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ANALYSIS OF A LARGE NUMBER OF INDEPENDENT TRANSGENIC RICE PLANTS PRODUCED BY THE BIOLISTIC METHOD

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SUMMARY

Over 500 independent transgenic rice plants have been obtained by the biolistic method with an average transformation frequency of 9.7% for japonica variety Taipei 309. A tight selection procedure using 50 mg/l of hygromycin B successfully prevented the growth of nontransformed tissues. Analysis of the T0 transgenic rice plants revealed that more than 97% of the transgenic plants were morphologically normal and more than 80% were at least partially fertile. The hyg^r trait was inherited as a dominant trait in a Mendelian manner in 8 out of 11 transgenic events assayed. Thirty-seven out of fifty transgenic plants were estimated to contain no more than five copies of the transgenes. In six out of seven transformation events, unlinked, co-transformed genes co-segregated in the T1 generation. The hyg^r trait has been stably inherited to the T4 generation. No chimerical transgenic plant has been found in an intensive search. Novel phenomena observed in transgenic rice plants are also reported.

Key words: chimera; hygromycin; Mendelian inheritance; microprojectile bombardment; rice transformation; transgene.

INTRODUCTION

Rice is an important cereal crop and the main food source for billions of people worldwide. Transformation of rice to introduce agronomically important genes is predicted to significantly strengthen rice breeding programs for rice improvement quantitatively and qualitatively (Khush and Toenniessen, 1991). Rice transformation has been achieved by uptake of DNA by protoplasts through polyethylene glycol (PEG) treatment or electroporation (protoplast-based methods) (*see* Vasil, 1994, for review), or by particle bombardment. Various devices for delivering particles have been successfully employed in rice transformation, including an electric-discharge system (Christou et al., 1991), a gun-powder-driven system (Cao et al., 1992; Li et al., 1993), and a high-pressure helium-driven system (Bio-Rad Laboratories, Hercules, CA) (this report). Recently, Hiei et al. (1994) reported efficient transformation of japonica rice mediated by *Agrobacterium*. Because rice can now be transformed at a relatively high frequency, transgenic rice will provide a model system for gene expression studies in monocot plants.

In the past few years, the biolistic method has emerged as a very promising method for the transformation of cereal crops (*see* Vasil, 1994, for review). In a previous communication, we reported the successful transformation of rice immature embryos and callus cultures by the biolistic method and the inheritance of the transgene to the next generation (Li et al., 1993). Three reports have so far provided detailed transgene inheritance information in rice. Goto et al. (1993) and Peng et al. (1995) studied transgene inheritance in transgenic rice plants produced by a protoplast-based co-transformation strategy (i.e., the selectable marker gene and the nonselectable gene were in two separate vectors). Cooley et al. (1995) obtained transgenic rice plants by electric-discharge particle acceleration, with two or three gene constructs in a single plasmid vector, and investigated the transgene behavior in progeny. In this communication, we report that hundreds of independent, transgenic rice plants have been obtained using the Bio-Rad Biolistic PDS-1000/He Particle Delivery System with the selectable and nonselectable genes in single or separate vectors. We also report selected features associated with the transformation system, including the efficiency of transformation, improved selection, fertility of transgenic plants, inheritance and stability of the transgenes, frequency of chimera, segregation of co-transformed genes, and a novel phenomenon observed in rice transformation.

MATERIALS AND METHODS

Plant materials. A japonica variety, Taipei 309 (or in brief, TP309), and an indica variety, Taichung Native 1 (TN 1), were used in the experiments. A few transgenic lines of variety 8706, a stable breeding line developed from another culture of progeny of crosses between japonica and indica varieties from previous experiments (Li et al., 1993) were also analyzed for transgene inheritance. Immature seeds approximately 10–15 d after pollination were dehulled, surface-sterilized in 25% of Clorox (containing 5.25% of sodium hypochlorite), and 0.1% of Tween 20 for 45 min, followed by rinsing five times with sterile water. The immature embryos were subsequently dissected and placed on culture medium with the scutellum side up.

Culture media and conditions. The tissue culture media and the culture conditions were described previously (Li et al., 1993). Before the grown calli were transferred to the regeneration medium, they were cultured in a pre-regeneration (PR) medium for 2 wk for improvement of the regeneration abil-

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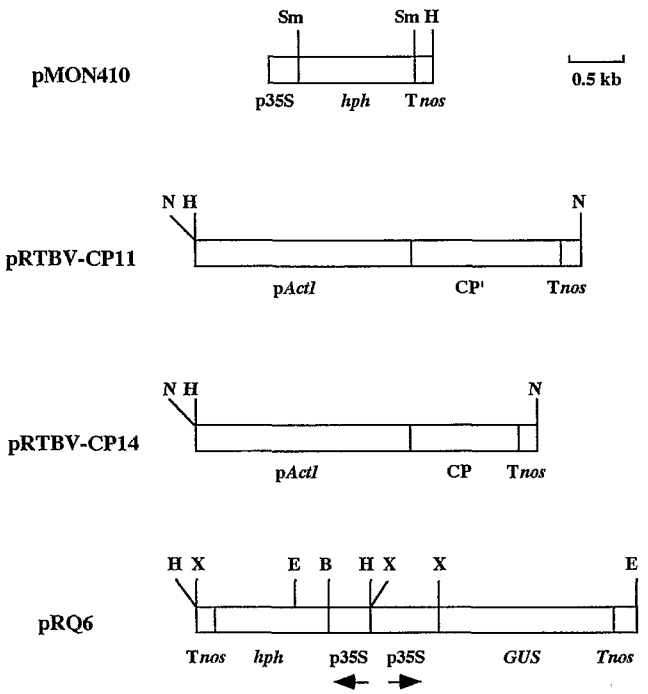

FIG. 1. Gene constructs used in the reported experiments. Abbreviations: p35S, CaMV 35S promoter; p*Act1*, rice *actin 1* gene 5′ region sequence; T*nos*, polyadenylation signal sequence of nopaline synthase gene of *Agrobacterium tumefaciens; hph*, hygromycin phosphotransferase of *Escherichia coli; GUS*, β-glucuronidase of *E. coli*; CP, coat protein gene (nt 2366–3373) of RTBV; CP′, coat protein precursor gene fragment (nt 2049–3581) of rice tungro bacilliform virus (RTBV); B, *Bam*H I; E, *Eco*R I; H, *Hin*d III; N, *Not* I.; Sm, *Sma* I; X, *Xba* I.

ity. PR medium contained the basal ingredients of the callus maintenance medium (Li et al., 1993), and the following plant growth regulators: 5 mg/l abscisic acid (ABA), 2 mg/l 6-benzylaminopurine (BAP), and 1 mg/l 1-naphthaleneacetic acid (NAA).

Plasmids. pMON410 (Fig. 1, Rogers et al., 1987) is the main source of selectable marker gene, *hph* (encoding hygromycin phosphotransferase). Plasmid pRQ6 (or pILTAB309) containing both *hph* and *Escherichia coli uidA* [encoding β-glucuronidase (GUS)] genes was also used in some experiments. pRQ6 was created by ligating pUC118, previously digested with *Bam*H I and *Eco*R I, with a 5 kb *Bgl* II/*Eco*R I fragment of plasmid pZA7 (courtesy of J. H. Zhou, Yale University). This fragment of DNA contained both *hph* and *uidA* coding sequences, each individually driven by a cauliflower mosaic virus (CaMV) 35S promoter (Benfey and Chua, 1990) and followed by the *Agrobacterium tumefaciens nos* polyadenylation signal (Rogers et al., 1987). In this plasmid, the genes were arranged in a head-to-head orientation (Fig. 1). Rice tungro bacilliform virus (RTBV) coat protein (CP) gene constructs pRTBV-CP11 and pRTBV-CP14 were also employed in some experiments. They are derivatives of pMON999 (Monsanto Co., St. Louis, MO) with coat protein gene fragments of RTBV (Qu et al., 1991) inserted in the polylinker region and rice *Act1* gene 5′ region (McElroy et al., 1990) substituted for the 35S promoter (Fig. 1).

The biolistic transformation. The Biolistic PDS-1000/He system (Bio-Rad) was employed for transformation experiments. The manufacturer's instructions were followed. In brief, approximately 3 mg of gold microcarriers, 1.0 μm in size, were coated with 5 μg of plasmid DNA for six shots. In case of co-transformation, 2.5 μg of each plasmid was used for coating. All of the plasmid DNAs were prepared with the Wizard Maxipreps DNA purification system (Promega, Madison, WI). Immature embryos, cultured for 1 d and placed at the center of the culture medium in a petri dish (10 cm in diameter), were positioned approximately 5.5 cm below the macrocarrier stopping plate and 1100 psi rupture discs were chosen to control the helium pressure. Each plate was bombarded twice.

Selection of transgenic calli and plants. Seven days after bombardment, the bombarded immature embryos were transferred to culture medium (Li et al., 1993) containing 30 mg/l equivalent of hygromycin B (hyg B, Calbiochem, La Jolla, CA) for preliminary selection. Two weeks later, freshly growing cell clusters were transferred to medium containing 50 mg/l of hyg B for selection of 4 additional wk (subcultured at 2 wk interval). Well-grown calli were cultured on the PR medium for 2 wk and subsequently transferred to the regeneration medium, both containing 50 mg/l hyg B. Regenerates were then transferred to the plant growth medium containing 50 mg/l hyg B in Magenta vessels (Sigma Chemical Co., St. Louis, MO). Plantlets with well-developed shoots and roots were transplanted to the soil, and grown to maturity in the greenhouse.

Molecular analysis of transgenic plants. Genomic DNA was prepared from leaf and stem tissues essentially as described by de Kochko and Hamon (1990). Exceptional care was given to prevent cross-contamination when genomic DNA was extracted for polymerase chain reaction (PCR) assays, in which DNA from nontransformed plants was included as a negative control in all experiments. PCR was performed in a volume of 50 μl with 1.5 m*M* of $MgCl_2$ using *Taq* DNA polymerase and the corresponding buffer from GIBCO-BRL (Gaithersburg, MD). Primers for detecting CP gene of RTBV were nucleotide (nt) 2505–2521 of the plus strand and nt 3175–3194 of the minus strand of the RTBV genome (Qu et al., 1991) to yield a 690 bp fragment. Primers for detecting the *hph* gene were nt 236–253 of the plus strand and nt 810–827 (to yield a 592 bp fragment) or nt 1166–1182 (to yield a 947 bp fragment) of the minus strand of the reported gene sequence (Gritz and Davies, 1983). The DNA was denatured at 94° C for 5 min followed by 35 cycles of amplification (1 min at 94° C, 1 min at 50° C, 1 min at 72° C). The final incubation at 72° C was extended to 7 min, and the reaction was cooled down to and kept at 4° C. Southern analysis of transgenic plants was basically performed as described by Maniatis et al. (1982). Five μg of rice genomic DNA, undigested or digested with appropriate restriction enzymes, were separated by electrophoresis in 0.8% agarose gels and transferred to Hybond-N nylon membranes (Amersham, Arlington Heights, IL). Hybridization was carried out in a buffer according to Church and Gilbert (1984). The probes were prepared as follows: PCR products of *hph* or RTBV-CP genes were recovered after electrophoresis from the agarose gels and purified with a Geneclean II kit (Bio 101, La Jolla, CA). The DNA fragments were labeled with 5′-[α-^{32}P]dATP or dCTP (Amersham) using a Prime-It II random primer labeling kit, and were subsequently purified through a Nuc-Trap probe purification column (both from Stratagene, La Jolla, CA) according to the manufacturer's instructions.

GUS assays. Gus assays were performed as previously described (Li et al., 1993).

Nomenclatures. A "transformation event" indicates the integration of the foreign genes into the genome of a single cell. An independent T0 transgenic plant is the plant eventually regenerated from a piece of callus originated from such a transformation event. T1, T2, . . . are the offspring generations followed-by. A "transgenic line" indicates the plants in the offspring generations of a single, independent transgenic T0 plant. A spikelet is defined as "fertile" when a seed is set and as "sterile" when no seed is set.

RESULTS

Transforming frequency and selection efficiency. By using the protocol reported previously (Li et al., 1993), frequency of transformation [calculated as the number of independently regenerated hygromycin B-resistant (hyg^r) plants over the total number of immature embryos bombarded] was computed from 11 transformation experiments (Table 1). The frequencies varied from 1.1% to 44.2%, with an average of 9.7%. Most of the transgenic plants were recovered from different embryos while some were from physically separated cell clusters on the same explant. In the latter cases, Southern hybridization was performed to confirm that the regenerated plants were from distinct transformation events based on the restriction digestion patterns.

The selection system for rice transformation experiments is the *E. coli* hygromycin phosphotransferase (*hph*) gene and the antibiotic

TABLE 1

TRANSFORMATION EFFICIENCY BY THE BIOLISTIC METHOD (VARIETY: TAIPEI 309)

Experiments	Explant No.	Hyg^r calli	Hyg^r plants	Overall %
T34	270	88	14	5
T35	180	52	10	5.6
T36	269	13	3	1.1
T37	321	90	13	4
T38	43	53	19	44.2
T39	109	49	2	1.8
T40	220	3	3	1.4
T41	292	86	56	19.2
T42	121	47	26	21.5
T44	345	106	53	15.4
T50	193	79	30	15.5
Total	2363	741	229	9.7

hygromycin B (hyg B) (Shimamoto et al., 1989; Li et al., 1993). It was found that selection at 50 mg/l of hyg B, but not 30 mg/l, especially during the stages of regeneration and seedling growth, was critical and prevented growth of nontransformants. We previously reported an 85% selection efficiency during our early transformation studies when selection in some experiments was carried out only at 30 mg/l of hyg B (Li et al., 1993). In a recently completed study of 63 hyg^r rice plants recovered from selection in which hyg B at 50 mg/l level was continuously applied, all contained the *hph* gene when analyzed by PCR reactions (data from 28 such plants are shown in Fig. 2), indicating that selection at 50 mg/l of hyg B was essential for recovering true transgenic rice plants. By using this protocol, six independently transformed plants of an indica rice variety, TN 1, an important parent line for many IR varieties, have been obtained though at a lower frequency (approximately 1%).

Transformation events on a single explant. We previously proposed, based on microscopic observations, that more than one stable transformation event could take place on a single immature embryo (Li et al., 1993). The proposed phenomenon can now be confirmed at the molecular level. Fig. 3 shows the results of Southern blot hybridization reactions of two pairs of such transgenic plants. Each pair of hyg^r plants was recovered from physically separated hyg^r calli excised from a single immature embryo in transformation experiments. Because the patterns of hybridization in each pair of plants were different, we conclude that the two plants were derived from separate transformation events.

Fertility of transgenic rice plants. More than 80% of the 99 hyg^r rice plants investigated were at least partially fertile with a broad range of fertility (Table 2); only 18% of the plants were completely sterile. Spikelet fertility was usually restored in the subsequent generation, indicating that partial sterility observed at T0 generation was not, in most of the cases, an inherited trait.

Inheritance, gene copy number, and stability of the transgenes. In eight of the eleven independently transformed hyg^r rice plants investigated, the hyg^r was inherited as a dominant trait in a Mendelian manner to the T1 generation while three plants showed non-Mendelian inheritance. Data of Mendelian inheritance of three transgenic plants were reported previously (Li et al., 1993), and the rest is listed in Table 3.

Fifty independently transformed plants were investigated for copy number of transgene (selectable marker gene or gene of interest) by Southern blot analyses in which a gene copy reconstruction lane was included. Eleven plants (22%) were estimated to contain one copy of the transgene per haploid genome, twenty-six (52%) had between two and five copies, nine (18%) had between six and ten copies, and four (8%) had more than ten copies. In cases in which the Southern hybridization patterns showed that there were multiple copies of the transgene, all of the copies were co-segregated, yielding progenies with all the copies or none (Fig. 4). This indicated that all the copies of the transgene were likely inserted at the same genetic locus.

In subsequent studies, it was determined that the hyg^r trait and CP or *uidA* transgene has been stably inherited and expressed to the T3 generation in 18 transgenic lines tested with one exception, in which *uidA* expression (measured by GUS activity) in plant leaves was gradually suppressed from one generation to another. Stable inheritance of hyg^r has been followed to the T4 generation in two trans-

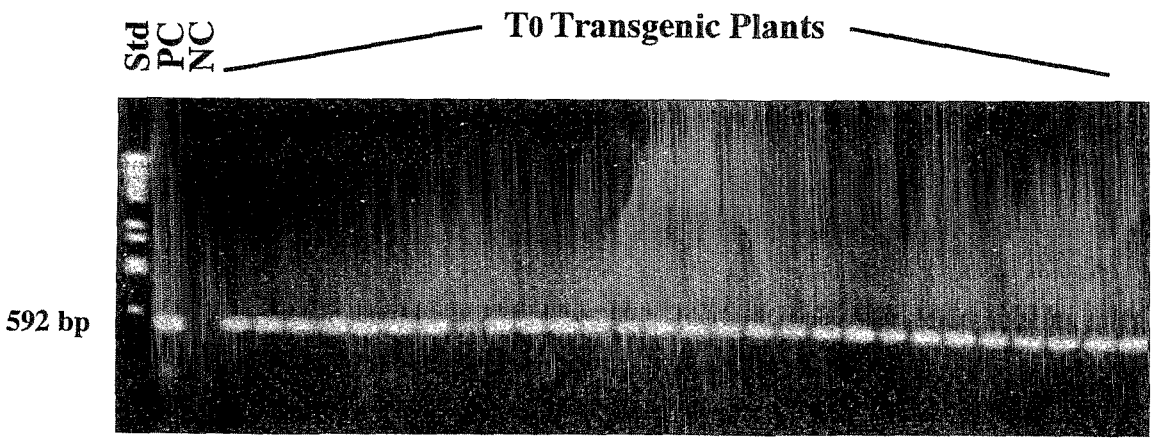

FIG. 2. Electrophoresis of polymerase chain reaction (PCR) products resulted from genomic DNA of 28 hyg^r plants (variety: TP309) using the *hph* gene primers (*see* "Materials and Methods"). NC: negative control, genomic DNA of TP309 as template; PC: positive control, pMON410 DNA as template; Std: molecular weight markers, λ DNA digested with *Bst* E II. The expected size of the resulting PCR fragments are indicated.

genic lines, in which all the plantlets germinated and grew well in hyg B-containing medium, indicating a homozygosity and stable expression of the *hph* transgene in the T4 generation (data not shown).

Co-segregation of unlinked, co-transformed genes. In most of the experiments performed, the selectable marker gene and the gene of interest were supplied on two vectors (co-transformation). Among seven transgenic lines investigated, six displayed co-segregation pattern of the two transgenes when assayed by phenotype or by PCR analyses. Co-segregation of the co-transformed genes can be demonstrated by the analysis of offspring of the transgenic plant T38-29, which was co-transformed with both *hph* and RTBV-CP genes. PCR reactions revealed that the T1 plants had either both genes or neither of the genes (Fig. 5). This result indicates that the two transgenes were closely linked to each other and co-segregated in offspring plants.

TABLE 2

SPIKELET FERTILITY OF TRANSGENIC RICE PLANTS

Fertility	No. of plants	%
>90%	9	9.1
70–90%	27	27.3
50–69%	7	7
30–49%	8	8.1
10–29%	18	18.2
<10% to trace	12	12.1
0%	18	18.2
Total	99	100

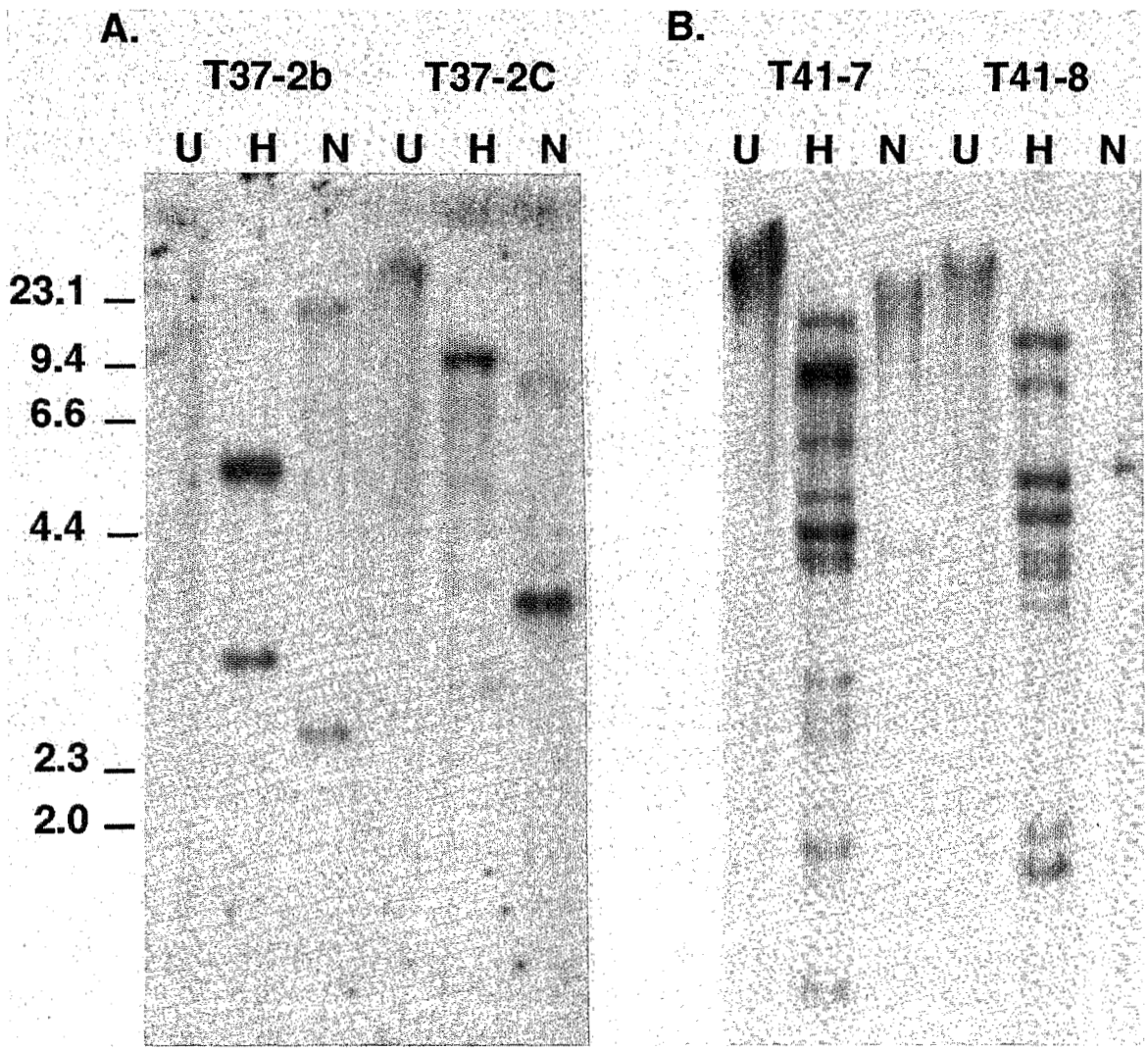

FIG. 3. Southern blot hybridization reveals more than one transformation event on an immature embryo after bombardment with vector DNA. Genomic DNA of two transgenic plants that were recovered from one embryo were undigested (U), or digested with *Hind* III (H) or *Not* I (N), respectively. *A.* DNA blot of two transgenic plants (T37-2b, T37-2c) obtained from a single immature embryo bombarded with pMON410, probed with a ^{32}P-labeled 947 bp polymerase chain reaction (PCR) product of the *hph* gene. *B.* DNA blot of another pair of such plants (T41-7, T41-8), bombarded with pRQ6 and pRTBV-CP11, probed with a ^{32}P-labeled 690 bp PCR product of the RTBV-CP gene. The molecular weight markers are presented on the *left*.

No chimerical transgenic plants found. To address the question of whether or not chimerical plants were produced in our studies, rice plants were transformed with the plasmid pRQ6, which contains both *hph* and *uidA* genes, each driven by the CaMV 35S promoter. Segments of approximately 400 leaves from a total of 119 tillers of 30 independently transformed plants were sampled and assayed for β-glucuronidase (GUS) activity. Among the 30 plants investigated, with one exception (*see* below), all leaf segments from a single plant uniformly showed either GUS or no GUS activity, suggesting that the transgenic rice plants were not chimerical.

One transgenic plant, however, showed an unusual pattern of GUS activity. GUS activity was observed in leaf segments from two tillers but not from the third one. However, to our surprise, Southern blot analysis revealed identical digestion patterns of the *uidA* transgene in these tillers (data not shown). This result suggests that all three tillers originated from the same transformation event and the plant was not chimerical. However, *uidA* gene expression was, for an unknown reason, not detectable in one of the three tillers.

Albino and abnormal transgenic plants. With the current transformation system, the frequency of albino transgenic plants was 1.7% (4 out of 232). In one case, green plantlets and albino plantlets were regenerated from the same piece of transgenic callus and shared identical digestion patterns as revealed by Southern blot hybridization reactions (Fig. 6), suggesting that the albino phenotype could develop later than the first division of the transformed cell.

Most of the transgenic rice plants looked morphologically normal. Thus far, in addition to albino, a strikingly abnormal phenotype has appeared three times among independently transformed rice plants (i.e., a frequency of approximately 0.6%). The variant plants had shorter internodes, resulting in a much shorter height (approximately 30 cm at maturity), with shorter and wider leaf blades and dark green streaks on the leaves. The inflorescences of these plants were also altered, exhibiting a variable degree of development of the spikelets. Some spikelets stopped development at very early stages, others were fully developed but often with an overdeveloped glume. Inside the spikelets, the pistils appeared more or less normal, whereas most of the stamens were morphologically abnormal with some having a stigmalike structure at the top, indicating a stamens-to-carpels conversion (Bowman et al., 1989). None of the spikelets were fertile, and

TABLE 3

INHERITANCE OF HYG^s TRAIT IN T_1 GENERATION OF TRANSGENIC RICE PLANTS

Plant lines	Varieties	Total	hyg^r	hyg^s	χ^2
K1-1-16(3a)	8706	35	22	13	2.752[a]
N2-3	TP309	36	23	13	2.37[a]
N2-14b	TP309	40	34	6	2.133[a]
T38-6	TP309	19	9	10	7.733
T38-28	TN1	45	19	26	25.785
T38-29	TN1	48	22	26	21.777
T38-36	TN1	40	31	9	0.133[a]
T41-36	TP309	23	16	7	0.363[a]

[a]In good agreement with 3:1 ratio at $P = 0.05$

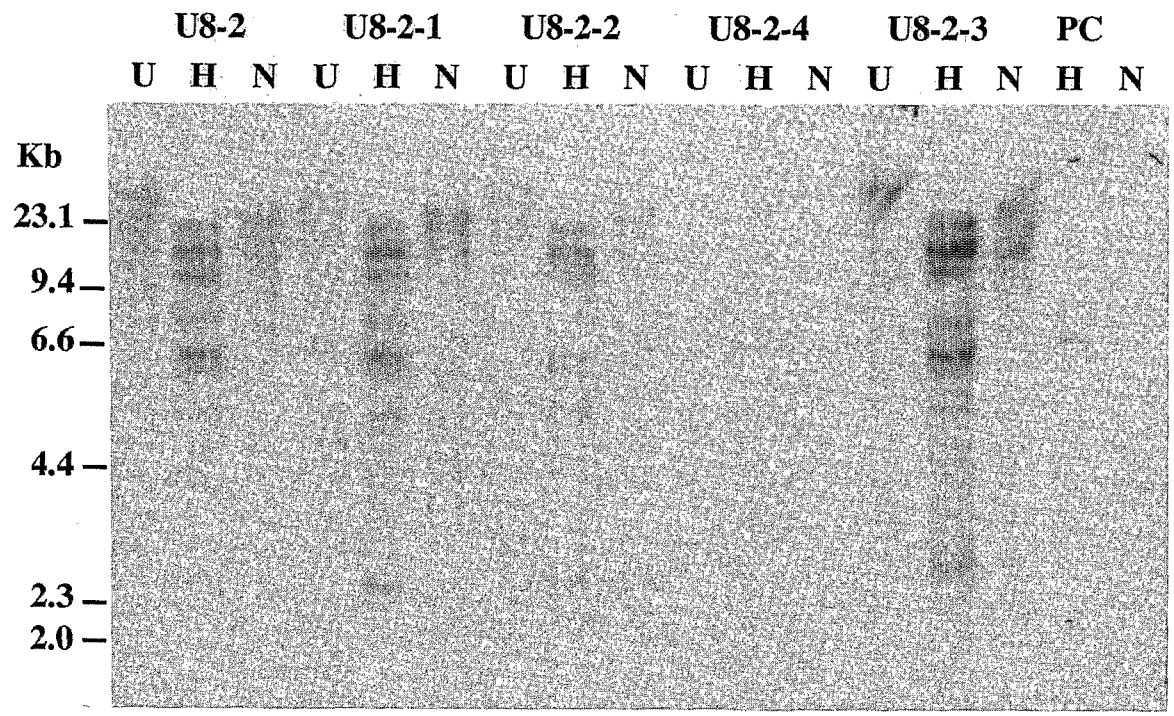

FIG. 4. Southern blot analysis of DNA isolated from T0 transgenic plant U8-2 and four T1 offspring of U8-2. The plant was transformed with pMON410 and pRTBV-CP14. The blot was probed with a ^{32}P-labeled 690 bp polymerase chain reaction (PCR) product of the RTBV-CP gene. U: undigested; H: digested with *Hind* III; N: digested with *Not* I. PC: positive control, DNA of pRTBV-CP14, digested with *Hind* III or *Not* I, respectively. The molecular weight markers are presented on the *left*.

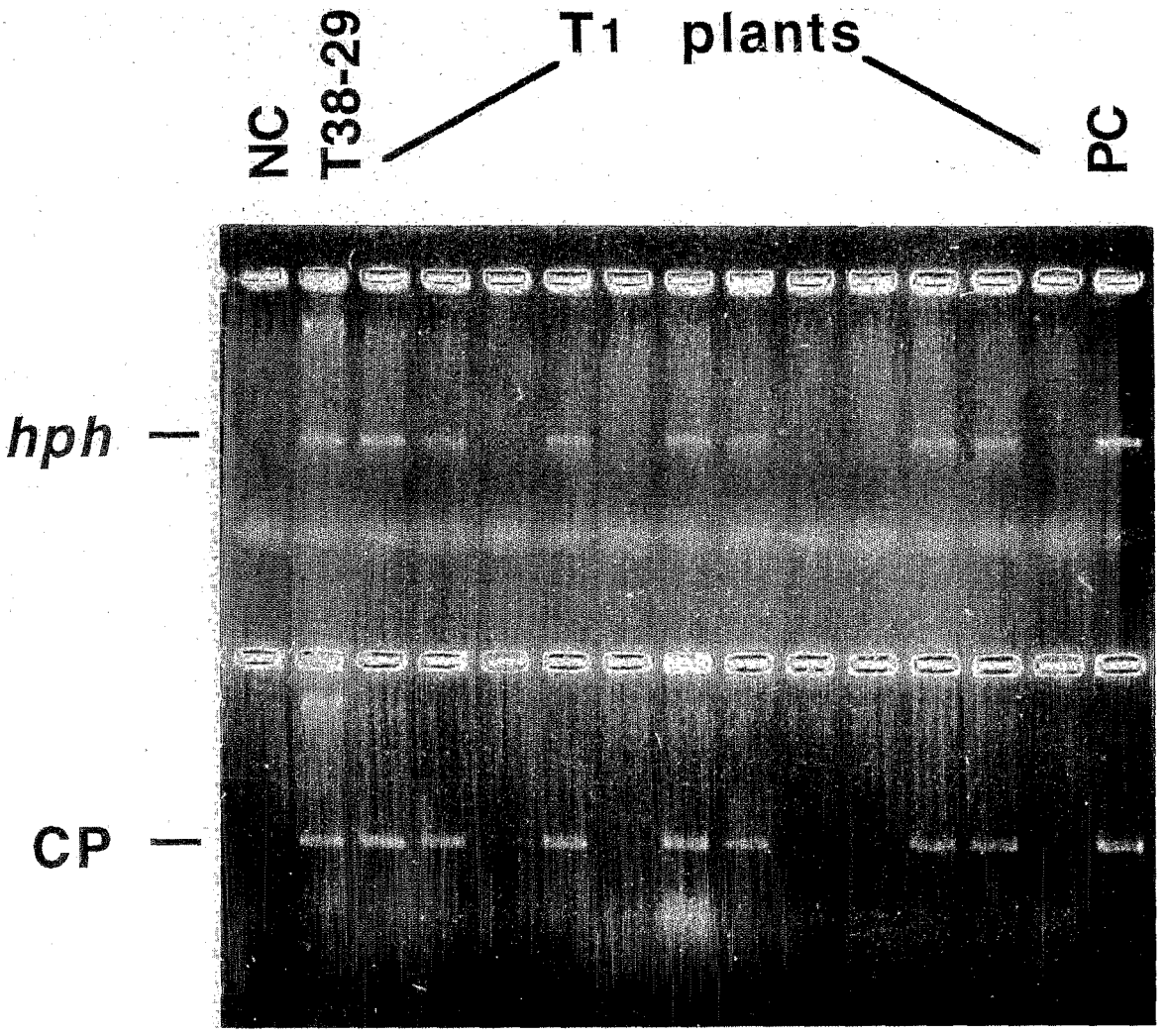

FIG. 5. Electrophoresis of polymerase chain reaction (PCR) products resulted from genomic DNA of T1 offspring of transgenic plant line T38-29. *Upper panel* presents the results of PCR using the *hph* gene primers while the *lower panel* is the results with the rice tungro bacilliform virus (RTBV)-CP gene primers (*see* "Materials and Methods"). NC: negative control, genomic DNA of TN1 as template; T38-29: the T0 plant DNA as template; T1 plants: genomic DNAs of T1 offspring of T38-29 as templates. PC: positive control, pMON410 DNA as template on the *upper panel* and pRTBV-CP5, a RTBV-CP construct which is similar to pRTBV-CP14 except that the p*Act1* was substituted with a RTBV promoter, as template for the *lower panel*. The expected positions of the corresponding PCR fragments are indicated.

cross-pollination attempts with pollen from nontransformed plants failed.

DISCUSSION

Over 500 independently transformed rice plants were obtained, many of which were analyzed for various features and the results are reported here. The system for the biolistic transformation of rice immature embryos is an efficient and reproducible one to yield fertile transgenic japonica rice plants. It is also useful in obtaining fertile transgenic indica rice plants though at a lower frequency. However, the frequency of transformation was inconsistent from one experiment to another. This most likely resulted from the current biolistic apparatus of which, though efficient in penetrating cell walls and delivering microcarriers to numerous cells, many factors are not controllable. Efficient DNA coating of the microcarriers and their even dispersal during bombardment affect the efficiency of transformation and largely depend on individual operators. Another possible factor that resulted in experimental inconsistency could be the donor plants (Vasil et al., 1985) which might, under different sets of environmental conditions, provide immature embryo cells in different status of competency.

In order to prevent escapes, it is important to apply 50 mg/l of hyg B during selection of the bombarded tissues. We found it highly effective to carry out selection at this concentration of the antibiotic during both regeneration and plantlet growth stages. In addition, it appears that some components in the medium may affect selection efficiency. For example, when sucrose was replaced with maltose, the nontransformed calli grew well in the presence of hyg B and could not be readily distinguished from the transformed calli (S. Z., un-

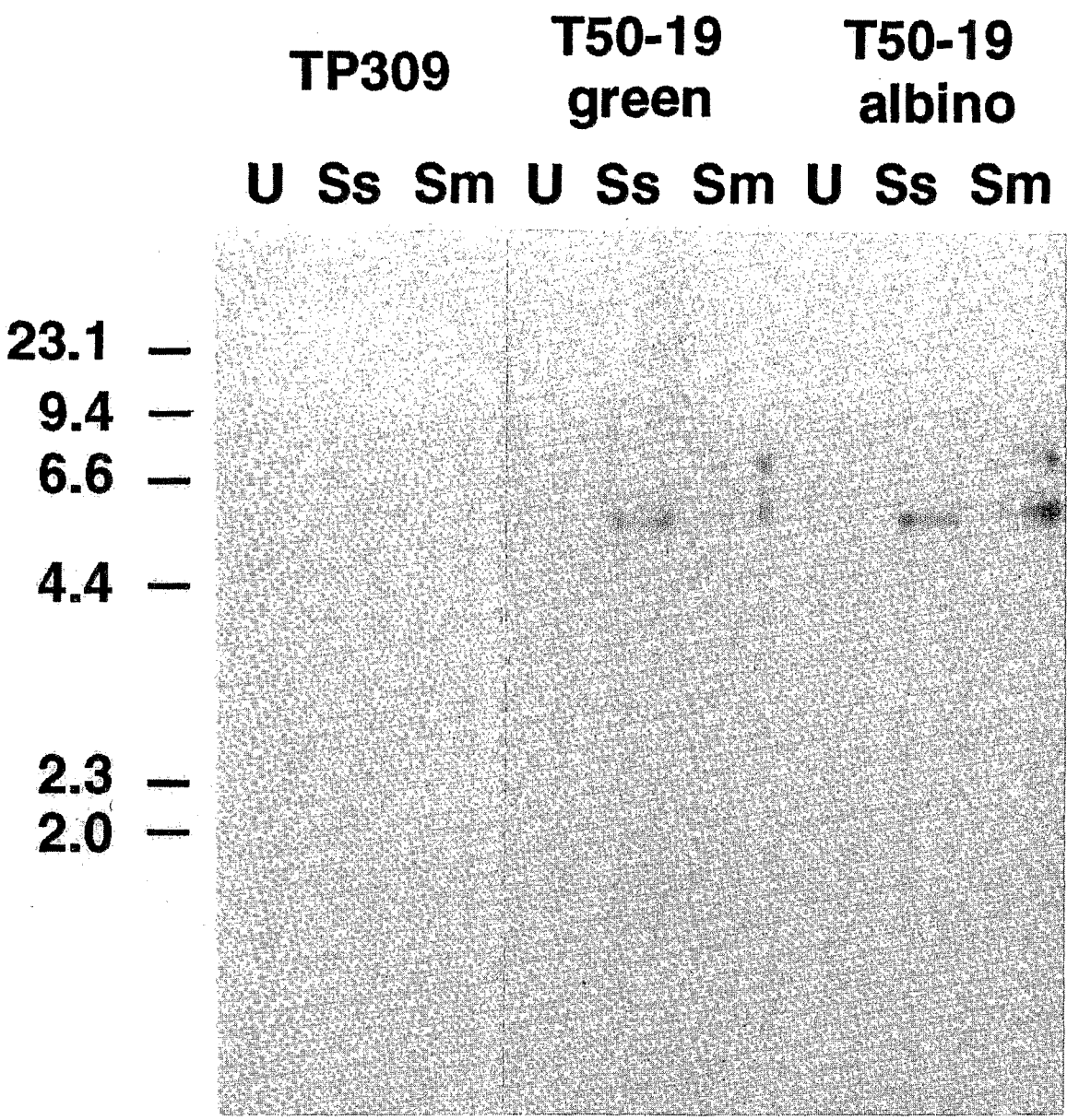

FIG. 6. Southern blot hybridization of DNA taken from green and albino plantlets derived from a single transformation event. Genomic DNAs of nontransformed Taipei 309 (TP 309) and the green and albino plantlets of a transgenic plant T50-19 (variety: Taipei 309) were undigested (U), or digested with *Sst* I (Ss) or *Sma* I (Sm), respectively. DNA blot was probed with a P^{32}-labeled 947 bp polymerase chain reaction (PCR) product of the *hph* gene. DNA molecular weight markers (in kb) are shown on the *left*.

published results). This result points out the need to carry out growth inhibition tests with hyg B for each medium used in the transformation experiments to identify the conditions that are best suited.

Fertility of the transgenic rice plants was first investigated in such a large scale and reported in detail. Most of the plants selected with hygromycin were partially or highly fertile. This is in contrast to the selection with *nptII* gene and kanamycin/G418, in which, almost all the plants were completely sterile (our unpublished data). On the other hand, it seemed that G418 selection did not affect fertility in transgenic wheat plants (Nehra et al., 1994). That makes us speculate that exposure at callus and seedling stages of rice to antibiotic(s) may have a long-term physiological impact in the development of reproduction organs, and the degree of such impact may depend on the antibiotic used for selection, as well as the species itself.

In most of the transgenic rice plants recovered, the number of transgene copies was relatively low, and rarely beyond 10. Multiple copies of a single transgene, or two co-transformed transgenes, were often linked to each other and behaved like a single genetic locus. This is consistent with other reports (Goto et al., 1993; Cooley et al., 1995) and implies recombination(s) between the vector DNAs before integration, and/or duplication of the transgene after integration.

Hyg^r trait resulted from *hph* transgene was inherited as a dominant Mendelian trait in approximately 70% of the transgenic events investigated. It was encouraging to find out that the *uidA* and *hph*

transgenes were stably inherited and expressed through the T3 and T4 generations. On the other hand, we did observe non-Mendelian inheritance of hyg^r trait, as well as suppression of the *uidA* gene expression in offspring of one transgenic line. Further investigation will be needed to study the mechanisms that caused the non-Mendelian inheritance and/or transgene suppression.

Christou and Ford (1995) reported that almost half transgenic rice plants were chimerical in nature when the selection was imposed 10 d after bombardment, whereas no chimerical plants were recovered if the selection started 2 d after bombardment. We have not recovered a single chimerical plant during the course of an intensive search when the selection usually started 7 d after bombardment. Vasil (1985) observed that regeneration of cereal plants from cultured calli or suspension cells was most likely through embryogenesis, in which the process initiates from a single cell, rather than organogenesis, in which multiple cells are involved. Because chimerical plants were not recovered in our transformation experiments, we support the hypothesis that the transgenic rice plants described here originated from single, stably transformed cells.

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REFERENCES

Benfey, P. N.; Chua, N.-H. The cauliflower mosaic virus 35S promoter: combinatorial regulation of transcription in plants. Science 250:959–966; 1990.

Bowman, J. L.; Smyth, D. R.; Meyerowitz, E. M. Genes directing flower development in *Arabidopsis*. Plant Cell 1:37–52; 1989.

Cao, J.; Duan, X.; McElroy, D., et al. Regeneration of herbicide resistant transgenic plants following microprojectile-mediated transformation of suspension culture cells. Plant Cell Rep. 11:589–591; 1992.

Christou, P.; Ford, T. L.; Kofron, M. Production of transgenic rice (*Oryza sativa* L.) plants from agronomically important indica and japonica varieties via electric discharge particle acceleration of exogenous DNA into immature zygotic embryos. Bio/Technology 9:957–962; 1991.

Christou, P.; Ford, T. L. The impact of selection parameters on the phenotype and genotype of transgenic rice callus and plants. Transgenic Res. 4:44–51; 1995.

Church, G. M.; Gilbert, W. Genomic sequencing. Proc. Natl. Acad. Sci. USA 81:1991–1995; 1984.

Cooley, J.; Ford, T.; Christou, P. Molecular and genetic characterization of elite transgenic rice plants produced by electric-discharge particle acceleration. Theor. Appl. Genet. 90:97–104; 1995.

de Kochko, A.; Hamon, S. A rapid and efficient method for the isolation of restrictable total DNA from plants of the genus *Abelmoschus*. Plant Mol. Biol. Rep. 7:3–7; 1990.

Goto, F.; Toki, S.; Uchimiya, H. Inheritance of a co-transferred foreign gene in the progenies of transgenic rice plants. Transgen. Res. 2:300–305; 1993.

Gritz, L.; Davies, J. Plasmid-encoded hygromycin B resistance: the sequence of hygromycin B phosphotransferase gene and its expression in *Escherichia coli* and *Saccharomyces cerevisiae*. Gene 25:179–188; 1983.

Hiei, Y.; Ohta, S.; Komari, T., et al. Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. Plant J. 6:271–282; 1994.

Khush, G. S.; Toenniessen, G. H. Rice biotechnology. Wallingford, UK: C. A. B. International; 1991:ix–x.

Li, L.; Qu, R.; de Kochko, A., et al. An improved rice transformation system using the biolistic method. Plant Cell Rep. 12:250–255; 1993.

Maniatis, T.; Fritsch, E. F.; Sambrook, J. Molecular cloning: a laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory; 1982.

McElroy, D.; Zhang, W.; Wu, R. Isolation of an efficient actin promoter for use in rice transformation. Plant Cell 2:163–171; 1990.

Nehra, N. S.; Chibbar, R. N.; Leung, N., et al. Self-fertile transgenic wheat plants regenerated from isolated scutellar tissues following microprojectile bombardment with two distinct gene constructs. Plant J. 5:285–297; 1994.

Peng, J.; Wen, F.; Lister, R. L., et al. Inheritance of gusA and neo genes in transgenic rice. Plant Mol. Biol. 27:91–104; 1995.

Qu, R.; Bhattacharyya, M.; Laco, G., et al. Characterization of the genome of rice tungro bacilliform virus: comparison with commelina yellow mottle virus and caulimoviruses. Virology 185:354–364; 1991.

Rogers, S. G.; Klee, H. J.; Horsch, R. B., et al. Improved vectors for plant transformation: expression cassette vectors and new selectable markers. Methods Enzymol. 153:253–277; 1987.

Shimamoto, K.; Terada, R.; Izawa, T., et al. Fertile transgenic rice plants regenerated from transformed protoplasts. Nature 338:274–276; 1989.

Vasil, I. K. Somatic embryogenesis and its consequences in the gramineae. In: Nenke, R. R., ed. Tissue culture in forestry and agriculture. New York: Plenum Press; 1985:31–47.

Vasil, I. K. Molecular improvement of cereals. Plant Mol. Biol. 25:925–937; 1994.