from mature embryos of japonica (A, E-yi 105) and indica (B, Pusa Basmati 1) rice, respectively, exhibiting GUS activity 5 d after cocultivation, bars = 1.0 mm; **(c)** Proliferation of the infected callus under hygromycin selection (50 mg/L), bar = 1.0 cm; **(d)** Chimeric hyg^R callus with transformed and non-transformed cell

(continued)

foci resulting from transient expression; this indicated that stable integration occurred at a correspondingly lower frequency. The average number of GUS foci on the callus of Pusa Basmati 1 was found to be lower than that on the three japonica cultivars. This observation coincides with the fact that callus of Pusa Basmati 1 grew more slowly than those of the three japonica cultivars during the cocultivation period. The frequencies the hyg^R calli from the four cultivars varied from 20–58% and the frequency of hyg^R calli of Pusa Basmati 1 was usually lower than those for the three japonica cultivars. However, the transformation frequency of transgenic plants of Pusa Basmati 1 was in the same range as those of the three japonica cultivars (**Table 1**). This is caused by a higher conversion rate of hyg^R calli to transgenic plantlets of Pusa Basmati 1 compared to the japonica cultivars (**Table 1**).

3.5. Southern Blot Analysis of T_0 Plants

Genomic DNA from the leaves of 10 randomly selected T_0 plants, as well as a non-transformed control plant, was subjected to Southern blot analysis. Integration of the *hpt*, *gus*A, and *npt*II genes into the genome of the transgenic plants was revealed by the corresponding probes (**Fig. 2A–C**). The copy number/sites of integrated genes among transgenic plants varied from one to four, based on the number of bands probed by *hpt* or *npt*II sequences (**Fig. 2A,C**). Each transgenic plant had a different hybridization pattern from the others. Two of the 10 plants revealed a single copy/site insertion of the transgenes.

3.6. Segregation of the hpt Gene in T_1 Plants

The results of a segregation analysis of the *hpt* gene in T_1 plants are presented in **Table 2**. The χ^2 analysis of segregation data indicated that the expected 3:1 segregation ratio was observed in 9 out of 11 independent transgenic T_1 plants (**Table 2**). Southern blot analysis also showed that hyg^R T_1 plants gave the same hybridization pattern as their parental line (**Fig. 2D,E**). These results confirmed the Mendelian inheritance of hygromycin resistance as a single, dominant trait in most progeny of independent transformants. Furthermore, the segregation of the *hpt* gene in T_1 seedlings of E-yi 105 collected from two discrete tillers of the same T_0 plant suggests that the primary transformants were genetically uniform. In two cases, the segregation pattern of the *hpt* gene was non-Mendelian (**Table 2**; E-yi 105-3 and ZSWG-3).

4. Discussion

An efficient *A. tumefaciens*-mediated transformation system has been established for one commercial indica cultivar and three élite japonica cultivars. The procedure is rapid, reproducible, and efficient; three bench workers have produced more than 600 independent T_0 transgenic rice plants within 7–8 mo from initiation of cultures.

Our results confirm that *A. tumefaciens* LBA4404 (pTOK233) ***(12)*** is very effective in transforming commercial japonica rice cultivars. In addition, we have successfully extended the use of this system by transforming the commercial indica rice, Pusa Basmati 1. During the preparation of this article, two other groups have independently

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Fig. 1. *(continued from previous page)* clusters, revealed by GUS activity 3 wk after hygromycin selection of E-yi105, bar = 0.17 mm; **(e)** Uniform expression of the *gus*A gene in hyg^R callus of Pusa Basmati 1 after 6 wk (2 cycles) selection, bar = 2.0 mm; **(f)** Differentiation of proliferated hyg^R calli on LSR medium after 3 wk, bar = 1.3 cm; **(g)** Hyg^R tissue undergoing regeneration of plantlets with GUS activity, bar = 4.0 mm; **(h)** Regeneration of transgenic plants with vigorous root systems on half strength LS0 medium containing hygromycin (50 mg/L), bar = 2.0 cm; **(i)** GUS activity in roots (bar = 2.2 cm) of a T_0 plant; **(j)** GUS activity in leaf (bar = 3.0 mm) of a T_0 plant; **(k)** Flowering of the transgenic plants, bar = 3.0 cm; **(l,m)** GUS activity in endosperm (**l**; bar = 0.1 mm) and immature embryo (**m**; bar = 0.3 mm) of transgenic plants.

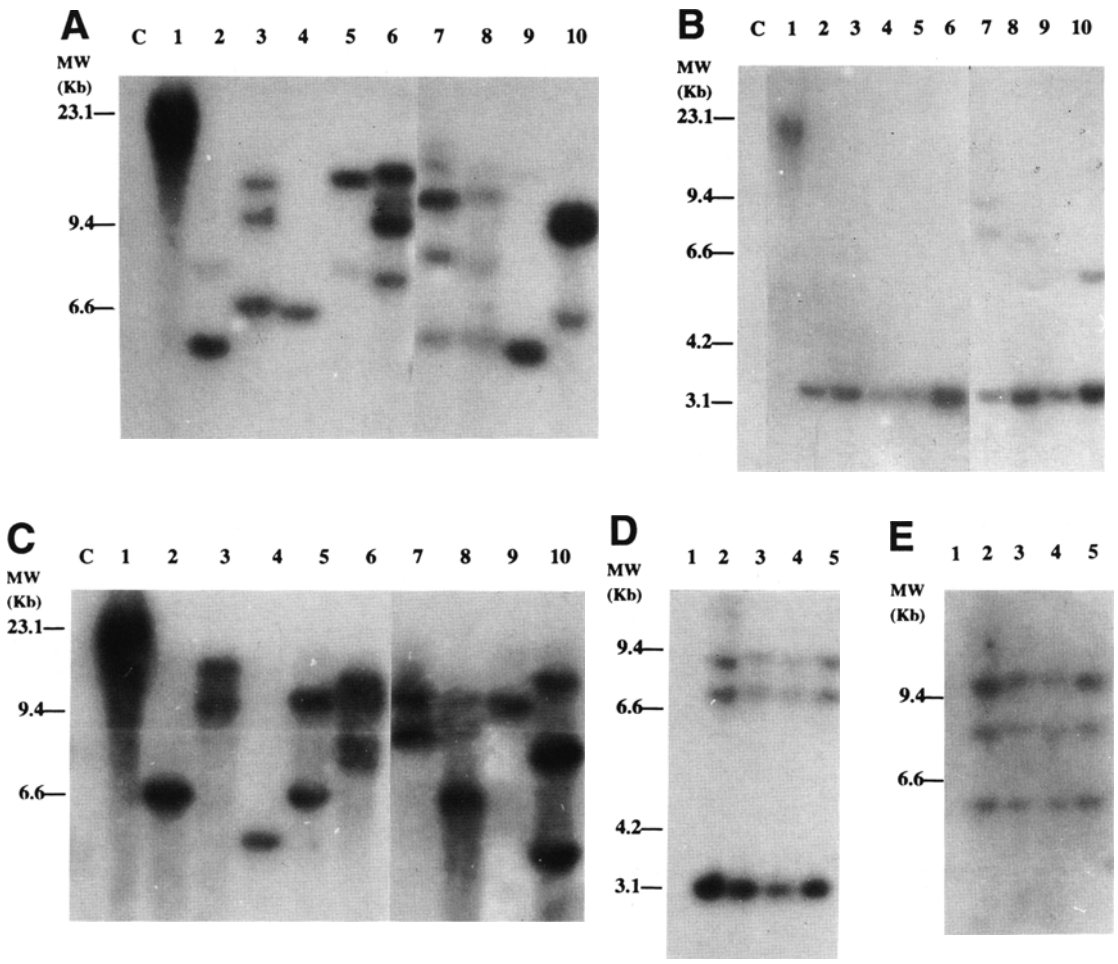

Fig. 2. Southern blot analysis of T_0 and T_1 rice plants. **(A–C)** Lane C, DNA from a nontransformed plant; lane 1, undigested DNA of a T_0 plant; lanes 2–10, DNA of T_0 plants were digested with *Hin*dIII, and hybridized to the *hpt* (A), *gus*A (B), or *npt*II (C) probes. Lanes 1–3, E-yi 105; lanes 4 and 5, E-wan 5; lanes 6–8, Pusa Basmati 1; and lanes 9 and 10, Zhong-shu-wan-geng. **(D,E)** Lane 5, DNA from a T_0 Pusa Basmati 1 plant (which is the same line as that in lane 7 of A–C); lanes 1–4, DNA from its progeny T_1 hygromycin-sensitive plant (lane 1) and hygromycin-resistant plants (lanes 2–4). DNAs were digested with *Hin*dIII and hybridized to the *gus*A (D) or *hpt* (E) probes.

reported *A. tumefaciens*-mediated transformation of indica rice varieties including IR72, TCS10 ***(14)***, and Basmati 370 and 385 ***(13)***. Very recently, this approach has also been applied successfully in javanica rice ***(21)***. Our work establishes the *Agrobacterium*-mediated transformation procedure for routine production of transgenic commercial indica and japonica rice cultivars.

In our study, we focused on simplifying the experimental process and achieving efficient recovery of transgenic plants of commercial lines of Pusa Basmati 1 and the three élite japonica cultivars. Rice seeds, as opposed to the popularly used immature embryos, were chosen as starting materials. This reduced the total length of experiments by 2–3 mo and also saved resources usually required for maintaining healthy plants to provide immature embryos.

The very efficient regeneration system via somatic embryogenesis has contributed significantly to the efficiency of the transformation procedure. The parameters increasing plant regeneration frequency are: a high concentration of the gelling agent (Gelrite 6.25 g/L) in the differentiation medium and maltose (30 g/L) as the carbon source in the culture medium.

Table 2
Segregation Analysis of the *hpt* Gene in T_1 Seedlings from Selfed-Seeds of the T_0 Plants

Transgenic rice lines	No. of T_1 seeds tested	Hyg^R	Hyg-sensitive seedlings	χ^2 (3:1)[a]	*p*
E-yi 105-1	Tiller 1 26	19	7	0.05	>0.05
	Tiller 2 29	21	8	0.10	>0.05
E-yi 105-2	Tiller 1 37	27	10	0.08	>0.05
	Tiller 2 23	17	6	0.01	>0.05
E-yi 105-3	Tiller 1 27	10	17	20.75	<0.001
	Tiller 2 28	9	19	27.43	<0.001
E-wan 5-1	57	45	12	0.47	>0.05
E-wan 5-2	68	54	14	0.71	>0.05
E-wan 5-3	63	36	17	1.42	>0.05
ZSWG-1	61	45	16	0.05	>0.05
ZSWG-2	62	55	17	0.07	>0.05
ZSWG-3	54	20	34	41.51	<0.001
Pusa Basmati 1-1	23	16	5	0.02	>0.05
Pusa Basmati 1-2	25	18	7	0.12	>0.05

[a]χ^2 = chi-square value for 3:1 ratio (hyg^R: hygomycin sensitive seedlings) with Yates (continuity) correction with one degree of freedom.

The interaction between *Agrobacterium* cells and plant cells determines successful T-DNA transfer and integration. Addition of AS (at 100 μ*M*) to the cocultivation medium is essential. However, we believe that the most important aspect of the protocol is recognition and selection of healthy, fast-growing embryogenic callus for inoculation. Continuous plant cell division during cocultivation and initial selection seems to be critical. It was evident that the blue GUS foci were observed only in rapidly growing areas of the infected calli.

Hygromycin has been used widely as a selection agent for rice transformation ***(1,2,12)***. In this investigation, continuous exposure to hygromycin in the culture medium for more than 8 wk was essential to eliminate nontransformed cells which may have been "nursed" by adjacent transformed cells (**Fig. 1D**). Inclusion of hygromycin in the regeneration medium further improved selection efficiency.

Most of the transgenic rice plants had less than three copies/sites of the T-DNA integration (**Fig. 2**). Each individual transgenic plant had a unique integration pattern, which suggested that the T-DNA had been inserted into the rice genome in a random manner. Moreover, the *hpt* gene segregated as a single dominant trait with a few exceptions (**Table 2**), indicating that the T-DNA sequences were integrated at the same or closely linked loci.

In summary, we have proved that an indica commercial cultivar, Pusa Basmati 1, and three élite japonica rice cultivars can be efficiently transformed using *A. tumefaciens*. The important factors in this system include:

1. Recognition and selection of healthy embryogenic callus derived from mature seeds as target material;
2. Use of *A. tumefaciens* LBA4404 (pTOK233) for inoculation;
3. Addition of AS to the cocultivation medium; and
4. Efficient recovery of transformed plantlets on optimized regeneration medium and use of continued hygromycin selection during regeneration.

The transgenic plants contain fewer copies/sites of integrated transgenes and exhibit no sterility problems. This system permits the production of a large number of transgenic plants with the desired gene expression and stability within 7–8 mo at low cost.

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References

1. Shimamoto, K., Teda, R., Izawa, T., and Fujimoto, T. (1989) Fertile transgenic rice plants regenerated from transformed protoplasts. *Nature* **338,** 274–277.
2. Datta, S. K., Peterhans, A., Datta, K., and Potrykus, I. (1990) Genetically engineered fertile indica-rice recovered from protoplasts. *Bio/Technol.* **8,** 736–740.
3. Christou, P., Ford, T., and Kofron, M. (1991) Production of transgenic rice (*Oryza sativa* L.) plants from agronomically important indica and japonica varieties via electric discharge particle acceleration of exogenous DNA into immature zygotic embryos. *Bio/Technol.* **9,** 957–962.
4. Cao, J., Duan, X. L., McElroy, D., and Wu, R. (1992) Regeneration of herbicide resistant transgenic rice plants following microprojectile-mediated transformation of suspension culture cells. *Plant Cell Rep.* **11,** 586–591.
5. Finnegan, J. and McElroy, D. (1994) Transgene inactivation: plants fight back. *Bio/Technol.* **12,** 883–888.
6. Flavell, R. B. (1994) Inactivation of gene expression in plants as a consequence of specific sequence duplication. *Proc. Natl. Acad. Sci. USA* **91,** 3490–3496.
7. Peng, J. Y., Wen, F. J., Lister, R. L., and Hodges, T. K. (1995) Inheritance of *gus*A and *neo* genes in transgenic rice. *Plant Mol. Biol.* **27,** 91–104.
8. Li, X. Q., Liu, C. N., Ritchie, S. W., Peng, J. Y., Gelvin, S. B., and Hodges, T. K. (1992) Factors influencing *Agrobacterium*-mediated transient expression of *gus*A in rice. *Plant Mol. Biol.* **20,** 1037–1048.
9. Liu, C. N., Li, X. Q., and Gelvin, S. B. (1992) Multiple copies of VirG enhance the transient transformation of celery, carrot and rice tissues by *Agrobacterium tumefaciens*. *Plant Mol. Biol.* **20,**1071–1087.
10. Raineri, D. M., Bottino, P., Gordon, M. P., and Nester, E. W. (1990) *Agrobacterium*-mediated transformation of rice (*Oryza sativa* L.). *Bio/Technol.* **8,** 33–38.
11. Chan, M. T., Chang, H. H., Ho, S. L., Tong, W. F., and Yu, S. M. (1993) *Agrobacterium*-mediated production of transgenic rice plants expressing a chimeric α-amylase promoter/beta-glucuronidase gene. *Plant Mol. Biol.* **22,** 491–506.
12. Hiei, Y., Ohta, S., Komari, T., and Kumashiro, T. (1994) Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. *Plant J.* **6,** 271–282.
13. Rashid, H., Yokoi, S., Toriyama, K., and Hinata, K. Transgenic plant production mediated by *Agrobacterium* in *Indica* rice. *Plant Cell Rep.* **15,** 727–730.
14. Aldemita, R. R. and Hodges, T.K. (1996) *Agrobacterium tumefaciens*-mediated transformation of japonica and indica rice varieties. *Planta* **199,** 612–617.
15. Chu, C. C., Wang, C. C., Sun, C. S., Hsu, C., Yin, K. C., Chu, C. Y., and Bi, F. Y. (1975) Establishment of an efficient medium for anther culture of rice through comparative experiments on nitrogen sources. *Scientia Sinica* **18,** 659–668.
16. Linsmaier, E. M. and Skoog, F. (1965) Organic growth factor requirement of tobacco tissue culture. *Physiol. Plant* **18:,** 100–127.
17. Chilton, M. D., Currier, T. C., Farrand, S. K., Bendich, A. J., Gordon, M. P., and Nester, E. W. (1974) *Agrobacterium tumefaciens* DNA and PS8 bacteriophage DNA not detected in crown gall tumours. *Proc. Natl. Acad. Sci. USA* **71,** 3672–3676.
18. Jefferson, R. A. (1987) Assaying chimeric genes in plants: the GUS gene fusion system. *Plant Mol. Biol. Rep.* **5,** 387–405.
19. Christou, P. and Ford, T. L. (1995) The impact of selection parameters on the phenotype and genotype of transgenic rice callus and plants. *Trans. Res.* **4,** 44–51.
20. Fromm, M. E., Taylor, L. P., and Walbot, V. (1986) Stable transformation of maize after gene transfer by electroporation. *Nature* **319,** 791–793.
21. Dong, J., Teng, W., Buchholz, W.G. and Hall, T.C. (1997) *Agrobacterium*-mediated transformation of Javanica rice. *Mol. Breeding* **2,** 267–276.