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Efficient regeneration of transgenic plants from rice protoplasts and correctly regulated expression of the foreign gene in the plants

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Summary. Rice is one of the most important crops in the world with 35% of the total population (over two billion people) depending on it as their source of food. It is therefore essential to develop efficient methods for the transformation and regeneration of rice plants in order to delineate the exact regulatory sequences responsible for gene expression and to transfer beneficial genes into this plant. Here, for the first time, we present definitive evidence for the regeneration of a large number of transgenic rice plants after introduction of the bacterial β-glucuronidase gene into rice protoplasts. The presence of integrated copies of this gene was detected in the genome of transgenic plants by DNA hybridization analysis. Furthermore, under the control of regulatory regions from a maize alcohol dehydrogenase sequence, β-glucuronidase gene expression was detected in the roots of transgenic plants. This expression was stimulated up to six fold under anaerobic conditions.

Key words: *Oryza sativa* – Transgenic rice – Gene transfer

Introduction

Spectacular progress has been made in gene transfer experiments over the past five years by introducing modified regulatory sequences and foreign genes into the genomes of dicotyledenous plants (Fraley et al. 1986; Goldberg 1986; Kuhlmeier et al. 1987; Schell 1987). This progress was made possible through readily available transformation and regeneration systems in dicots, such as tobacco, using *Agrobacterium* with its associated Ti plasmids (Bevan and Chilton 1982; Gheysen et al. 1985). However, most monocots, especially economically important crops such as rice, wheat, and corn, are not normally susceptible to *Agrobacterium*-mediated transformation (Goodman et al. 1987).

Success in the regeneration of plants from rice protoplasts has been achieved only within the last 2 years (Abdullah et al. 1986; Coulibaly and Demarly 1986; Fujimura et al. 1985; Yamada et al. 1986; Kyozuka et al. 1987). Protoplasts isolated from cultured rice cells show the transient expression of a foreign gene after electroporation (Ou-Lee et al. 1986) and stable integration (Uchimiya et al. 1986) using polyethylene glycol. However, there has been no published report on the regeneration of transgenic rice plants.

Materials and methods

Two varieties of rice (*Oryza sativa* L.), 'Pi-4' and 'Taipei 309' of the *Sinica* subspecies (also known as *Japonica*), were used as the source of protoplasts. Scutella of mature rice seeds were grown into calli and then maintained as suspension cultures in AA2 liquid medium (Abdullah et al. 1986). Serial subculture was carried out once a week by adding 5 volumes of fresh medium. After subculturing for 3 months, protoplasts were isolated according to the method of Abdullah et al. (1986) from suspension cultures 3–5 days after subculturing. For each experiment, protoplasts were resuspended in one of four media to 10^6 per ml (Table 1). One milliliter protoplast suspension was incubated at 45 °C for 5 min, followed by 10 sec at 0 °C, and then brought to 25 °C. The plasmid pAI_1GusN (Klein et al. 1988) (10 μg/ml), carrier calf thymus DNA (50 μg/ml), and 1 volume of a 38% polyethylene glycol-4000 (Sigma) solution were added sequentially at 10 min intervals. The mixture was incubated at 25 °C for 30 min, diluted with CPW13 medium (Abdullah et al. 1986), and centrifuged. The protoplasts were washed 3 times in CPW13 medium, resuspended in a simplified KPR liquid medium (Kao 1977) to 10^6 per ml, and incubated at 27 °C in the dark for 3 days. Triplicate samples of 0.1 ml each (containing 10^5 protoplasts) were assayed for β-glucuronidase (GUS) activity.

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The plasmid pAI_1GusN (Klein et al. 1988) includes the β-glucuronidase (Gus) gene (which served as the reporter gene), the promoter region and intron 1 of the maize alcohol dehydrogenase (*Adh*1) gene, and the 3′ non-coding region of the nopaline synthase gene (N). The circular plasmid (6.5 Kb) was linearized by digestion with EcoRI enzyme for experiments listed under the column "linear plasmid".

Total DNA from a portion of each plantlet was isolated according to a method of Moon et al. (1987), with slight modifications. About 20–40 mg wet weight of fresh tissue from the plantlets was used, and about 2 μg of DNA subsequently isolated. In slot-blot experiments, 0.7 μg DNA was spotted on a piece of nitrocellulose filter using a slot-bot apparatus (Schleicher and Schuell). The filter was hybrized to a 1.8 Kb PstI fragment containing the Gus gene. The Gus probe was isolated by gel electrophoresis after cutting pAI_1GusN DNA with the PstI enzyme, and labeled with ^{32}P-dATP by nick translation (10^8 cpm/μg DNA). Overnight hybridization in 50% formamide and 5 × SSC (Kao et al. 1984) was carried out at 42 °C, and the filter was washed 3 times in 0.2% SSC and 0.1% SDS at 70 °C. The DNA used in Southern blot was isolated from the leaves of plants 15 weeks after the gene transfer step.

Table 1. Efficiency of gene transfer into protoplasts in different media

Media[a]	Number of blue cells[b]	
	Linear plasmid	Circular plasmid
W5	120	42
MaMg	607	49
CPW13	265	15
KPR	15	5

[a] W5 and MaMg were recommended for stable transformation in tobacco (Negrutiu et al. 1987). W5: 125 m*M* $CaCl_2 \cdot 2H_2O$, 154 m*M* NaCl, 5 m*M* KCl, 5 m*M* glucose, pH 5.6. MaMg: 15 m*M* $MgCl_2$ 0.1% morpholinoethanesulphonic acid, 400 m*M* mannitol, pH 5.6. CPW13: 0.16 m*M* K_2HPO_4, 1 m*M* KNO_3, 10 m*M* $CaCl_2 \cdot 2H_2O$, 1 m*M* $MgSO_4 \cdot 7H_2O$, 1 μ*M* KI, 0.1 μ*M* $CuSO_4 \cdot 5H_2O$, 710 m*M* mannitol, pH 5.8. KPR, a medium for protoplast culture (Kao 1977), was simplified by omitting minor vitamins, organic acids, sugar alcohols, and coconut milk. It is referred to as a simplified KPR medium throughout this paper

[b] Each value is the mean of triplicate assays with Pi-4 protoplast-regenerated cells. Detection of the β-glucuronidase activity in rice cells was carried out by an in situ staining method (Jefferson et al. 1987) using 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-GLU) substrate. Triton X-100 (0.03%) was added to allow the substrate to enter the cells. Another set of experiments using protoplasts from 'Taipei 309' gave similar results

Results and discussion

For the regeneration of plants from protoplasts, we essentially followed the method of Abdullah et al. (1986) with one important modification, which will be described later. Protoplasts were incubated with a plasmid containing the β-glucuronidase gene. The efficiency of the DNA uptake by protoplasts was tested first by comparing four commonly used media, and the results are given in Table 1. The efficiency of gene transfer was determined by counting the number of blue cells in an in situ assay for β-glucuronidase activity (Jefferson et al. 1986) 48 h after the addition of its substrate. We found that the MaMg medium (Negrutiu et al. 1987) was the best among the four tested, and that DNA derived from EcoRI-digested linear plasmids gave at least 10 times more blue cells than the circular plasmids. In this experiment, up to 0.6% of the protoplasts showed transient expression when assayed 3 days after gene transfer.

For the regeneration of transgenic plants, the MaMg medium, linear pAI_1GusN plasmid (Klein et al. 1988) and 10^7 protoplasts from 'Pi-4' were used. After the gene transfer step, the protoplasts were resuspended in a simplified KPR medium (Kao 1977) containing 1.2% agarose, and 0.5 ml aliquots (5×10^5 protoplasts) were placed in 12-well plates (COSTAR) and sealed with parafilm. Ten days later, agarose-embedded calli (regenerated from protoplasts) were transferred to 6-well plates, and 1 ml of a simplified KPR liquid medium containing 1 mg/l 1-naphthylacetic acid (NAA) and 0.5 mg/l filter-sterilized zeatin was added per well. Two weeks later, visible calli were transferred into MS medium (Murashige and Skoog 1962) containing 0.8% agarose, 2 mg/l kinetin, and 0.5 mg/l NAA. This step is different from that of Abdullah et al. (1986) who did not use plant growth regulators at this stage. We found that adding growth regulators at this stage was essential in subsequent plant regeneration from calli of 'Pi-4' (data not shown). As can be seen in Fig. 1 D, the size of the agarose-embedded calli 24 days after gene transfer was much larger than those grown in liquid medium (Fig. 1 A, B, C, and legend to Fig. 1).

For plant regeneration, the calli which gave rise to small shoots (around 35 days after gene transfer) were grown in light. After green shoots had grown to a length of 2 cm, plantlets were transferred into fresh MSO solid medium (Abdullah et al. 1986). A part of the callus from the base of each plantlet was excised for assays of β-glucuronidase enzyme activity. Figure 1 E shows the results of assays on six calli; three of them had enzyme activity as indicated by the blue areas. A total of 61 calli out of the 378 assayed showed enzyme activity.

In the plantlet on the right-hand side of Fig. 1 F, both shoots and a portion of the callus turned blue 48 h after the addition of the substrate. As a negative control, a non-transformed shoot (on the left-hand side) did not turn blue.

Six to seven weeks after the gene transfer step, the shoots of certain regenerated plants had reached a height of 4 cm (Fig. 1 G). Two weeks later, some shoots were 7–8 cm tall (Fig. 1 H). The large callus (right-hand plant in Fig. 1 H) gave at least six shoots, but we counted them

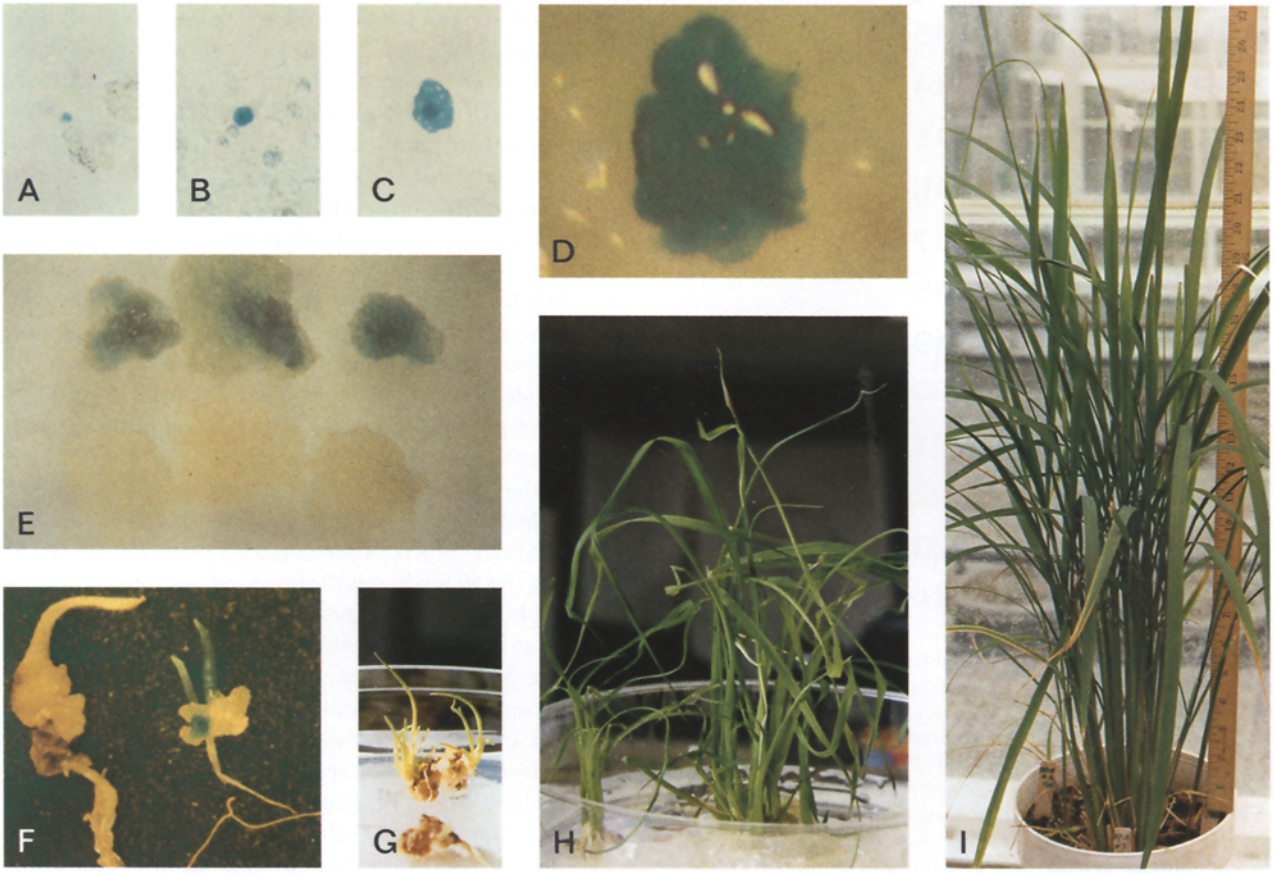

Fig. 1 A–I. Rice cells, calli, plantlets, and plants regenerated from protoplasts. For the in situ β-glucuronidase (GUS) assay, 0.03% Triton X-100 was used for **A, B** and **C,** and 0.5% Triton X-100 was used for **D, E** and **F. A–D** GUS activity-positive cells and calli regenerated from Pi-4 rice protoplasts: **A** three days; **B** 10 days, and **C** 24 days after gene transfer and grown in a simplified KPR liquid medium. **D** 24 days after gene transfer and grown in KPR medium in the presence of 1.2% low-melting point agarose (BRL). The diameter of the cell in **A** is around 0.02 mm, and that of the calli is around 0.05 mm, 0.15 mm, and 1.2 mm for **B, C,** and **D,** respectively. **E** Assay for GUS activity in a portion of each six calli (1–2 mm diameter) after the shoots were around 2 cm in length. The three yellow calli represent non-transformed controls. **F** Assay for GUS activity when the shoots were around 1–2 cm in length. Albino shoots with calli were chosen because it is easier to see the blue color for positive GUS activity. **G** Two calli with shoots around 4 cm in length. **H** Plants grown from two calli in a petri dish (8–9 weeks after gene transfer); the tallest plant was 7–8 cm in length. **I** 20-week-old green plants (70 cm tall) grown in a greenhouse for 12 weeks, this and other transgenic plants produced viable seeds 3 months later

as one regenerated plant. All green plants 7–8 cm in height were transferred to soil and placed directly in a greenhouse (day temperature 28 °C, night 22 °C; 14 h day/10 h night cycle) without the need for hardening the plantlets. Eleven weeks after the gene transfer step, a total of 343 regenerated green plants were planted in soil; 35 albino plants were used for assays only. Out of these 378 plants, at least 86 were transgenic plants as determined by DNA hybridization analysis. Twenty weeks after the gene transfer step, many regenerated plants were about 70 cm in height (Fig. 1 I), which approaches the adult height. The transgenic plants looked healthy and grew as fast as non-transformed 'Pi-4' plants.

The presence of the β-glucuronidase gene in the shoots of the regenerated plants was revealed by DNA-DNA hybridization using a purified restriction fragment carrying the β-glucuronidase gene as the probe. DNA was isolated from 100 plants, including the 61 plants whose calli showed β-glucuronidase activity. The results of slot-blot hybridization on 24 DNA samples are given in Fig. 2. In this analysis, most samples gave positive hybridization signals. The copy number of the β-glucuronidase gene ranges from one to ten per cell, with the majority of plants carrying one to two copies per cell (a copy number of much less than one was considered negative). Several albino shoots showed a relatively high copy number (data not shown). The ratio of calli that gave rise to green shoots: albino shoots is 10:1; thus, most plantlets have the potential for growing into healthy mature plants.

The number of stable transformants, as detected by assaying calli for β-glucuronidase activity, was 61 out of

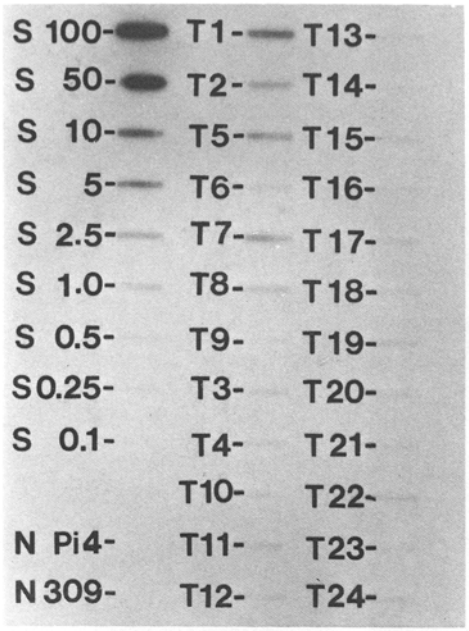

Fig. 2. Analysis of the β-glucuronidase gene in transgenic rice plantlets by DNA-DNA slot-blot hybridization. *S*, copy number standard using the labeled Gus gene, e.g. S10 means DNA equivalent to 10 copies per rice genome. *N*, DNA from non-transformed rice plants, 'Pi-4' and 'Taipei 309'. *T*, DNA from transformed rice plants. Only 24 samples (out of 100 tested) are shown here. Samples giving much less than the equivalent of one copy of the Gus gene per cell were considered negative. These include samples T6, T9, T14, T16 and T23

378. However, slot-blot DNA hybridization analysis of 100 plants, including the 61 which showed positive enzyme activity in the calli, gave 86 positives. Thus, the slot-blot hybridization is a more sensitive assay for plant transformation than the assay for enzyme activity. If we were to carry out slot-blot assay on all 378 plants, we would expect to find more than 86 transgenic plants. On the other hand, it is likely that some transgenic plants may not produce β-glucuronidase or give enzyme activity too low to be detected by the enzyme assay employed.

Genomic blot experiments (Southern 1975) were next carried out with total DNA isolated from the leaves of a transgenic plant (T) and a non-transformed control (N), as shown in Fig. 3, using the same β-glucuronidase gene probe as that used for experiments in Fig. 2. Figure 3 A shows that the size of the undigested chromosomal rice DNA (lane U), as visualized by ethidium bromide staining, is about 50 Kb. After digestion with EcoRI (E) or PstI (P), the 50 Kb bands disappeared, and lower molecular weight DNA fragments, represented by a smear, appeared as expected. Hybridization results (Fig. 3 B) show that the 50 Kb band near the top of the gel in lane U hybridized to the probe only in the transformed plant (T), but not in the non-transformed control (N). Thus, the β-glucuronidase gene appears to have integrated into the rice genome. When the genomic DNA was digested with the restriction enzyme PstI (P), the high molecular weight hybridizing band disappeared, and the probe now hy-

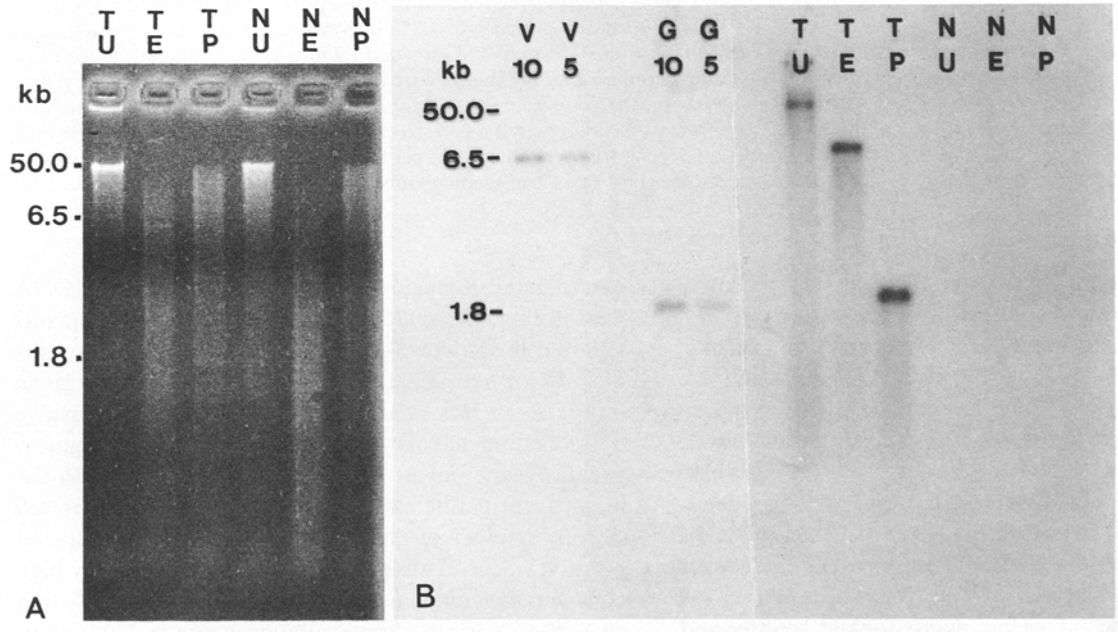

Fig. 3 A, B. Genomic blot hybridization to detect the β-glucuronidase gene in transgenic rice plants. DNA was isolated from leaves and shoots of 15-week-old plants (Moon et al. 1987). Undigested rice DNA samples (*U*; 5 μg per lane) from a transgenic plant (*T*) and a non-transformed (*N*) control 'Pi-4' plant, and samples digested with EcoRI (*E*) or PstI (*P*) enzyme were fractionated on an 1% agarose gel (**A**) and transferred to a nitrocellulose filter by Southern blot method (**B**). Labeled Gus gene (1.8 Kb) was used as the probe (*lane G*) and hybridization was carried out as described under Materials and methods

Table 2. β-glucuronidase activity in the Pi-4 rice plants. Plant materials (roots or leaves) taken from non-transformed control plants and transgenic plants were divided into two parts. One part was anaerobically induced in 10 m*M* *Tris* buffer, pH 8, containing 0.5 mg ampicillin per ml. The buffer with plant material was bubbled with nitrogen for 20 min, wrapped in aluminium foil, and incubated for 46 h at 30 °C. The other part was treated in the same way except that it was not bubbled with nitrogen. Tissue extracts were made and assayed according to Jefferson et al. (1987). The β-glucuronidase activity was determined after 24 h of incubation with the substrate, 4-methyl umbelliferyl-glucuronide (1 m*M*), using a fluorometric assay to determine the amount of product, 4-methyl umbelliferone (MU), produced

Experiment	Rice plant	Tissue assayed	Induction	β-glucuronidase activity (ng MU produced/20 mg protein)
1	Control A	Leaves	None	4
			Anaerobic	4
		Roots	None	31
			Anaerobic	25
1	Transgenic 2	Leaves	None	6
			Anaerobic	8
		Roots	None	29
			Anaerobic	175
2	Control B	Roots	None	37
			Anaerobic	34
2	Transgenic 7	Roots	None	34
			Anaerobic	138
	Transgenic 50	Roots	None	31
			Anaerobic	71
	Transgenic 12	Roots	None	31
			Anaerobic	31

bridized to a 1.8 Kb band (lane T-P), which has the same mobility as the probe carrying the β-glucuronidase gene (lane G). When DNA was digested with EcoRI (E), a 6.5 Kb hybridizing band appeared (lane T-E), which corresponds to the size of the linear pAI_1GusN plasmid (lane V) used for this transformation experiment.

The assay for β-glucuronidase activity in the transgenic rice plant was carried out using a sensitive fluorometric assay (Jefferson et al. 1987). Since the plasmid pAI_1GusN used for transformation includes the promoter of maize *Adh*1 gene, which is known to be highly induced by anaerobiosis (Okimoto et al. 1980), β-glucuronidase activity was measured with or without anaerobic stressing of rice plants. It is also known that maize alcohol dehydrogenase activity is expressed in the roots, but not in the leaves (Okimoto et al. 1980). Results in Table 2 show that in a control 'Pi-4' rice plant, the endogenous β-glucuronidase-like activity in the leaf is very low, but it is several fold higher in the roots. However, the level of activity was the same with or without anaerobic induction. Significantly, in two transgenic 'Pi-4' plants (numbers 2 and 7), the β-glucuronidase activity was induced about five- or six-fold under anaerobic conditions in the roots. No anaerobic induction of enzyme activity was found in plant number 12, and a two-fold induction was seen in plant number 50. Thus, in some of the transgenic rice plants, the β-glucuronidase gene was expressed, and it was highly stimulated by anaerobiosis in a manner expected for that of the maize *Adh*1 gene.

In conclusion, we have regenerated transgenic green plants from protoplasts at a high frequency. This success will open up unlimited opportunities for studies aimed at defining the exact regulatory sequences responsible for the expression of important rice genes, and for transfer of agronomical beneficial genes into rice by genetic engineering.

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