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Regeneration of fertile transgenic indica (group 1) rice plants following microprojectile transformation of embryogenic suspension culture cells

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Abstract: Regenerable embryogenic suspensions of elite Indica (group 1) rice varieties IR24, IR64, IR72 and an advanced Indica rice breeding line IR57311-95-2-3 were established within 6-8 weeks from 3-4 week old calli derived from mature seeds. Transgenic rice plants were obtained by introducing a plasmid carrying genes encoding hygromycin phosphotransferase (*hph*, conferring resistance to hygromycin B) and ß-glucuronidase (*uid*A), both driven by the CaMV 35S promoter, via particle bombardment of embryogenic suspensions. The effect of osmotic conditioning on transformation was evaluated. Regenerated plants were resistant to hygromycin B and expressed the *uid*A (GUS) gene. The growth of mother plants (R_0) was normal and seeds were produced. Southern blot analysis of R_0 and R_1 plants showed that hygromycin resistant plants contained intact *hph* genes that were inherited in a Mendelian fashion. A protocol for a simple, efficient, repeatable, genotype- and environment-independent Indica rice transformation system is described.

Key words: Indica rice, cell suspension, transformation

Abbreviations: 2,4-D: 2,4-dichlorophenoxy acetic acid; NAA: α-naphthalene acetic acid; kb: kilobase; GUS: ß-glucuronidase; hph: hygromycin B phosphotransferase.

Introduction:

Rice (*Oryza sativa* L.) is the major nutritional source for about 40% of the world's population. Japonica and Indica rice are the two major subspecies grown in different regions of the world. The Indica varieties are the most widely-grown, covering temperate to tropical regions, and occupying 80% of the cultivated rice areas in the world (Swaminathan, 1982; Wu *et al.* 1990). The development of plant transformation techniques has made it possible to improve crop plants by the introduction of cloned genes. In recent years, a number of laboratories have reported transformation of rice by the direct delivery of DNA into protoplasts (Toriyama *et al.* 1988; Zhang *et al.* 1988; Zhang and Wu, 1988; Shimamoto *et al.* 1989; Datta *et al.* 1990, 1992; Hayashimoto *et al.*. 1990; Peng *et al.* 1990, 1992; Terada and Shimamoto, 1990; Cao *et al.* 1992; Hayakawa *et al.* 1992; Fujimoto *et al.* 1993; Rathore *et al.* 1993). Other protocols that describe transformation of rice by biolistics (Christou *et al.* 1991; 1992; Li *et al.* 1993), *Agrobacterium*-mediated methods (Hiei *et al.* 1994) and electroporation of intact seed embryo cells (Xu and Li, 1994) have been published. For the Indica rice designated as group 1 by Glaszmann (1987), four laboratories have reported transformation (Peng *et al.* 1990, 1992; Christou *et al.* 1991, 1992; Datta *et al.* 1992; Xu and Li, 1994). However, there has been limited success in obtaining fertile transgenic Indica (group 1) rice plants. At the present time, such fertile rice lines could be regenerated only by bombarding immature embryos (Christou *et al.* 1991, 1992) or by electroporating seed embryo cells (Xu and Li, 1994). The limiting factor for group 1 Indica rice transformation is apparently related to genotype- and culture-dependent components. The use of immature and mature embryos as target tissue for bombardment and electroporation is both time consuming and labor-intensive. Since the supply of immature embryos is environment-dependent it is difficult to maintain a continuous supply of suitable explants.

In this paper, a genotype- and environment-independent transformation system for Indica rice that uses regenerable embryogenic suspensions as target tissue for bombardment is described. Fertile transgenic plants of Indica (Group 1) elite rice varieties IR24, IR64, IR72 and an advanced breeding line IR57311-95-2-3 have been obtained using this system. It is a simple, efficient, rapid, repeatable system and can be widely used for introduction of genes of interest into elite varieties of rice.

Material and Methods:

Callus induction and initiation of cell suspension culture. Protocols for the induction of callus and the initiation of cell suspension cultures were identical to those reported by Zhang (1995). Mature seeds were surface-sterilized in 70% (v/v) ethanol for 1 min, followed by 2.6% (w/v) sodium hypochlorite for 45 min. The seeds were then rinsed three times with sterile distilled water. The explants were plated on Petri dishes containing MS (Murashige and Skoog 1962) medium with 30 g/l sucrose, 2 mg/l 2,4-D, and 0.4% (w/v) agarose (type 1, Sigma). After 2 weeks of culturing in the dark at 25-26 °C developing calli were either left on the same medium or subcultured on fresh MS medium with 30 g/l

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maltose and 2 mg/l 2,4-D, solidified with 0.4% (w/v) agarose and kept under the same environmental conditions.

Three to four weeks old small, compact, embryogenic calli with loosely attached globular embryos were selected and placed into a 125 ml Erlenmeyer flask containing 30-40 ml liquid R_2 medium (Ohira *et al.* 1973) supplemented with 20 g/l sucrose and 2 mg/l 2,4-D on a gyratory shaker at 110-130 rpm in order to establish suspension cultures. The medium was replaced at intervals of 1-5 days (depending on the degree of browning) for 2-3 weeks. Liquid cultures were maintained by subculture every 5-7 days, in 125 ml Erlenmeyer flasks containing R_2 medium supplemented with 2 mg/l 2,4-D and 20 g/l sucrose or maltose on a gyratory shaker at 110-130 rpm. The establishment of Indica (group 1) rice suspension took about 6-8 weeks.

Particle bombardment. All experiments were conducted with the Biolistic PDC-1000/He system (BIO-RAD, Hercules, CA), and the plasmid pILTAB227 was used. This plasmid contained both the *uid*A (GUS) and the *hph* gene, each individually driven by the CaMV 35S promoter and terminated by the *nos* 3' end. Genes were arranged in a head-to-head orientation (Figure 1a). The manufacturer's instructions were followed for coating the mixture of 1.0 µm (BIO-RAD) and 1.8-2.3 µm (Johnson Matthew, Ward Hill, MA) gold microcarriers with plasmid DNA that was prepared using the Magic Maxipreps DNA purification system (Promega, Madison, WI). After the cell suspension cultures were established, a half gram sample of cell suspensions that had been subcultured 3-5 days before was plated in the center of petri dishes containing R_2 medium with 30 g/l mannitol, 30 g/l sorbitol, 30 g/l sucrose, 2 mg/l 2,4-D, and 0.25 % (w/v) phytagel (Sigma) for 4 h prior to bombardment. The plates were placed 8 cm beneath the stopping plate of the gun. A helium pressure of 1100 psi was used to accelerate the particles. After bombardment, the cultures were maintained on the same medium in the dark at 25-26 °C.

Growth and selection of bombarded cells. Sixteen to twenty hours after bombardment, the cell aggregates were transferred to CC medium (Potrykus *et al.* 1979) containing 300 mg/l casein hydrolysate, 2 mg/l 2,4-D and 30 mg/l hygromycin B (hyg B.; Calbiochem, La Jolla, CA), solidified with 0.4 % (w/v) agarose. Cell aggregates were cultured in the dark at 25-26 °C for 2-3 weeks, after which growing cell clusters were selected and transferred to CC medium containing 50 mg/l hyg B. Two weeks later, the hygromycin resistant (hyg^r) calli were transferred to CC medium containing 300 mg/l casein hydrolysate, 2 mg/l kinetin, 1 mg/l NAA, 5 mg/l ABA and 50 mg /l hyg B solidified with 0.4 % (w/v) agarose for the pre-regeneration treatment.

Regeneration of putative transgenic plants. Hyg^r calli measuring 2-3 mm in diameter were transferred to R_2 medium containing 2.5 mg/l kinetin, 0.1 mg/l NAA, 30 g/l maltose and 50 mg/l hyg B, solidified with 0.4 % (w/v) agarose. Cultures were kept in darkness for 3 days, and then maintained at 25-26 °C under a photoperiod of 16 h at 110-130 $\mu M/m^2/S$ PAR using Sylvania F40/CW cool white fluorescent tubes. As plantlets were regenerated, they were transferred to Magenta boxes containing a medium with half-strength MS salts, 30 g/l sucrose, 0.1 mg/l NAA and with 0.25 % (w/v) phytagel. When plants were 8-10 cm high, they were transferred to Yoshida's culture solution (Yoshida *et al.* 1976) for efficient root development. Two weeks later, the plants were transferred to pots and grown to maturity in the greenhouse. The plants (R_0) were checked for fertility, and seeds of R_0 plants were planted for morphological and molecular analysis of R_1 plants.

GUS assays. Histochemical assays to monitor GUS expression of R_0 and R_1 transgenic plants were performed basically as described by Klein *et al* (1988). Transient GUS expression assays were carried out 48 h after bombardment.

Southern blot analyses. Genomic DNA was extracted from leaf tissues based on the method of Dellaporta *et al.* (1983). DNAs from each sample were separately digested with HindIII (cuts only the plasmid at the beginning and the end of the cassette) and BamHI (cuts once in the plasmid) restriction endonucleases (Gibco-BRL, Gaithersburg, MD) (Figure 1b legends). Undigested and digested DNAs (5 µg per well) were loaded on a 0.8% agarose gel. Electrophoresis was carried out at 5 volt/cm for 6 h. DNAs were then transferred onto a Hybond-N^+ nylon membrane (Amersham, Arlington Heights, IL) according to instructions of the manufacturer. The *hph* probe was a purified PCR product of pMON410 using internal hygromycin primers. Labeling was done by random 9-mer priming with the Exo- klenow fragment of DNA polymerase (Stratagene, La Jolla, CA) and using 5'-[α-32P]dCTP. Hybridization was carried out at 65°C in hybridization buffer (3 x SSC, 2 x Denhardt solution, 0.1 % SDS, 6 % PEG) overnight. The final wash was done at 65°C with (0.5 x SSC ; 0.1 % SDS). The washed membrane was then exposed to X-ray film overnight.

Results and Discussion:

Callus induction and cell suspension

An experiment was conducted to compare the production and quality of small, compact embryogenic calli containing globular embryos, induced from mature seeds and immature embryos using the methods described by Zhang (1995). The results of this study indicated that there were few differences between immature embryos and mature seeds when one considers the quality of the induced calli and the time required to initiate cell suspensions. To reduce the dependence on a consistent supply of immature embryos. mature seeds were routinely used for callus induction.

For the establishment of Indica (group 1) rice embryogenic suspensions, the most difficult step and a limiting factor is browning and cell death in liquid media at the stage of suspension initiation. In order to protect cells from browning and dying, small, compact embryogenic calli with loosely attached globular embryos should be carefully selected and cultured in R_2 medium with 2 mg/l 2,4-D and 20 g/l sucrose. If callus begins to brown, the medium should be changed to R_2 medium with 2 mg/l 2,4-D and 20 g/l maltose and cultures should be subcultured at short intervals (1-5 days) for 2-3 weeks.

One of the limiting factors for the efficient transformation of Indica (group 1) rice so far has been the inability to produce large quantities of regenerable target tissues for transformation experiments. In this study Indica (group 1) rice suspensions were established in 6-8 weeks (Figure 2a & b). They were used as target tissue for transformation until they were 3.5 months old. However, there were no problems in regenerating transgenic calli even at later stages. More importantly, a large quantity of embryogenic suspension can be easily produced to scale up transformation experiments as necessary.

Improvements of biolistic transformation efficiency

Osmotic treatment (30 g/l mannitol, 30 g/l sorbitol) of cell suspension cultures for 4 h before and 16-20 h after bombardment using the methods described by Vain *et al.* (1993) enhanced transformation efficiency of rice (Table 1 and Figure 2d). Table 1 shows results similar to those of Vain *et al.* (1993) reported for maize. These workers proposed that osmotic treatment enhances transient expression and stable transformation of maize by facilitating plasmolysis of the target cells. Plasmolyzed cells are less likely to extrude their protoplasm following penetration of the cell by particles (Armaleo *et al.* 1990). The plasmolyzed state should be maintained for a few hours before and after bombardment for maximum effectiveness (Vain *et al.* 1993). Figure 2c shows transient expression of the *uid*A (GUS) gene 48 hours after bombardment with osmotic treatment.

Table 1. Effect of osmotic treatments on transient *uid*A expression in rice suspension cells after particle bombardment.

Variety	Treatment	# of blue foci for 0.5 g of cells[2]
IR64	With osmotic treatment[1]	594 ± 78
IR64	Without osmotic treatment	258 ± 42
IR57311-95-2-3	With osmotic treatment[1]	1152 ± 202
IR57311-95-2-3	Without osmotic treatment	324 ± 48

[1]Osmotic treatment consisted of cell storage on an R_2 medium containing 0.4 M osmoticum.

[2]Each value is the mean of 4 replications.

Transformation and selection of cell suspension cultures

Hygromycin B was used as the selection agent for these studies. The effect of hyg B on the growth of embryogenic suspensions of each cultivar was examined prior to conducting experiments. Cell growth was significantly reduced on solid medium containing 30 mg/l hyg B, and growth was greatly inhibited on medium containing 50 mg/l hyg B. However, if cells were plated from suspension cultures on the solid growth medium without hyg B for a few days, the selection on hyg B containing medium became more difficult, presumably because the vigorous growth of the cell clusters caused the cells to be resistant to high concentration hyg B. Therefore, bombarded cell suspension cultures were kept on high osmotic pressure medium for 16-20 h, then transferred directly to selection medium. In some experiments, immature embryos were also used as the target tissue for bombardment and development of transgenic plants. Because immature embryos produce calli very slowly compared to cell suspension cultures, they were cultured for 5-7 days on solid medium without hyg B before applying selection pressure by growth on hyg B containing medium.

Sixteen to twenty hours after bombardment with the plasmid pILTAB227, the cultures were transferred directly to selection medium containing 30 mg/l hyg B. Most explants gradually turned brown and 2 to 3 weeks later white, growing cell clusters were identified (Figure 2d). At this stage, not all growing clusters were transgenic, so they were carefully removed from dying explant tissue and transferred to fresh selection medium containing 50 mg/l hyg B for further selection. In order to increase plant regeneration, after 2 weeks of selection on 50 mg/l hyg B, the hyg^r calli were transferred to pre-regeneration medium containing 50 mg/l hyg B for further growth prior to transfer to regeneration medium.

Regeneration of hygromycin resistant plants

When the size of the hyg^r calli reached approximately 2 to 3 mm in diameter, they were transferred to regeneration medium containing 50 mg/l hyg B. In preliminary experiments, it was observed that if hyg B was not added to the regeneration medium, some of the regenerated plants were not transgenic, although the calli were previously grown on medium with hyg B for more than 1 month. After plantlets were produced on the regeneration medium containing hyg B (Figure 2e), they were transferred to rooting medium without hyg. B; rooting in the absence of hyg B may be important to retain fertility of the R_0 plants.

In total, 46 independent transgenic lines, and 148 green plants of IR24, IR64, IR72 and IR57311-95-2-3 were regenerated in 10 independent experiments. Most were grown to maturity and produced seeds (Figure 2f).

Fertility of transgenic rice plants and germination of transgenic seeds

Transgenic Indica (group1) rice plants that were developed from protoplast transformation protocols using cell suspensions are reported to be sterile (Peng *et al.* 1990, 1992; Datta *et al.* 1992). This may be due in part to the length of time that was involved in establishing cell suspension cultures. In our experiments, cell suspensions were used for transformation 6-8 weeks after initiation . All of the regenerated transgenic plants tested were fertile. Table 2 shows the degree of fertility of three independent transgenic R_0 IR72 lines. The fertility of transgenic IR72 plants (R_0) is lower than non-transgenic plants, perhaps due to the effects of the tissue culture protocols used (Zhang 1995). Normal fertility was, however, restored in the R_1 plants (data not shown). The germination rate of seeds from R_0 transgenic rice lines is comparable to that of seeds from non-transgenic plants (Table 2).

Table 2. Transgenic IR72 plant fertility and germination rates.

Transgenic rice line	Fertility[1] %	Seeds germinated /seeds planted[2]
IR72-1	NT	47/50
IR72-2	NT	139/150
IR72-5	45.1 ± 14	47/49
IR72-7	40.1 ± 17	55/56
IR72-10	49.5 ± 18	47/47
IR72(control)	65.3 ± 7	63/63

[1] mean of full set seeds per hundred seeds collected from 6 random panicles. NT: not tested.

[2] seeds were germinated in magenta boxes containing half-strength MS salts, 30 g/l sucrose, 0.1 mg/l NAA and with 0.25 % (w/v) phytagel medium.

Inheritance of hph and uidA genes

Integration of the introduced *hph* gene into the genome of transgenic rice plants was confirmed by Southern blot hybridization reactions using the *hph* sequence as probe. When comparing patterns of undigested and digested DNA (Figure 1b), each IR64 and IR72 transgenic line examined had a different pattern. All lines tested contained the intact cassette (promoter, *hph* gene and nos terminus), as expected. Some lines such as IR64-2 (Figure 1b) seemed to have integration of the active *hph* gene at a single locus. The segregation of the hyg^r trait and *uid*A gene expression among offspring of the transgenic plants was demonstrated by germinating R_1 seeds on medium containing 50 mg/l hyg B and by GUS assays, respectively (Table 3). Results showed that *hph* and *uid*A genes segregated together in offspring tested. Most of the lines exhibited 3:1 segregation ratios among the offspring, indicating Mendelian inheritance from a single genetic locus of functional *hph* and *uid*A genes. Inheritance of the site of integration of the *hph* gene by southern blot pattern was also demonstrated (Figure 1b).

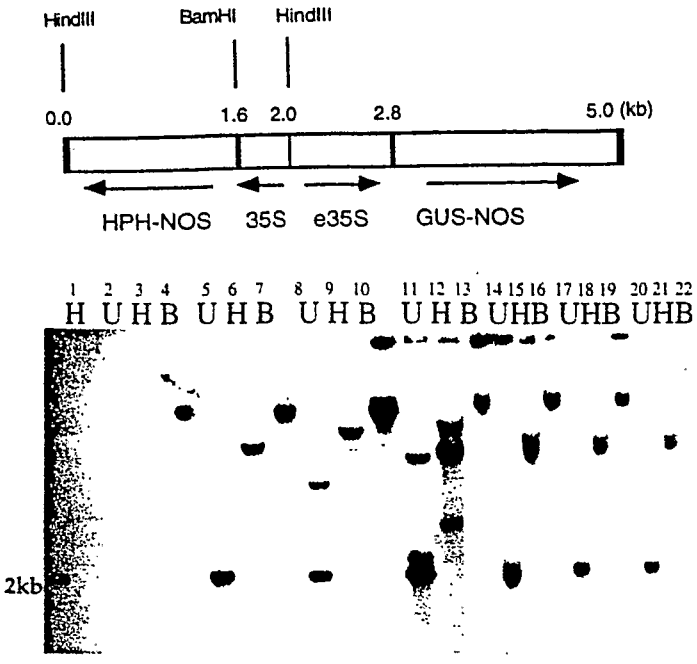

Figure 1: a) Linearized map of the cassettes of the construct: the two genes of interest are in a different orientation and both are driven with 35S promoter (with an enhancer for the GUS gene). HindIII releases the entire cassette for the *hph* gene: BamHI cut only once within the construct.

b) Southern blot analysis of genomic DNA from leaves of rice plants. Purified PCR product of pMON410 using internal hygromycin primers was used as *hph* probe. Line 1: plasmid pILTAB 227. Lines 2-4: non-transgenic rice IR72. Lines 5-13: three transgenic IR64 (R0) rice lines: IR64-2; IR64-4; IR64-26. Lines 14-16: transgenic rice IR72-27 (R0). Lines 17-22: two IR72-27 progenies; IR72-27-15 and IR72-27-17. U: undigested DNA; H: DNA digested with HindIII; B: DNA digested with BamHI.

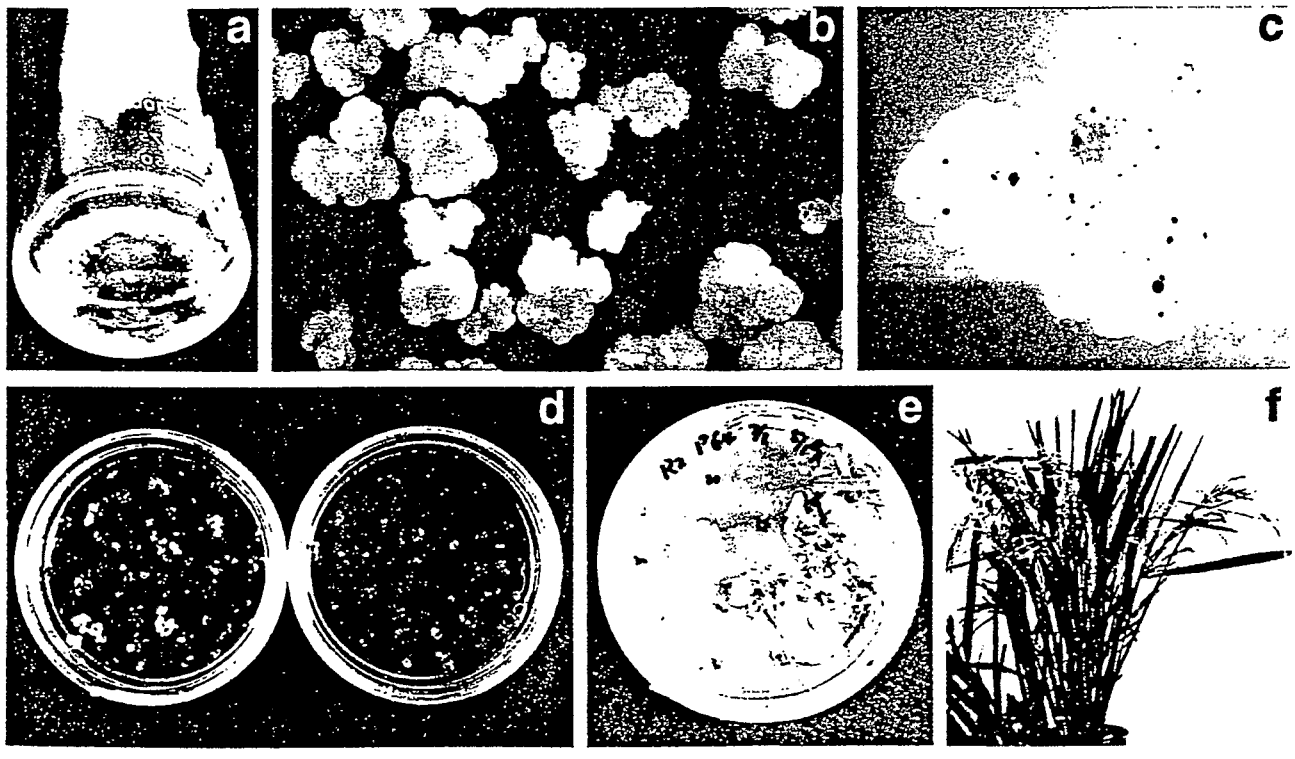

Figure 2: Regeneration of fertile transgenic Indica (group1) rice plants following microprojectiletransformation of embryogenic suspension culture cells. (a) A newly established cell suspension culture of IR64, 6 weeks after initiation. (b) a close-up the newly established cell suspension culture of IR64. (c) GUS activity 2 days after bombardment of IR 64 suspension culture with plasmid pILTAB 227. (d) Cell cultures bombarded with plasmid pILTAB 227, 20 days after selection on the medium containing hyg B, Left: without osmotic treatment, Right: with osmotic treatment. (e) Hyg^r plants regenerated from regeneration medium containing hyg B. (f) Fertile transgenic IR72 plants.

Table 3. Inheritance of the hyg^r trait and *uid*A gene expression in transgenic IR72

Transgenic rice line	Seeds planted	R1 germin. seedl.*	No. of hyg^r seedlings and %	GUS positive seedl.	X^2
IR72-1	50	47	35 (74.5)	35	0.007
IR72-2	100	91	76 (83.5)	76	5.280
IR72-5	49	47	34 (72.3)	34	0.177
IR72-7	56	55	39 (70.9)	39	0.491
IR72-10	47	47	35 (74.5)	35	0.007
IR72(control)	63	63		0	--

* Transgenic rice seeds (R_1) were planted on 1/2 MS salts medium for 6 days, then transferred to hyg B (50 mg/l)-containing medium.
X^2 tests indicate good agreement with segregation ratios of 3:1 except the line IR72-2.

In conclusion, we have taken advantage of the utility of the microprojectile-mediated DNA delivery to Indica (group 1) rice suspension cultures. Transgenic Indica (group 1) rice plants of the cultivars IR24, IR64, IR72 that are widely used elite varieties in southeast Asia and IR57311-95-2-3 (an advanced breeding line) were obtained from bombarded cell suspension cultures. Some of these plants were grown to maturity, and to date all mature transgenic plants produced exhibit full or partial fertility. This system provides the opportunity to introduce agronomically useful genes directly to elite rice cultivars.

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