Plant Molecular Biology **21**: 871–884, 1993.

Use of *bar* as a selectable marker gene and for the production of herbicide-resistant rice plants from protoplasts

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Received 2 September 1992; accepted in revised form 10 December 1992

Key words: bar, herbicide resistance, phosphinothricin acetyltransferase, rice, selectable marker, transformation

Abstract

We have used the *bar* gene in combination with the herbicide Basta to select transformed rice (*Oryza sativa* L. cv. Radon) protoplasts for the production of herbicide-resistant rice plants. Protoplasts, obtained from regenerable suspension cultures established from immature embryo callus, were transformed using PEG-mediated DNA uptake. Transformed calli could be selected 2–4 weeks after placing the protoplast-derived calli on medium containing the selective agent, phosphinothricin (PPT), the active component of Basta. Calli resistant to PPT were capable of regenerating plants. Phosphinothricin acetyltransferase (PAT) assays confirmed the expression of the *bar* gene in plants obtained from PPT-resistant calli. The only exceptions were two plants obtained from the same callus that had multiple copies of the *bar* gene integrated into their genomes. The transgenic status of the plants was varified by Southern blot analysis. In our system, where the transformation was done via the protoplast method, there were very few escapes. The efficiency of co-transformation with a reporter gene *gusA*, was 30%. The T_0 plants of Radon were self-fertile. Both the *bar* and *gusA* genes were transmitted to progeny as confirmed by Southern analysis. Both genes were expressed in T_1 and T_2 progenies. Enzyme analyses on T1 progeny plants also showed a gene dose response reflecting their homozygous and heterozygous status. The leaves of T_0 plants and that of the progeny having the *bar* gene were resistant to application of Basta. Thus, the *bar* gene has proven to be a useful selectable and screenable marker for the transformation of rice plants and for the production of herbicide-resistant plants.

Introduction

Rice is one of the most important crops in the world, and is a major source of nutrition especially for people living in poor countries. Thus, there is a major emphasis on the improvement of rice through breeding, improved husbandry, and more recently genetic engineering. For genetic engineering, transformation of rice cells and regeneration of transgenic plants is now possible with a variety of means. These include PEG [12, 25, 39] and electroporation-mediated uptake of DNA by protoplasts [29, 34] and particle bombardment of immature embryos [1]. The transformed cells

and protoplasts can subsequently be regenerated into fertile plants.

The choice of selectable markers to produce transgenic plants at high efficiency is rather limited. In most attempts to obtain transgenic rice plants, antibiotic resistance genes (neomycin phosphotransferase II and hygromycin B resistance genes) in combination with the antibiotics have been used as selectable markers. In this report we show that the *bar* gene, which confers resistance to phosphinothricin (PPT), the active ingredient in the broad-spectrum herbicide Basta, can be used effectively to produce transgenic rice plants that are also resistant to the herbicide. The *bar* gene, isolated from *Streptomyces hygroscopicus*, encodes for phosphinothricin acetyltransferase (PAT) [22]. This gene in conjunction with bialaphos or phosphinothricin has been shown to be an effective selectable marker in obtaining transgenic plants of many species including maize and wheat [2, 3, 6, 7, 9, 10, 36]. Bialaphos is a tripeptide which is composed of PPT, an analogue of glutamic acid, and two *L*-alanine residues. PPT, released by the action of peptidases on bialaphos, is a powerful inhibitor of glutamine synthetase [33]. It is believed that inhibition of glutamine synthetase leads to ammonia accumulation resulting in death of the plants [32]. However, on the basis of their results on ammonia accumulation in alfalfa tissue cultures, induced by PPT or ammonium nitrate, Krieg *et al.* [15] questioned whether the accumulation of ammonia was really the cause of cell death. Whatever the mechanism, it is clear that PPT is a strong inhibitor of plant growth. PAT acetylates the free NH_2 group of PPT, thereby rendering it nontoxic. Dekeyser *et al.* [5] reported that the *bar* gene could be used as a selectable marker for obtaining transgenic calli from rice protoplasts. However, this study was limited to the production of PPT-resistant calli and plant regeneration from these calli was not shown. The *bar* gene has also been used as a selectable marker for obtaining transgenic maize and wheat plants by the particle bombardment technique [9, 10, 36]. Recently, Christou *et al.* [1] reported that both hygromycin and *bar* genes could be used as selectable markers for the transformation of rice following introduction of the DNA by electric discharge particle acceleration into immature zygotic embryos. However, the regenerated plants from the hygromycin-and bialaphos-resistant tissue included escapes. They attributed this problem to the cross-protection of untransformed regions of tissue by the detoxifying enzymes coming from the transformed cells. This problem of cross-protection of untransformed cells by the transformed ones, in combination with regeneration via organogenesis could accentuate production of chimeric regenerants following particle bombardment. Transformation of protoplasts followed by selection of microcalli should avoid these problems because of better separation of transformed and untransformed cells in this system. Thus, the *bar* gene has the potential to be a good selectable marker gene for obtaining transgenic rice plants from protoplasts transformed by direct DNA uptake into protoplasts. In addition, the *bar* gene could be useful agronomically since it can provide resistance to the herbicides Basta and Herbiace. The active ingredient in Basta is glufosinate ammonium which is the ammonium salt of PPT (Hoechst AG, FRG) and in Herbiace the active ingredient is bialaphos (Meiji Seika, Japan).

Materials and methods

We made two constructs, pG35barA and pG35barB, each with a CaMV 35S promoter and *nos* 3' transcription termination sequence. Figure 1 shows schematic diagrams of both constructs. The *bar* gene used to construct the pG35barA was derived from pGSFR1 which we obtained from Plant Genetic Systems, Gent, Belgium. pGSFR1 contains an intact *bar* gene with an ATG translation initiation codon instead of the original GTG [2]. In the second construct pG35barB, the source of *bar* gene was pIJ4104 which we received from Dr M. Bybb of John Innes Institute, Norwich, England. In the pIJ4104 plasmid there was a further modification, in that the sequence immediately preceding the ATG start codon had been changed for optimal trans-

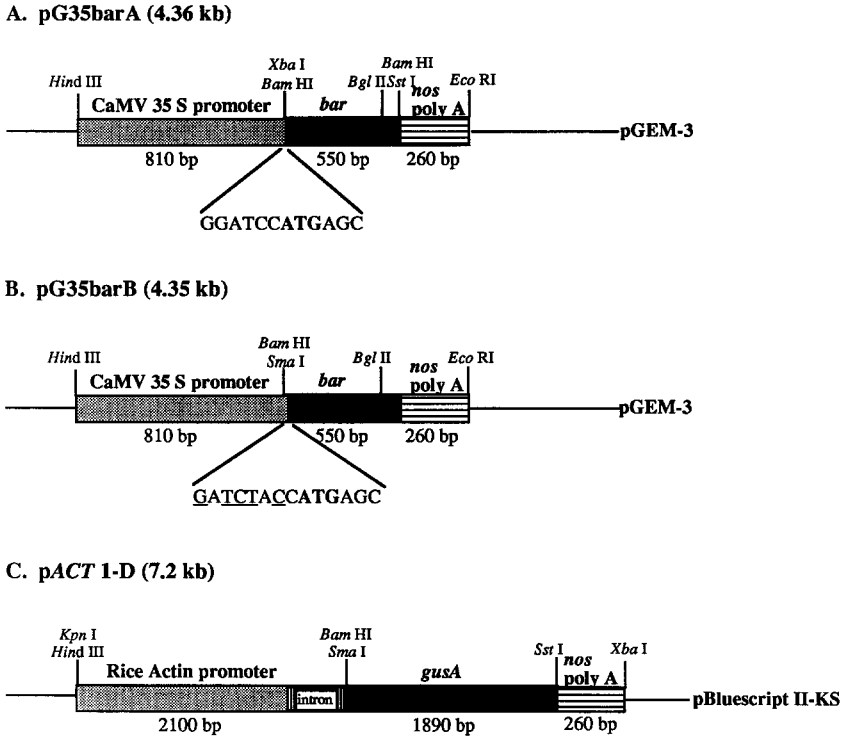


Fig. 1. Structure and partial restriction maps of plasmids (A) pG35barA, (B) pG35barB, and (C) p*Act1*-D. Nucleotide sequence on either side of the translation initiation codon is shown for the plasmids pG35barA and pG35barB. The modifications carried out by White *et al.* [38] are underlined.

lational initiation in eukaryotes [38]. Protoplasts were cotransformed with pG35barB and p*Act1*-D in experiment 6. p*Act1*-D contains the *gusA* gene, which encodes for β-glucuronidase (GUS) which was driven by the rice actin promoter [21].

Protoplasts used for this study were from embryogenic suspension cultures of cvs. Radon and Nortai (both are japonica subspecies). Radon seeds were obtained from Dr R. Osler, Universita degli Studi di Udine, Italy. Nortai rice has been developed jointly by ARS, USDA and the Arkansas Agricultural Experiment Station. Radon suspension culture was initiated from callus obtained from immature embryos [37] and those of Nortai from callus derived from microspores of young anthers [31]. The procedures for suspension maintenance and protoplast isolation were as described by Lee *et al.* [17]. Freshly isolated protoplasts were suspended in protoplast wash medium [8] at a density of 1.5×10^7 cells/ml. The transformation procedure used was as described by Peng *et al.* [25]. Each plasmid was used at 50 μg per ml of protoplast suspension. Following transformation, the protoplasts were suspended in modified Kao medium [14, 17] at a density of 5×10^6 protoplasts per ml. 200 μl of this suspension was plated on Millipore filters (Type AA, 0.8 μm) that were placed on top of IR52 nurse cells embedded in 0.8% Sea Plaque agarose in Kao medium [14, 17]. After 3–4 weeks growth on the nurse cells, the filters supporting the growing micro-calli were transferred to Petri dishes containing PPT. Linsmaier and Skoog [18] medium (LS) supplemented with 0.5 mg/l 2,4-D and 2.5–5 mg/l glufosinate ammonium (PPT, M_r 198) (pH 5.7) was used as the selection medium. After 3–4 weeks on selection medium the PPT-resistant calli were selected with the aid of a microscope and transferred to plates containing non-selective LS medium supplemented with 0.5 mg/l 2,4-D

and 50 mg/l tryptophan (pH 5.7) for proliferation. After 2–3 weeks calli containing embryo-like structures were selected and placed on regeneration medium which consisted of MS medium [23] supplemented with 50 mg/l tryptophan, 10 mg/l kinetin and 0.1 mg/l NAA, 0.6% agarose (pH 5.7). Regenerated plantlets were transferred to hormone-free agarose-solidified MS medium to allow further root development before transferring them to soil.

PAT activity assays were performed essentially according to the method described by De Block *et al.* [3]. Transient gene expression assays were carried out on protoplasts 48 h after DNA treatment. Protoplasts were scraped off 3–4 filters, suspended in 100 µl extraction buffer, and sonicated to disrupt the cells. For stable gene expression assays PAT activity was measured in 2–3 month old callus weighing 30–40 mg and in leaf pieces weighing 30–50 mg. The tissue was ground with acid-washed sand in 400 µl of extraction buffer [3]. Protein concentrations of the extracts were determined using the BioRad protein assay reagent (BioRad Laboratories, Richmond, CA). Reactions were carried out using 24 µl of extract (10 µg protein in 24 µl extract unless otherwise stated), 4 µl of 1 mM PPT, and 4 µl of [^{14}C]-acetyl coenzyme A (55.9 mCi/mmol, NEN Products, Boston, MA) at 37 °C for 30 min (unless otherwise stated). Regenerated plants were tested for their resistance to PPT by dipping the apical 8–10 cm portion of a leaf (while still on the plant) into 0.25% Basta (500 mg/l PPT) briefly. The leaves were then monitored daily for signs of damage.

DNA was extracted from the leaf tissue following the method of McCouch *et al.* [20]. The procedures for restriction enzyme digestion, electrophoresis and Southern blot analysis were according to Sambrook *et al.* [28] or the manufacturer's recommendations. Southern analysis was done on 20 µg of genomic DNA (except in the case of plants TR5-6A and TR5-6B where only 4 µg of DNA was used) either undigested or digested with *Hin*d III and *Eco* RI, or *Hin*d III only. The *Bgl* II-*Bam* HI fragment (563 bp) from pG35barB, which represents most of the *bar* coding sequence, was used as the radioactive probe.

β-glucuronidase (GUS) activity was measured in leaf extracts by the fluorescence assay described by Jefferson [13]. Leaf tissue weighing 30–40 mg was ground in 400 µl of extraction buffer [13]. The enzyme activity was expressed as nmol MU produced per mg protein per hour. We also used a rapid, convenient method of GUS assay based on the above method to screen a large number of plants for expression of the *gusA* gene. This involved taking a 2 cm long piece of leaf and cutting it into thin strips and incubating them in 1 ml of GUS assay buffer containing 1 mM 4-methylumbelliferyl β-D-glucuronide (MUG). A handheld UV lamp was used to visualize the development of fluorescence in this MUG buffer. 200 µl of this solution was removed at different time points and mixed with 300 µl of stop buffer. The stopped reactions were photographed under UV light for a permanent record.

Results

The results of an experiment to compare the transient expression of the *bar* gene when protoplasts were transformed with pG35barA or pG35barB are presented in Fig. 2. It shows that PAT activity was higher in the protoplasts transformed with pG35barB compared to protoplasts that were transformed with pG35barA. The modification of the *bar* gene in pG35barB for optimal translation in eukaryotes [38] presumably accounted for the higher PAT activity. In fact, several attempts to obtain PPT-resistant calli after transformation of protoplasts with pG35barA had failed prior to the above experiment. On the basis of these observations we chose the plasmid pG35barB for subsequent transformation experiments to select PPT-resistant calli. Figure 3 (A, B, C) shows that PPT was quite effective in inhibiting the growth of untransformed calli. It also shows that transformed calli can overcome the inhibitory effects of PPT and continue to grow. These resistant calli were easily identified by microscopic observation. The concentration of PPT which was toxic to the cells varied depending on the cell line and geno-

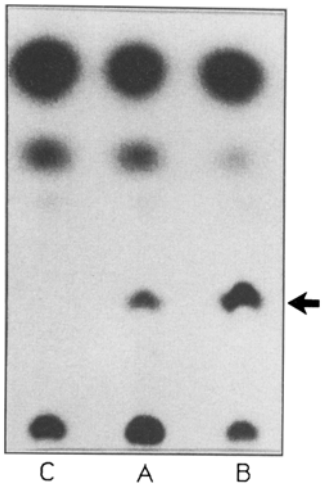


Fig. 2. Analysis of transient PAT activity in extracts of protoplasts 48 h after transformation. Lane C, untransformed protoplasts; lane A, protoplasts transformed with pG35barA; lane B, protoplasts transformed with pG35barB. The arrow indicates the position of acetylated PPT.

type. Table 1 lists several experiments on the selection of PPT-resistant calli in two rice genotypes. Nortai was used for experiments 1 to 5 and Radon was used for experiments 6 and 7. Selection efficiency (number of PPT-resistant calli/number of plated protoplasts) ranged from 0.8×10^{-6} to 9.7×10^{-6} in different experiments. This selection efficiency was in the range of that reported for transformation of rice calli with the *bar* gene [5]. Out of 18 PPT-resistant calli, from two different experiments (1 and 7 in Table 1), 17 proved to be positive for stable integration of the *bar* gene as confirmed by Southern analysis (data not shown). Regeneration from the Nortai lines was not possible. The reason for this was the loss of embryogenic capacity of this line and not an inhibitory effect of PPT on regeneration since the control calli that were not kept on selection medium also failed to regenerate. Protoplast calli from the Radon line (experiments 6 and 7) were embryogenic and regenerated plants. Plants could be regenerated from the PPT-resistant calli as readily as from the control calli that had not undergone the selection regimen.

About 3 to 4 months after the regenerated plants were transferred to soil they were tested for their resistance to Basta. The method of dipping a single leaf in Basta solution enabled us to apply the herbicide to the plants without taking them out of the growth chambers. Furthermore, treatment of only a small portion of a leaf on each plant avoided damaging or killing the whole plant (the control and transgenics with low level of resistance) so that these plants could be used subsequently for PAT assay, DNA isolation, and seed production. Figure 3D shows results of such a test one week after treatment of a control plant and 5 randomly chosen transgenic plants. The treated portion of the control leaf showed severe damage, whereas the leaves of transgenic plants showed high levels of protection.

We also monitored expression of the *bar* gene by measuring PAT enzyme activity in leaf extracts of plants. Figure 4 shows the results of such measurements on leaf extracts of plants from two different experiments. Plants B4, B11, B22, and B25 from experiment 6 exhibited high PAT activity as indicated by the intensity of the acetylated-PPT spots (Fig. 4A). B17 expressed PAT, but at a relatively lower level, and the leaf extract of a control plant did not show any PAT activity. Figure 4B shows PAT activity in plants from experiment 7. Two of the transgenic plants TR3-18 and TR5-N showed high PAT activity but the other two transgenic plants, TR5-6A and TR5-6B, did not exhibit PAT activity. This is interesting because TR5-6A and TR5-6B, which originated from the same callus, have several copies of the *bar* gene integrated into their genome (Fig. 6), but they have stopped expressing this gene. Again, the control plant in this experiment did not exhibit PAT activity.

Southern blot analyses were performed on DNA from leaves of putatively transformed plants regenerated from PPT-resistant calli and from leaves of control plants obtained from untransformed protoplasts (Figs. 5 and 6). DNA from the putative transformants, but not from control plants, hybridized with the *bar* probe. Hybridization occurred in the high-molecular-weight regions in lanes containing undigested DNA (Fig. 5B). When DNA was digested with *Eco* RI and *Hind* III, which cleave the intact expression

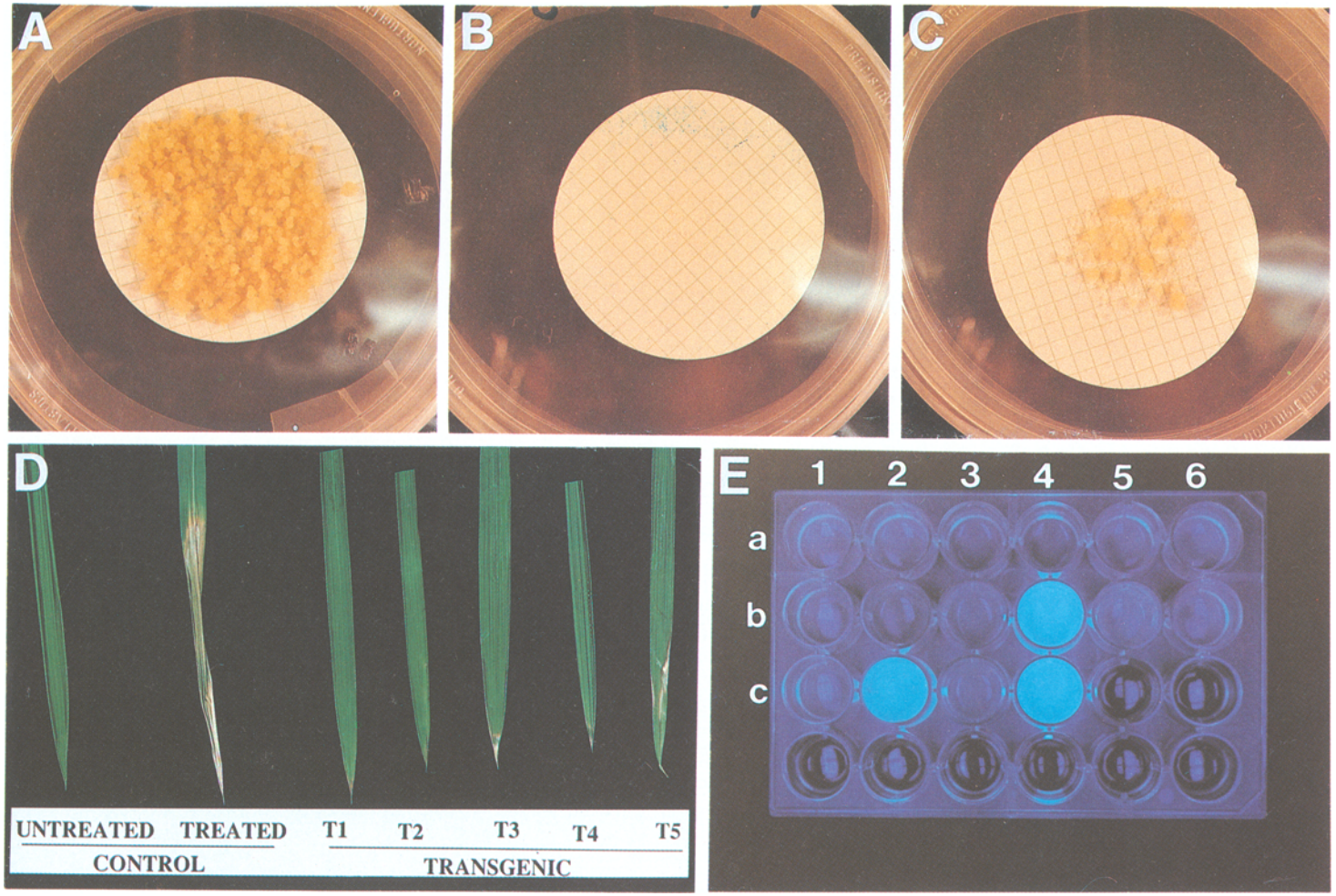


Fig. 3. A, B, C. Growth of protoplast-derived microcalli on medium with or without PPT; A, untransformed control calli growing on PPT-free medium; B, untransformed control calli, showing growth inhibition on medium containing 4 mg/l PPT; C, resistant calli, putatively transformed with pG35barB, growing on medium containing 4 mg/l PPT. D. Demonstration of resistance to 0.25% Basta (500 mg/l PPT) in leaves of T_0 plants; control leaves from plants regenerated from untransformed protoplasts, treated and untreated with Basta; T1–T5, leaves from 5 different putative transgenic plants regenerated from PPT-resistant calli. E. Demonstration of results from rapid GUS assay; a1 to a6 represent 6 different untransformed control plants; b1 to c4 show activity in plants regenerated from 10 different PPT-resistant calli from experiment 6; b1 = B3, b2 = B4, b3 = B7, b4 = B11, b5 = B13, b6 = B17, c1 = B19, c2 = B21, c3 = B22, c4 = B25. B11, B21, and B25 are the only plants exhibiting GUS activity.

Table 1. Efficiency of selection of PPT-resistant calli.

Exp.	Genotype (line)	PPT concentration (mg/l)	Selection efficiency[1]
1	Nortai	3	4×10^{-6}
2	Nortai(100)	3	0.8×10^{-6}
3	Nortai	2.5	6.5×10^{-6}
4	Nortai	2.5	9.7×10^{-6}
5	Nortai (2)	3	7×10^{-6}
	Nortai (2)	5	9×10^{-6}
6	Radon	4	3.5×10^{-6}
7	Radon	3	4.2×10^{-6}
	Radon	5	2×10^{-6}

[1] Selection efficiency = number of PPT-resistant calli selected/ number of protoplasts plated.

unit consisting of CaMV 35S promoter-*bar-nos* poly(A) (1.64 kb) from the rest of the plasmid, a variety of hybridization patterns were observed from DNA of different transgenic plants (Fig. 5A). B4 and B22 showed a single band at 1.64 kb. However, the integration pattern of DNA from other plants was more complex. In addition to the 1.64 kb fragment, B11 showed a few larger and one smaller fragments. This suggests multiple sites of integration, rearrangements and loss of one of the restriction sites for some of the copies. B17 and B25 did not show any hybridizing bands at 1.64 kb, but they both had larger and smaller bands than the 1.64 kb fragment indicating multiple sites of integration and rearrangements. These plants, however, did exhibit PAT activity even though one or both restriction sites had been altered. Southern analysis following the double digestion with *Eco* RI and *Hin*d III included DNA from three plants regenerated from callus B11, two from callus B17, and two from callus B22. In each case the clones showed similar hybridization patterns. These results suggested that there were no untransformed escapes associated with the resistant callus. These results, taken together with our results from other experiments where we found 17 out of 18 PPT-resistant calli to be positive for integration of the *bar* gene, indicated that transformation of protoplasts with the *bar* gene and selection on PPT, results in few escapes. The plasmid pG35barB is 4.35 kb in length and has a single *Hin*d III site. When genomic DNA from the plants was restricted with *Hin*d III, the restriction fragments that bound to the probe were larger than 4.35 kb except in the case of B22 (Fig. 5B). B22 had a band corresponding to the intact expression unit (Fig. 5A), which indicated that some part of the plasmid other than the chimeric gene must have been lost before integration. Since none of the transformed plants showed a band at 4.35 kb, concatamerization before integration into the genome must not have occurred. These results, along with the results from undigested DNA that showed hybridization of the *bar* probe in the high-molecular-weight region, indicated integration of the *bar* gene into the rice genome.

Figure 6 shows Southern blot analysis of DNA from plants of experiment 7. The untransformed plant did not show any hybridization to the *bar* probe but the putative transformants showed a variety of hybridization patterns. Unrestricted

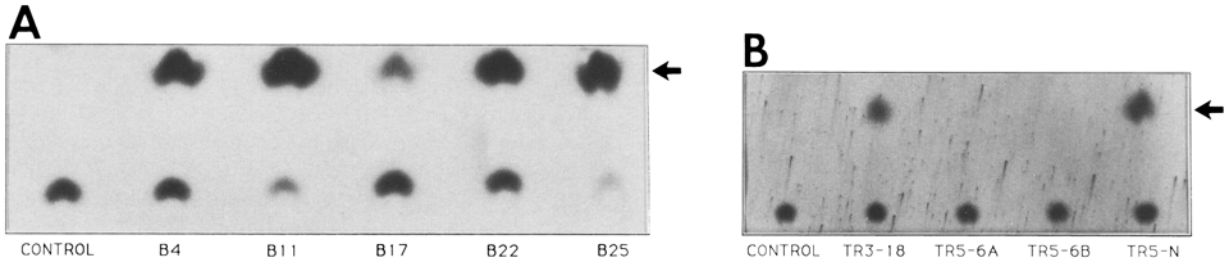
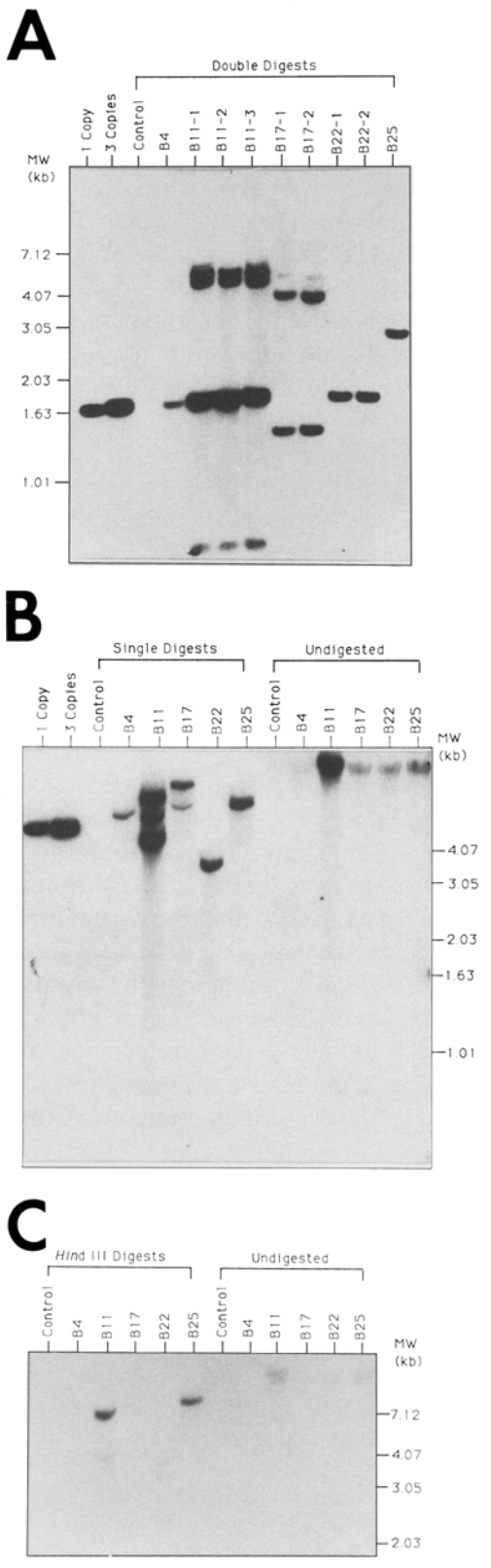


Fig. 4. Analysis of PAT activity in leaf extracts from untransformed control plants and plants regenerated from PPT-resistant calli. (A) Control and putative transgenic plants (B4, B11, B17, B22, B25) from experiment 6; (B) control and putative transgenic plants (TR3-18, TR5-6A, TR5-6B and TR5-N) from experiment 7. The arrow indicates the position of acetylated PPT.



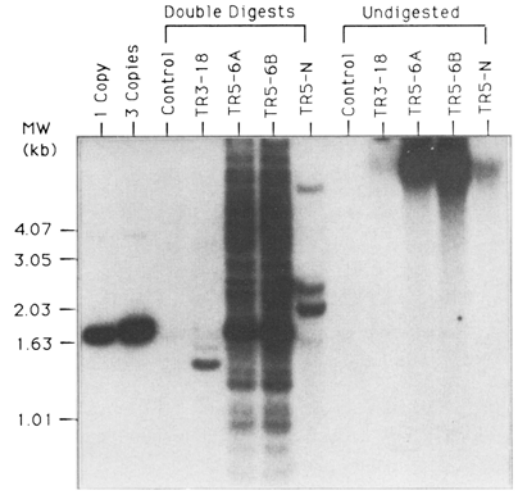


Fig. 6. Southern blot analysis of genomic DNA from leaves of rice plants regenerated from control and PPT-resistant calli (TR3-18, TR5-6A, TR5-6B, TR5-N) from experiment 7. TR5-6A and TR5-6B are plants regenerated from the same callus. Lanes 1 and 2 are 1 and 3 copies, respectively, of the intact expression unit consisting of CaMV 35S promoter-*bar-nos* poly(A) (1.64 kb). Left half of the blot shows DNA restricted with *Hind* III and *Eco* RI. Undigested DNA from these plants is on the right side of the blot. The *Bam* HI-*Bgl* II fragment of the *bar* gene (563 bp) was used as the probe. Only 4 μg of DNA was used in the case of TR5-6A and TR5-6B.

DNA showed hybridization in the high molecular weight region. Restricted DNA from TR3-18 showed a band smaller than 1.64 kb suggesting loss of part of the chimeric gene, yet it was still functional. Two of the restriction fragments, both

Fig. 5. Southern blot analysis of genomic DNA from leaves of rice plants regenerated from control and PPT-resistant calli (B4, B11, B17, B22, B25) from experiment 6. A. Genomic DNA was restricted with *Hind* III and *Eco* RI; lanes 1 and 2 are 1 and 3 copies, respectively, of the intact expression unit consisting of CaMV 35S promoter-*bar-nos* poly(A) (1.64 kb); lane 3: DNA from control plant; lanes 4–12: DNA from plants regenerated from PPT-resistant calli (B4, B11, B17, B22, B25), including three regenerated plants from callus B11, two from callus B17, and two from callus B22. B. DNA samples on the left side of the blot were digested with *Hind* III, while those on the right side were undigested; lanes 1 and 2 are one and three copies, respectively, of the plasmid pG35barB digested with *Hind* III. The *Bam* HI-*Bgl* II fragment of the *bar* gene (563 bp) was used as the probe. C. Blot shown in B washed and reprobed with *gusA* gene (1870 bp).

larger than 1.64 kb, from TR5-N hybridized to the *bar* probe, suggesting the loss of one of the restriction sites and integration of more than one copy. Plants TR5-6A and TR5-6B were clones which regenerated from the same callus. This was confirmed by the hybridization pattern which was similar in both cases. Despite the fact that only 4 μg of DNA was used (20 μg of DNA was used in all the other cases) from these two plants, the hybridization was most intense with DNA from these two plants. The 1.64 kb band was the most intense band but several larger and smaller bands were also seen. These results suggest integration of many copies and multiple rearrangements of the plasmid DNA. More interesting was the fact that the *bar* gene was not being expressed in these two plants as shown by the results of the PAT assay (Fig. 4B).

Protoplasts in experiment 6 were cotransformed with pG35barB and p*Act1*-D. Table 2 shows GUS activity in leaf extracts of two control plants and from 10 plants that were regenerated from 10 different PPT-resistant calli. Plants B11, B21, and B25 expressed the *gusA* gene to varying degrees. These results, obtained using the quantitative fluorimetric method, confirmed the results of our rapid GUS assay shown in Fig. 3E. The control and the untransformed tissue produced negligible fluorescence after 5 h of incubation of leaf strips in the MUG buffer. At 20 h, the solution containing the untransformed and control tissue developed some fluorescence; however, the *gusA*-expressing tissue had produced significantly more intense fluorescence by this time. In order to confirm that results of the GUS assay were due to stable integration of the *gusA* gene, the *bar* probe was washed from the blot shown in Fig. 5B and the blot was reprobed with the *gusA* gene. The *gus* probe hybridized to the DNA from B11 and B25 suggesting that the cotransformed *gusA* gene was stably integrated into the genome of these two plants (Fig. 5C).

Table 2. GUS activity in leaf blades of two control plants and of T_0 plants regenerated from ten different PPT-resistant calli from experiment 6.

	GUS activity (nmol MU per mg protein per hour)
Control 1	0.5
Control 2	3.2
B3	7.2
B4	0.4
B7	12.9
B11	1459.4
B13	17.8
B17	0.6
B19	13.0
B21	1712.6
B22	0.8
B25	9408.0

Definitive evidence for the transgenic status of a plant is transmission of the transgene to its progeny. We chose plants B11 and B17 for analysis of inheritance of *bar* and *gusA* in their progeny. Six-week old progeny plants were used for evaluating their resistance to Basta using the leaf test method as described before. The progeny of B17 segregated as 20 resistant plants and 7 susceptible plants. However, in the case of B11, 27 of the T1 plants were resistant and 3 were susceptible to Basta. As mentioned earlier, the B11 plant also contained functional *gusA* gene. Its progeny was tested for GUS activity using the rapid GUS assay. All the T1 plants that were resistant to Basta also exhibited GUS activity, whereas the 3 plants that were susceptible to Basta were negative for GUS activity. In order to confirm the presence of transgenes in the T1 generation plants, Southern analysis was performed on DNA from twelve progeny of B11 and twelve progeny of B17. The results shown in Fig. 7A show the presence of *bar* gene in the progeny of B11 that were resistant to Basta treatment. Progeny numbers 1, 4, 19, and 21 from the B11 parent appeared to be homozygous for the *bar* gene, whereas progeny numbers 2, 3, 7, 8, and 17 appeared to be heterozygous for the *bar* gene as indicated by the intensity of the bands. DNA from progeny numbers 9, 14, and 22 from B11 did not hybridize to the *bar* probe and these plants were also susceptible to Basta treatment. The same progeny plants appeared to be homozygous and heterozygous for the *gusA* gene as indicated

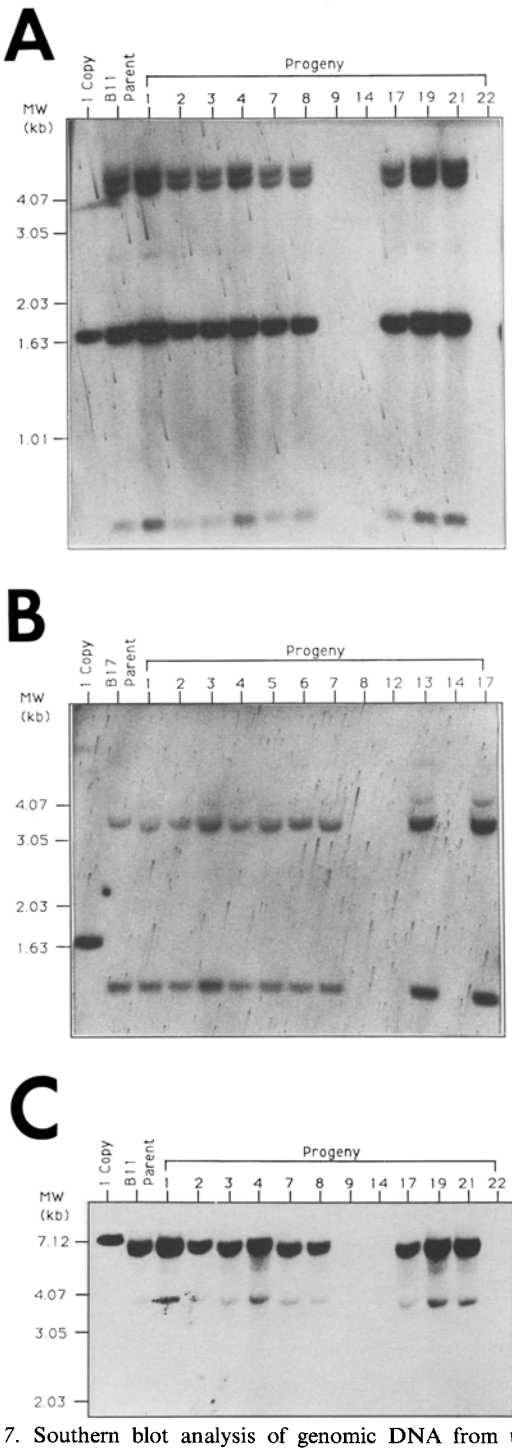


Fig. 7. Southern blot analysis of genomic DNA from two transgenic plants and their progenies. A. DNA from B11 parent and 12 of its progeny probed with the *bar* gene. B. DNA from B17 parent and 12 of its progeny probed with the *bar* gene. The first lane in both blots has one copy of the intact expression unit consisting of CaMV 35S promoter-*bar*-*nos* poly(A) (1.64 kb). The genomic DNA was digested with *Hin*-d III and *Eco* RI. The *Bam* HI-*Bgl* II fragment of the *bar* gene (563 bp) was used as the probe. C. DNA from B11 parent and 12 of its progeny probed with *gusA* gene. The genomic DNA was digested with *Hin*d III. Lane 1 has one copy of p*Act1*-D digested with *Hin*d III. A *gusA* gene fragment of 1870 bp was used as the probe.

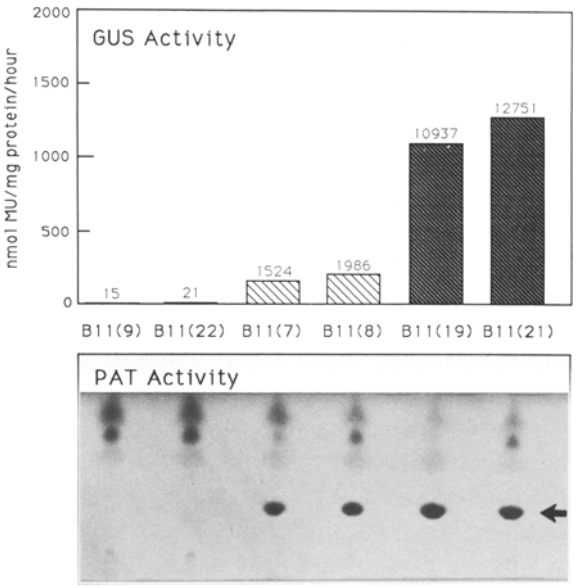


Fig. 8. Analysis of GUS and PAT activity in leaf extracts of some of the B11 progeny. B11(9) and B11(22) are homozygous (–), B11(7) and B11(8) are putatively heterozygous, and B11(19) and B11(21) are putatively homozygous (+) for the *gusA* and *bar* genes based on the Southern blots shown in Fig. 7. PAT assays were carried out with the leaf extract that was diluted to 1 μg protein per 24 μl extract and the reaction was terminated after 10 min. The arrow indicates the position of acetylated PPT.

by the intensity of bands on Southern blots (Fig. 7C). We selected two progeny from plant B11 for their apparent homozygous (–) (9 and 22), heterozygous (7 and 8), and homozygous (+) (19 and 21) status for the *bar* and *gusA* genes for further evaluation. Results of GUS assays and PAT assays on leaf extracts of these plants are shown in Fig. 8. The putative homozygous (+) plants had higher GUS and PAT activities in their leaf extracts than the putative heterozygous plants. The homozygous (–) plants did not ex-

hibit GUS and PAT activity. In order to fully confirm the heterozygous status of T1 progeny 7 and 8, and the homozygous (+) status of T1 progeny 19 and 21, we carried out segregation analyses on the T2 generation from these four plants. The progeny of B11 (7) segregated as 26 herbicide resistant and GUS^+ and 7 susceptible and GUS^- plants, and those of B11(8) segregated as 24 herbicide resistant and GUS^+ and 6 susceptible and GUS^- plants. Again, herbicide-resistance and GUS activity cosegregated in every instance. All the 35 progeny of B11(19) and the 35 progeny of B11(21) were resistant to the herbicide treatment and were positive for GUS activity. These results confirmed our assumptions based on Southern blot and enzyme analyses that B11(7) and B11(8) were heterozygous and B11(19) and B11(21) were homozygous (+). Progeny numbers 1, 2, 4, 5, 6, and 7 from the B17 parent appeared to be heterozygous for the *bar* gene and the progeny 3, 13, and 17 appeared to be homozygous (+) (Fig. 7B). DNA from progeny numbers 8, 12, and 14 did not hybridize to the *bar* probe and were also susceptible to Basta. These results clearly indicate transmission and expression of the transgenes to the T1 and T2 generations.

Discussion

The results presented in this paper show that the *bar* gene can be used as a selectable marker to obtain transgenic rice plants from protoplasts that were transformed via direct DNA uptake. Stably transformed microcalli were easily selected under microscopic observation. The timing of application of PPT was important. If microcalli were allowed to grow until they formed a thick mat over the filter, it was difficult to distinguish resistant calli from the untransformed ones. However, if the timing of the selection pressure was right, i.e. about 3–4 weeks after transformation for our culture line, there were few escapes. This was indicated by our results from initial experiments which showed that out of 18 selected calli 17 were positive for the integration of the *bar* gene. Although we did not continue the selection pressure at the stage of callus proliferation, such a treatment would further minimize escapes. Indeed problems of escapes, which are more common with microprojectile transformation methods [1, 9], were overcome by maintaining selection pressure right up to the regeneration stage [9]. In our protoplast studies the PPT-resistant transgenic calli were able to regenerate plants as well as the untransformed control calli that had been grown on PPT-free medium indicating that transformation with the *bar* gene and selection on PPT does not interfere with the ability of the transformants to regenerate plants.

A comparison of transient gene expression following transformation with constructs pG35barA and pG35barB showed higher PAT activity in the cell extract when pG35barB was used. We were unable to get any PPT-resistant calli when we used pG35barA for transformation and were successful in obtaining PPT-resistant calli only when we used pG35barB which contained the *bar* gene that was modified for more efficient translation in eukaryotes [38]. Thus, in our system, the CaMV 35S promoter in combination with the modified *bar* gene was sufficient to produce transformants that were capable of growing on PPT-containing medium. The selection efficiency when the *bar* gene was used in combination with PPT was in the same range of that reported for transformation of rice with the *neo* gene [25] and the *hpt* gene [34]. However, selection efficiencies one order of magnitude higher have been reported [12] for hygromycin selection. The discrepancies in selection efficiencies in various reports may be due to differences in cultivars used, methods of transformation, and methods of protoplast culture. It may be possible to achieve higher selection efficiencies with a stronger promoter driving the *bar* gene; however, this could also increase the number of escapes.

All transgenic plants, except two, continued to express the *bar* gene as indicated by PAT activity measurements. In one of the experiments where the reporter gene *gusA* was used for cotransformation, 3 out of 10 PPT-resistant calli produced plants that expressed the reporter gene.

We used five transgenic plants from experiment 6 and four transgenic plants from experiment 7 for detailed analysis of stable integration of the *bar* gene into their genomes. Southern analysis of undigested DNA showed hybridization of the *bar* probe to DNA in the high-molecular-weight region indicating integration of the *bar* gene into the genome. Results of double digestion with restriction enzymes showed a variety of integration patterns ranging from integration of a single copy to multiple insertions with rearrangements. Such variation in integration patterns have been observed in many other studies where transgenic plants were produced using a variety of transformation methods [3, 10, 25, 27, 34].

Transmission of the transgenes to the T1 generation was studied in progeny of transformants B11 and B17. Resistance to Basta segregated in a Mendelian fashion in progeny of B17. However, the segregation ratio in progeny of B11 was 27 herbicide-resistant plants to 3 susceptible plants. When GUS activity and herbicide resistance were present in the parent, GUS activity and herbicide resistance cosegregated in the progeny. Transmission of both genes to progeny provides definitive evidence that the genes were integrated into the chromosomes and in close proximity to each other in the T_0 plants. It is interesting that progeny of B11, which were homozygous for the *bar* and *gusA* gene exhibited higher PAT and GUS activities than progeny that were heterozygous. Such a correlation between homozygosity and higher gene expression has been previously reported at the mRNA level [4] and at the protein level [16, 26].

Two of the transgenic plants from experiment 7, which regenerated from the same PPT-resistant callus, did not show PAT activity in leaf extracts, and Southern blot analysis of the DNA from these two plants showed integration of many copies of the *bar* gene into their genomes. Northern blot analysis of RNA from the leaves of these two plants showed a complete absence of message for the *bar* gene (data not shown). The silencing of the *bar* gene must have occurred some time after the selection of callus on PPT-containing medium since expression of the gene was necessary initially for the callus to overcome the inhibitory effect of the selection agent. There are many reports on suppression or silencing of homologous genes in transgenic plants that have been either re-transformed with the same bacterial genes or transformed with extra copies of endogenous plant genes [4, 11, 19, 24, 30, 35]. In some cases it was observed that the newly introduced genes do not even have to be full length copies to suppress the expression of the original gene [11, 30]. Various possible explanations have been given for gene silencing, such as, methylation of promoters [19], interference of RNA strands with the transcription process [35], and degradation of mRNA [30]. This suppression of gene expression appears to be related to transgene dose in the genome of the transgenic plants [4]. Whatever the mechanism, it is clear that plants possess some system to silence transgenes whose copy number exceeds certain limits.

All except two of the regenerated T_0 plants continued to express *bar* gene as determined by the PAT assay. These plants and their progeny which had the *bar* gene showed resistance to herbicide Basta. This raises the possibility of using Basta or Herbiace as post-emergence herbicides on rice plants transformed with the *bar* gene. However, concerns about the *bar* gene outcrossing into weeds such as red rice, need to be addressed before such an application for the *bar* gene in rice is attempted.

Acknowledgements

We thank Dr F. Wen and R.C. Su for providing the suspension culture lines used in this study. We also thank Dr L. Lyznik for suggesting the use of the rapid GUS assay and critical reading of the manuscript, Lynn Hirayama for technical assistance, Drs R. Wu and D. McElroy for providing us with the construct p*Act1*-D. This research was supported by a grant from the Rockefeller Foundation. This is Journal Paper No. 13531 from the Purdue Agricultural Experiment Station.

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