J. Plant Biochemistry & Biotechnology Vol. **10**, 121 - 126, July 2001

Agrobacterium tumefaciens - mediated Transformation of Rice Using Coleoptile and Mature Seed-derived Callus

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Morphologically normal, fertile transgenic rice plants (*Oryza sativa* L *cv* Taipei 309) were obtained using *Agrobacterium tumefaciens* strain LBA4404 harbouring the plasmid pTOK233. Two transgenic systems were developed. The first involved callus derived from mature seeds (scutellum) and, the second, used callus derived from 4-d-old coleoptiles. This is the first time that a coleoptile-based system has been used for producing transgenic rice plants. In the development of coleoptile based system, we have evaluated the effect of the length of callus induction period of the coleoptiles on transformation efficiency. The proportion of GUS positive plants was 23% in coleoptile experiment while in mature seed experiments it was 21%. Southern analyses were done to confirm the presence of the transgene. It was found that one to three copies of the transgene integrated in the transgenic plants.

Key words : transgenic plants, *Oryza sativa,* transgene, gene transfer.

It has been difficult until recently to transform monocotyledonous plants with good efficiency using *Agrobacterium* on account of the fact that monocots fall outside the natural host range of *Agrobacterium* (1,2). The gene transfer techniques developed over the years for transforming monocots have, therefore, depended more on direct gene transfer methods using protoplasts (3-7) or biolistics (8,9). However, *Agrobacterium*-mediated transformation method is preferred, as it does not compulsorily require the protoplasts, and in general results in higher transformation efficiencies with a more predictable pattern of transgene integration, compared to all other available technique (10,11). Moreover, with *Agrobacterium*-mediated transformation, only a few copies of the disarmed T-DNA, carrying the transgene(s), are integrated into the host genome and the transgenic plants are generally fertile (12,13).

Recently, there have been several reports on successful transformation of monocots by *Agrobacterium*. These include maize (14), wheat (15) and barley (16). In rice, successful transformation and DNA integration has been reported in *japonica* rice (10,12). Report by Hiei *et al* (10) is of significance as they achieved efficient transformation of *japonica* varieties of rice and confirmed T-DNA integration into the genome by Southern analysis and by sequencing the T-DNA-genomic junctions (10). These reports were confirmed and further extended to include more-difficult-to-transform *indica* rice varieties (11-13). Various explants have been used in *Agrobacterium*-mediated transformation studies to initiate the cultures, which include, mature embryo (10) immature embryos (12), and scutellum (13). We report the use of coleoptile tissue of *japonica* rice variety Taipei-309, as the starting material in *Agrobacterium*-mediated transformation at a fairly high frequency.

Materials and Methods

Plant material — Four-day-old coleoptiles of *Japonica* rice (*Oryza sativa* L *cv* Taipei 309) were used to develop a system for *Agrobacterium*-mediated transformation. In the coleoptile based experiments, dehusked, surface sterilized seeds were allowed to germinate under submerged condition in dark. On the fourth day, coleoptiles were excised out from the base and the apical tips were cut-off. Five different set of experiments were carried out as follows: Freshly excised 4-d-old coleoptiles were directly co-cultivated with *Agrobacterium*;

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Abbreviations: AS: acetosyringone; 2,4-D: 2,4-dichlorophenoxyacetic acid; BAP: 6-benzylaminopurine; CB: Carbenicillin; CF: Cefotaxime: HYG: hygromycin-B; Kn: Kinetin; MES: 2-(N-morpholino) ethanesulphonic acid; MS: Murashige and Skoog (1962); NAA: 1-naphthaleneacetic acid; PGR: Plant growth regulator.

4-d-old coleoptile were placed on callus induction medium for one, two or three weeks before co-cultivation or 4-d-old coleoptile-derived primary callus was sub-cultured and maintained for an additional three weeks on callus proliferation medium before inoculation with *Agrobacterium*. Callus induction and callus proliferation media were MSS2.5D and MSM2.5D, respectively (Table 1). Cultures were incubated in darkness in growth chamber at 26 ± 2°C for 3 weeks on each medium (Fig.1a).

Parallel to the coleoptile experiment, another set of experiment was performed using mature seed. All media and culture conditions used were identical to those used in the coleoptile-based experiments. However, callus derived from mature seed had been sub-cultured on MSM2.5D before co-cultivation.

***Agrobacterium tumefaciens* strain and plasmid** — The *A. tumefaciens* strain LBA4404 harbouring the plasmid pTOK233 was obtained from Japan Tobacco Inc. (10). The binary vector pTOK233 contains *vir* B, *vir* C, *vir* G genes derived from the *KpnI* fragment of supervirulent Ti-plasmid pTiBo542 and *hpt*, *npt*II and *gus*A with an intron-*gus* within the T-DNA region (10).

Table 1. Media used for tissue culture and transformation of japonica rice cultivar Taipei-309

Medium	Composition
MSS2.5D	MS salts and vitamins (20), sucrose (30g/l), 2,4-D (2.5mg/l), 6g/l agarose, pH 5.8
NSM2.5D	MSS2.5D medium with Maltose (30g/l) in place of sucrose.
MS-CC	MS salts and vitamins, sucrose (30g/l), glucose (10g/l), casamino acids (1g/l), 2,4-D (2.5mg/l), gelrite (3g/l), AS(100µM), pH 5.8
MSS2.5D 1KCBCF	Ms Salts and vitamins, casamino acids (1g/l), sucrose (30g/l), CB (250mg/l), CF (100mg/l), gelrite 3g/l pH 5.8
MS50HYG	MS salts and vitamins, casamino acids (1g/l), sucrose (30g/l), 2, 4-D (2.5 mg/l) Kn (1mg/l), HYG (50mg/l), gelrite (3g/l), pH 5.8
MS 100HYG	GYG50MS medium with HYG (100mg/l instead of 50mg/l)
MS(3)BD	MS salts and vitamins, Sucrose (20g/l), sorbitol (30g/l), BAP (0.5mg/l), 2,4-D (2mg/l), gelrite (3g/l), pH 5.8
MSS0.5N2B	MS salts and vitamins, tryptophan (1g/l, sucrose (30g/l), NAA (0.5mg/l) BAP (2mg/l), agarose (6g/l), pH 5.8
3SMS0	MS salts and vitamins, tryptophan (1g/l), sucrose (30g/l), phytagel (2g/l), pH 5.8

Preparations of rice cultures for co-cultivation — This step was identical for both coleoptile-based transformation experiments and mature seed-based experiments. The proliferated calli were cut into small (1-2 mm) pieces, and plated on fresh callus proliferation medium. These were incubated in dark at 26 ± 2°C for four days. On the fourth day, these calli were transferred to the co-cultivation medium (MS-CC) just prior to co-cultivation (Table 1).

Preparation of Agrobacterium suspension — *Agrobacterium* cultures were initiated on ABG plates (AB minimal medium (17) having 5 g l^{-1} glucose) from glycerol stock stored at – 80°C, and after four days single colonies were cultured in YEP (Yeast Extract Peptone) rich medium supplemented with hygromycin-B (50 mg l^{-1}). After 30 h of incubation, bacteria were pre-induced overnight with 100 mM Acetosyringone (AS) in PIM2 medium modified slightly from original preparation (18,19) by increasing the concentration of MES buffer from 20 mM to 75 mM. An additional 100 mM AS was added to the bacterial suspension and its concentration was adjusted to give A_{600} between 1.6-2.0. At this stage *Agrobacterium* was ready for co-cultivation.

Co-cultivation, selection and plant regeneration — Ten ml of *Agrobacterium* suspension was placed individually on each callus piece on co-cultivation media. The cultures were incubated in dark at 26 ± 2°C.

Three days after these callus pieces were inoculated with *A. tumefaciens*, they were transferred to MSS2.5D1KCBCF medium (Table 1) for stopping the growth of *Agrobacterium* while promoting the growth of the callus. At this stage however, no selection agent was added to the medium. After 3 weeks the calli were cut into small 1-2 mm pieces and transferred to the first selection medium (MS50HYG) (Table 1) containing hygromycin-B (50 mg l^{-1}). This medium also contained carbenicillin (250 mg l^{-1}) and cefotaxime (100 mg l^{-1}) to stop the growth of any remaining *Agrobacterium*. Callus pieces surviving the first selection were subjected to a more stringent second selection on MS100HYG (Table 1), which contained 100 mgl^{-1} hygromycin-B. Both the selections were carried out for 3 weeks each. Finally, the callus pieces that survived the second selection (Fig. 1b) were transferred to MS(3)BD medium (Table 1) for proliferation without selection pressure. After 3 weeks the calli were transferred to the regeneration medium, MSS0.5N2B (Table 1). The cultures were initially kept in dark for one week before exposure to light conditions. Regenerated plants were transferred to a rooting medium, 3SMS0 (Table 1), devoid of any PGR. Putative transformants were then transferred to steam-sterilized soil in the green house.

β-Glucuronidase assay — Histochemical GUS assay was performed on callus pieces after 3 days of co-

cultivation with *Agrobacterium* for monitoring transient GUS activity and after 12 weeks for monitoring stable GUS activity. Assay was also performed on all the putative transformed plants using leaf segments. For the histochemical staining, tissues and cells were incubated in X-Gluc solution (21) at 37°C from few min to overnight and scored for evidence and extent of GUS activity.

Genomic DNA isolation and Southern blot — Genomic DNA was isolated from putative transformed plants following the method of McCouch *et al* (22) The procedures for restriction enzyme digestion, gel electrophoresis and Southern blot analyses were according to Sambrook *et al* (23). 20 mg of genomic DNA was used from each plant for preparing Southern blots. For each plant, genomic DNA was digested in two sets. First set was digested with enzyme *Hin*dIII, which cuts out cleanly the 3.14 kb 35S-IG-*nos* fragment from the integrated T-DNA. The second set was cut with enzyme *Xba*I which has a unique site at 8.01kb within the T-DNA. On a Southern blot, (probed by ^{32}P-labled *gusA* fragment), the *Hin*dIII digested DNA produced a band at 3.14, while *Xba*I digested sample produced band(s) of variable sizes, an indication of the number of copies of the T-DNA integrated into the genome.

Results and Discussion

Coleoptile based system — It has been earlier established in *indica* rice cultivar CH1039 (24) and later confirmed for the same variety that plant regeneration was dependent on the age of coleoptile and that the highest frequency of plant regeneration was from 4-day-old coleoptiles. These results were found to apply for *japonica* cultivar Taipei 309. Starting from 4-d-old coleoptiles in Taipei-309 we studied the effect of length of incubation period of these explants (on callus induction medium, MSS2.5D), on transformation efficiency using *Agrobacterium tumefaciens* strain LBA4404 harbouring the plasmid pTOK233.

Callus initiation from 4-day-old coleoptiles plated onto MSS2.5D medium was visible from both basal and apical cut ends after a week of incubation. In some cases however, only basal end show callus initiation. While in some cases callusing even starts from the coleoptile surface. After two weeks of incubation callusing was noticeable through most of the coleoptile surface (Fig. 1a) and after three weeks, callusing is visible throughout the coleoptile surface. This callus on being sub-cultured on MSM2.5D media for three weeks grew further and became embryogenic.

Freshly excised coleoptiles did not survive the co-cultivation stress and turned black within 3 days. Transient GUS expression (3 d after co-cultivation) was observed in all experiments (Fig. 1b) except for 4 day-old freshly excised coleoptile co-cultivated with

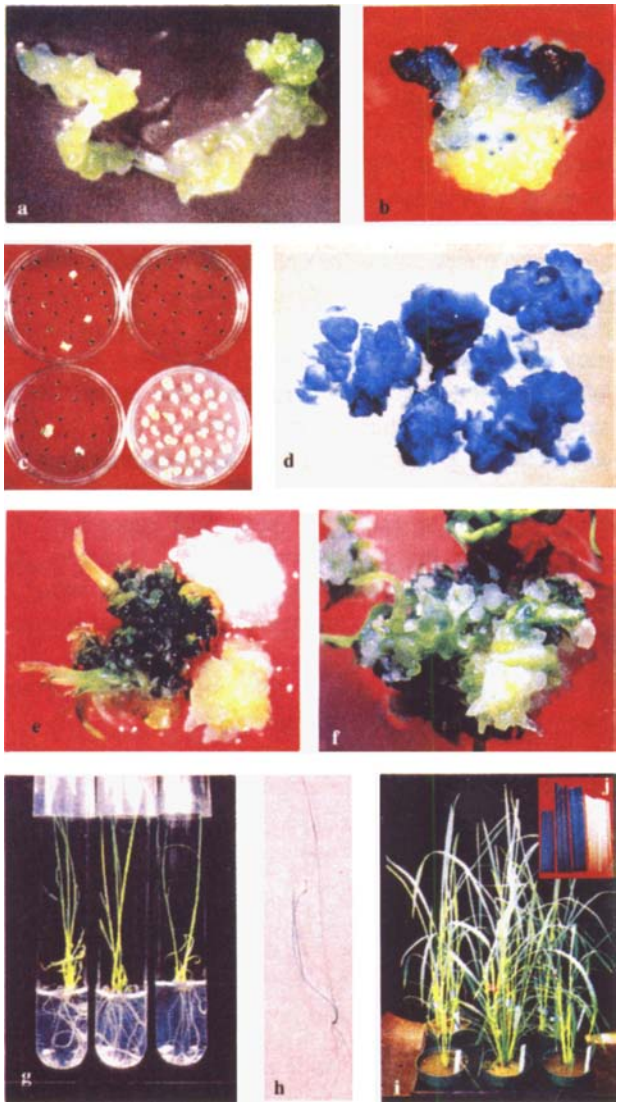

Fig. 1. Genetic transformation of rice using the coleoptile system. **a:** Callus induction from 4-day-old coleoptile of rice (*c.v.* Taipei 309) after two weeks on callus induction medium; **b:** Callus after 3 days of co-cultivation with *Agrobacterium* showing transient GUS expression; **c:** Co-cultivated calli at the end of second selection on medium containing 100mg/l Hyg (Petri-dishes on the left), right upper petri-dish is the negative control and the right lower one is the positive control; **d:** Hyg^R Calli showing a very strong GUS reaction (stable GUS expression) after 12 weeks of co-cultivation; **e, f:** Regeneration in Hyg^R GUS$^+$ callus; **g:** Rooting in putative transgenic shoots; **h:** Entire transgenic plantlets showing strong and uniform GUS expression; **i:** Putative transformants growing in the green house; **j (inset):** Leaf segments of mature transformants showing strong uniform GUS expression. The white leaf segment is the negative control.

Agrobacterium from the beginning. Stable GUS expression (12 weeks after co-cultivation) was observed in co-cultivated calli from the experiment where callus was 3 week and 3+3 week-old (Fig. 1d). However, regeneration occurred only in the 3+3 week-experiment, where primary callus was sub-cultured on MSM2.5D medium for an additional 3 week before co-cultivation (Fig. 1 e,f,g).

Based on GUS histochemical assay performed on the leaf segments (Fig. 1 h,i,j) of the regenerated plants growing in the green house, it was found that 23% plants were GUS positive. GUS staining was repeated several times and the results were uniform each time. DNA was isolated and Southern analysis of the plants confirmed the presence of the *gusA* gene (Fig. 2). For the confirmed transgenic plants, only one copy of the T-DNA integration into the host genome was observed. The results of the experiments are summarized in Table 2. Copy number was determined by counting the bands generated in the lane carrying one cut DNA for each plant since the length of the fragments generated will depend on the position where the second cut is made. Therefore, the number of bands generated will reflect the number of copies of the transgene integrated into the host genome since each additional copy of the transgene is most likely to get integrated at different position thereby creating a fragment of different size.

It was observed that, when 3 week-old primary callus obtained on MSS2.5D medium, was sub-cultured for another 3 week on MSM2.5D medium, containing maltose in place of sucrose as the carbon source, the callus grew faster, became more compact, with shining nodular structures and appeared highly embryogenic. On the other hand when the same 3-week-old primary callus was cultured on the same MSS2.5D medium on which it was induced, it did not grow as good nor did it turn compact and nodular. From these observations it seems that maltose played an important role in formation of embryogenic callus, which presumably became more receptive to *Agrobacterium.*

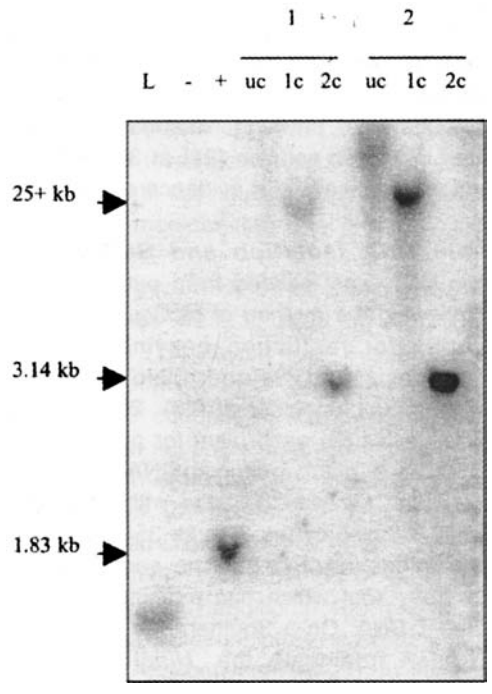

Fig. 2. Southern blot analysis of regenerated T_0 plants of Taipei 309 from coleoptile-based transformation mediated by *Agrobacterium.* Each plant represented by 3 lanes carrying respectively: uncut (uc), once cut (1c) within T-DNA (by *Xba*I) and twice cut (2c) within T-DNA (by *Hin*dIII). "L" indicates lane with 1 kb ladder. "+" indicates lane carrying positive control (1.83 kb *gusA* fragment). "-" indicates negative control. Probe = 1.83 kb *gusA* fragment.

Table 2. Correlation between stage of coleoptile at which co-cultivation was done with *Agrobacterium* and transformation frequency (T.F.)

Stage at which co-cultivated	No. of coleoptiles (a)	Transient GUS expression	No. of calli surviving I selection	No. of calli surviving II selection	Stable GUS expression (no. +ve)	No. of HYG^R GUS^+ plants (b)	T.F.% (b/a x100)
4d col.	72	-	-	-	-	-	-
4d col.+1w CI	80	+	55	23	-	-	-
4d col.+2w CI	77	+	41	29	-	-	-
4d col.+3w CI	102	+	96	83	+(7)	-	-
4d col. +3w CI+3w CP	60	+	39	33	+(19)	14	23.3

4d col.=4 day old coleoptile; w = week, CI = callus induction on MSS2.5D medium;
CP = callus proliferation on MSM2.5D medium.

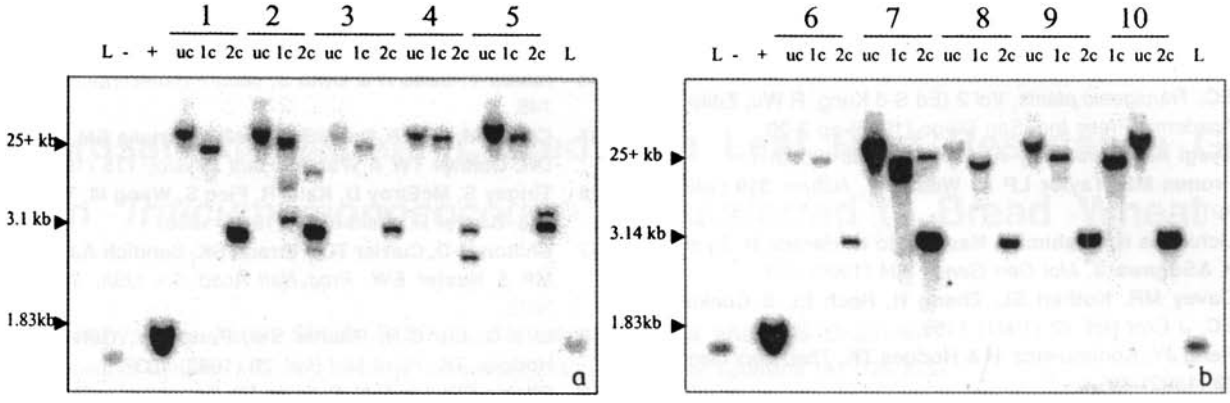

Fig. 3. Southern blot analysis of regenerated T_0 plants of Taipei 309 from mature seed-derived callus based transformation mediated by *Agrobacterium*. Each plant represented by 3 lanes carrying respectively: uncut (uc), once cut (1c) within T-DNA (by *Xba*I) and twice cut (2c) within T-DNA (by *Hin*dIII). "L" indicates lane with 1 kb ladder. "+" indicates lane carrying positive control (1.83 kb *gus*A fragment). "–" indicates negative control. Probe = 1.83 kb *gusA* fragment. Fig.**a**: Southern blot for plant number 1-5; Fig. **b**: Southern blot for plant numbers 6-10.

Mature seed based experiment — Callus induction was very efficient from mature seeds with an efficiency of 96%. Callus was always originating from scutellum only and no part of the endosperm appeared to be involved.

After 3 days of co-cultivation, GUS assays to determine transient GUS expression were all positive. The intensity of the blue colour varied from scattered light spots to deep blue uniform staining of the entire callus pieces. After 12 weeks of co-cultivation, and 2 cycles of selection, as done in the coleoptile experiments, the callus pieces were scored for stable GUS expression and it was found that all except one were GUS^+. Frequency of transformed calli based on stable GUS expression was found to be 83%, while 2.4% callus escaped the selection. Most of the calli turned uniformly deep blue. After selection, the HYG^R calli were first transferred to MS(3)BD medium for proliferation and then transferred to regeneration medium. The regenerated plantlets were transferred to the rooting medium followed by the transfer of the plants to the green house. GUS assays on the leaf segments of these plants revealed that 23% out of all regenerated plants were GUS^+. DNA was isolated and Southern analyses (Fig. 2) revealed that the transformation frequency was 21%. Since there is a large difference between frequency of GUS^+ calli (83%) after second selection and transformation frequency (21%), it seems that the efficiency of *Agrobacterium* to transfer the gene into the host system is high but the regeneration system does not match the same efficiency. It was also found that 79% of the regenerated plants from the HYG^R callus had actually escaped transformation. A high frequency of 83% of GUS^+ calli and yet 79% untransformed plants produced can probably be correlated to a loss of regeneration potential of the transformed cells in the callus. While, it is quite natural for some of the untransformed cells to have bypassed the selection pressure, these wild-type cells seem to grow faster after the selection pressure is removed.

Southern analysis after digestion of the genomic DNA of the HYG^R and GUS^+ plants with *Xba*I, which produces a single cut within the T-DNA at 8.01 kb produced 1-3 bands when probed with *gus*A fragment. Most of the bands were larger than 8.3 kb, indicating integration into the genome. Thus, (usually) 1 to (rarely) 3 copies of the foreign gene got integrated into the host genome. Integration of the *gus*A gene into the rice genome is presumably random, as in dicotyledonous plants, however, compared to the direct DNA transfer methods (6,9), the number of copies of the gene integrated, and frequencies of gene rearrangement appears to be much fewer.

The successful use of coleoptile tissue for rice transformation opens up new possibilities as coleoptiles can be obtained by germinating rice seeds anywhere without any seasonal constraints.

Acknowledgements

TK thanks the Rockefeller Foundation, USA for providing the Ph.D. Dissertation Research Fellowship. We thank Dr T Komari, Japan Tobacco Inc. for providing *Agrobacterium tumefaciens* strain LBA4404 (pTOK233).

Received 18 May, 2001.

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