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TRANSFORMATION OF INDICA AND JAPONICA RICES AT IRRI USING MARKER AND AGRONOMICALLY-IMPORTANT GENES[1]

F.J. ZAPATA
Corresponding Author
E.M. ABRIGO
N.P. OLIVA
M.F. ALAM
C.Y. WU
S. ZHANG
J. BENNETT
Tissue Culture Laboratory
Plant Breeding, Genetics and Biochemistry Division
International Rice Research Institute
Los Baños, Laguna
T. OKITA
Institute of Biological Chemistry Washington State University, WA, U.S.A.

ABSTRACT

The novel technique of direct gene transfer, which allows the incorporation and expression of foreign genes into crops such as rice, a method not possible through conventional breeding, is an alternative for crop improvement.

PEG-mediated cotransformation of protoplasts of several japonica and indica cultivars was performed using gene constructs containing either the β-glucoronidase gene (pCAL1GC) and hygromycin-resistance gene (pTRA132), or the soybean trypsin inhibitor gene (pSBTI) and the hygromycin-resistance gene. More than 300 putative transformants were regenerated from six varieties.

Histological and molecular analyses were performed either at the callus stage or whole plant level. Plants of IR58 stained with X-gluc solution showed positive GUS reaction. On the other hand, Southern analysis for hygromycin B of 81 Zhonghua 10 plants indicated that 13 contained the gene for antibiotic resistance. All the transformed Zhonghua 10 plants set seeds. Integration of selectable markers into the plants demonstrates the possibility of introducing agronomically-important genes from sources other than rice.

The agronomically-important gene from soybean, soybean trypsin inhibitor, which confers resistance to yellow stemborers, is being used to transform the japonica cultivar Zhonghua 6. Molecular analysis of the transformants demonstrated the incorporation of the gene.

Keywords: Rice, transformation, polyethylene glycol, protoplasts, plasmid, transgenic plants

INTRODUCTION

Rice (*Oryza sativa* L.) is the most important food crop in the world, with more than two billion people, predominantly in developing countries, depending on it. Rice improvement through conventional breeding methods has had considerable success. However, development of novel techniques for the genetic manipulation of this crop offers rapid production and improvement of genotypes to meet the even faster increase in the world's population (Swaminathan, 1982; Khush, 1984; Toenniessen, 1990). The introduction of foreign genes into crop plants promises to overcome some of the substantial agronomic and environmental problems that have not been solved using genes currently available in plant breeders' germplasms.

Several techniques have been employed for rice genetic transformaiton such as the PEG-mediated method, electroporation, biolistic method, pollen tube pathway and *Agrobacterium*-mediated gene transfer system. Despite the availability of various methods, the production of transgenic plants is still considered inefficient due to low recovery and poor fertility (Hodges et al., 1991).

Foreign genes are exploited in the plant transformation process. Since very few cells in a target population become transformed, selection of transformants demands the use of selectable markers. Typically, selectable marker genes encode enzymes which detoxify antibiotics such as hygromycin and thus permit only transformed cells to survive and grow on media containing the antibiotic.

This study reports the production of transgenic plants of the japonica cultivars Zhonghua 6, Zhonghua 10, Taipei 309 and the indica cultivars IR58, IR64 and IR57311-95-2-3 carrying single or two genes of either hygromycin phosphotransferase (hph), β-glucoronidase (GUS) or soybean trypsin inhibitor (SBTI). Evidence of integration of the genes is also presented.

MATERIALS AND METHODS

Plant Materials

The cultivars used in this study were: the japonica cultivars Taipei 309, a variety considered a model system in rice, Zhonghua 6 and Zhonghua 10 (which are high-yielding Chinese varieties developed through another culture); and the indica cultivars IR58, IR64 and the promising line IR57311-95-2-3.

Explant Sterilization

Immature embryos or mature seeds were used as explants. Immature embryos were collected seven days after pollination. The explants were sterilized with 2.6% sodium hypochlorite for 45-60 min with stirring under vacuum, then washed three times with sterile distilled water. These were then placed onto sterile filter papers to absorb excess water.

Callus Induction

Explants were plated in petri plates or 50-ml flasks containing MS (Murashige and Skoog, 1962) medium with 30 g/l of sucrose or maltose, 2 mg/l 2,4-D and solidified with 0.8% agar. Cultures were kept in the dark for 3-4 weeks at 25 ± 1°C. For direct isolation of protoplasts from primary calli, the cultures were kept under diffused light for the first 10 days, then transferred to dark conditions.

Initiation and Maintenance of Cell Suspension Cultures

Ttransformation of Zhonghua 6 utilized protoplasts isolated directly from primary calli (Wu and Zapata, 1992). Suspension cultures were established in the other varieties.

Three- to four-week-old primary calli were used for the initiation of cell suspensions. Embryogenic regions of primary calli were selected and placed into a 50-ml Erlenmeyer flask containing 15 ml liquid R2 medium (Ohira et al., 1973) with 20 g/l sucrose and 2 mg/l 2,4-D. Approximately 0.5g of embryogenic calli was inoculated per flask. The suspension cultures were placed on a gyratory shaker at 100 rpm and cultured in the dark at 25 ± 1°C. The medium was replaced at intervals of 3-7 days until fast-growing cell suspensions were established.

For maintenance of cell suspension, the cells were transferred to 125-ml Erlenmeyer flasks containing 30-ml medium. The medium was replaced every 5-10 days.

Protoplast Isolation

One gram of 3- to 5-day-old suspension cells or 1-month old embryogenic primary calli was incubated in 20 ml filter-sterilized cell-protoplast washing solution, CPW (Zapata et al., 1977) containing 1-2% (w/v) cellulase RS (Yakult Honsha Co., Tokyo, Japan) and 0.05-0.2% pectolyase Y-23 (Seishin Pharmaceutical Co., Tokyo, Japan). The mixture was kept in the dark at 25-26°C for 2-5 hours. Enzymatic digests were filtered through four layers of 25 μm nylon mesh and the protoplasts were washed three times with CPW by centrifugation at 800 rpm for 5 min each. Protoplast yield was determined using a haemocytometer.

Plasmids Used

Protoplasts were cotransformed with the gene constructs containing either the β-glucoronidase gene (pCAL1GC) and hygromycin-resistance gene (pTRA132), or the soybean trypsin inhibitor gene (pSBTI) and pTRA132. β-glucoronidase is a reporter gene which could be monitored histochemically (Jefferson, 1987). GUS catalyzes the hydrolysis of a wide variety of β-glucoronides and makes it excellent for gene fusion experiments. Hygromycin-β-phosphotransferase gene (hph) is a

selectable marker which is responsible for resistance to hygromycin. It modifies the antibiotic by phosphorylation making it non-toxic (Singh et al., 1979). Soybean trypsin inhibitor is an agronomically-important gene as this confers resistance to yellow stemborer. Protein inhibitors are some of the plant chemicals in storage tissues that limit predation by insects and other herbivores.

Transformation Using PEG

About $8x10^6$ protoplasts were resuspended in 1 ml of CPW solution and incubated with 50 μg each of the plasmids used for cotransformation for 10 min to allow sufficient contact between the DNA and the plasma membrane. One ml of 40% (w/v) PEG 8000 Mannitol-Magnesium solution was added dropwise, with gentle mixing between each addition, to 20% final concentration. The mixture was incubated at 25°C for 5-30 min and then diluted slowly with 30 ml of Krens, F buffer (Krens et al., 1982), followed by centrifugation at 780 rpm for 15 min to pellet the protoplasts. The supernatant was removed, and the protoplasts were washed once more with CPW solution.

Protoplast Culture

Purified protoplasts were gently mixed with modified R2 containing 137 g/l sucrose and 1 mg/l 2,4-D or Kao's modified medium (Kao et al., 1970), KPR-2 (Thompson et al., 1986) medium containing 1.2% Sea Plaque agarose. Density of protoplasts was adjusted to $1x10^6$ per ml. One ml of the mixture was placed into a 60 x 15 plastic petri dish. When the mixture was completely solidified, 100 mg of nurse cells (Oc rice cell line graciously provided by Dr. K. Syono, University of Tokyo) suspended in 5 ml of the protoplast liquid medium was added into each petri dish. The petri dishes were placed on a slow shaker (40 rpm) and incubated in the dark at 25 $\pm$ 1°C for one week and then cultured without shaking. The nurse cells were removed at 12-15 days after culture and fresh protoplast medium was added to the agarose beads.

Selection of Transformed Calli

Hygromycin selection for resistant colonies was performed 14 days after protoplast culture. The protoplast-derived colonies embedded in agarose blocks were immersed in protoplast medium containing different concentrations of hygromycin B (25-100 μg/ml) for four weeks. The calli were allowed to proliferate in semisolid medium with 1 mg/l 2, 4-D for 7-10 days. The hygromycin-resistant colonies were counted and transformation efficiency was calculated as the percentage of the number of hygromycin-resistant colonies per total number of protoplasts originally plated in the agarose block.

Regeneration of Selected Transgenic Calli

Hygromycin-resistant calli 1-2 mm in diameter were transferred to N6 or R2 regeneration medium. Plants 10-20 cm in height were transferred to culture solution (Yoshida et al., 1976) and reared in the Phytotron at 21/29°C night/day temperature under natural light. After 3-4 weeks, the plants were transferred to pots and kept in the Phytotron until maturity. Seeds were collected from the fertile plants and the vegetative portions were burned. The plant height and tiller number relative to control were noted.

Histochemical Analysis for GUS Gene

Histochemical analysis of transgenic plants for the presence of GUS gene was conducted on various tissues such as calli, roots, leaves, stems, leaf sheath and spikelets (Jefferson, 1987).

Molecular Analysis of Putative Transformants

Molecular analysis to confirm the integration of foreign genes was performed on 3 IR58, 81 Zhonghua 10 and 24 Zhonghua 6 putative transformants sampled at random. DNA was extracted from leaf blades collected 1-2 months after transplanting following the procedure of Dellaporta et al. (1983). DNA extracts were subjected to restriction enzyme digestion, electrophoresis and Southern blot analysis according to the protocol of Maniatis et al. (1982). The DNA probes used for hybridization were the 1.1 Kb BamHI fragment of pTRA132 for the hph gene, the 1.8 Kb GUS fragment of pCAL1GC for the GUS gene and the 0.7 Kb fragment of pSBTI for the SBTI gene.

RESULTS AND DISCUSSION

Callus Induction and Establishment of Cell Suspension

Production of sufficient embryogenic calli from all the varieties was observed at 3-4 weeks after plating (Fig. 1). Embryogenic cell suspension lines were established in R2 medium within one month after initiation (Fig. 2). Establishment of cell suspension within the shortest time possible is very critical for high efficiency of plant regeneration.

Suspension cultures consisted of spherical cells and the cell fresh weight was doubled in 3-7 days. The clusters, which represented growth centers, differed in size, ranging from a few to 100 or more cells per cluster.

Protoplast Isolation, Culture and Selection

Sufficent amounts of protoplasts for transformation were isolated from primary calli or cell suspensions (Fig. 3). A protoplast population with a 50% survival rate after PEG treatment is considered optimal for transformation studies. In this case, the suitable PEG incubation period was 15-25 min. The absolute transformation efficiency did not increase when PEG incubation was beyond 25 min. Longer periods of incubation decreased protoplast survival rate and colony formation, although transformation efficiency based on total colony number was higher.

Survival of colonies decreased with increasing hygromycin B concentration (Fig. 4). Likewise, the number of regenerated putative transgenic plants was inversely proportional to the concentration of hygromycin in selection medium (Table 1).

Histochemical Analysis for GUS

Histochemical analysis of transgenic plants such as roots, leaves, stems, leaf sheaths and spikelets of IR58 and Zhongua 10 demonstrated the expression of the GUS gene (Fig. 5).

Molecular Analysis of Putative Transformants

Southern blot anlaysis of DNA isolated from leaf blades of 81 plants regenerated from Hygromycin-resistant colonies confirmed the presence of the introduced Hph genes in 13 plants. Untransformed plants did not show any hybridization band.

Integration of the foreign gene was illustrated by the formation of a smear in only the high molecular weight region when undigested genomic DNA was hybridized with the ^{32}P-labelled 1.1 Kb BamHI fragment of pTRA132 (Fig. 6'). Five of six plants (Nos. 1, 4, 5, 6 and 8) showed hybridization signals in the 1.1 Kb region corresponding to the intact Hph coding sequence. Plant No. 7 showed a single hybridization band higher than 1.1 Kb indicating that at least one of the BamH1 restriction sites of pTRA132 was mutated. Plant Nos. 4, 5 and 8 had multiple hybridization bands at molecular weight regions higher than 1.1 Kb indicating multiple insertions of foreign gene and at least one of the BamH1 restriction sites of the plasmid was altered during integration of the gene into the genome. This alteration apparently did not interfere with the hph function since the colonies were selected in the presence of the antibiotic.

It was observed that the introduced hph gene was integrated at various patterns. The presence of multiple bands at molecular weights higher than expected is an indication of multiple insertion events and rearrangements of the integrated gene, both of which have been commonly observed in transformed plant materials (Rhodes et al., 1988; Lyznik et al., 1989; Gordon-Kamm et al., 1990).

Using the 1.8 Kb GUS fragment of pCAL1GC as a probe, Southern blot analysis of transgenic IR58 plants also confirmed the presence of GUS gene in the high molecular weight region, and in the 1.8 Kb band corresponding to the intact GUS gene coding sequence (Fig. 7).

Twenty of the 24 Zhonghua 6 plants analyzed for the presence of SBTI gene showed positive signals in both total and digested DNA (Fig. 8). The 20 plants were regenerated from seven individual calli. This result demonstrates the enormous possibility of incorporating foreign genes of agronomic importance into rice where the source of resistance is not available in the cultivated rice germplasm. Soybean trypsin inhibitor was found to be inhibitory to the midgut trypsin of the yellow stem borer, the major lepidopteran pest of rice. About 18% of the area devoted to rice in Southeast Asia is affected by this insect pest. Actual screening under proper containment facilities for the resistance of the transgenic plants to yellow stemborer will be done.

Performance of Transgenic Plants

More than 300 putative transformants have been regenerated from these experiments (Table 2). The transformants were generally inferior in morphology with less tiller number than the control. The Zhonghua 6 plants are now flowering in the Phytotron, while all the Zhonghua 10 plants have been harvested and all of them set seeds. The number of seeds produced per plant varied from 1-106.

The success of this technique allows use of other available plasmids which carry genes of agronomic importance such as those conferring resistance to pests and diseases.

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Table 1. Putative transgenic plants of Zhonghua 10 regenerated from hygromycin-resistant colonies

Hygromycin Concentration (μg/ml)	*Calli giving Plants (No.)*	*No. of Plants*		
		Regenerated	*Analyzed*	*With HYG*
25	77	167	60	6
50	16	20	4	1
75	25	27	13	4
100	7	10	4	2

Table 2. Molecular analysis of transgenic plants and putative transformants in six rice varieties

Variety	*Putative transformants (No.)*	*Plants analyzed (No.)*	*Genes for cotransformation*[1]	*Plants positive for Southern (No.)*			
				GUS	*Hyg*	*Kan*	*SBTI*
IR58	1	1	GUS + Kan	1	–	*	–
	2	2	GUS + hph	2	2	–	–
IR64	9	9	GUS + hph	0	0	–	–
Zhonghua 10	224	81	GUS + hph	13	13	–	–
Zhonghua 6	17	0	GUS + hph	*	*	–	–
	39	24	SBTI + hph	–	*	–	> 5
Taipei 309	1	0	SBTI + hph	–	*	–	*
IR57311-95-2-340		0	GUS + hph	*	*	–	–

[1]GUS = β-glucoronidase; Kan = Kanamycin; Hyg = Hygromycin; SBTI = Soybean trypsin inhibitor

– = Not studied; * = Being analyzed

Figure 1. Embryogenic calli used for the establishment of cell suspension or direct isolation of protoplasts from primary calli

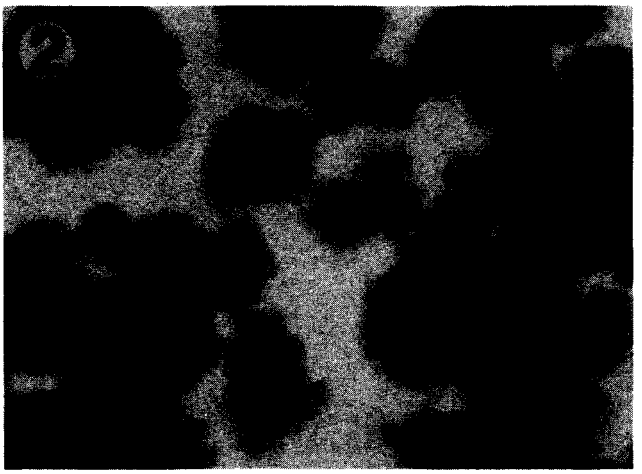

Figure 2. Embryogenic cell suspension used for protoplast isolation

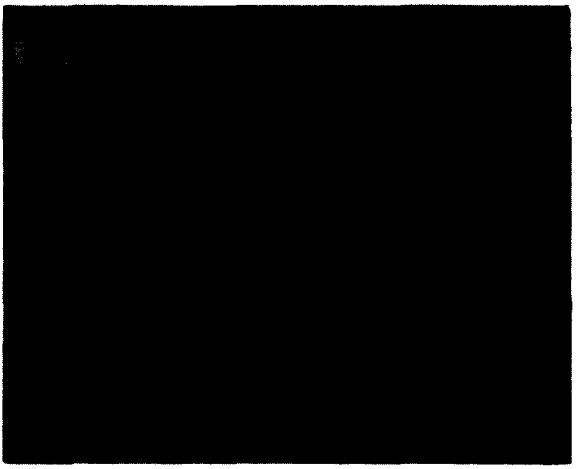

Figure 3. Freshly-isolated rice protoplasts

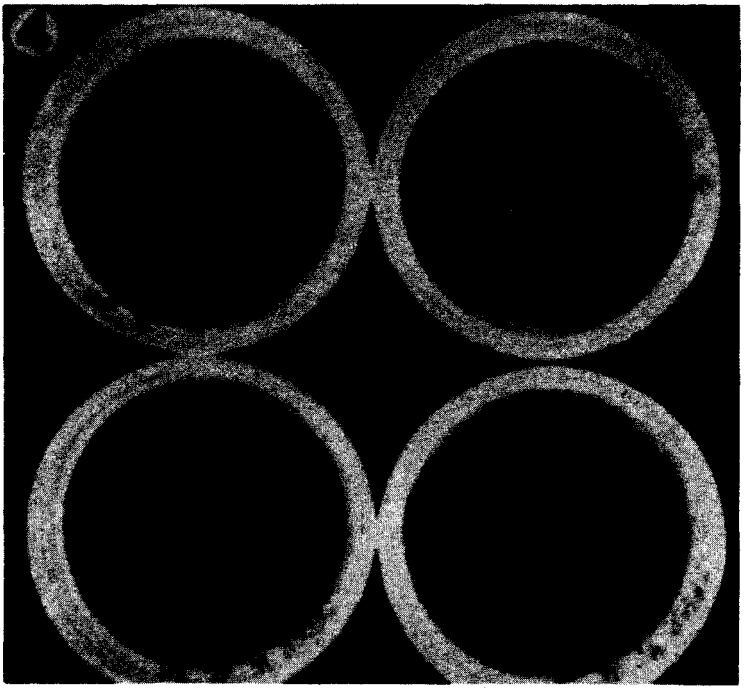

Figure 4. Hygromycin-resistant colonies of Zhonghua 10 calli after 25 days in selection medium with different hygromycin concentrations. a = 100 μg/ml; b = control; c = 75 μg/ml; d = 50 μg/ml.

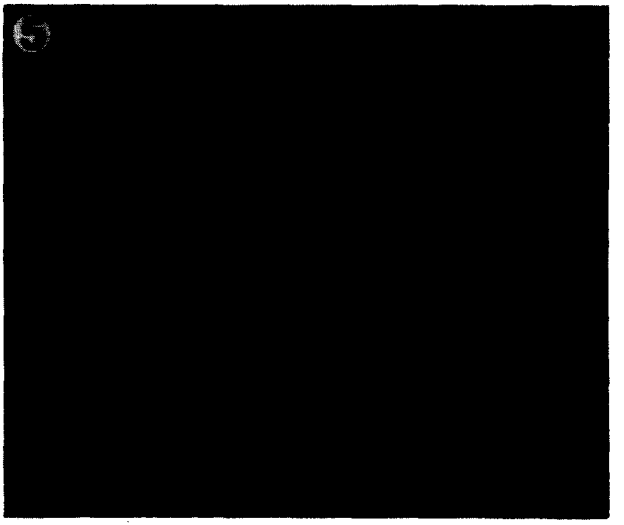

Figure 5. Histochemical analysis for the GUS gene showing its integration into the plant

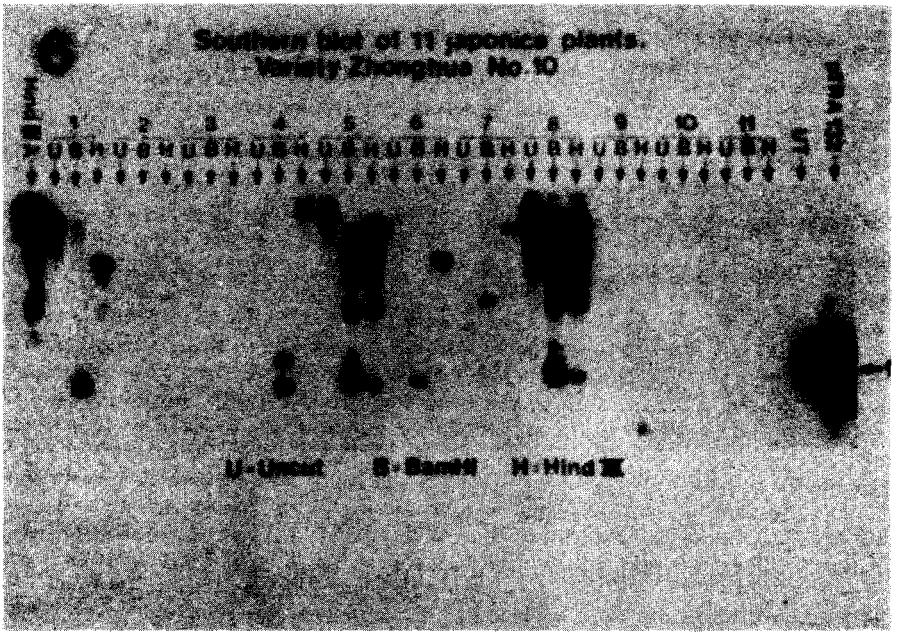

Figure 6. Southern blot analysis of Zhonghua 10 plants showing integration of the hygromycin-resistance gene in some of the plants

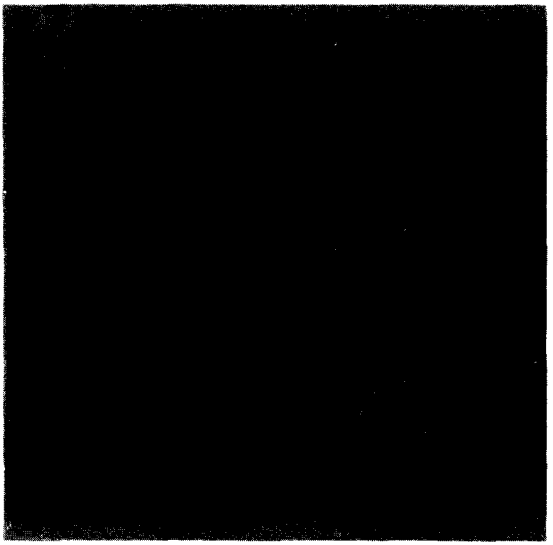

Figure 7. Southern blot analysis of IR 58 showing the integration of the GUS gene in both the total and digested DNA

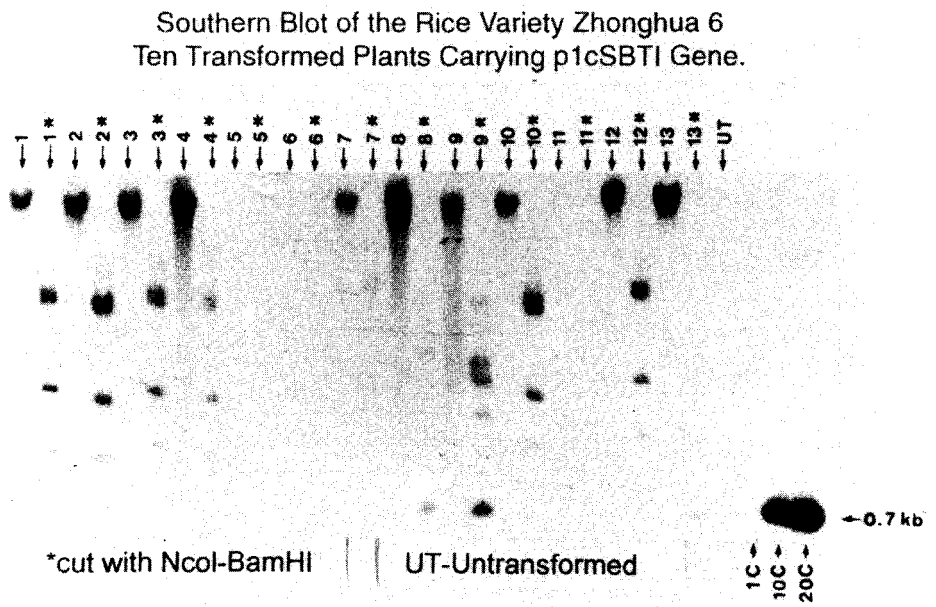

Figure 8. Southern blot analysis of Zhonghua 6 transformants showing the integration of the SBTI gene in both the total and digested DNA

REFERENCES

Dellaporta, S.L., J. Wood and J.B. Hicks. 1983. A plant minipreparation: version II. *Plant Mol. Biol. Rep.* 1(4): 19-21.

Gordon-Kamm, W.J., T.M. Spencer, M. Mangano, T.R. Adams, R.J. Daines, W.G. Start, J.V. O'brien, S.A. Chambers, W.R. Adams Jr., N.G. Willetts, T.B. Rice, D.J. Mackey, R.W. Krueger, A.P. Kausch and P.G. Lemaux. 1990. Transformation of maize cells and regeneration of fertile transgenic plants. *The Plant Cell.* 2:603-618.

Hodges, T.K., J. Peng, L.Z. Lyznik, and D.S. Koetje. 1991. Transformation and regeneration of rice protoplasts. In: **G.S. Khush and G.H. Toenniessen** (eds.), Rice Biotechnology. Oxford: C.A.B. International and IRRI.

Kao, K.N. W.A. Keller and R.A. Miller. 1970. Cell division in newly-formed cells from protoplasts of soybean. *Exp. Cell Res.* 62:338-340.

Khush, G.S., 1984. IRRI breeding program and its worldwide impact on increasing rice production. In: **J.P. Gustafson** (ed.) *Gene Manipulation in Plant Improvement.* New York: Plenum Press.

Krens, R.A., L. Molendij, G.J. Wullems and R.A. Schilperoort. 1982. *In vitro* transformation of plant protoplasts with Ti-plasmid DNA. *Nature.* 298:72-74.

Jefferson, R.A. 1987. Assaying chimeric genes in oplants: the gus gene fusion system. *Plant Mol. Biol. Rep.* 5:387-405.

Lyznik, L.A., K.K. Kamo, H.D. Grimes, R. Ryan, K.L. Chang and T.K. Hodges. 1989. Stable transformation of maize: the impact of feeder cells on protoplast growth and transformation efficiency. *Plant Cell Rep.* 8:292-295.

Maniatis, T., E.F. Fitsch and J. Sambrook, 1982. *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15:473-497

Ohira, K., K. Ojima and A. Fujiwara. 1973. Studies on the nutrition of rice cell culture. I. A simple, defined medium for rapid growth in suspension culture. *Plant Cell Physiol.* 14:1113-1121.

Rhodes, C.A., D.A. Perce, I.J. Mettler, D. Mascarenhas and J.J. Detmer. 1988. Genetically transformed maize plants from protoplasts. *Science.* 240:204-207.

Singh, A., D. Ursie and J. Davies. 1979. Phenotypic suppression and misreading in Saccharomyces cereviseae. *Nature.* 277:146-148.

Swaminathan, M.S. 1982. Biotechnology research and third world agriculture. *Science.* 218:967-972.

Thompson, J.A., R. Abdullah and E.C. Cocking. 1986. Protoplast culture of rice (*Oryza sativa* L.) using media solidified with agarose. *Plant Sci.* 47:123-133.

Toenniessen, G.H. 1990. In: **K. Higi** (ed.) *Second International Workshop on Molecular Biology of Rice.* Okayama: Kawasaki Medical School. p. 39.

Toriyama, K., Y. Arimoto, H. Uchimiya and K. Hinata. 1988. Transgenic rice plants after direct gene transfer into protoplasts. *Bio/Technology* 6:1072-1074.

Wu, C. and F.J. Zapata. 1992. Plant regeneration from protoplasts isolated from primary callus of four japonica rice (*Oryza sativa* L.) varieties. *Plant Science.* 86:83-87.

Yoshida, S., D.A. Forno, J.H. Cock and K.A. Gomez. 1976. *Laboratory Manual for Physiological Studies of Rice.* Los Banos, Philippines: The International Rice Research Institute.

Zapata, F.J., P.K. Evans, J.B. Power and E.C. Cocking. 1977. The effect of temperature on the division of leaf protoplasts of Lycopersicon esculentum and Lycopersicon peruvianum. *Plant Sci. Lett.* 8:119-124.