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A protocol for consistent, large-scale production of fertile transgenic rice plants

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Abstract A protocol for consistent production of fertile transgenic rice plants was established utilizing microparticle bombardment of embryogenic tissues (*Oryza sativa* L. japonica cv. Taipei 309). This system has been employed to produce several thousand independently transformed plant lines carrying the hygromycin phosphotransferase (*hph*) gene and various genes of interest. The most efficient target tissue was highly embryogenic callus or suspension cell aggregates, when they were given an osmotic pre- and post-transformation treatment of 0.6 M carbohydrate. By optimizing the age of the tissue at the time of gene transfer and applying an improved selection procedure, transgenic plants were recovered in 8 weeks from the time of gene transfer, at an average of 22.3±9.7 per 100 calli and 22.4±8.0 plant lines per dish of suspension cell aggregates. This system has facilitated a number of studies using rice as a model for genetic transformation and will enable the large-scale production of transgenic rice plants for genomic studies.

Key words Rice · Microparticle bombardment · Genetic transformation · Embryogenic tissues

Abbreviations *ABA* Abscisic acid · *BAP* 6-Benzylaminopurine · *2,4-D* 2,4-Dichlorophenoxyacetic acid · *hyg B* Hygromycin B · *NAA* α-naphthaleneacetic acid

Introduction

During the last decade, considerable research has been directed at developing genetic transformation technologies for rice. The ultimate goal is to use biotechnology to secure increased yields and improved quality from agronomically important varieties. A result of these efforts has been that genetic transformation capabilities are becoming more highly developed in rice than for the other cereal crops.

Early gene transfer procedures for rice relied on protoplast-based systems (Toriyama et al. 1988). However, these resulted in large numbers of infertile plants and have been superseded by microparticle bombardment (Christou 1997), and more recently by *Agrobacterium*-mediated gene transfer (Hiei et al. 1997). Although plant fertility has improved, detailed information regarding transformation efficiency, reproducibility, and required inputs are still lacking. These questions must be addressed before the full potential of rice as a model system can be realized.

We report here the development of a low-cost, highly reproducible protocol for rice transformation, which has been employed to produce over 5800 transgenic plant lines containing various genes of interest. Its robust nature has been instrumental in the recovery and characterization of transgenic rice plants expressing the *Xa21* rice bacterial leaf blight resistance gene (Song et al. 1995), and for developing the capability to regenerate fertile rice plants containing as many as 12 transgenes (L. Chen et al. 1998). We present here details of the procedures which are applicable for use both with microparticle bombardment and with *Agrobacterium*-mediated gene transfer systems.

Materials and methods

Culture media and conditions

Culture media employed in these studies were adjusted to pH 5.8 with 1 M KOH and solidified with 2.5 g/l Phytagel (Sigma) unless otherwise stated. Embryogenic calli were cultured in 100 mm × 20 mm petri dishes containing 40 ml semi-solid medium. Rice plantlets were grown on 50 ml medium in Magenta boxes. Cell suspensions were maintained in 125-ml conical flasks containing 35 ml liquid medium and rotated at 125 rpm. Induction and maintenance of embryogenic cultures took place in the dark at 25–26°C, and plant regeneration and whole-plant culture took place in a 16-h photoperiod (Zhang et al. 1996).

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Table 1 The composition of the modified NB medium (mg/l) [*B5 vitamins* Gamborg et al. (1968) vitamins (sigma)]

KNO_3	2 830	$MnSO_4 \cdot H_2O$	7.58	L-Proline	500
$(NH_4)_2SO_4$	463	H_3BO_3	3	L-Glutamine	500
KH_2PO_4	400	$ZnSO_4 \cdot 7H_2O$	2	B5 vitamins	112
$CaCl_2 \cdot 2H_2O$	165	KI	0.75	2,4-D	2
$MgSO_4$	90	$Na_2MoO_4 \cdot H_2O$	0.25	Sucrose	30 000
FeNa-EDTA	41.5	$CuSO_4 \cdot 5H_2O$	0.025	Casein, enzymatic hydrolysate	300
		$CoCl_2 \cdot 6H_2O$	0.025		

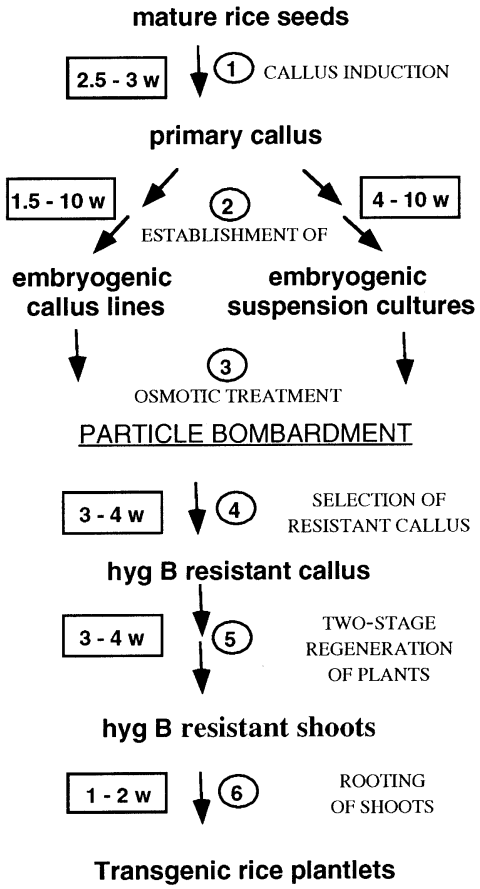


Fig. 1 Flow diagram illustrating the standard protocol for the recovery of transgenic rice plants (*w* week)

Induction and maintenance of embryogenic callus took place on NB basal medium as described previously (Li et al. 1993), but adapted to contain 500 mg/l glutamine. The composition of the modified NB medium is presented in Table 1. Suspension cultures were initiated and maintained in SZ liquid medium (S. Zhang et al. 1998) with the inclusion of 30 g/l sucrose in place of maltose.

Osmotic medium (NBO) consisted of NB medium with the addition of 0.256 M each of mannitol and sorbitol. Hygromycin-B-resistant (hyg^r) callus was selected on NB medium supplemented with 40 or 50 mg/l (NBH40 or NBH50) hygromycin B (hyg B; Calbiochem, La Jolla, Calif.) for 3–4 weeks. Regeneration of plantlets took place on pre-regeneration medium (PRH50) consisting of NB medium without 2,4-dichlorophenoxyacetic acid (2,4-D), but with the addition of 2 mg/l 6-benzylaminopurine (BAP), 1 mg/l α-naphthaleneacetic acid (NAA), 5 mg/l abscisic acid (ABA) and 50 mg/l hyg B for 1 week, followed by culture on regeneration medium (RNH50) comprising NB medium without 2,4-D, and supplemented with 3 mg/l BAP, 0.5 mg/l NAA, 50 mg/l hyg B and 5 g/l agarose (type 1 Sigma; or GibcoBRL, Gaithersburg, Md.) until shoots regenerated. Shoots were transferred to rooting medium with half-strength Murashige and Skoog (1962) basal salts and Gamborg's B5 vitamins (Sigma), supplemented with 1% sucrose and 50 mg/l hyg B (1/2 MSH50). The selection and culture procedures are summarized in Fig. 1.

Experimental plant tissues

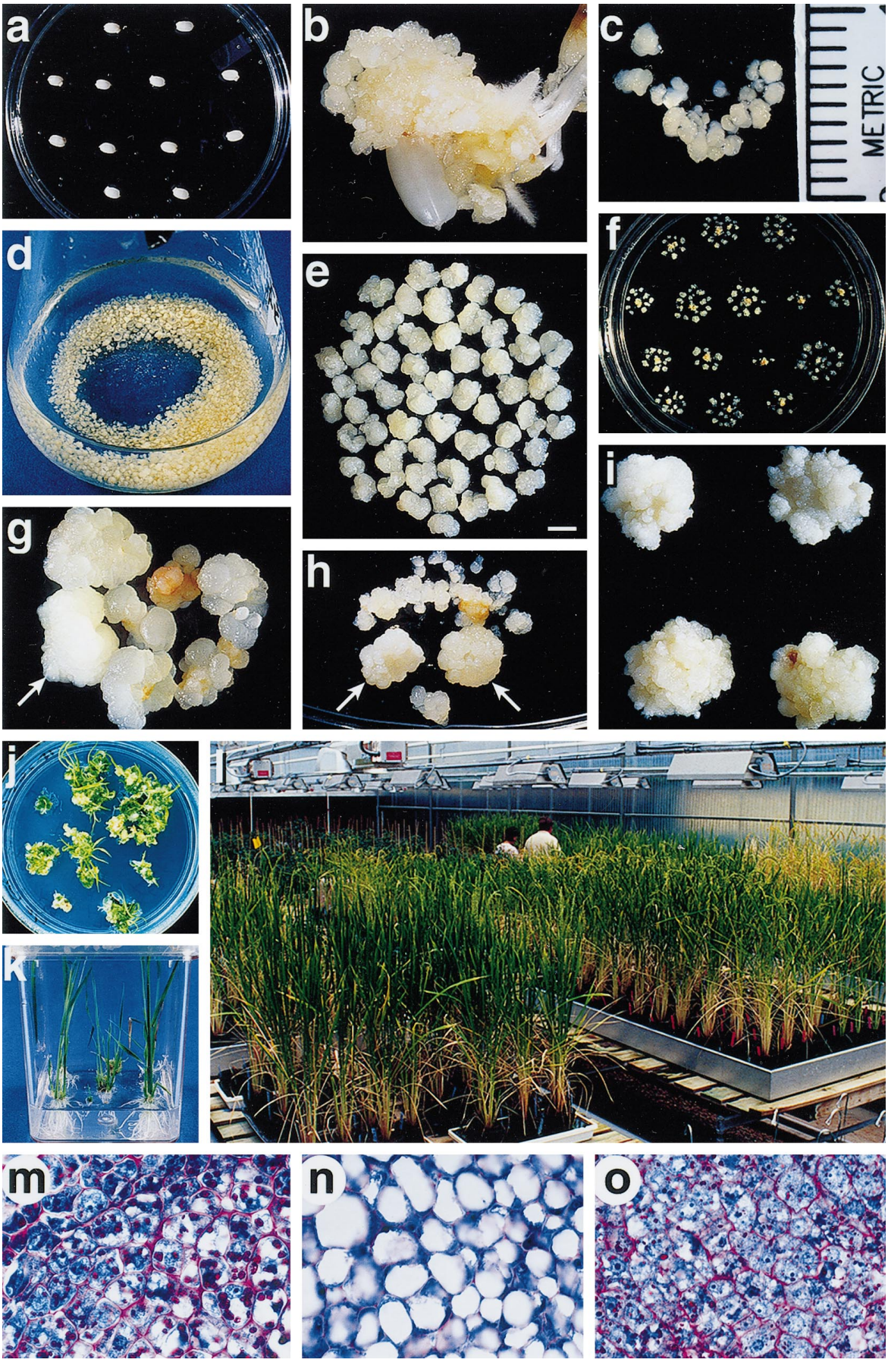
Oryza sativa L. japonica cv. Taipei 309 (TP309) was used throughout these studies. Mature desiccated seeds were sterilized as described previously (Zhang et al. 1996). Immature seeds were collected from greenhouse-grown plants and treated according to Li et al. (1993).

Embryogenic tissues were induced by culturing sterile mature rice seeds on NB medium in the dark (Fig. 2a). After 18–21 days, primary callus approximately 1 mm in diameter, was removed from the scutellum and used to establish embryogenic callus lines and to initiate embryogenic suspension cultures (Fig. 2b, c). Embryogenic callus, 0.5–1 mm in diameter, was subcultured at 2-week intervals onto solidified NB medium. Embryogenic suspension cultures were initiated by placing 10–15 calli in each flask and culturing in SZ liquid medium. Suspensions were then maintained as described previously (Zhang 1995) (Fig. 1, steps 1, 2).

Callus tissues were subjected to particle bombardment 9–14 days after the previous subculture. Embryogenic calli, between 1 and 3 mm in diameter, were placed on NBO osmotic medium to form a circle about 2.5 cm across in a petri dish and cultured for 4 h prior to bombardment (Fig. 2e). Suspension-derived embryogenic tissues (Fig. 2d) were removed from liquid culture 3–5 days after the previous subculture and subjected to the same treatment (Fig. 1, step 3).

Sixteen to 20 h after bombardment, tissues were transferred from NBO medium onto NBH40 or NBH50 hyg B selection medium, ensuring that the bombarded surface was facing upward, and incubated in the dark for 14–17 days. Newly formed callus was then separated from the original bombarded explants and placed nearby on the same medium (Fig. 2f). Following an additional 8–12 days, relatively compact, opaque callus was visually identified (Fig. 2g, h), and transferred to PRH50 pre-regeneration medium for 7 days in the dark. Growing callus, which became more compact and opaque (Fig. 2i), was then subcultured onto RNH50 regeneration medium for a period of 14–21 days under a 16-h photoperiod (Fig. 2j). Regenerating

Fig. 2 a–o Rice tissues from callus induction to the recovery of fertile transgenic plants in the transformation protocol. **a** Dehusked mature rice seeds on callus induction medium. **b** Callus formation from the scutella of a mature seed after 3 weeks culture. **c** Primary callus for initiation of embryogenic tissues. **d** Six-week-old embryogenic suspension cultures in liquid medium. **e** Embryogenic callus arranged for bombardment (*bar* 2 mm). **f** Callus separated and grouped after 2 weeks culture on selection medium. **g, h** Compact and opaque hyg^r callus (*arrows*) on selection medium 3–4 weeks after bombardment. **i** More compact and opaque hyg^r callus 1 week after transfer onto pre-regeneration medium. **j** Shoot production on regeneration medium. **k** Growth of healthy plantlets and root systems on rooting medium. **l** Fertile transgenic rice plants under greenhouse conditions at ILTAB. **m** Cross-section of a hyg^r callus growing on selection medium (×50). **n** Cross-section of a non-transformed callus growing on the selection medium (×50). **o** Cross-section of a non-transgenic callus on non-selective callus maintenance medium (×50)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shoots were transferred to Magenta boxes containing 1/2 MSH50 medium (Fig. 2k; Fig. 1, steps 4–6).

Multiple plants regenerated from a single explant are considered siblings and treated as one independent plant line. A plant was scored as positive for the *hph* gene if it produced thick, white roots and grew vigorously on 1/2 MSH50 medium. Once plantlets had reached the top of Magenta boxes, they were transferred to soil in a 6-cm pot under 100% humidity for a week, then moved to a growth chamber with a 14-h light period at 30°C and in the dark at 21°C for 2–3 weeks before transplanting into 13-cm pots in the greenhouse (Fig. 2l). Seeds were collected and dried at 37°C for one week prior to storage at 4°C.

Genetic constructs

Plasmid pMON410 (Monsanto) or pHX4 (Dr. J. J. Finer, Ohio State University), both containing the *hph* gene driven by the CaMV 35S promoter, was co-transformed as described by L. Chen et al. (1998), with a variety of constructs carrying different genes of interest and/or promoters. Plasmid pILTAB227, containing the *hph* gene and the *uidA* gene, each under control of the CaMV 35 S promoter, was used as a visual reporter (Zhang et al. 1996).

Microprojectile bombardment

All bombardments were conducted with the Biolistic PDC-1000/He system (BIO-RAD, Hercules, Calif.). Three milligrams of 1.0-µm-diameter gold particles was washed once with 100% ethanol, twice with sterile distilled water and resuspended in 50 µl water in a siliconized Eppendorf tube. Five micrograms plasmid DNA, 20 µl spermidine (0.1 M) and 50 µl calcium chloride (2.5 M) were added to the gold suspension. The mixture was incubated at room temperature for 10 min, pelleted at 10000 rpm for 10 s, resuspended in 60 µl cold 100% ethanol and 8–9 µl was distributed onto each macrocarrier. Tissue samples were bombarded twice at 1100 psi and 27 in of Hg vacuum as described by Zhang et al. (1996).

GUS assays and tissue examination

Histochemical assays to assess expression of the *uidA* gene in rice tissues were carried out by staining with 5-bromo-4-chloro-3-indolyl-beta-D-glucuronide acid (Biosynth) as described by Jefferson et al. (1987). Tissues were fixed, sectioned, and stained as described by Konan et al. (1997).

Results and discussion

Comparison of target tissues

Published protocols for the recovery of transgenic rice plants utilize either protoplasts, immature seeds, or embryogenic tissues for gene transfer (Christou 1997). In this study, embryogenic callus, immature embryos, and embryogenic suspension cell aggregates were compared for their ability to act as efficient target tissues for the production of transgenic rice plants. All of the tissues were given a 0.6 M osmotic treatment and selected with a single cycle on NBH40 medium for 4 weeks, followed by plant recovery on PRNH50 pre-regeneration and RNH50 regeneration media (Fig. 1).

The efficiency of stable transformation was defined as the number of independent hyg^r plant lines regenerated per 100 bombarded explants. Plant lines were scored as positive for the *hph* gene only if they rooted and grew strongly in 1/2 MSH50 medium. This criterion, initially reported by Li et al. (1993), is used routinely to confirm expression of the *hph* gene in regenerated plantlets and seedlings. Data gathered from three experiments, where a total of 209 hyg^r plant lines were regenerated and grown on 50 mg/l hyg B were found to contain no escapes. All of the plants that produced strong, white root systems on rooting medium were found to be positive for the *hph* gene when tested by polymerase chain reaction assays (results not shown).

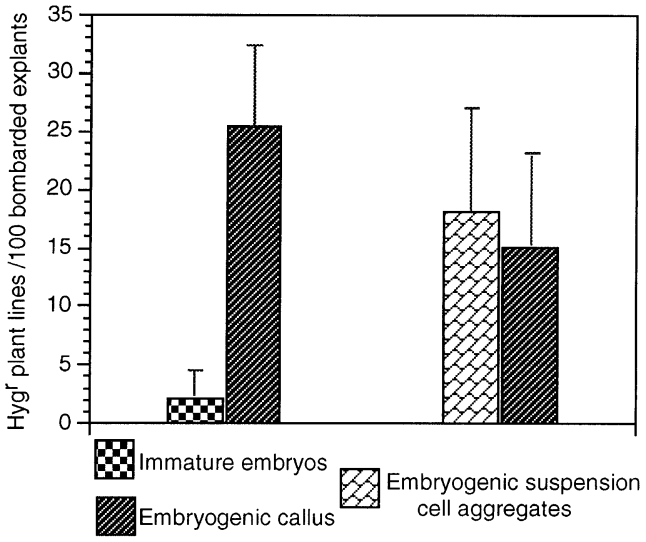

Fig. 3 Comparison of immature embryos, embryogenic callus, and embryogenic suspension cultures as target tissues for the recovery of transgenic rice plants. Each value is the mean of three replicates. For the comparison of immature embryos and embryogenic callus, 30 explants were used per replicate, and the *hph* gene was provided by plasmid pILTAB227; for the comparison of embryogenic callus and suspension cell aggregates, 90 explants were used per replicate, and the *hph* gene was contained within plasmid pHX4

Embryogenic suspension cultures, initiated from mature seeds, were found to be significantly more efficient target tissues than immature zygotic embryos for the production of transgenic rice plants. An average of 25 hyg^r plant lines were regenerated per 100 calli when compared to only 2 or 3 plant lines per 100 immature embryos, and embryogenic callus and suspension cell aggregates were found to be equally effective in a separate experiment (Fig. 3). With data gathered from 42 consecutive experiments, the recovery of transgenic plant lines has averaged 22.3±9.7 per 100 calli.

Cell suspensions were initially established and maintained using R2 liquid medium (Ohira et al. 1973). However, problems were often encountered using this medium, the tissues, for example, ceasing growth and turning brown. SZ liquid medium, originally developed for use with indica rice (S. Zhang et al. 1998), was adopted for use with japonica suspension culture, instead of R2 medium. Reliable establishment and maintenance of high-quality suspensions became routine once SZ medium was used, having a doubling time of approximately 5 days, and persisting for up to 3 months without deterioration of the tissues.

Suspension cell aggregates cultured in SZ medium have proven to be competent tissues for transformation in our hands, resulting in the production of 939 transgenic plant lines from 42 dishes in 21 experiments, at an average of 22.4±8.0 hyg^r plant lines per bombarded sample.

The results presented above also show that mature seeds are excellent starting material for the production of the large quantities of embryogenic tissue needed for genetic transformation in rice. From a practical standpoint, mature seeds are preferable to immature embryos as they provide tissues of a uniform physiological state, do not require non-stop production of flowering rice plants on site, and are easily stored until required.

The ideal embryogenic tissues for gene transfer, both from callus and suspension cell aggregates, were vigorously growing and pale yellow. After bombardment and culture for 3–4 weeks on selection medium containing 40 or 50 mg/l hyg B, putative hyg^r callus could be observed growing from the separated primary callus (Fig. 2g, h). Likewise, the hyg^r callus was opaque, compact, highly embryogenic in appearance, and contrasted with the softer, more translucent, non-transformed callus. When the tissues were fixed and examined, hyg^r callus was seen to consist of cells with dense cytoplasm and prominent nuclei (Fig. 2m). These cells were similar in appearance to non-transgenic, embryogenic callus maintained on hyg B-free medium (Fig. 2o), contrasting with translucent, non-transformed callus that developed on the selection medium (Fig. 2n). The latter consisted of vacuolated cells with little cytoplasmic content.

The ability to recognize embryogenic tissues and hyg^r callus by eye at different stages of the procedure is an important factor in obtaining high frequencies of transgenic plant recovery.

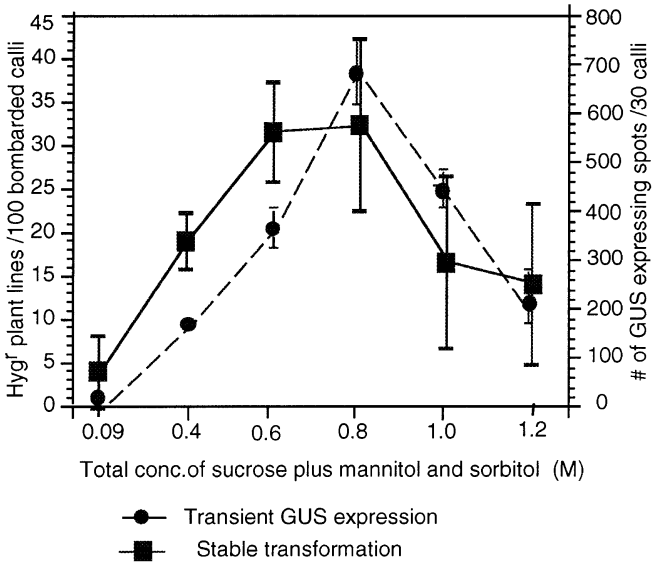

Fig. 4 Effect of culturing embryogenic rice callus on media with varying carbohydrate concentrations on the efficiency of both transient and stable transformation. Each value is the mean of four replicates with 30 calli per replicate. Media contained the indicated concentrations of 0.09 M sucrose alone or 0.09 M sucrose in combination with equimolar amounts of mannitol and sorbitol from 0.4 to 1.2 M. Callus was transferred to the osmotic medium 4 h before and 16–20 h after bombardment. Plasmid pILTAB227 was used for the transient GUS expression assays, and the selectable marker was provided by plasmid pMON410. GUS assays were carried out 24 h after bombardment

Effect of an osmotic treatment

Subjecting embryogenic tissues to an osmotic pre- and post-transformation treatment has been shown to increase the efficiency of both transient and stable transformation in a number of crop species including maize (Vain et al. 1993) and rice (Zhang et al. 1996). For indica rice, cultures were treated with 0.4 M osmoticum, but the optimum conditions have not been determined. To investigate the optimal conditions for rice, embryogenic callus tissues were subjected to culture on NB medium containing either 0.09 M sucrose alone, or 0.09 M sucrose plus equimolar amounts of mannitol and sorbitol, raising the total carbohydrate concentrations to between 0.4 and 1.2 M.

Increasing the osmotic potential of the medium was found to have a significant effect on both transient and stable transformation (Fig. 4). The greatest number of transient GUS-expressing spots recorded was between 650 and 700 per plate, and took place when the medium was supplemented with sorbitol, mannitol, and sucrose to a total of 0.8 M carbohydrate. Tissues which underwent bombardment without an osmotic treatment produced only around 6 GUS-expressing spots per plate. Culturing callus on the medium containing 0.6 or 0.8 M total carbohydrate was also found to be the most effective treatment, and resulted in eight times as many plant lines when compared to the use of 0.09 M sucrose alone (32.1±4.7% and 33.8±10.6% versus 4.1±4.2%). Culture on osmotic medium with concentrations of sugar greater than 0.8 M was detrimental to both transient and stable transformation. When the results of transient and stable transformation were compared, the two curves correlated with a value of 0.82, indicating that the results obtained for transient gene expression assays could, in this study, be extrapolated to the recovery of transformed plants.

Age of the target tissue

To determine the optimum time for gene transfer with regards to subculture duration, embryogenic callus was bombarded at 4, 9, 14 and 19 days after transfer to fresh NB medium. Time of incubation since the previous subculture was found to have a significant effect on transgenic plant recovery (Fig. 5). Tissues were most competent for transformation between 9 and 14 days after subculture, producing 22.2±3.9 and 18 ±1.9 hyg^r plant lines, respectively, per 100 calli. In addition, no effect was observed on transgenic plant production as a result of up to five subculture cycles on solidified medium, or after four to eight subculture cycles in liquid medium prior to gene transfer.

Table 2 Effect of hyg B concentrations in the production of transgenic rice plants from embryogenic suspensions. Data represent the mean±SD of four petri dish replicates, each containing 30 bombarded embryogenic suspension cell aggregates. *a* and *b* indicate significant differences at the 5% level by Student's *t*-test. Tissues were cultured on NB medium containing 30, 40, and 50 mg/l hyg B prior to subculture onto pre-regeneration, regeneration, and rooting media each containing 50 mg/l hyg B. Plasmid pMON410 was used to introduce the selectable marker

hyg B (mg/l)	Number of calli on pre-regeneration medium	Number of calli on regeneration medium	Number of hyg^r callus lines regenerating plants	Number of hyg^r plant lines growing on rooting medium	Number of hyg^r plant lines/100 bombarded explants
30	85	17	11	11	9.2 ± 4.3a
40	74	50	35	35	29.2 ± 5.5b
50	68	44	30	30	25.0 ± 8.3b

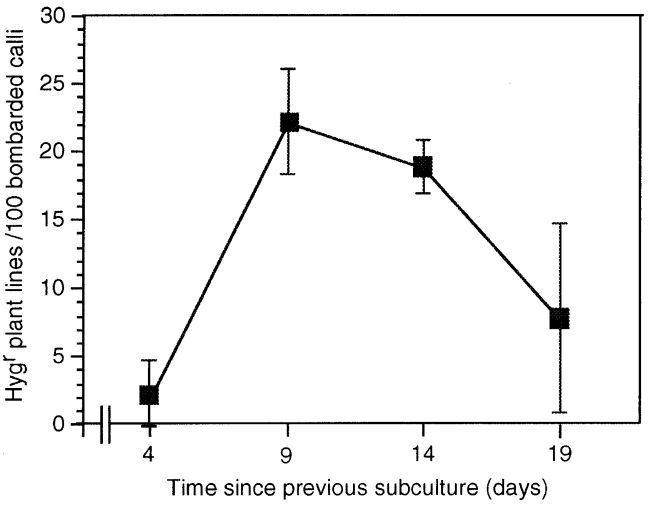

Fig. 5 Effect of time since previous subculture on the competence of embryogenic callus to act as target tissues for the production of transformed rice plants. Each value represents the average of three replicates with 30 calli per replicate. The *hph* gene was provided by plasmid pMON410

Optimizing the selection and regeneration procedures

In previous reports (Christou et al. 1991; Li et al. 1993; Qu et al. 1996), the selection procedures used to recover transgenic rice plants required numerous cycles on selection medium. This took place prior to transferring tissues to the regeneration media. Because of the large amounts of embryogenic tissue produced, it became essential to simplify the selection procedure to reduce the labor required. The effect of applying hyg B a single selection cycle, prior to the regeneration stages, was therefore investigated. Antibiotic was added at 50 mg/l to the regeneration and rooting stages as first described by Li et al. (1993). Recovery of transgenic plants was possible from a single selection cycle of 4 weeks on medium containing 30, 40, or 50 mg/l hyg B, prior to the pre-regeneration stage (Table 2). Nevertheless, 40 and 50 mg/l hyg B were found to be significantly more effective than 30 mg/l for the recovery of transgenic plants. This may be due to suppression of the transformed tissues by the more vigorous non-transformed callus at the lower antibiotic concentration.

Table 2 also shows the effectiveness of the antibiotic during each stage in the selection process. All of the non-transformed tissues had been eliminated by the end of the culture period on regeneration medium (RNH50). Therefore, the selection process was completed before the rooting stage in this experiment, and the inclusion of hgy B in rooting medium was considered as a final screen to ensure that 100% of the rooted plants were transgenic.

Concluding remarks

Figure 1 illustrates the protocol used for the large-scale production of fertile transgenic rice plants at ILTAB. With the combination of a single selection cycle and visual assessmen for hyg^r callus, we can now routinely obtain transformed plants in 7–10 weeks from embryogenic tissues treated with osmoticum. In a 3-year period, more than 5800 transformed rice plant lines have been recoverd from an estimated labor input of below 60 h per week. Fertility of these R0 plants ranged from 47 to 80% with an average of 61.1±9.6%. In addition, this protocol has been applied for the production of fertile transgenic plants from an additional seven elite japonica varieties and one Basmati (indica) variety. Although originally developed for use with the biolistic system, the embryogenic tissues and the selection and regeneration procedures have also been utilized, with minimal adaptation, to recover several hundred rice plant lines transformed by *Agrobacterium tumefaciens* (unpublished results).

The availability of an efficient system for producing large numbers of transgenic rice plants offers new opportunities to study transgenes and their expression in rice. It has already facilitated investigations involving numerous genes of interest under the control of a range of promoters (Verdaguer et al. 1996; Yin et al. 1997) and in a recently completed study, to demonstrate the integration of as many as 13 different transgenes into the rice genome (L. Chen et al. 1998). Training given by the Rockefeller Rice Transformation Center based at ILTAB has resulted in the transfer of this technology to more than 35 laboratories in industrialized and less-developed countries.

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