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Evaluation of Selectable Markers for Rice Transformation[1]

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ABSTRACT

A variety of expression systems and selection régimes have been developed to transform plants such as tobacco, petunia, and tomato. We investigated several of these to determine whether the promoters and selectable markers used in dicotyledonous plants are suitable for selecting transformed rice callus. We compared transient expression driven by constitutive and regulated promoters in rice (*Oryza sativa*) protoplasts and found that the 2′ promoter of the octopine T-DNA is approximately 3 to 4 times more efficient than the CAMV *35S* promoter, 10 times more efficient than the *nos* promoter and the 1′ promoter, and more than 100 times better than two other regulated plant promoters. Similar results were obtained in tobacco (*Nicotiana tabacum*) protoplasts with the exception that the *nos* promoter was expressed nearly 10 times better in rice. Further studies demonstrated that rice callus growth is sensitive to low concentrations of methotrexate, phosphinothricin, and bleomycin, and to moderate concentrations of G418 and hygromycin, but is only partially inhibited by relatively high concentrations of kanamycin. Finally, we tested the ability of stably introduced resistance genes to protect callus against some of the selective agents. Genes that inactivated phosphinothricin or G418 permitted transformed calli to grow almost unimpeded on toxic concentrations of these selective agents. However, a gene conferring resistance to methotrexate could not be used to select for activily growing transformants. Southern analysis of the transformed cell lines demonstrated that 50% of the transformants contained a single plasmid copy and that nearly all integrated copies showed rearrangements. These results on the use of selectable markers in rice should facilitate efforts to obtain transformants of this important grain.

The tissue culture techniques for rice are some of the best elaborated among the Gramineae. Plants can be regenerated from callus tissue initiated from immature and mature embryos, inflorescence, leaf bases, and root tips. Recently, several groups have gone even further toward the goal of transforming cereals by regenerating plants from rice protoplasts isolated from suspension cultures (1, 34). Similar improvements in tissue culture techniques have also permitted plants to be regenerated from maize (24) protoplasts.

Since grasses are recalcitrant to *Agrobacterium*-mediated transformation, other gene transfer techniques must be employed. One such approach combines protoplast regeneration with direct DNA uptake techniques, making it possible to obtain transformed calli of *Zea mays* (7), *Triticum monococcum* (17), *Lolium multiflorum* (22), *Panicum maximum* (10), and *Oryza sativa* (29). Recently, Rhodes *et al.* (25) have recovered transformed maize plants using naked DNA transformation.

A number of selective agents and suitable resistance genes have been investigated concurrently with the studies on gene transfer and cell culture. The most widely used inhibitors are kanamycin, G418, and hygromycin. All of them are aminoglycoside antibiotics which interfere with the translation machinery of prokaryotic and eukaryotic cells. In addition, all of them can be inactivated by phosphorylation reactions mediated by the products of either the Tn*5* neomycin phosphotransferase II (*npt*-II) gene (12) or the hygromycin B resistance gene from *Escherichia coli* (32).

Less commonly used selective agents are bleomycin, methotrexate, and phosphinothricin. Bleomycin creates single- and double-stranded breaks in eukaryotic DNA (20), making it a very potent mutagen. The introduction into *Nicotiana plumbaginifolia* of a chimeric bleomycin resistance gene whose product binds the drug (8) conferred resistance to high concentrations of bleomycin (13). Methotrexate binds to the catalytic site of the dihydrofolate reductase enzyme, resulting in a deficiency of thymidylate and subsequent cell death (33). The introduction of a chimeric construct built from the coding region of a mutant mouse *dhfr* gene (26) has been used to produce an enzyme with a very low affinity for methotrexate so that it is possible to select for transformed *Panicum maximum* calli (10).

Phosphinothricin inhibits glutamine synthase, causing a rapid accumulation of ammonia that leads to plant cell death (27). The bialaphos resistance (*bar*) gene codes for a phosphinothricin acetyl-transferase, and acetylated phosphinothricin is no longer inhibitory to glutamine synthase. A chimeric *bar* gene has been used to confer resistance to transformed tobacco, potato, and tomato plants (5).

While the kind of resistance is determined by the coding region of the chimeric gene, the level of resistance can be influenced by the transcription signals, as illustrated by Nutter *et al.* (21). Thus, it is necessary to test several gene constructs in order to find one that permits transformed cells to outgrow nontransformed cells. Graminaceous plants show a high natural resistance to kanamycin, making it very difficult to separate transformed from nontransformed tissue with chimeric *npt*-II gene constructions (10, 22). In an effort to develop more effective gene transfer systems for rice, we have determined the expression levels of six different promoters in rice protoplasts and assessed the utility of four chimeric resistance genes as selectable markers.

MATERIALS AND METHODS

Protoplast Isolation and Protoplast Culture

Protoplasts were isolated from suspension cultures kindly provided by Dr. E. C. Cocking (Nottingham University,

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U.K.). Isolation and subsequent culture was according to the method of Thompson *et al.* (28), including heat shock and embedding in 1% SeaPlaque agarose.

Leaf protoplasts were obtained from leaf bases of 7-d-old rice (*Oryza sativa*) seedlings by adding 0.5% Macerozyme R10 to the enzyme solution used for suspension protoplast isolation, incubating the digest for 16 h, and pelleting the protoplasts at 250 g instead of 80 g. Tobacco (*Nicotiana tabacum*) leaf protoplasts were prepared according to De Block *et al.* (4).

Transient Expression Assay

Protoplasts were prepared using the standard protocol, but the last two washes were done in 0.5 M glucose (or 0.4 M sucrose for tobacco protoplasts), 4 mM $CaCl_2$, and 10 mM Hepes (pH 7.2). After the last wash, protoplasts were counted with a hemocytometer and pooled to 5 to 7.5 × 10^6/mL. Two hundred μL protoplast suspension was transferred to disposable cuvettes, and 10 μg supercoiled pLD1 DNA (or equimolar amounts of DNA if other plasmids were used) and 11 μL of a 3 M NaCl stock were added. After a 10-min incubation time on ice, an electrical shock of 375 V/cm from a 200 μF capacitor was delivered to the mixture. The τ value was 54 msec. The postincubation time on ice was 15 min. After a 48-h incubation time in 5 mL liquid culture medium, the protoplasts were collected by centrifugation and proteins were extracted by sonication. We determined the protein concentrations in each of the samples using the Bio-Rad assay, and equal amounts of protein from each sample were analyzed.

Transformation Experiments

The procedure was identical to the transient expression experiments, except that after electroporation, the protoplasts were resuspended in 3 mL KpR[2] medium (28), subjected to a heat shock, and embedded in KpR medium with 1% SeaPlaque agarose. After 1 week, the agarose segments were transferred to 5-cm Petri dishes containing 3 mL KpR medium with or without the selective agent. After three more weeks the developing microcalli were transferred to LS medium (16) with 3% sucrose, 2 mg/L 2,4-dichlorophenoxyacetic acid, and 0.4% agarose (Sigma type I) with or without inhibitor.

Plasmid DNA

Plasmid DNA was prepared according to Birnboim and Doly (2) and purified by CsCl gradient centrifugation. Equimolar amounts of plasmid DNA were obtained by spectrophotometrical determination of DNA concentrations followed by adjustment for size differences using ethidium bromide-staining of plasmid DNA digested with restriction enzymes generating similarly sized fragments, and electrophoresed on agarose gels.

Plasmids

For the transient expression experiments, the nopaline synthase promoter (6), the 4.7 promoter (3), and the 700-base pair *Rsa*I fragment of the 5′ region of the zein *4* gene (18) have been cloned in the *Bgl*II site 5′ of the promoterless *npt*-II gene of pGVL150 (11). The 1-kb *Bam*HI-*Bgl*II fragment of the 5′ region from the cauliflower mosaic virus 35S gene (9) and the transcript 1′ and transcript 2′ promoters (32) were fused to the same *npt*-II-*ocs* 3′ construct, and were carried on the pSP64 vector (19) and the pLD1 plasmid (Fig. 1), respectively.

Plant DNA

To prepare DNA from rice callus tissue, we followed the RNA preparation method described by Jones *et al.* (14) up to the nucleic acid precipitation step. Twenty μg of DNA digested with restriction enzymes was electrophoresed through a 1% agarose gel, blotted onto Amersham nylon filters, and hybridized for 16 h to radioactive probes generated by a multiprime kit (Amersham, U.K.). The filters were exposed to x-ray films using an intensifying screen at −70°C.

NPT-II and PAT Assays

Neomycin phosphotransferase II and phosphinothricin acetyltransferase activities in rice callus were assayed by the methods of Van den Broeck *et al.* (30) and De Block *et al.* (5), respectively.

Growth Rate of Rice Calli on Selective Media

Callus tissue was subcultured on a basal LS medium with 3% sucrose, 2 mg/L 2,4-dichlorophenoxyacetic acid, and 0.4% agarose (Sigma type I). At time zero, 10 to 15 calli were subdivided into a number of pieces equaling the number of parameters to be tested, and the experiment was repeated three times. The average initial fresh weight of one callus piece was 70 ± 20 mg. The fresh weight of the calli was measured after 10 to 12 d, 20 to 24 d, and 28 to 31 d. After each measurement, the calli were returned to fresh medium.

RESULTS

Promoter Choice via Transient Gene Expression Assay

Our initial concern was to determine the most suitable conditions to introduce DNA into rice protoplasts by means of electroporation. This was accomplished by optimizing the conditions for transient expression of the reporter gene *2′-npt*-II in tobacco leaf and rice suspension protoplasts. Varying voltage and capacitance proved that a 375 V/cm shock from a 200 μF capacitor gave reasonable protoplast survival (65% after 48 h as measured by fluoresceine diacetate staining) and high NPT-II signal (data not shown) for both types of protoplasts. Under these conditions 50% of maximal NPT-II activity was reached after 24 h and maximal expression was obtained after 48 h.

To compare the expression of different promoters in protoplasts, we fused the 5′ region of six genes to the coding region of the bacterial neomycin phosphotransferase II gene (*npt*-II). All constructs were terminated with the 3′ region of the octopine synthase gene (*ocs* 3′). The promoters we tested are isolated from the zein *4* gene (18), an uncharacterized rice gene called *4.7* (3), the nopaline synthase gene (6), the *1′*

[2] Abbreviations: KpR, protoplast culture medium containing coconut water and casamino acids (28); LS, Linsmaier and Skoog medium (16).

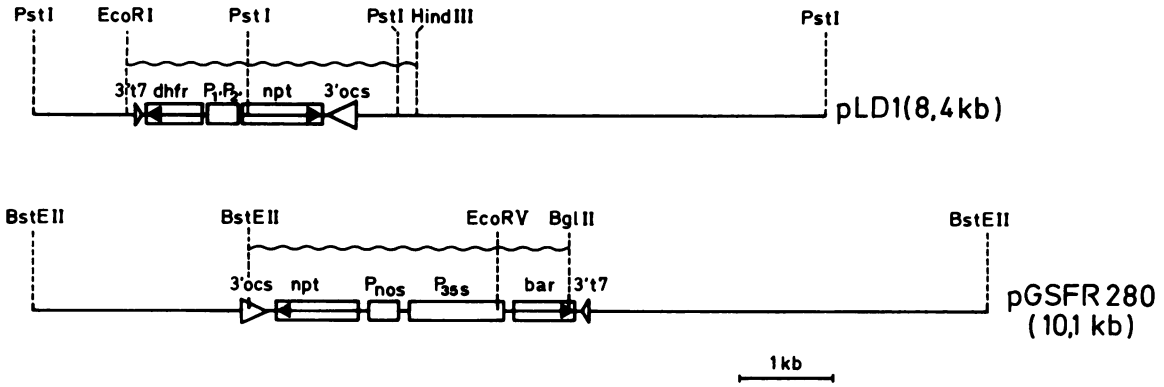

Figure 1. Schematic presentation of the plasmids pLD1 and pGSFR280. Only pertinent restriction sites are shown. The fragments used as probes are indicated by the wavy line. *Pnos*, promoter from nopaline synthase; P_{35S}, promoter from cauliflower mosaic virus 35S transcript; $P_{1'}$, and $P_{2'}$, promoters from *1'* and *2'* transcripts from T_R-DNA; *3'ocs*, 3' region of octopine synthase gene; 3't7, 3' region of transcript 7; *bar*, bialaphos resistance gene; *npt*-II; neomycin phosphotransferase II gene; *dhfr*, mutant dihydrofolate reductase gene.

transcript, and *2'* transcript of the octopine T_R-DNA (31), and the cauliflower mosaic virus 35S gene (9). The first two have a monocotyledonous origin, the last four have evolved to function efficiently in dicotyledonous plants. The resulting chimeric constructs are abbreviated *z4-npt*-II, *4.7-npt*-II, *nos-npt*-II, *1'-npt*-II, *2'-npt*-II, and *35S-npt*-II. Equimolar amounts of the constructs were electroporated into rice suspension protoplasts, rice leaf protoplasts, and tobacco leaf protoplasts, and NPT-II activity was determined after 48 h (Fig. 2). The *2'-npt*-II construct gives the highest level of NPT-II activity regardless of the source of protoplasts. If we quantify the NPT-II activity according to Reiss *et al.* (23) and normalize the level of NPT-II activity directed by *2'-npt*-II to 100% in the different types of protoplasts, then the relative level of NPT-II activity produced using *4.7-npt*-II, *z4-npt*-II, and *1'-npt*-II is constant (0.2, 0.4, and 10%, respectively) in the different protoplast types (Table I). The *35S-npt*-II construct reaches 40 and 28% of the NPT-II activity obtained with *2'-npt*-II in rice and tobacco protoplasts, respectively. Unexpectedly, the *nos-npt*-II construct shows a significant differential expression. The relative NPT-II activity obtained with this gene is nearly 10 times higher in rice suspension and rice leaf protoplasts than in tobacco leaf protoplasts (Table I). These experiments were repeated twice for leaf protoplasts of rice and three times with each of the other preparations, with little variation between experiments (Table I). Furthermore, when a *35S-cat* gene is included in each plasmid and the expression level of this gene is used to calibrate the amount of protein loaded in the NPT-II assay, similar activity ratios are obtained (I Ingelbrecht, personal communication). Although parts of some of the plasmids used in these experiments do differ, the eukaryotic-derived sequences in each construction are the same. It seems unlikely that distantly placed prokaryotic sequences could account for the differences in expression of the chimeric genes, and therefore we believe these NPT-II activity ratios primarily reflect differences in promoter activity.

Table I. *Relative NPT-II Activity (%) Directed by Different Promoters in Tobacco and Rice Protoplasts*

NPT-II activity was measured according to Reiss *et al.* (23). The amount of phosphorylated kanamycin produced in each assay was normalized to that produced by the *2'-npt*-II construct, analyzed simultaneously. Plasmid pGVL150 (11) contains a promoterless *npt*-II gene and served as negative control.

Chimeric Gene	Rice Suspension	Leaf	Tobacco Leaf
2'-npt-II	100	100	100
35S-npt-II	40 ± 4	ND[a]	28 ± 1
nos-npt-II	11 ± 2	8 ± 1	1.2 ± 0.4
1'-npt-II	10 ± 2	ND	9 ± 2
z4-npt-II	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
4.7-npt-II	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
pGVL 150	UD[b]	UD	UD

[a] Not determined. [b] Undetected.

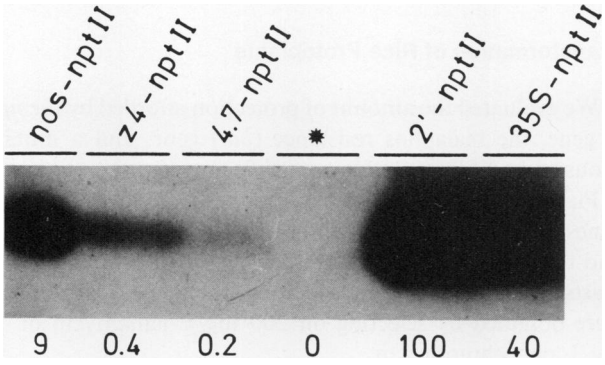

Figure 2. NPT-II activity in rice protoplasts electroporated with different chimeric constructs. Protoplasts were electroporated with equimolar amounts of plasmid DNA carrying the different chimeric constructs. After a 48-h culture period, proteins were isolated from the protoplasts and equal amounts of proteins were subjected to an NPT-II assay. Shorter exposures of the autoradiogram, suitable for visualizing *2'-npt*-II, show no cross-contamination of samples in adjacent lanes. The kanamycin-$^{32}PO_4$ spots were quantified (23) and normalized to the activity produced by the *2'-npt*-II construct. The asterisk designates an electroporation with pGVL150 containing a promoterless *npt*-II gene.

Sensitivity of Rice to Selective Agents

A variety of selective agents and corresponding resistance markers have been developed to distinguish transformed from untransformed plant cells. To be able to judge which of these selection régimes was most efficient for rice, we determined the sensitivity of rice tissue to six different toxic compounds: kanamycin, G418, hygromycin, bleomycin, methotrexate, and phosphinothricin.

Three series of 12 calli of an established callus line were transferred from nonselective basal medium to basal medium with different concentrations of the selection agents. The growth rate was defined as the ratio of the total fresh callus weight at day x after transfer to the initial weight. Figure 3 shows that the growth of rice callus tissue could be fully inhibited by 2 mg/L methotrexate, 10 mg/L phosphinothricin, 20 mg/L bleomycin, 50 mg/L hygromycin, or 100 mg/L G418. After 1 month, the growth of calli on 500 mg/L kanamycin reached 40% of the growth on unselective medium. In a second experiment, a suspension culture was sieved through 500 μm mesh and embedded in LS medium with 2 mg/L 2,4-D, 3% sucrose, and 0.6% SeaPlaque agarose. As shown in Figure 4, similar concentrations of phosphinothricin, G418, and hygromycin were needed to stop growth. Addition of 300 mg/L kanamycin was sufficient to inhibit callus growth. Protoplast division could be arrested with 200 mg/L kanamycin.

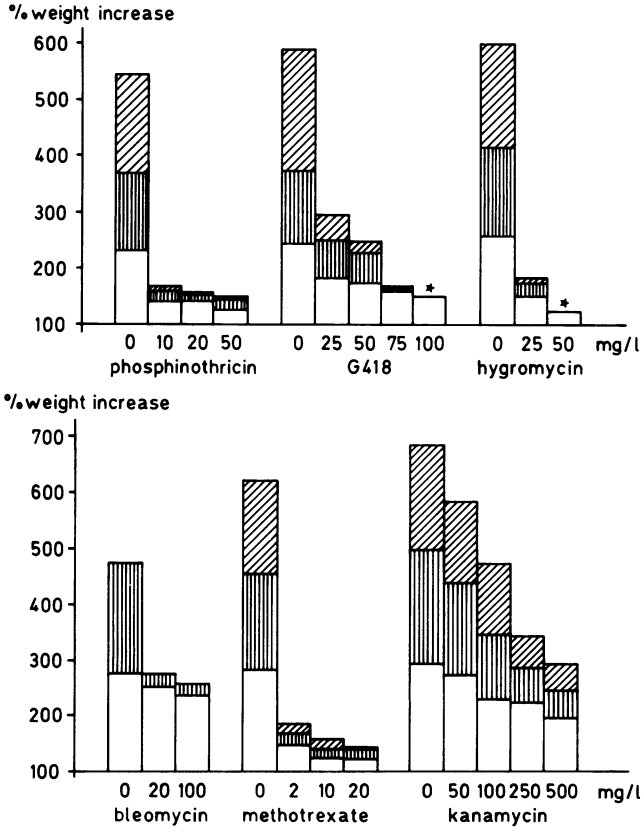

Figure 3. Sensitivity of rice calli to different selective agents. Ten to 15 calli were transferred to media containing different concentrations of the selective agent and the initial fresh weight was determined. The calli were reweighed after 10 to 12 d (white), 20 to 24 d (vertical stripes), and 28 to 31 d (slanted stripes) incubation. The growth rate is defined as 100 times the ratio of fresh weight at day *x* to the initial fresh weight. The values are the averages of three independent experiments. An asterisk means there was no further increase in weight.

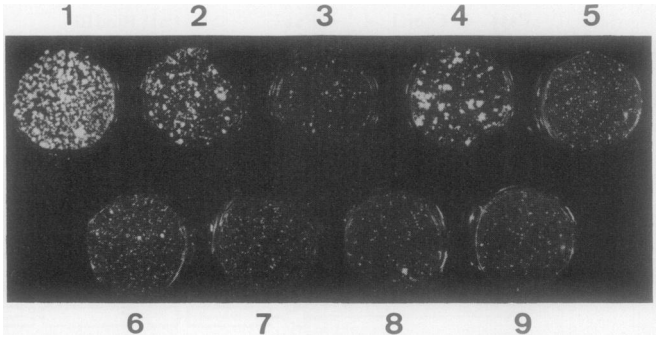

Figure 4. Endogenous resistance of rice calli to different selectable markers. Calli from a suspension culture were sieved through a 500-μm mesh and embedded in basal medium (LS with 3% sucrose, 2 mg/L, 2,4-D, and 0.6% SeaPlaque agarose) without selection (1), with 100 mg/L (2), or 500 mg/L (3) kanamycin; with 5 mg/L (4) or 10 mg/L (5) phosphinothricin; with 25 mg/L (6) or 50 mg/L (7) hygromycin; and with 50 mg/L (8) or 100 mg/L (9) G418. Colonies are shown 3 weeks after transfer.

To have an effective phosphinothricin selection it is important to omit not only glutamine from the selective medium, but also several other amino acids. Addition of 10 mM glutamic acid, 25 mM proline, 10 mM arginine, or 25 mM proline in combination with 10 mM arginine to medium with 20 mg/L phosphinothricin restores the growth rate from 9% to 31, 49, 65, or 80%, respectively, of the growth on medium without selection (data not shown). For this reason, we excluded both coconut water and casamino acids from the phosphinothricin culture medium during the transformation experiments with pGSFR280. The frequency of protoplast division remained unchanged under these conditions (Table II).

Transformation of Rice Protoplasts

We evaluated the amount of protection afforded by the *npt*-II gene, the bialaphos resistance (*bar*) gene, and a mutant mouse dihydrofolate reductase (*dhfr*) gene.

Plasmid pLD1 (Fig. 1) carrying the *2'-npt*-II and *1'-dhfr* genes and plasmid pGSFR280 (Fig. 1) carrying the *35S-bar* and the *nos-npt*-II genes were electroporated into rice protoplasts. Transformants regenerating from these protoplasts were obtained by selecting on 200 mg/L kanamycin or 10 mg/L phosphinothricin.

The plating efficiency of untreated protoplasts on KpR medium was 3×10^{-3}. Our electroporation conditions reduced the plating efficiency by approximately 40%. The transformation frequency obtained with pLD1-treated protoplasts and kanamycin selection was 1.7% of the calli obtained on kanamycin-free medium. Protoplasts electroporated with pGSFR280 and subjected to phosphinothricin selection resulted in a 0.5% transformation frequency (Table II). No calli were recovered from untreated protoplasts subjected to kanamycin or phosphinothricin selection.

Table II. *Number of Protoplasts Regenerating to Calli*

Treatment	Medium[a]			
	KpR	KpR-cw-ca	KpR + Km_{200}	KpR-cw-ca + ppt10
Unelectroporated	3.0×10^{-3}[b]	3.0×10^{-3}	0	0
Electroporated				
Without DNA	1.8×10^{-3}		0	0
With pLD1	1.8×10^{-3}		3.0×10^{-5}	
With pGSFR280		1.6×10^{-3}		7.0×10^{-6}

[a] KpR, protoplast culture medium containing coconut water and casamino acids (28); −cw, without coconut water; −ca, without casamino acids; Km_{200}, 200 mg/L kanamycin; ppt10, 10 mg/L phosphinothricin. [b] The frequencies are the average of two independent experiments.

Analysis of Transformants

NPT-II assays were performed on six pLD1 and six pGSFR280 randomly chosen transformants, called K1 to K6 and P1 to P6, respectively. All clones, except P2 and P3, showed NPT-II activity (Fig. 5). Clones P2, P3, and the four other pGSFR280 clones demonstrated PAT activity, supplied by the *35S-bar* gene on pGSFR280. We expected that the NPT-II$^-$, PAT$^+$ calli resulted from a deletion of the nonselected *nos-npt*-II marker on pGSFR280 during integration into the plant chromosome. This was confirmed by a series of Southern blot analyses summarized in Table III.

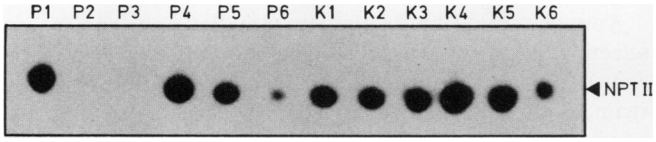

Figure 5. Autoradiogram of an NPT-II assay carried out on rice calli transformed with pGSFR280 (P1–P6) or pLD1 (K1–K6). Clones P2 and P3 do not contain NPT-II activity. Equal amounts of protein are loaded in each lane.

Table III. *Southern Analysis of Rice Transformants*

DNA isolated from	*Eco*RI-Digested DNA with the pGSFR280 Probe	*Bst*EII-*Eco*RV-Digested DNA with the 3.4-kb *Bst*EII-*Bgl*III Fragment of pGSFR280 as Probe
	number and size of bands observed after hybridizing	
pGSFR280	No restriction sites	2 (5.1 and 2.7 kb)
P1[a]	1 (>20 kb; >5 copies integrated vector)	3 (3.8[b], 2.7, and 1.9 kb[b])
P2	1 (8 kb)	None
P3	1 (11 kb)	None
P4[a]	1 (>20 kb; >5 copies integrated vector)	4 (5.1, 3.7[b], 2.7, and 2 kb[b])
P5	ND[c]	2 (5.1 and 2.7 kb)
	*Bgl*III-Digested DNA with the pLD1 Probe	*Eco*RI-*Hin*dIII-Digested DNA with the 3-kb *Eco*RI-*Hin*dIII Fragment of pLD1 as Probe
pLD1	No restriction sites	1 (3 kb)
K3	1 (9 kb)	1 (3.4 kb[b])
K4	3 (10.5, 9, and 4.3 kb)	2 (6.6[b] and 3 kb)

[a] The number of copies in P1 and P4 are estimated from relative hybridization intensity. [b] Restriction fragments not present in original vector. [c] Not determined.

DNA from the clones P1, P2, P3, and P4 was digested with *Eco*RI, a restriction enzyme that does not cut pGSFR280. Hybridization of P1 and P4 DNA with a probe of pGSFR280 revealed one high mol wt band (>20 kb) that contains, based on hybridization intensity, approximately five copies of the integrated vector. Clone P2 contains an insert smaller than 10.1 kb, while clone P3 shows one band of about 11 kb (Table III).

The restriction enzyme *Bgl*II does not cut the pLD1 plasmid and was used to estimate the number of integration sites in the DNA from clones K3 and K4. After hybridization with the pLD1 probe, one insert of 9 kb can be detected in clone K3. Digestion of DNA from clone K4 generates bands of 4.3, 9, and 10.5 kb. Further analysis of the DNA restriction pattern (Table III) provided more evidence that clones P2, P3, and K3 contain only one insert, while clones P1, P4, P5, and K4 contain several copies of the introduced DNA. Furthermore, six out of the seven analyzed calli contain at least one rearranged plasmid sequence. In particular, the NPT-II$^-$, PAT$^+$ clones P2 and P3 lack the sequences corresponding to the *npt*-II gene. Similarly, frequent truncations and rearrangements have been reported by other investigators (15).

Resistance of Transformed Rice Calli to Selective Agents

We used the transformed calli to determine which selectable markers work best in discriminating transformed from untransformed rice calli. Both transformed and nontransformed calli were grown on basal medium and basal medium supplemented with different concentrations of the selection agents. As demonstrated in Figure 6, the discrimination between transformed and untransformed rice callus tissue is optimal with 10 mg/L phosphinothricin or 100 mg/L G418. At these concentrations the growth of transformed calli is minimally reduced and the growth of untransformed calli is fully inhibited. Higher concentrations reduce the growth of transformed tissue; lower concentrations (75 mg/L G418, 5 mg/L phosphinothricin) allow growth of untransformed tissue.

As noted earlier, kanamycin was not an efficient selection agent for calli, although it could be used to select for transformed cells immediately after protoplast regeneration. Nevertheless, the growth of untransformed calli was 30% slower than that of the calli transformed with either *2′-npt*-II or *nos-npt*-II constructs on medium containing 500 mg/L kanamycin. In tobacco, the *2′-npt*-II construct can confer resistance to 1000 mg/L kanamycin (31; our unpublished data), possibly indicating that the endogenous resistance of

rice and the resistance supplied by the *2'-npt*-II construct are not additive. Levels of 500 mg/L G418 are necessary to obtain growth inhibition of both tobacco (data not shown) and rice tissue transformed with the *2'-npt*-II gene.

On methotrexate, untransformed calli are killed within 1 week. Calli transformed with pLD1 stay alive but grow very slowly; upon transfer to media with 500 mg/L kanamycin, they grow rapidly again. Based upon these results, it may be possible to use a short incubation time on methotrexate to eliminate potential escapes from other selection régimes. Replacing the *1'* promoter by the *2'* promoter might improve the protection against methotrexate; however, the *1'* promoter directs a level of expression similar to the *nos* promoter in rice protoplasts and the *nos-npt*-II constructs supplies full protection against kanamycin and G418.

It is also valuable to point out that (a) under selective conditions the average growth rate of clones containing single copies of the chimeric genes is similar to the average growth rate of those clones with multiple copies, and (b) the average growth rate of the *2'-npt*-II transformants on kanamycin and G418 is only slightly higher (≤3%) than the average growth rate of the *nos-npt*-II transformants (the result shown in Figure 6 is the average growth rate of both cell lines).

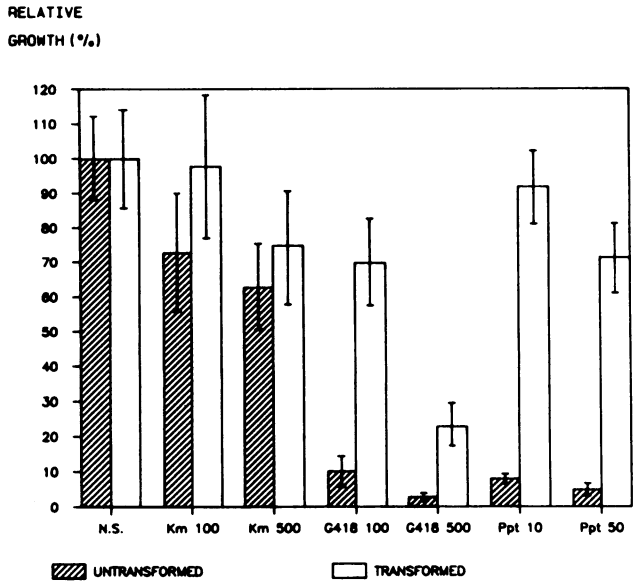

Figure 6. Relative growth of transformed and untransformed rice calli on selective media. *2'-npt*-II-transformed calli, *nos-npt*-II-transformed calli, and untransformed calli (regenerated from protoplasts) were transferred to basal medium (LS with 3% sucrose, 2 mg/L 2,4-D, and 0.4% Sigma type-I agarose) without selection or with different concentrations of kanamycin or G418. The average initial fresh weight and the average fresh weight after 20 d of incubation was determined. The relative growth on the different media has been calculated with the formula:

$$\left[\left(\frac{FW_{20} \text{ on selective medium}}{FW_0 \text{ on selective medium}} - 1\right) \div \left(\frac{FW_{20} \text{ on basal medium}}{FW_0 \text{ on basal medium}} - 1\right)\right] \times 100$$

with FW_0, initial fresh weight; FW_{20}, fresh weight at day 20. *35S-bar*-Transformed calli and untransformed calli (regenerated from protoplasts) were transferred to basal medium without selection or with different concentrations of phosphinothricin and the relative growth rate was determined with the same formula.

DISCUSSION

A variety of techniques have been developed to introduce DNA into cereals. In most cases, the resulting transformants have been selected using the neomycin phosphotransferase II gene transcriptionally driven by either the nopaline synthase or CaMV 35S promoters (7, 10, 17, 22, 29). Our results demonstrate that, at least in rice, the *2'* transcript promoter functions 3- to 10-fold better in transient expression assays than these more commonly used promoters. This means that constructs using this transcriptional control could provide more protection against selective agents during the initial stages of protoplast regeneration and callus growth, and thus, under severe selection régimes, permit more transformants to be recovered.

Rice and other grasses (10, 22) have a high tolerance to kanamycin. Chimeric *npt*-II genes can, nevertheless, be used to select transformants by using stringent selection conditions (200 mg/L kanamycin) 1 week after protoplast electroporation. Once microcalli are greater than 500 μm in diameter, the largest survivors may be transferred to 100 mg/L G418 which is more effective at inhibiting the growth of nontransformed material.

Aminoglycoside antibiotics are not the only choice for selecting transformed rice. Our results with the *bar* gene have shown that the transformation frequency with a phosphinothricin selection on electroporated protoplasts approximately equals the number of transformants obtained with kanamycin selection. In addition, the sensitivity of rice to hygromycin and bleomycin suggests the hygromycin phosphotransferase (32) and bleomycin resistance (13) genes will also be suitable for distinguishing transformed from untransformed rice cells. The hygromycin resistance genes have been shown to permit *Triticum monococcum* transformants to be selected using 100 to 300 mg/L of the antibiotic (10), while the bleomycin resistance gene described by Hille *et al.* (13) permits *Nicotiana plumbaginifolia* to grow on 10 mg/L bleomycin which is in the range of the dose needed to kill rice cells. In order to obtain the maximum number of transformants, it is important to optimize DNA concentration and to use selection conditions that employ the lowest level of inhibitor needed to prevent growth of untransformed tissue. By using higher concentrations of selective agent, we presume that transformants containing a higher gene copy number are recovered preferentially, especially when chimeric genes with low transcription levels are used. With our experimental conditions, three out of seven clones contained a single copy of the gene.

Although it may be advantageous to have transformants containing single copies of foreign DNA in order to correlate a particular phenotype with gene dosage, our results indicate that these transformants must be carefully analyzed by Southern analysis. All the cases where single copies of the plasmids were integrated contained aberrant fragments, and two independently isolated pGSFR280 clones lacked the nonselected *npt*-II gene. This result is consistent with previous reports. For example, Potrykus *et al.* (22) reported that for two genes on the same plasmid, the nonselectable gene was lost in approx-

imately 50% of the transformants. This means that as long as one depends on techniques such as direct gene transfer to transform cells, decreasing the copy number may inadvertently decrease the chances that a nonselected gene will be expressed.

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