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Transgenic rice cell lines and plants: expression of transferred chimeric genes

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Abstract

Cell suspension-derived rice (*Oryza sativa* L.) protoplasts were transformed by direct gene uptake. PEG-mediated transformation was more efficient than electroporation. Plasmid DNA containing a hygromycin phosphotransferase (HPT) gene (which confers hygromycin resistance) driven by the CaMV 35S promoter and a β-D-glucuronidase (GUS) gene under control of the 1′, 2′ double promoter of the mannopine synthase (*mas*) locus of *Agrobacterium tumefaciens* was introduced into rice protoplasts. Southern analysis of DNA from transformed cell lines showed that the HPT and GUS genes were present intact. Both genes were expressed in transgenic cell suspensions. GUS activity was detected by histochemical staining of the cells and by enzyme assays. During a 12-day culture period the proportion of stained cells rose to a maximum and then decreased again. Considerably higher numbers of blue-stained cells were obtained when the transgenic cell lines were grown in the presence of 5-azacytidine. Transcripts of the GUS gene could not be detected, in contrast with the HPT gene. Plantlets were regenerated from one transgenic cell line. GUS activity was found in both leaf and root tissues of these plants, particularly, but not exclusively, in vascular bundles.

A mouse dihydrofolate reductase coding sequence (DHFR), conferring methotrexate resistance, fused to the CaMV 35S promotor and the wild-type nopaline synthase (NOS) gene of *A. tumefaciens* were also introduced into rice protoplasts. Stable integration of both genes was confirmed by Southern analysis. Expression of the DHFR gene was demonstrated by high levels of resistance to methotrexate of the transgenic cell suspensions and by the presence of DHFR transcripts. Expression of the NOS gene at enzyme or RNA level was not detected. Southern analysis suggests that this gene was probably either methylated or scrambled in these lines.

Introduction

Transformation of *japonica* rice via direct DNA uptake by protoplasts and the subsequent regeneration of transgenic plants has been reported by a number of authors [31, 33, 38, 39]. As selectable marker systems the neomycin phosphotransferase II gene (*nptII*) [38, 33], coding for kanamycin or G418 resistance, and the hygromycin phosphotransferase (HPT) gene, coding for hygromycin resistance [31, 39], were used; both genes are driven by the CaMV 35S promoter.

Uchimiya *et al.* [34] introduced an *nptII* gene under the control of the promoter of the *Agrobacterium tumefaciens* nopaline synthase (NOS) gene into rice protoplasts and obtained kanamycin-resistant transgenic callus. Zhang and Wu [39] reported the production of transgenic rice plants from transformed protoplasts without the use of a selectable marker. Only Shimamoto *et al.* [31] presented molecular evidence for the genetic transmission of the introduced genes to the progeny. They also used the β-D-glucuronidase (GUS) gene under the control of the CaMV 35S promoter as reporter gene and showed it to be active in endosperm and embryo tissue of rice seeds [31]. Zhang and Wu [39] reported the introduction of the GUS gene under the control of regulatory sequences from a maize alcohol dehydrogenase gene into rice protoplasts. These authors observed expression of this gene in transgenic rice callus and root tissue.

We have found the *nptII* system to be unsatisfactory for rice protoplast transformation. Similar observations were made Shimamoto *et al.* [31]. Methotrexate resistance, conferred by an altered mouse dihydrofolate reductase (DHFR) coding sequence under control of a suitable promoter, could be an alternative second selectable marker. It has been used to transform *Petunia hybrida* [6], *Brassica napus* [29] and to produce transgenic *Panicum maximum* (a graminaceous species) callus [8]. Dekeyser *et al.* [5] used the DHFR gene as a second (unselected) marker in rice protoplast transformation experiments but found no expression of this gene in transgenic rice callus.

The 1′, 2′ dual promoter of the mannopine synthase (*mas*) locus of *A. tumefaciens* was shown to be an effective promoter in transient expression assays with rice protoplasts [5]. It has been studied in detail in transgenic tobacco plants, in which it was found to be wound-inducible [17]. In addition, the activity of the MAS promoter appeared to be regulated by plant growth regulators [17].

Now we report on the production (through direct DNA uptake by protoplasts) of transgenic rice cell suspensions containing either the mouse DHFR gene and the wild-type NOS gene or the HPT gene in combination with a *GUS* gene driven by the MAS 1′, 2′, double promoter. The *GUS* gene and the NOS gene were used as reporter genes. In addition, data on the expression of the introduced genes in transgenic cell lines and of the chimeric GUS gene in transformed rice plantlets are presented.

Materials and methods

Plasmids

Plasmid DNA was isolated by the alkaline lysis method according to Birnboim [3] followed by two subsequent CsCl gradient centrifugation steps.

Construct HH271 (Fig. 1; Hensgens *et al.*, in preparation) containing the HPT gene and the GUS reading frame was made in pIC plasmids [20]. The *HPT* (hygromycin phosphotransferase) gene under control of the 35S CaMV promoter and the nopaline synthase polyadenylation signal were cloned from pMOGEN 24 (a gift from Dr P.J.M. Van der Elzen, MOGEN Int., Leiden). The GUS reading frame was taken from pBI101.2 [12, 13] as a *Bam* HI-*Sac* I fragment. The *Sac* I site was blunted by S_1 nuclease treatment. As promoter a 521 bp Klenow filled in *Nla* IV-*Cla* I gene 1′/2′ promoter fragment from pRAL3267 ([2], sequence 20649–20128) was fused. As polyadenylation signal a 749 bp long *Pst* I/*Nla* IV fragment (T-DNA sequence 22456–21708), which spans the region 23 bp downstream of the TGA of gene 0′ and the last 194 nucleotides of the gene 1′ reading frame, was used. Fragments were subcloned individually in different sites of several pIC plasmids [20] and ultimately assembled into pHH271.

Plasmid pMON806 (Fig. 1; a gift from Dr R. Fraley, Monsanto Co., St. Louis, MO) contains a mutant mouse DHFR gene conferring methotrexate resistance) under control of the 35S CaMV promoter and nopaline synthase NOS termination sequences. It also contains the NOS gene itself and the right border of the nopaline T-DNA. Between the two genes the bacterial gene coding for streptomycin resistance is present.

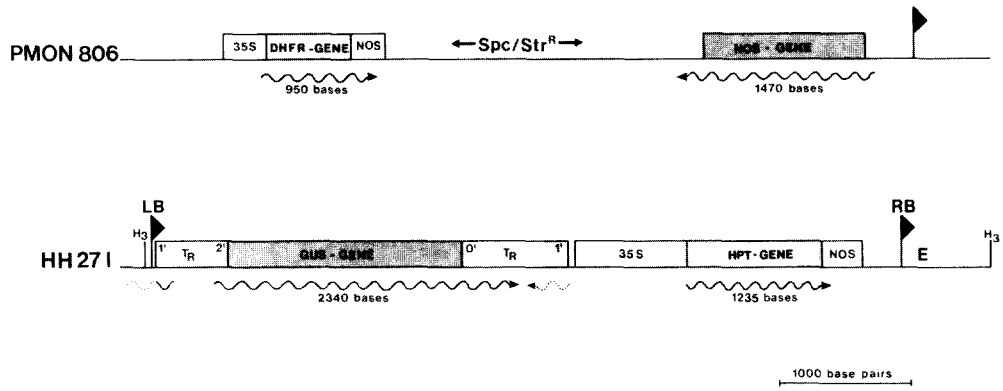


Fig. 1. Plasmids used for the transformation of rice. pMON806 contains the mutated mouse dihydrofolatereductase (DHFR) gene (conferring methotrexate resistance) under control of the 35S CaMV promoter and nopaline synthase (NOS) termination sequences. It also contains the NOS gene itself and the right border of the nopaline T-DNA. Between the two genes the bacterial gene coding for streptomycin resistance is present. pHH271 was made at the Dept. of Plant Molecular Biology, Leiden University. It contains the hygromycin phosphotransferase (HPT) gene under control of the 35S CaMV promoter and NOS termination sequence, and the reporter gene coding for β-D-glucuronidase (GUS), under control of the promoter of gene 2′ and termination sequence of gene 0′. Gene 2′, 0′ and 1′ are from the TR-DNA of the octopine plasmid pTiAch5. Reading frames and regulatory sequences are indicated by boxes. Direction of transcription and size of transcripts are indicated by the wavy lines. Triangles indicate border sequences involved in the transfer of T-DNA from *Agrobacterium* to plant cells. E indicates sequence which enhances this transfer [27].

Cell culture and transformation

Cell suspensions of *Oryza sativa* (Japonica type cv. Taipei 309) were initiated from scutellum-derived callus and maintained in AA2 medium [23, 32] according to Thompson *et al.* [32]. Serial subculture was carried out weekly by adding 3 volumes of fresh medium to one volume of cell culture. Protoplasts from cell lines T4 or T5, which had been in culture for 10–18 months, were isolated 4 days after transfer and cultured as described [1, 32] with the following modifications: purification was through 31 and 22 μm mesh sieves and in some experiments protoplast isolation was overnight in a 1 : 1 mixture of enzyme solution [1, 32] and KpR culture medium [14, 32]. Casein hydrolysate and coconut water were omitted from the KpR medium in all experiments. Protoplasts were washed twice in W5 salt solution [24] and suspended in transformation buffer (150 mM NaCl, 5 mM $CaCl_2$, 0.75 mM Na_2HPO_4, 5 mM KCl, 0.3 M mannitol, pH 7.1) at a density of 2×10^6 protoplasts/ml in 1 ml aliquots. Supercoiled circular DNA of plasmids pHH271 (Fig. 1) or pMON806 (Fig. 1) was added (final concentration 50 μg/ml) and mixed. Then 0.5 ml 40% polyethylene glycol 6000 (PEG) in transformation buffer was added. After 30 min 8.5 ml W5 solution was added slowly, followed by centrifugation (5 min, 80 *g*). This is essentially a modification of the procedure of Krens *et al.* [15]. After transformation the protoplasts were suspended (3×10^5 protoplasts/ml) in KpR medium solidified with 1.2% SeaPlaque low-melting agarose and cultured in the dark at 26–27 °C [cf. 1, 32]. Alternatively, protoplasts were electroporated prior to a PEG treatment of 15–20 min. A rectangular pulse (5–20 ms, 400 V/cm, 1500 μF) from stainless steel electrodes (d = 2 mm) was applied to 0.5 ml protoplast suspension in 1 ml disposable cuvettes. After PEG treatment the electroporated protoplasts were treated as described above. Preliminary transformation experiments with the low salt $MgCl_2$ buffer [24] were unsuccessful due to poor protoplast survival and were therefore abandoned.

After 15 days agarose blocks were transferred to liquid KpR medium containing 0.3 M glucose and 100 μg/ml hygromycin (pHH271) or 0.5 μg/ml methotrexate (pMON806). The

medium was refreshed at weekly intervals and the concentrations of hygromycin and methotrexate were increased stepwise to 200 μg/ml and 2.5 μg/ml respectively. Approximately 5–8 weeks after transformation resistant calli were transferred to N6 medium [4] containing 60 g/l sucrose, 2 mg/l, 2,4-D, 6 g/l Sigma agarose type I (N6d) and 200 μg/ml hygromycin or 2.5 μg/ml methotrexate. The calli were subcultured at weekly intervals. From individual putative transformants cell suspensions were initiated by transferring callus to liquid AA2 medium containing the appropriate selective agent. The resulting cell lines were used for DNA/RNA analysis, determinations of GUS activity and plant regeneration. Transformed callus and the corresponding cell lines derived from line T4 were designated 4H (hygromycin) or 4M (methotrexate) and each independent transformant was numbered (e.g., 4H1, 4H2 etc. represent lines obtained from individual hygromycin-resistant transformants). A similar coding system was used for T5-derived transformed cell lines. During the course of regeneration experiments with the transgenic line 5H2 two new 'protoclonal' cell lines, 5H2A and 5H2B, were produced from two 5H2 protoplast-derived calli.

In order to examine a possible role of DNA methylation in GUS gene expression 30 μM 5-AZA (5-azacytidine) (as a filter-sterilized solution) was added to a number of transformed cell lines. Higher concentrations were toxic and led to reduced growth rates.

Plant regeneration

Protoplasts from hygromycin- and methotrexate-resistant cell lines were isolated and cultured and the resulting drug-resistant calli were used for regeneration experiments in which published protocols [1, 16, 20, 31, 35] and combinations thereof were evaluated. Only line 5H2A (as well as its 'parental' line 5H2) was regenerable.

Protoplast-derived 5H2A calli were cultured on N6d containing 200 μg/ml hygromycin in the dark for 4–8 weeks, followed by culture on N6 with 60 g/l sucrose, 2 mg/l kinetin and 7 g/l agarose in a 12 h photoperiod for 10 days and finally culture on growth regulator free N6 containing 60 g/l sucrose and 6 g/l agarose in a 12 h photoperiod. Plantlets were cultured on N6 with 30 g/l sucrose and 4 g/l agarose for further development and eventually transferred to a hydroponic system.

Glucuronidase and nopaline synthase assays

GUS activity in callus tissue, cell suspensions, leaf tissue and root tissue was determined histochemically (without embedding and sectioning) with 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) and enzymatically (in cell suspensions only) with p-nitrophenol-β-D-glucuronide (PNPG) as substrate essentially as described by Jefferson [11] and Rueb and Hensgens [30]. PNPG reactions were stopped after 2 and 4 h.

Nopaline synthase activity in pMON806-transformed cell lines was assayed according to Otten and Schilperoort [25] using 250 mg cells (fresh weight). Reactions were stopped after 3 and 20 h.

DNA and RNA isolation

DNA/RNA was isolated from 3–5 g cell suspension according to Hensgens and van Os-Ruygrok [9]. Cells were harvested by vacuum filtration on a Büchner funnel and immediately frozen with liquid nitrogen. 5 g cells were ground in a pre-cooled mortar and transferred to an Erlenmeyer flask. Ten ml of 50 mM Tris-HCl, 50 mM EDTA, 500 mM NaCl (pH 8.5) and 10 mM β-mercaptoethanol were added to the partly frozen powder. After thawing of the material, 10 ml of 6% (v/v) 2-butanol, 1% (w/v) tri-isopropylnaphtalene disulfonic acid disodium salt, 2% (w/v) 4-amino-2 hydroxybenzoic acid sodium salt and 4% (w/v) SDS were added and gently mixed for 2 minutes. Proteins were removed by extraction with 20 ml phenol equilibrated with 1 M Tris-HCl (pH 8.5). Extraction was repeated twice. LiCl was added to the clear DNA/RNA supernatants to a final concentration of 2 M.

RNA was precipitated overnight at 4 °C. After centrifugation at 12500 rpm the RNA pellet was washed several times with 70% ethanol, dried and taken up in 50 mM Tris, 10 mM EDTA (pH 8.0) and 0.1% SDS. To the LiCl supernatant, 1 g/ml CsCl was added followed by 0.1 ml of a 5 mg/ml ethidium bromide solution per ml supernatant. After ultracentrifugation, in a Beckmann Type 65 rotor at 45000 rpm for 42 h, DNA bands were recovered with syringes. Ethidium bromide was removed by extraction with CsCl-saturated isoamyl alcohol and dialyzed extensively against 10 mM Tris-HCl, 0.5 mM EDTA (pH 8.5). DNA from methotrexate-resistant pMON806 transformed cells was isolated according to Mettler [22] since DNA isolated from these cells stayed on top of the CsCl gradients.

DNA and RNA analysis

Five μg DNA was digested with *Xba* I or *Cla* I according to the conditions described by the manufacturer, electrophoresed on 0.7% agarose gels in 1 × TBE [19], transferred to GeneScreen Plus membranes as described [19]. DNA was fixed by UV cross-linking and hybridized with nick-translated probes for 40–50 h at 42 °C in 50% formamide, 5 × SSC and 5% SDS. Filters were washed in 2 × SSC, 0.5% SDS at 50 °C with four subsequent changes of fluid. A final wash was preformed at 63 °C for 30 minutes with 0.1 × SSC and 0.5% SDS. Exposure was at − 80 °C for 48 hours using an intensifying screen and Fuji RX film.

Total RNA (5 μg) was separated on a 1.25% non-denaturating agarose gel in 1 × TBE. After electrophoresis, RNA was stained by washing the gel in 50 mM NaH_2PO_4, 5 mM EDTA (pH 6.5) containing 0.1 μg/ml ethidium bromide. After photography of the gels, RNA was transferred onto GeneScreen Plus membranes by capillary blotting using 50 mM NaH_2PO_4 and 5 mM EDTA (pH 6.5) as blotting buffer. After transfer, membranes were washed, dried and baked for 2 hours at 80 °C. Hybridization and washing conditions were as described for Southern analysis.

Results

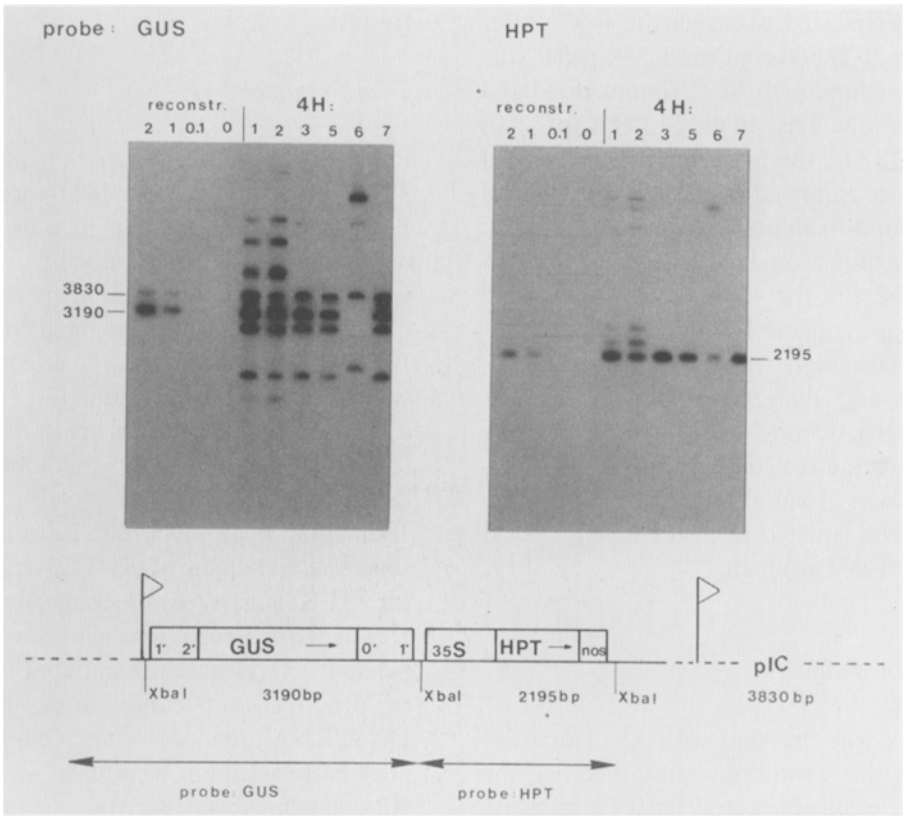
Transformation

PEG-mediated transformation usually yielded 20–25 transformants per 10^6 treated protoplasts. Electroporation gave rise to only 0–3 transformants per 10^6 protoplasts. When PEG treatment was performed after electroporation the frequency increased but remained lower than with PEG treatment only. Early selection (15 days) was essential as late selection (30 days after transformation) could lead to up to 40% non-transformed hygromycin- or methotrexate-resistant calli; non-transformed calli growing on 1000 μg/ml hygromycin can be obtained with late selection. Screening of pHH271-transformed calli for GUS activity by histochemical procedures was negative, except for callus line 5H2. Cell suspensions were therefore initiated from a number of drug-resistant callus lines for GUS and DNA/RNA analysis. Only cell lines obtained after early selection were used for these studies. All these lines were resistant to the highest concentrations of selective agent tried (1000 μg/ml hygromycin or 20 μg/ml methotrexate).

DNA/RNA analysis

Southern blot analysis of total DNA showed that in all suspensions analyzed, except 4M5, the resistance gene was detected as a fragment of the expected size (Figs. 2 and 3). This shows that no false-positive 'transformants' escaped the (early) selection procedure. It can be estimated from the intensity of the 2195 bp band that the 4H cell lines contain 1–5 copies of the HPT gene per diploid complement. No scrambling of the HPT gene was detected for lines 4H3, 4H5, 4H7, 5H2A and 5H2B (Figs. 2 and 9). Several high molecular weight bands, however, were found in lines 4H1, 4H2 and 4H6 (Fig. 2). Such bands could be due to scrambling or to methylation of the introduced genes.

In the methotrexate-resistant suspensions the number of introduced DHFR genes was esti-



Figs. 2 and 3. Southern blot analysis of transgenic rice cell suspensions derived by chemical transformation of *japonica* rice cv. Taipei 309 protoplasts with pHH271 or pMON806. For each cell line 5 μg total DNA was digested with *Xba* I or *Cla* I, separated, blotted onto GeneScreen Plus and hybridized.

Fig. 2. A pure hpt-gene probe and a mixed GUS/pIC plasmid probe were used for analysis of hygromycin resistant cell suspensions. Reconstr.: For pHH271 transformed suspensions, 5 μg DNA isolated from non-transformed T309 suspensions was digested with *Xba* I and mixed with *Xba* I digested pHH271 plasmid DNA derived from a *dam*$^-$ *E. coli* strain. 2, 1, 0.1 or 0 copies of pHH271 plasmid DNA were mixed per diploid T309 genome. pHH271 DNA used in the transformation of the rice cells was methylated by the *dam* methylation system of *E. coli* on the first two *Xba* I sites.

mated at 1–3 copies per diploid complement (Fig. 3). High molecular weight bands were also observed (Fig. 3), and these could have the same possible causes as comparable bands in the HPT transformed cell lines (see above). No hybridization was observed with DNA from non-transformed rice cells indicating that the DHFR probe does not detect the endogenous rice DHFR gene. The suspension line 4M5 did not display hybridization with an intact 1285 bp DHFR fragment. Since the probes used in the hybridizations with the 4M and 5M series share the NOS termination sequence, the NOS probe used in the left panel of Fig. 3 also detects the 1285 bp DHFR fragment. The relative strengths of the signals indicate, however, that the strongly hybridizing bands obtained with the DHFR probe in 4M5 (Fig. 3, right panel) are due to the presence of introduced DHFR sequences in this line.

With the GUS specific probe, 5 of the 6 4H lines analyzed displayed hybridization with the expected 3190 bp *Xba* I fragment (Fig. 2, left panel). Copy numbers are estimated to range from 2 to 4. Only line 4H6 lacks an intact GUS fragment. The GUS probe used contains pIC plasmid sequences and therefore also detects the 3830 bp pIC plasmid *Xba* I fragment. In addition, smaller fragments (bands of ca. 2000 and 2700 bp) were

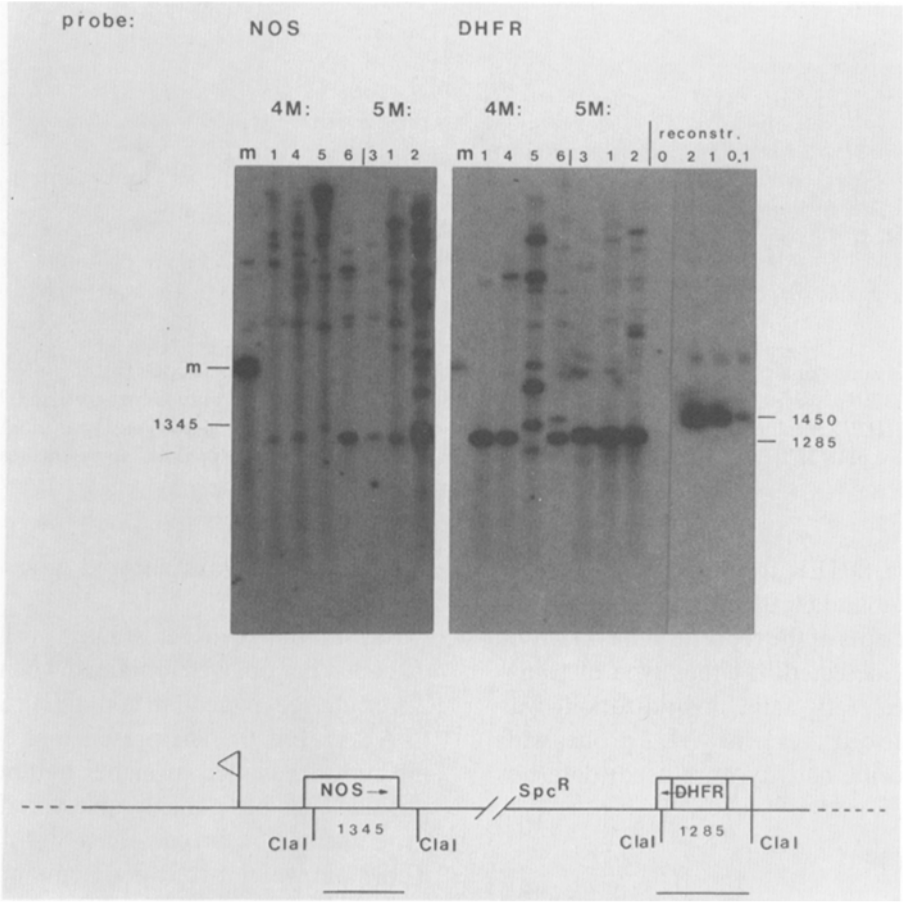
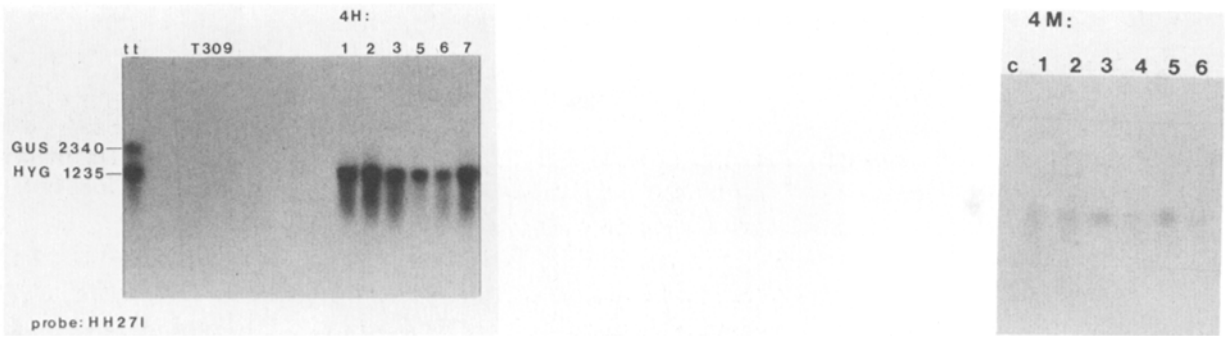


Fig. 3. The methotrexate-resistant lines were analyzed with a pure NOS-gene and a pure DHFR-gene probe (indicated by the horizontal bars). The two probes share the NOS termination sequences. Therefore the DHFR-gene probe also detects the NOS gene band although less efficiently. For pMON806-transformed lines, 5 μg control Taipei 309 was digested with *Cla* I and mixed with an isolated dhfr gene fragment (1450 bp). 2, 1, 0.1 and 0 copies per diploid T309 genome were mixed. Sizes of hybridizing bands are indicated in bp. m = marker.

detected with this probe in all lines except 4H6 (Fig. 2). This could indicate that plasmid sequences are integrated in the rice genome as repeats whereas multimerization has taken place in a rather sequence-specific manner.

The banding patterns observed for the NOS gene in the M lines were even more complex than those found for the GUS gene in the H lines (Fig. 3). A band corresponding to the 1345 bp NOS fragment was detected only in line 5M2. All M lines displayed a large number of high molecular weight bands with varying intensities (Fig. 3). Such bands could have been caused by similar factors as listed above for the HPT and DHFR genes.

Northern analysis (Fig. 4) of total RNA isolated from the H cell lines revealed the presence of an abundant 1235 nucleotides long HPT transcript of the same size as the HPT transcript in SR1 tobacco plants transformed with pHH271 (Fig. 4, lane TT) (L.A.M. Hensgens *et al.*, in preparation). The relative abundances in the different cell lines appear to parallel the estimated copy number of HPT genes in these lines (Figs. 2 and 4). Southern analysis indicated a lower copy number in lines 4H5 and 4H6 and also less HPT transcript is detected within these lines. This suggests that the observed steady-state concentration of this transcript is at least partly directed by the copy number of its gene. In the methotrexate-



Figs. 4 and 5. Transcript analysis of transgenic hygromycin-resistant (4H:) or methotrexate-resistant (4M:) rice cell suspensions. 5 μg total RNA was separated on a 1.25% non-denaturing agarose gel, blotted onto GeneScreen and hybridized with nick-translated pHH271 plasmid (Fig. 4) or with a pIC plasmid containing the dhfr gene (Fig. 5). tt, RNA from tobacco transformed with the pHH271. T309 and c, RNA from non-transformed Taipei 309 cell suspensions and plants respectively. Sizes of transcripts are indicated in nucleotides.

resistant lines a DHFR transcript was detected which was less abundant than the HPT transcript (Fig. 5). Transcripts of the reporter genes GUS or NOS were not detected in either type of transgenic cell line. Very faint hybridization was detected in some cell lines when GUS probes with a very high specific activity were used (data not shown).

Gus activity in pHH271-transformed cell lines

Figure 6 shows the patterns of histochemical staining for GUS activity in four 4H cell suspensions over a 12-day period during which the cells proliferated rapidly. The wells contained approximately equal amounts of cells. Cells from non-transformed cell suspensions were always negative in these GUS assays. Not every transgenic cell line with an intact GUS gene (as deduced by Southern analysis) showed blue staining. For example, no staining could be detected in line 4H2 (not shown), although Southern analysis showed a pattern identical to that of 4H1 (Fig. 2), a cell line with relatively high levels of GUS activity (Fig. 6).

The patterns observed during the 12-day period were similar in all cell lines: a gradual increase in the proportion of blue cells to a maximum followed by a decrease (Fig. 6). There were, however, reproducible differences between cell lines; e.g. line 4H1 always showed more blue-stained cells than 4H5.

Substantially more stained cells, over an extended period, were obtained when the 4H cells were cultured in medium containing 5-azacytidine (5-AZA) (Fig. 6). The presence of 5-AZA in the medium always led to higher numbers of GUS-positive cells but considerable variation between experiments occurred. This is illustrated in Table 1 in which two experiments with lines 4H1 and 5H2B are summarized. Exp. 2 with 4H1 in this table corresponds to Fig. 6. These quantitative data represent estimated counts based on microscopic observations which were, however, somewhat impeded by the presence of cell clumps. Unexpectedly, no correlation between the proportion of blue-stained cells (in the presence or absence of 5-AZA) and the GUS activity in protein extracts from the two cell lines studied was observed (Table 2).

Northern analysis of RNA isolated from cell suspensions 4H1 and 4H3 incubated with 5-AZA, and for which a substantial increase in GUS activity was observed histochemically, failed to show detectable gus transcripts in 4H1 (not shown). A faint band was detected in line 4H3 but the strength of the observed signal was not increased by the addition of 5-AZA (not shown).

Southern analysis of DNA isolated from the two 'protoclonal' transgenic cell lines 5H2A and

Table 1. Percentages of GUS-positive cells obtained in representative experiments with pHH271-transformed cell lines 4H1 and 5H2B. Cells were cultured in the presence (AZA) and absence (C) of 30 μM 5-azacytidine over a period of 12 days. No blue-stained cells were found in non-transformed cell lines.

Number of days in culture	GUS-positive cells (%)							
	4H1				5H2B			
	Exp. 1		Exp. 2		Exp. 1		Exp. 2	
	C	AZA	C	AZA	C	AZA	C	AZA
0	0	0	30	30	<1	<1	5	5
3	10	25	30	35	60	30	65	35
6	1	15	40	40	10	40	50	100
9	0	2	5	90	5	20	40	85
12	0	1	<1	75	1	10	20	45

Table 2. GUS activity with PNPG as substrate in protein extracts from two transgenic cell lines. Highest value obtained with a non-transformed control cell line T4 was 10 pmol/min per mg protein.

Cell line	5-AZA	Day	% positive cells	GUS activity (pmol/min per mg protein)
4H1	–	3	10	95
	+	3	25	120
	–	6	0	120
	+	6	15	120
5H2B	–	3	60	170
	+	3	30	30
	–	6	10	145
	+	6	40	245

5H2B showed identical patterns (Fig. 9). The proportion of blue-stained cells, however, differed considerably in the two lines (Fig. 6). Higher values were obtained consistently with 5H2B than with 5H2A. Line 5H2B also showed exceptionally high levels of gus activity in the absence of 5-azacytidine (Tables 1 and 2).

Expression of GUS in transgenic plants

GUS gene expression was examined in five 5H2A regenerants (Fig. 7) maintained *in vitro* which had reached a shoot height of 6–12 cm. Mature transgenic plants could not be studied as the 5H2A plants did not survive transfer to a hydroponic system in a growth chamber under conditions which are suitable for rice cultivation.

Intense staining was detected in leaf and root tissue of transgenic plantlets but never in non-transformed regenerants from other rice cell lines or in control seedlings. In 5H2A leaf tissue GUS activity was found to be localized in the vascular bundles and their transverse connections, in mesophyll cells or other non-vascular tissue, in trichomes and stomatal cells (Figs. 8A–E) as well as in epidermal cells located near the tip of the leaf (not shown). Staining often appeared to spread from the vascular bundles (Figs. 8B–D) and was particularly prominent near the cut surface of the

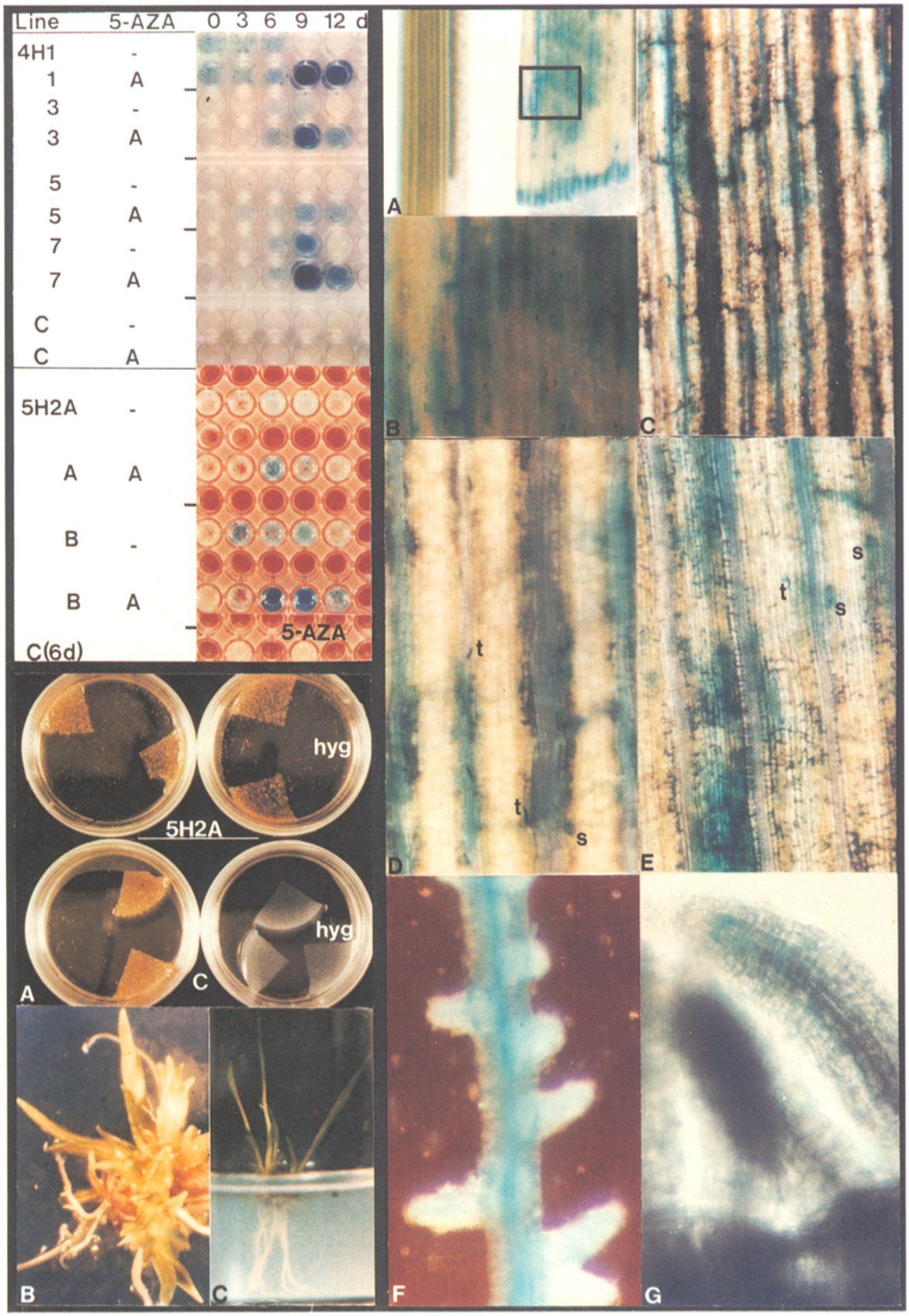


Fig. 6

Fig. 7

Fig. 8

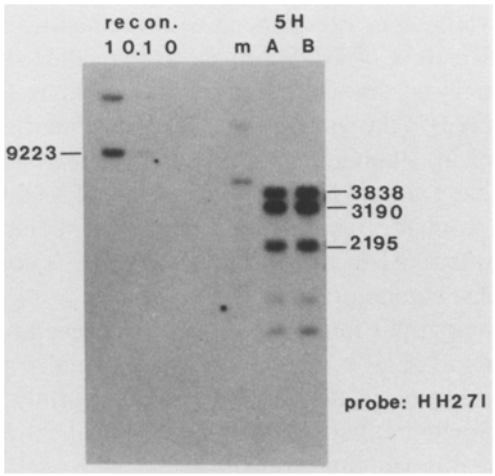


Fig. 9. Southern blot analysis of the protoclonal lines 5H2A and 5H2B. Probe was pHH27L. Five μg DNA was digested with *Xba* I. (See Fig. 2 for further details). 1 and 0.1 copy linearized pHH271 was mixed with DNA from a non-transformed Taipei 309 cell suspension and digested with *Xba* I. m = marker DNA.

leaf (Figs. 8A). GUS staining was also prominent in vascular bundles of roots (Fig. 8F). Expression could also be detected in other types of root tissue and in lateral roots (Fig. 8G). Exact cellular localization of GUS activity in root and leaf tissue, however, was not always possible with the histochemical methods employed in these studies.

Expression of the NOS gene in pMON806-transformed cell lines

Cell lines for which Southern analysis indicated the presence of the intact nopaline synthase gene (Fig. 3) were cultured in the presence and absence of 30 μM 5-AZA, in order to investigate whether the expression of this gene could be stimulated by 5-AZA. No nopaline synthase activity was detected, however, in cells grown in the presence (for 6 and 9 days) or absence of 5-AZA.

Discussion

Transformation and regeneration

Substantially higher [37, 39] or similar [31, 33] transformation frequencies of rice protoplasts with electroporation have been reported by others. Also, the transformation frequencies after PEG-mediated gene transfer in our study were similar to or lower than those reported by other workers [34, 37]. The extremely high transformation frequency (18%), based on dot blot assay) reported by Zhang and Wu [39] remains unexplained. Exact comparisons are difficult to make, however, because of the use of different transformation buffers and electroporation apparatus by the various authors. Moreover, calf thymus carrier DNA was used in all these experiments. We consider the use of carrier DNA less desirable as it can be co-transferred to plant protoplasts [26].

The protocol for regeneration of plants from 5H2A protoplasts is essentially a combination of two previously published methods [1, 16, 31]. Interestingly, the untransformed line T5, from which 5H2A originates, did not yield regenerants [21]. All cell lines had been cultured *in vitro* as callus, cell suspension and protoplasts for long periods and loss of regenerative potential is

Fig. 6. Pattern of histochemical gus staining in transgenic cell lines of rice over a 12-day period. C, control (non-transformed) cell line T4; A, medium contained 30 μM 5-azacytidine.

Fig. 7. Regeneration of plantlets from protoplasts isolated from transgenic cell line 5H2A. A, protoplast cultures after 30 days in medium with and without 200 μg/ml hygromycin; C, control line T5. B, shoot development after somatic embryogenesis in 5H2A cultures. C, 5H2B plantlet showing root and shoot system.

Fig. 8. Gus-staining in leaf (A–E) and root (F, G) tissue of 5H2A plantlets. A, left leaf from non-transformed seedling, right leaf from 5H2A plantlet; B, boxed area of A. Trichomes (t) and stomata (s) show GUS activity.

expected under such circumstances. The regenerative capacity of 5H2A can possibly be regarded as a form of somaclonal variation. The failure of these plants to develop to maturity could be due to genetic changes leading to disturbed developmental regulation.

Selectable markers

The observed steady-state concentrations of the HPT transcript are in the same range as in tobacco transformed with the same construct (Fig. 4, lane TT; L.A.M. Hensgens *et al.*, manuscript in preparation). This finding suggests that the combination of CaMV 35S promoter and NOS polyadenylation signal works equally well in rice and tobacco.

This report shows that methotrexate resistance can be used as a selectable marker system for rice protoplast transformation and that the mouse DHFR gene is functional in rice cells. Expression was confirmed by the presence of transcripts. In mammalian cells, however, other enzymes involved in the synthesis of nucleotides, such as thymidylate synthetase and serine-hydroxymethyl transferase, are also inhibited by methotrexate [10]. This could lead to an unbalanced nucleotide pool within the cells grown in the presence of methotrexate. Furthermore methotrexate was shown to be recombinogenic in yeast [28]. A disturbance of the nucleotide pool could have been the cause of the abnormal CsCl banding of DNA isolated from suspensions grown on methotrexate (data not shown) and the rather complex Southern patterns obtained with the methotrexate-resistant transformants. The physical properties of this DNA are currently under investigation. It can be concluded, however, that these complicating factors seem to make methotrexate resistance a less suitable selectable marker system than hygromycin resistance.

Reporter genes

The difficulties experienced with the histochemical detection of GUS activity at the callus stage (virtually all lines) and in cell suspensions (e.g. line 4H2) in the transformants suggest that (histochemical) staining for GUS may not always be a reliable method for early screening of putative (rice) transformants. Also, GUS activity could be demonstrated in extracts of cells which showed no blue staining (Table 2).

Comparable findings were reported recently by Harris *et al.* [7]. They found that clonal transgenic maize callus did not stain uniformly for GUS activity, but fluorimetric assays of extracts from non-staining transformed callus sectors showed considerable gus activity [7]. This finding is consistent with our observations (as shown in Table 2). Sectioning of the callus prior to staining did not overcome the presence of such non-staining sectors and it was therefore concluded that negative staining was not due to impermeability to the substrate [7]. Possibly certain cell types contain unknown inhibitors of the GUS staining reaction which are not active or not present in protein extracts.

The consistent presence of detectable GUS activity in the regenerated plantlets is interesting since regeneration took place without selection pressure. The possibility that the stability of the GUS protein is higher in the plantlets than in cultured cells cannot be excluded.

The stimulating effect of 5-AZA on histochemically detected GUS activity in the transgenic cell suspensions suggests that DNA methylation may affect the expression of this reporter gene. However, no indication for this was found in the Southern analysis. Furthermore, no GUS transcripts could be found in cells grown in the presence of 5-AZA. Recently it was shown that in *Daucus carota* cells the proportion of methylated cytosine increases 2.5-fold when the 2,4-D concentration in the culture medium is raised to 2 mg/l [18]. The rice cells were grown in medium containing 2 mg/l 2,4-D and it is possible that 5-AZA acts as an antagonist of 2,4-D in this respect, causing hypomethylation which in turn

affects gene expression. From our data it cannot be concluded, however, whether 5-AZA affects the transcription of the gus gene directly or indirectly. The possibility that 5-AZA influences other factors controlling the staining process cannot be excluded either. The exact nature of the observed effects of 5-AZA remains therefore to be elucidated.

Figure 6 and Table 1 clearly demonstrate that the moment of screening for GUS is important, particularly in the absence of 5-AZA, and that 'false negatives' are easily obtained (cf. [7]). The consequence of these findings is that caution is required in the interpretation of data on the expression of reporter genes. The data clearly demonstrate, however, that the 1′, 2′ MAS promotor of *A. tumefaciens* can be functional in cells of a graminaceous monocot. The failure to detect GUS transcripts, despite the presence of GUS activity, suggests that GUS mRNA is rather unstable in cultured rice cells. This is in contrast to tobacco, transformed with the same construct, in which high steady-state levels of the GUS transcript were found (Fig. 4, lane TT). The histochemical data suggest, however, that the chimeric GUS gene was expressed in a wide variety of rice cell types. The prominent staining of vascular bundles near the cut end of the leaves (Fig. 8A) suggests that the degree of substrate uptake by different cell types appears to affect the observed GUS staining. Caution in the interpretation of histochemical data seems therefore necessary.

Our results suggest that the NOS gene may not be very active in rice cell suspensions. The NOS promoter seems to be functional, however, in combination with the nptII gene in kanamycin-resistant transgenic rice callus [34].

Conclusions

- Chimeric hygromycin and methotrexate resistance genes are expressed phenotypically and at the RNA level in transgenic rice cells.
- The MAS promoter of *Agrobacterium tumefaciens* is functional in transgenic rice cells suspensions and in a wide variety of cell types in transformed rice plants.
- β-glucuronidase can be used as reporter gene in rice but screening of transformants by histochemical methods may be unreliable.

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