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An improved rice transformation system using the biolistic method

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Abstract. Immature embryos and embryogenic calli of rice, both *japonica* and *indica* subspecies, were bombarded with tungsten particles coated with plasmid DNA that contained a gene encoding hygromycin phosphotransferase (HPH, conferring hygromycin resistance) driven by the CaMV 35S promoter or *Agrobacterium tumefaciens* NOS promoter. Putatively transformed cell clusters were identified from the bombarded tissues 2 weeks after selection on hygromycin B. By separating these cell clusters from each other, and by stringent selection not only at the callus growth stage but also during regeneration and plantlet growth, the overall transformation and selection efficiencies were substantially improved over those previously reported. From the most responsive cultivar used in these studies, an average of one transgenic plant was produced from 1.3 immature embryos or from 5 pieces of embryogenic calli bombarded. Integration of the introduced gene into the plant genome, and inheritance to the offspring were demonstrated. By using this procedure, we have produced several hundred transgenic plants. The procedure described here provides a simple method for improving transformation and selection efficiencies in rice and may be applicable to other monocots.

Abbreviations: bp, base pairs; CaMV, cauliflower mosaic virus; GUS, β-glucuronidase; HPH, hygromycin phosphotransferase; hyg B, hygromycin B; hyg^r, hygromycin resistance; NOS, *Agrobacterium tumefaciens* nopaline synthase; PCR, polymerase chain reaction; X-Gluc, 5-bromo-4-chloro-3-indolyl-β-D-glucuronide.

Introduction

The development of plant transformation techniques during the past decade has made it possible to improve crop plants by introduction of cloned genes. However, since the number of genes inserted and chromosomal locations of the integrated genes are not controllable, the expression of the introduced gene varies among individual transformants. Therefore, a relatively large number of transgenic plants must be developed in order to select desirable transformants as well as to study the expression of the introduced gene. For dicots, the *Agrobacterium*-mediated transformation system (Hooykaas, 1989) can be used to generate many transformants while for monocots, especially for the agronomically important cereal plants, current transformation systems still need to be improved.

Transgenic cereal plants were first obtained in rice from protoplast transformation systems, with DNA uptake mediated by electroporation or/and polyethylene glycol (PEG) (Toriyama *et al.* 1988; Zhang *et al.* 1988; Zhang and Wu, 1988; Shimamoto *et al.* 1989; Datta *et al.* 1990; Peng *et al.* 1992). However, since this method requires delicate manipulation of protoplasts as well as embryogenic suspension cell cultures, which are generally genotype-dependent, it is not readily applied to all cereal species. In the past few years, the biolistic method has emerged as a simple and promising alternative for cereal transformation, and has been successfully used in maize, rice, sugarcane and wheat transformation (Fromm *et al.* 1990; Gordon-Kamm *et al.* 1990; Christou *et al.* 1991; Bower and Birch, 1992; Vasil *et al.* 1992). Procedures using organized tissues such as immature embryos (Christou *et al.* 1991) or embryo slices (Cao *et al.* 1991) as target tissues for rice transformation may make the system less genotype-dependent. However, the reported transformation efficiency by the biolistic method is still low, and the probability of producing non-transformants (Christou *et al.* 1991) or chimeric plants (Cao *et al.* 1991) is high.

In our attempts to transform rice with coat protein genes of rice tungro bacilliform and spherical viruses (to be reported elsewhere), we have developed a procedure using the biolistic method to improve transformation and selection efficiencies and to reduce the numbers of non-transformants and chimeric rice plants. The target tissues used were immature embryos and embryogenic calli. The main differences between our procedure and those previously published include: the use of embryogenic rice calli, the excision of the hyg^r cell clusters at an early stage of selection, and the continuous selection on hyg B during regeneration and plantlet growth. This procedure is simple, efficient, and may have general application in the transformation of other monocot species by the biolistic method.

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Materials and Methods

Materials The following rice (*Oryza sativa*, L.) varieties and lines were used: Taipei 309 (*japonica*), 77125 (*japonica*, bred by Dr. Shanbao Chen, Institute of Crop Cultivation and Breeding, Chinese Academy of Agricultural Sciences, Beijing, China), Tetep (*indica*), TN1 (*indica*) and 8706 (the latter is a stable line developed through anther culture of progeny of a cross between *japonica* and *indica* varieties, identified as morphologically intermediate but relatively *indica*-like. Line 8706 was bred by Dr. Ying Chen, the Institute of Genetics, Academia Sinica, Beijing, China). The target tissues used for bombardment were: (1) immature embryos dissected from immature seeds approximately 10-15 d after pollination. Tissue was placed on media with the scutellar side up for bombardment; (2) primary embryogenic calli induced from immature or mature embryos, or calli subcultured on solid medium for no longer than 3-4 months. All the seeds used in the experiments were dehulled and surface-sterilized according to Thompson *et al.* (1986).

Particle bombardment The particle gun employed in the experiments was a home-made apparatus, similar to that described by Klein *et al.* (1987), and the macroprojectile was driven by gun powder. Target explants, approximately 30-40 immature embryos or 50-80 pieces of calli (approximately 0.5 cm in size and total 2 g by weight), were centered on the medium in a Petri dish 10 cm in diameter. The plate was placed 8 cm beneath the stopping plate of the gun with a layer of metal net (120-180 mesh per linear inch) 1.5 cm above the target materials for even dispersion of tungsten particles. Plasmids used in the experiments included: pAct1D which contains the rice actin1 gene promoter linked to the GUS gene (McElroy *et al.* 1990; a gift from Dr. R. Wu); pNG3 which contains the NOS promoter driving HPH gene (a gift from Dr. M. C. Van Montagu); and pMON410 which contains CaMV 35S promoter driving the HPH' gene (Rogers *et al.* 1987; a gift from Monsanto Company). All plasmids were purified by CsCl gradient centrifugation. Coating of the tungsten particles (M10, Dupont, Wilmington, DE) was basically as described by Klein *et al.* (1988). In co-transformation experiments, 2.5 μg of each plasmid was used in coating of approximately 40 μl of particle suspension. Two and one-half μl of suspended particles was loaded per shot; explants on each plate were bombarded twice.

Culture media The basic ingredients of the media included macroelements of N6 medium (Chu *et al.* 1975), microelements and vitamins of B5 medium (Gamborg *et al.* 1968), proline (500 mg/l), enzymatic casein hydrolysate (300 mg/l), and sucrose (30 g/l). The callus growth medium contained 2 mg/l of 2,4-D (2,4-dichlorophenoxyacetic acid). The regeneration medium contained 3 mg/l of BAP (6-benzylaminopurine). The same basal medium or MS medium (Murashige and Skoog, 1962) was used for plantlet growth without addition of phytohormones.

Growth and selection of transgenic tissues The bombarded materials and subsequent calli were cultured in the dark at 25°C. Regeneration and plantlet growth were carried out under 16 h photoperiod with light intensity of 110-130 $\mu Mm^{-2}s^{-1}$PAR using Sylvania F40/CW cool white fluorescent tubes. Hyg B (Calbiochem, La Jolla, CA) was added to the selection, regeneration, and plantlet growth media at the concentration of 30-50 mg/l. When well-grown plantlets reached 6-10 cm, they were transplanted to soil and transferred to a greenhouse.

GUS assays Histochemical GUS expression (Jefferson *et al.* 1987) assays were performed basically as described by Klein *et al.* (1988). Methanol was added to a final concentration of 20% in the buffer to suppress endogenous β-glucuronidase activity (Kosugi *et al.* 1990) Transient GUS expression assays were carried out 48 h after bombardment.

PCR assays of the putative transgenic plants DNA samples of the putative transgenic rice plants were extracted from leaf or leaf sheath tissues based on McGarvey and Kaper (1991). PCR assays were performed in 50 μl of each reaction with AmpliTaq DNA polymerase, Stoffel Fragment (Perkin Elmer Cetus, Norwalk, CT) according to the manufacturer's instruction. Two 18-mers (nt 236-253 of plus strand and nt 810-827 of minus strand) were designed as PCR primers based on the published sequence of the HPH gene (Gritz and Davies, 1983). A 592 bp amplified fragment of DNA was expected from transgenic plants. Ten μl of each reaction was used for electrophoresis in a gel of 1% agarose (Gibco BRL, Gaithersburg, MD) and TBE buffer (Sambrook *et al.* 1989).

Progeny test for resistance to hyg B Seeds from self-pollinated hyg^r rice plants were dehulled and surface-sterilized as described above. They were germinated on MS medium without hormones but with the addition of 50 mg/l of hyg B, and incubated under conditions for plantlet growth as described above.

Southern analyses of transgenic plants Genomic DNA was extracted from leaf and stem tissues of rice plants based on Dellaporta *et al.* (1983) with modifications. Five μg of genomic DNA from each sample, undigested or digested with restriction endonuclease (see relevant Figure Legends), was used for electrophoresis in an 0.8% agarose gel. DNA was blotted to Hybond-N nylon membrane (Amersham, Arlington Heights, IL) according to instructions of the manufacturer. Hybridization was carried out according to Church and Gilbert (1984). The HPH probe was a 1.1 kb *Sma* I fragment of pMON410 labeled by random hexamer priming with Klenow fragment of DNA polymerase I (Promega, Madison, WI) and 5'-[α-^{32}P]dATP (Amersham) based on the manufacturers' instructions.

Results and Discussion

Transient GUS gene expression after bombardment

After bombardment with tungsten particles coated with pAct1D, a high frequency of GUS expression was observed from both types of target materials (Fig. 1A, 1B). The average number of blue units per plate for each material were: >750 (for immature embryos) and >1100 (for calli), respectively (strain 77125). However, variation among different shots was high, e.g. from 176 to 2920 per plate for embryogenic calli, probably reflecting the variation in manipulation of the particle gun.

Appearance and separation of hyg B-resistant cell clusters during early selection

In a control experiment, we examined the effect of hyg B on the growth of calli and regenerated plantlets from each of the rice varieties used. Callus growth was greatly inhibited when 30 mg/l of hyg B was added into the medium, and the growth was completely inhibited at a concentration of 50 mg/l (data not shown). Plantlets regenerated from non-transformed calli died when the medium contained 50 mg/l of hyg B. Based on these observations, the procedure for selecting stable transformants was designed.

One week after bombardment with the plasmid pMON410 or pNG3, immature embryos or embryogenic calli were transferred to selection medium containing 30 mg/l of hyg B. The explants gradually turned brown. Two weeks later, fresh white cell clusters were identified on the surface of the bombarded tissues when viewed under a dissection microscope. Hyg^r cell clusters which could be distinguished from each other (Figs. 1C, 1D) presumably originated from different transformation events. At this stage, the fresh cell clusters were carefully removed from the dying explant tissues and transferred to medium containing 30 mg/l of hyg B for further selection. Table 1 compares the yield of hyg^r cell clusters of two different types of explants from various rice varieties after bombardment with a plasmid containing HPH gene.

This table shows that hyg^r cell clusters can be obtained from both immature embryos (1.7-2.7 clusters on average per embryo bombarded) and embryogenic calli (0.4-0.5 clusters per piece of callus bombarded). Not all the explants were responsive to the bombardment. Hyg^r cell clusters

For Figs. 1-4 see p. 255

were found from approximately 40% of the bombarded immature embryos or some 20% of the embryogenic calli. Since the cell clusters were separated at an early stage, fusion or co-mingling of cell clusters from different transformation events on the same piece of explant was prevented. Therefore, not only was the transformation efficiency substantially increased from that previously reported (Christou et al. 1991), but also the possibility of regenerating chimeric plants from fusion of distinct transformation events was greatly reduced.

Table 1. Hyg^r cell clusters obtained from transformation after 2 weeks of selection on hyg B

Variety	Explant	No. of explants bombarded	No. of hyg^r cell clusters obtained	Hyg^r clusters / explant
Taipei 309	im.e.	34	57	1.7
8706	im.e.	75	189	2.5
Tetep	im.e.	22	60	2.7
8706	callus	94	43	0.5
77125	callus	160	57	0.4

im. e.: immature embryo

When the explants were bombarded with tungsten particles coated with both pMON410 (or pNG3) and pAct1D, some of the hyg^r cell clusters turned blue after incubation with X-Gluc, indicating that they resulted from co-transformation events (Figs. 1C, 1D, 1E). In some clusters both blue and white sectors were seen. Such clusters may originate from two different transformation events, or, from cases in which non-transgenic cells survived due to detoxification of hyg B by the adjacent transformed cells (Christou *et al.* 1991; Fig. 1E). However, we did not see blue-white chimeric calli after growing under continuous selection. It appeared that the surrounding non-transformed cells were gradually degenerated under selection pressure.

Yield of hyg^r callus lines and regeneration of hyg^r plants

When the hyg^r cell clusters were carefully excised under a dissection microscope, a brown scar could usually be seen at the base of the clusters, especially when immature embryos were used as target tissues. This is the junction between the cell cluster and the "parental" explant tissue, and can be generally used as a criterion for identifying individually originated cell clusters. The clusters usually grew well when transferred to medium without hyg B, whereas 45-65% survived when grown on medium containing 30 mg/l of hyg B (Table 2). After further selection at 50 mg/l of hyg B, the cell clusters grew to form hyg^r callus lines (Fig. 2A, left).

When the size of the hyg^r calli reached approximately 5 mm in diameter, they were transferred to regeneration medium containing 50 mg/l of hyg B. We did not observe detrimental effects by addition of hyg B to the regeneration medium (Fig. 2A, right). Usually 1-5 plantlets (or plantlet clusters) were obtained from a piece of hyg^r callus; this formed a plant line. The regenerated plantlets were then transferred to plantlet growth medium that also contained 50 mg/l of hyg B. We found that selection at the plantlet growth stage was quite efficient: the control plantlets (i.e. those regenerated from non-transformed calli) did not grow and finally died whereas the putative transformants grew normally (Fig. 2B). In PCR assays using primers derived from the HPH coding sequence, 30 out of 35 plants that survived selection showed the expected size of the amplified DNA fragment, indicating that at least 85% of the hyg^r plants are true transgenic plants. Figure 5 shows the result of PCR assay of eight putative transgenic plants: seven of them have the expected size of the amplified DNA.

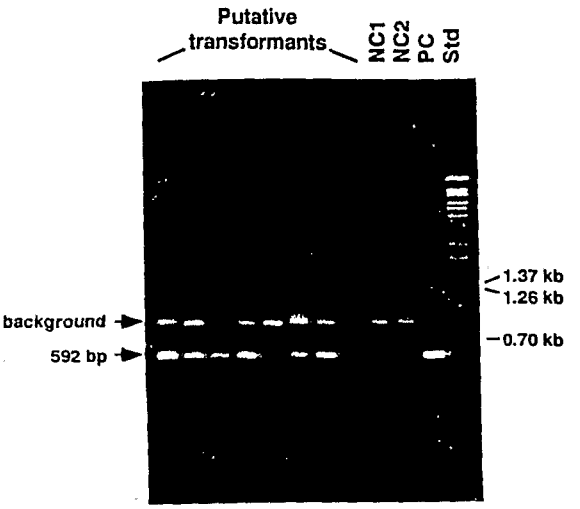

Figure 5. Agarose gel electrophoresis of PCR-amplified DNA from putatively transformed plants. Each lane represents an individual plant. NC1 and NC2: non-transformed Taipei 309 and 77125 plants, respectively. PC: 1 ng of the plasmid pMON410 was used in the reaction. The 592 bp fragment represents the amplified fragment of the HPH gene. The band above is apparently a specific sequence found in all plant DNA samples tested and is not related to the transformation events.

Table 2. Survival and regeneration of hyg^r cell clusters

Variety	Explant	Hyg^r cell clusters	Hyg^r callus lines	Hyg^r plant lines No.	%
Taipei 309	im.e.	39	18	10	25.6
8706	im.e.	87	52	27	31.0
8706	callus	26	17	11	42.3

im.e.: immature embryo

Table 2 reveals the results of selection and plant regeneration after bombardment of two rice varieties. Approximately 30% of the originally separated hyg^r cell clusters were finally regenerated into plantlets. Calculations based on the data in Tables 1 and 2 indicated that, from the best responsive variety used in the experiments (line 8706), one resistant plant line was obtained from 1.3 immature embryos or from 5 pieces of embryogenic calli bombarded, which is substantially higher than that previously reported (Christou *et al.* 1991).

Co-transformation

In co-transformation experiments using constructs pMON410 (containing the HPH' gene) and pAct1D (containing the GUS gene), 33 hyg^r plants were examined and 12 showed GUS activity (Fig. 3), giving a 36% co-expression efficiency. This is substantially lower than that reported in rice protoplast co-transformation experiments (Peng *et al.* 1990).

Inheritance of the HPH gene

Integration of the introduced HPH gene into the genome of transgenic rice plants was shown by Southern blot hybridization reactions of the undigested genomic DNA of the plants (Fig. 6). When the genomic DNAs were digested with either *Hin*d III or *Not* I, different transformants showed various digestion patterns. Since neither of the restriction enzymes used in the experiments has a cleavage site within the 1.8 kb HPH gene construct, it seems that at least some lines carry multiple copies of the gene. The segregation of the hyg^r trait among offspring of the transgenic plants was demonstrated by germinating R1 seeds on medium containing 50 mg/l of hyg B (Table 3, Fig. 4). Table 3 shows 3 : 1 segregation among offspring of three transgenic plants, indicating Mendelian inheritance from a single genetic locus of a functional HPH gene.

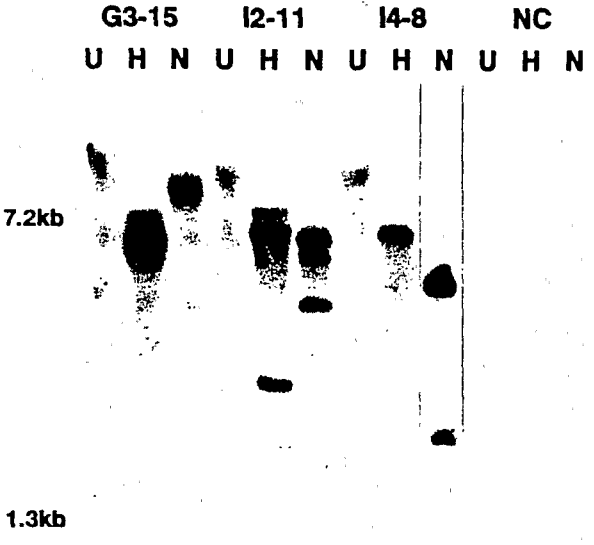

Figure 6. Southern blot hybridization reaction of total DNA of 3 R_0 transgenic rice plant lines, demonstrating integration of the HPH gene. U: undigested DNA; H: DNA digested with *Hin*d III; N: DNA digested with *Not* I. NC: Taipei 309. The low intensity band of undigested genomic DNA may have resulted from inefficient transfer of high molecular weight DNA.

Concluding remarks

The generation and maintenance of embryogenic suspension cell cultures for rice transformation experiments are labor intensive and generally genotype-dependent. The practice of using immature embryos as target tissue for rice transformation by particle bombardment, as reported by Christou *et al.* (1991), made rice transformation independent of the suspension cell cultures and simplified the procedure. In our experiments, we found that by excising hyg^r cell clusters at an early stage from tissues bombarded with HPH gene (two weeks after applying hyg B selection), and by continuous selection during regeneration as well as plantlet growth, the transformation and selection efficiencies were substantially improved. Figure 7 outlines the procedure we used in our studies. By employing this proce dure, we have been able to develop several hundred transgenic plants which can grow normally on medium containing 50 mg/l of hyg B.

Table 3. Inheritance of the hyg^r trait in R_1 generation

Transgenic rice line	R_1 seeds germinated	Well-grown seedlings	Dead seedlings	χ^2
I4-29 (Taipei 309)	40	31	9	0.133
K1-1-45a (8706)	48	36	12	-
M7-1a (TN1)	48	37	11	0.111

Data were collected 2 weeks after the seeds were germinated on hyg B (50 mg/l)-containing medium. All control seedlings were dead. χ^2 tests indicate good agreement with segregation ratios of 3:1.

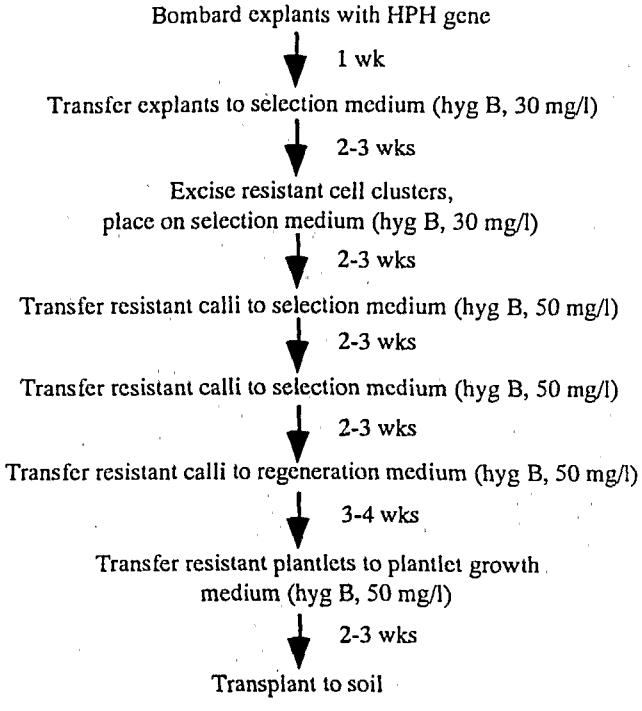

Fig. 7. Outline of the rice transformation-selection procedure

Our work also demonstrates that embryogenic calli can be used for rice transformation by the biolistic method. Subcultured calli, or primary calli from mature seeds, do not depend on the availability of flowering plants. This advantage makes calli an alternative material for rice transformation. However, long-term subcultured calli may lose regeneration ability, or the plants regenerated from such cultures may be sterile (our unpublished data). Therefore, when using embryogenic calli for transformation, primary calli from immature or mature embryos, and short-term (no longer than 3-4 months) subcultured calli are preferred.

Although varieties of both *japonica* and *indica* rice can be transformed in our experiments, the capacity for plant regeneration is quite genotype-dependent, and is still a limiting factor especially for *indica* rice transformation. We are currently trying to improve the regeneration rate of *indica* varieties by testing various factors.

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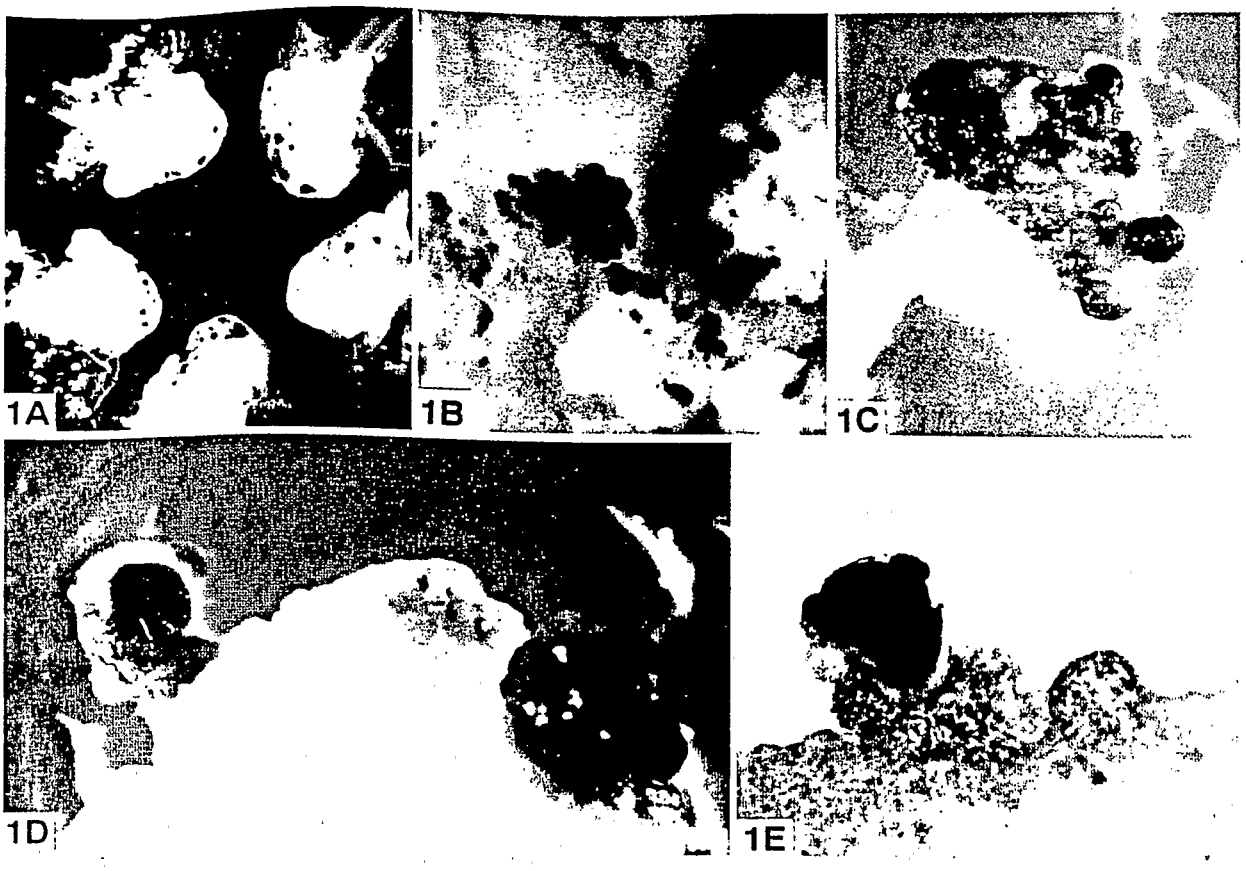
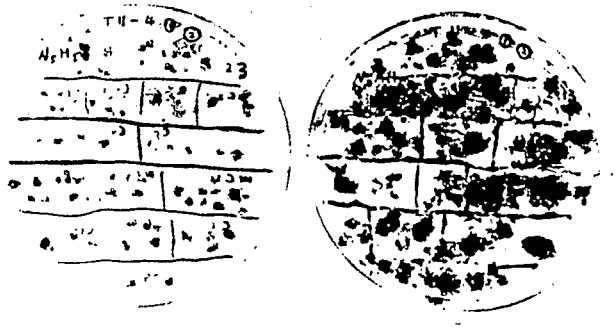
References

Bower R, Birch RG (1992) Plant J 2: 409-416
Cao J, Zhang W, McElroy D, Wu R (1991) In: Rice Biotechnology, GS Khush, GH Toenniessen (eds), CAB International, Wallingford, UK, pp 175-198
Christou P, Ford TL, Kofron M (1991) Bio/Technology 9: 957-962
Chu CC, Wang CC, Sun CS, Hsu SC, Yin KC, Chu CY, Bi FY (1975) Sci Sin 18: 659-668
Church GM, Gilbert W (1984) Proc Natl Acad Sci USA 81: 1991-1995
Datta SK, Peterhans A, Datta K, Potrykus I (1990) Bio/Technology 8: 736-780
Dellaporta SL, Wood J, Hicks JB (1983) Plant Mol Biol Rep 1(4): 19-21
Fromm, ME, Morrish F, Armstrong C, Williams R, Thomas J, Klein TM (1990) Bio/Technology 8: 833-844
Gamborg OL, Miller RA, Ojima K (1968) J Exp Res 50: 151-158
Gordon-Kamm WJ, Spencer TM, Mangano ML *et al.* (1990) Plant Cell 2: 603-618
Gritz L, Davies J (1983) Gene 25: 179-188
Hooykaas PJJ (1989) Plant Mol Biol 13: 327-336
Jefferson RA, Kavanagh TA, Bevan MW (1987) EMBO J 6: 3901-3907
Klein TM, Wolf ED, Wu R, Sanford JC (1987) Nature 327: 70-73
Klein TM, Gradziel T, Fromm ME, Sanford JC (1988) Bio/Technology 6: 559-563
Kosugi S, Ohashi Y, Nakajima K, Arai Y (1990) Plant Sci 70: 133-140
Li Z, Burow MD, Murai N (1990) Plant Mol Biol Rep 8: 276-291
McElroy D, Zhang W, Cao J, Wu R (1990) Plant Cell 2: 163-171
McGarvey P, Kaper JM (1991) BioTechniques 11: 428-432
Murashige T, Skoog F (1962) Physiol Plant 15: 473-497
Peng J, Lyznik LA, Lee L, Hodges TK (1990) Plant Cell Rep 9: 168-172
Peng J, Kononowicz H, Hodges TK (1992) Theor Appl Genet 83: 855-863
Rogers SG, Klee HJ, Horsch RB, Fraley RT (1987) In: Methods in Enzymology, vol 153, R Wu, L Grossman (eds), Academic Press, San Diego, pp 253-277
Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York
Shimamoto K, Terada R, Izawa T, Fujimoto H (1989) Nature 338: 274-276
Thompson JA, Abdullah R, Cocking EC (1986) Plant Sci 47: 123-134
Toriyama K, Arimoto Y, Uchimiya H, Hinata K (1988) Bio/Technology 6: 1072-1074
Vasil V, Castillo AM, Fromm ME, Vasil IK (1992) Bio/Technology 10: 667-674
Zhang HM, Yang H, Rech EL, Golds TJ, Davis AS, Mulligan BJ, Cocking EC, Davey MR (1988) Plant Cell Rep 7: 379-384
Zhang W, Wu R (1988) Theor Appl Genet 76: 835-840

Figures 1-4. 1. (A) GUS activity 2 days after bombardment of immature embryos with the plasmid pAct1D. (B) GUS activity 2 d after bombardment of embryogenic calli with the plasmid pAct1D. (C) An immature embryo bombarded with plasmids pMON410 and pAct1D 17 d after selection on the medium containing hyg B. The embryo stained with X-Gluc indicated multiple transformation events. (D) A piece of callus tissue bombarded and treated as described in (C), illustrating two hyg^r cell clusters which also showed GUS activity. (E) A cross section through an immature embryo bombarded and treated as described in (C), in which the central sector of the hyg^r cell cluster showed GUS activity while the surrounding cells did not. The scars caused by bombardment are illustrated as well. See text for details. **2.** (A) Left: callus lines formed from hyg^r cell clusters grown on medium containing 50 mg/l of hyg B. One callus line for each grid (line 8706). Right: plantlet regeneration of hyg^r calli on regeneration medium containing 50 mg/l of hyg B. One plant line for each grid. (B) Regenerated plantlets (line 8706) from hyg^r calli (right box) and non-transformed calli (left box) on medium containing 50 mg/l of hyg B. **3.** Plantlets regenerated from hyg^r calli after bombardment of immature embryos with plasmids pMON410 and pAct1D, stained with X-Gluc, indicating they were from co-transformation events (line 8706). **4.** Seeds (R1) of transgenic rice plant line I4-6 germinated on medium containing 50 mg/l of hyg B, indicating segregation of hyg^r among the offspring (left box). The seeds from control (non-transformed) plant were all dead 2 weeks after germination on the same medium (right box) (variety: Taipei 309).





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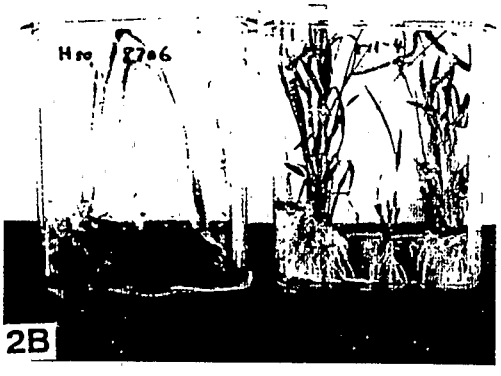

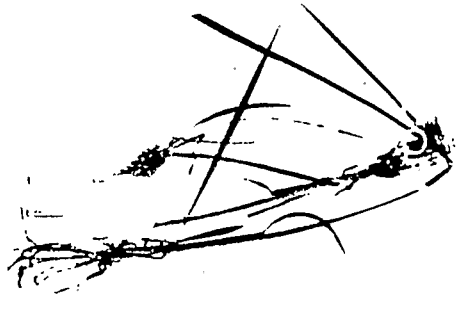

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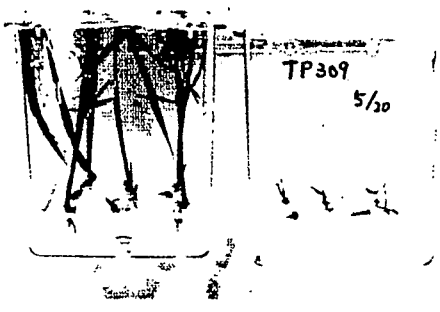

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