Plant Cell Reports (1997) 16: 363–367

S. Yokoi · T. Tsuchiya · K. Toriyama · K. Hinata

Tapetum-specific expression of the *Osg6B* promoter-β-glucuronidase gene in transgenic rice

Received: 18 March 1996 / Revision received: 30 May 1996 / Accepted: 21 August 1996

Abstract The promoter of an anther tapetum-specific gene, *Osg6B*, was fused to a β-glucuronidase (*GUS*) gene and introduced into rice by *Agrobacterium*-mediated gene transfer. Fluorometric and histochemical GUS assay showed that GUS was expressed exclusively within the tapetum of anthers from the uninucleate microspore stage (7 days before anthesis) to the tricellular pollen stage (3 days before anthesis). This is the first demonstration of an anther-specific promoter directing tapetum-specific expression in rice.

Key words *Agrobacterium* · Tapetum-specific promoter · Transformation · Rice

Abbreviations *GUS* β-Glucuronidase

Introduction

Rice transformation is not easy and rice genes have sometimes been analyzed in tobacco, which is readily transformed (Leisy et al. 1989; Yamaguchi-Shinozaki et al. 1990; Kano-Murakami et al. 1993). We have used tobacco to analyze the promoter activity of tapetum-specific genes isolated from rice and demonstrated that the *Osg6B* promoter directed β-glucoronidase (GUS) expression specifically in the tapetum of transgenic tobacco (Tsuchiya et al. 1994). However, the activity of the *Osg6B* promoter has not yet been demonstrated in transgenic rice. Recently, Hiei et al. (1994) reported a simple and efficient method for transformation of rice by *Agrobacterium*-mediated gene transfer.

Communicated by A. Komamine

S. Yokoi · T. Tsuchiya · K. Toriyama (✉) · K. Hinata
Laboratory of Plant Breeding, Faculty of Agriculture, Tohoku University, Aobaku, Sendai 981, Japan

In this report, we employed *Agrobacterium* for rice transformation and describe successful expression of the *Osg6B* promoter-GUS gene in the anther tapetum of transgenic rice plants.

Materials and methods

Construction of the chimeric gene

A chimeric *Osg6B* promoter-*GUS* gene fusion plasmid was constructed previously (Matsuda et al. 1996) and its essentials are illustrated in Fig. 1A. A binary vector, pIG121Hm, containing genes for GUS as well as for hygromycin resistance (Hiei et al. 1994), was also tested for optimizing the transformation conditions. The binary vectors were transferred to *Agrobacterium tumefaciens* strain EHA101 (Hood et al. 1986) using freeze-thaw methods (An et al. 1988).

Transformation

A cultivar of *japonica* rice [*Oryza sativa* (L.) Yamahoushi] was used for transformation by the method of Hiei et al. (1994) with some modifications. Media used for tissue culture and transformation are listed in Table 1. When calli were infected, a wide-mouth tube (4 cm high and 3 cm diameter) with a 30 μm mesh in the bottom was used for convenience with *Agrobacterium* infections and washings of calli. Hygromycin-resistant plants were transferred to soil in pots and grown in a greenhouse.

DNA gel blot analysis

Total genomic DNA was isolated from young leaves (1–2 g) by the CTAB method (Murray and Thompson 1980), 3 μg DNA from each sample was digested with *Bam*HI or *Eco*RI. After electrophoresis, the DNA was transferred onto a nylon membrane (Nytran NY13N; Schleicher & Schuell). An *Eco*RI fragment of the *Osg6B-GUS* gene fusion plasmid, which covers the entire GUS gene, was labeled with digoxigenin and was used as a probe. Hybridization and washing were performed according to the instruction manual of the DIG Luminescent Detection Kit (Boehringer Mannheim).

GUS assay

Fluorometric and histochemical GUS assays were performed essentially as described by Jefferson (1987). Soluble protein was extract-

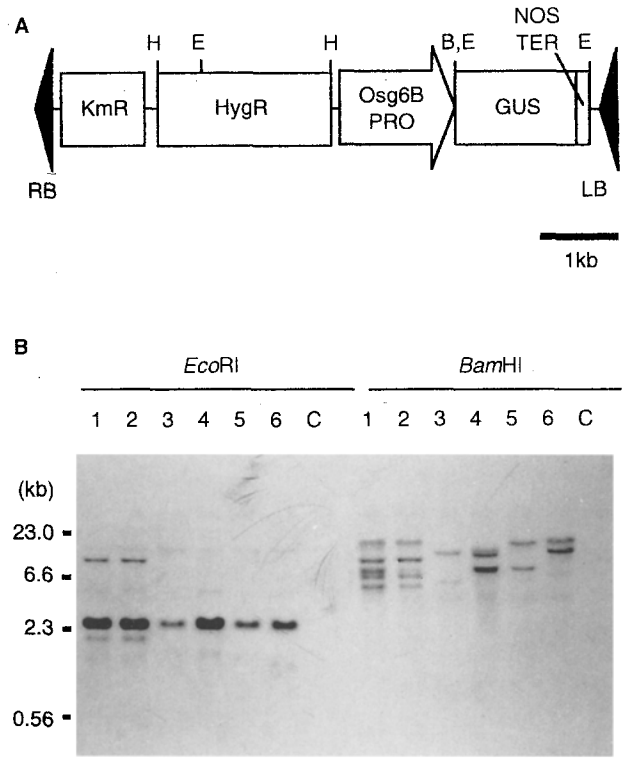

Fig. 1 A, B T-DNA region of the *Osg6B* promoter-β-glucuronidase GUS gene and Southern blot analysis. **A** Schematic diagram of a part of the T-DNA region of the transformation vector *Osg6B-GUS*. (*RB* right border, *LB* left border, *KmR* gene for kanamycin resistance, *HygR* gene for hygromycin resistance, *Osg6B PRO Osg6B* promoter, *GUS* β-glucuronidase, *NOS TER* terminator of nopaline synthase, *H Hin*dIII, *E Eco*RI, *B Bam*HI) **B** Southern blot analysis of six transgenic plants (lanes 1–6) and an untransformed plant (lane C). DNA was digested with *Eco*RI or *Bam*HI and allowed to hybridize with GUS probe. Molecular markers are indicated on the left

ed from leaves, roots, and anthers at different developmental stages for fluorometric GUS assay. The solution used for this purpose contained 50 mM sodium phosphate (pH 7.0), 10 mM EDTA, 0.1% Triton X-100, 0.1% sarcosyl, 10 mM 2-mercaptoethanol, and 20% methanol. The reaction was carried out using 4-methylumbelliferyl glucuronide, as detailed in Jefferson (1987).

Spikelet's tips were removed with scissors for histochemical GUS assay of spikelets and incubated in a solution containing 1 mM 5-bromo-4-chloro-3-indoryl–D-glucuronide, 50 mM sodium phosphate (pH 7.0), 10 mM EDTA, 0.1% Triton X-100, and 20% methanol. The reaction mixture was placed under a mild vacuum for a few minutes and then incubated for 8 h at 37°C. Spikelets were fixed in ethanol and acetic acid (3:1 vol/vol), dehydrated and embedded in paraffin by standard methods (Tsuchiya et al. 1994). They were sliced into 15-μm thick cross sections.

Results and discussion

Transformation of rice using *A. tumefaciens*

Successful tranformation of rice using *Agrobacterium* was recently reported by Hiei et al. (1994). Their transformation frequency of *japonica* rice was as high as that of dicots, and they demonstrated Mendelian transmission of the introduced DNA to the progeny.

The method in this study was essentially the same as that reported by Hiei et al. (1994). First, a binary vector, pIG121Hm, was used to optimize the transformation conditions. A transformation frequency of 26% was obtained using the media described in Table 1. GUS expression was observed from leaves of 36 of 37 hygromycin-resistant plants.

A total of 22 hygromycin-resistant plants were obtained when 144 calli were infected with EHA101 carrying the *Osg6B-GUS* gene using the conditions optimized with

Table 1 Media used in study (*2,4-D* 2,4-dichlorophenoxyacetic acid; *NAA* naphthaleneacetic acid; *BAP* 6-benzylaminopurine)

Medium	Composition
AB[a]	3 g/l K_2HPO_4, 1 g/l NaH_2PO_4, 1 g/l NH_4Cl, 0.3 g/l $MgSO_4 \cdot 7H_2O$, 0.15 g/l KCl, 0.01 g/l $CaCL_2$, 2.5 mg/l $FeSO_4 \cdot 7H_2O$, 5 g/l glucose, 15 g/l agar, pH 7.2
AA suspension	AA salts and amino acids, B5[b] vitamins, 20 g/l sucrose, 2 mg/l 2,4-D, 0.2 mg/l kinetin, 10 mg/l acetosyringone, pH 5.8
N6 callus induction (N6Cl)	N6[c] salts and vitamins, 30 g/l sucrose, 2 mg/l 2,4-D, 2 g/l gelrite, pH 5.8
N6 coculture (N6CO)	N6[c] salts and vitamins, 30 g/l sucrose, 10 g/l clucose, 2 mg/l 2,4-D, 10 mg/l acetosyringone, 2 g/l gelrite, pH 5.2
N6 selection (N6SE)	N6[c] salts and vitamins, 30 g/l sucrose, 2 mg/l 2,4-D, 2 g/l gelrite, 500 mg/l carbenicillin, 50 mg/l hygromycin, pH 5.8
MS regeneration (MSRE)	MS[d] salts and vitamins, 30 g/l sucrose, 30 g/l sorbitol, 2 g/l casamino acids, 1 mg/l NAA, 2 mg/l BAP, 250 mg/l carbenicillin, 50 mg/l hygromycin, 4 g/l gelrite, pH 5.8
MS horomone free (MSHF)	MS[d] salts and vitamins, 30 g/l sucrose, 50 mg/l hygromycin, 8 g/l agar, pH 5.8

[a] AB from Chilton et al. (1974)
[b] B5 from Gamborg et al. (1968)
[c] N6 from Chu et al. (1975)
[d] MS from Murashige and Skoog (1962)

pIG121Hm. All the plants exhibited a normal phenotype and grew to maturity. They had the same seed fertility as untransformed control plants.

Integration of the GUS gene in the genome of transgenic plants

To provide physical evidence for the integration of the *Osg6B-GUS* gene into the genome, Southern blot analysis was carried out on six hygromycin-resistant plants (Fig. 1B, lanes 1–6) and an untransformed control (Fig. 1B, lane C). *Eco*RI digestion, which was designed to give a 2.3-kb fragment of the GUS gene and NOS terminator, showed a band of 2.3 kb in all the transgenic plants tested (Fig. 1B, lanes 1–6). Some extra bands of unexpected size, which might represent the integration of part of the T-DNA, were also observed from two plants (Fig. 1B, lanes 1, 2). Since the *GUS* DNA probe only hybridized to DNA from transgenic plants but not to the DNA from untransformed control plants (Fig. 1B, lane C), the result indicated that the *GUS* DNA was integrated into the rice genome. *Bam*HI digestion was also carried out to obtain a unique fragment for each integrated copy, because there is only one *Bam*HI site in the T-DNA (Fig. 1B). The number and strength of bands produced by *Bam*HI indicated the copy number of the integrates, and was estimated to be one to five.

Osg6B promoter activity in transgenic rice

To investigate the spatial and temporal regulation of the *Osg6B* promoter in rice, a GUS assay was carried out from 22 hygromycin resistant plants into which the *Osg6B* promoter-GUS gene had been introduced. All plants exhibited GUS activity in immature anthers. No activity was detected in other tissues of spikelets, leaves, or roots (data not shown).

GUS activity in anthers at different developmental stages was examined in detail from five transgenic plants and an untransformed control plant by fluorometric assay. The activity was restricted to specific stages of anther development. At the meiotic stage taken from spikelets 10 days before anthesis, GUS activity was not detected (Fig. 2, stage A). GUS expression was first detected from anthers of two transgenic plants (no. 4 and 5) at the middle uninucleate microspore stage taken from spikelets 7 days before anthesis (Fig. 2, stage B). The most intense activity was observed at the stages between the early bicellular pollen stage (Fig. 2, stage D) and the late bicellular pollen stage (Fig. 2, stage F). High GUS activity was also observed in anthers at the early tricellular pollen stages 3 days before anthesis (Fig. 2, stage G). GUS activity suddenly disappeared in anthers 2 days before anthesis. In subsequent stages, no GUS activity was detected in anther tissue or mature pollen (Fig. 2, stage H). Anthers of an untransformed control plant did not exhibit GUS activity throughout the developmental stages.

A histochemical GUS assay showed that the activity was restricted to within the tapetum. Figure 3 shows the

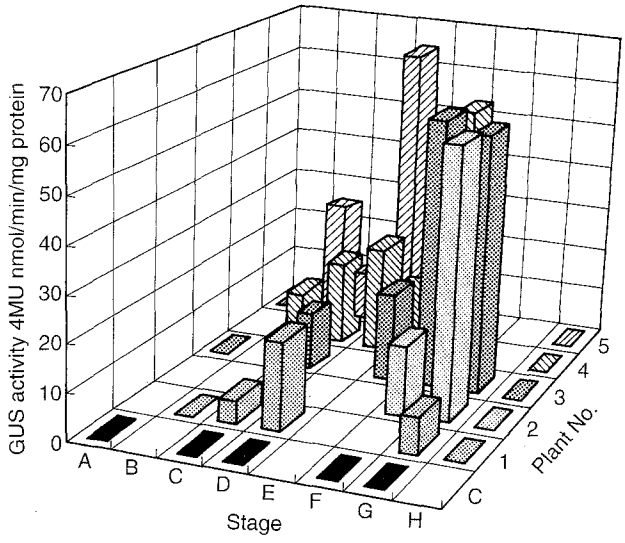

Fig. 2 Fluorometric analysis of GUS activity in anthers at different developmental stages from five transgenic plants (1 to 5) and an untransformed plant (C). Stages: *A* meiotic stage, *B* middle uninucleate microspore stage, *C* late uninucleate microspore stage, *D* early bicellular pollen stage, *E* middle bicellular pollen stage, *F* late bicellular pollen stage, *G* early tricellular pollen stage, *H* mature pollen stage

cross section of anthers at the middle bicellular pollen stage. Blue staining, GUS activity, was observed exclusively in the tapetal cells (Fig. 3A). Weak blue coloration was also found in the pollen grains of this figure, but this coloration was revealed to be an artifact, because washed pollen grains squeezed out of the anthers showed no GUS activity. Our results indicate that the *Osg6B* promoter is active only in tapetal cells. The tapetal cells started to degenerate at the bicellular pollen stage. GUS activity was obtained in the remnant of a thin layer of tapetum on inner anther walls until the tricellular pollen stage, 3 days before anthesis. Untransformed rice anthers did not exhibit detectable GUS activity (Fig. 3B).

Previous study of in situ hybridization demonstrated that the mRNAs of the *Osg6B* gene was localized within the tapetum in anthers at the uninucleate microspore stage (Tsuchiya et al. 1992, 1994). Northern blot analysis demonstrated that the *Osg6B* gene was expressed most strongly in anthers at the uninucleate microspore stage. The steady-state levels of the transcripts decreased slightly in anthers at the bicellular pollen stage and a few transcripts were also detected at the mature stage (Tsuchiya et al. 1992). The GUS activity results in this study are consistent with the expression pattern of the *Osg6B* gene, although the GUS activity in transgenic rice appeared about 1 day later than that of *Osg6B* mRNA. We presume that it might take a day before enough GUS protein has been synthesized from mRNA to become detectable.

The *Osg6B* promoter-GUS gene was previously introduced into tobacco (Tsuchiya et al. 1994). The *Osg6B* promoter was active in the tapetum of transgenic tobacco from the tetrad stage to the uninucleate microspore stage just before microspore mitosis (Tsuchiya et al. 1994). In contrast,

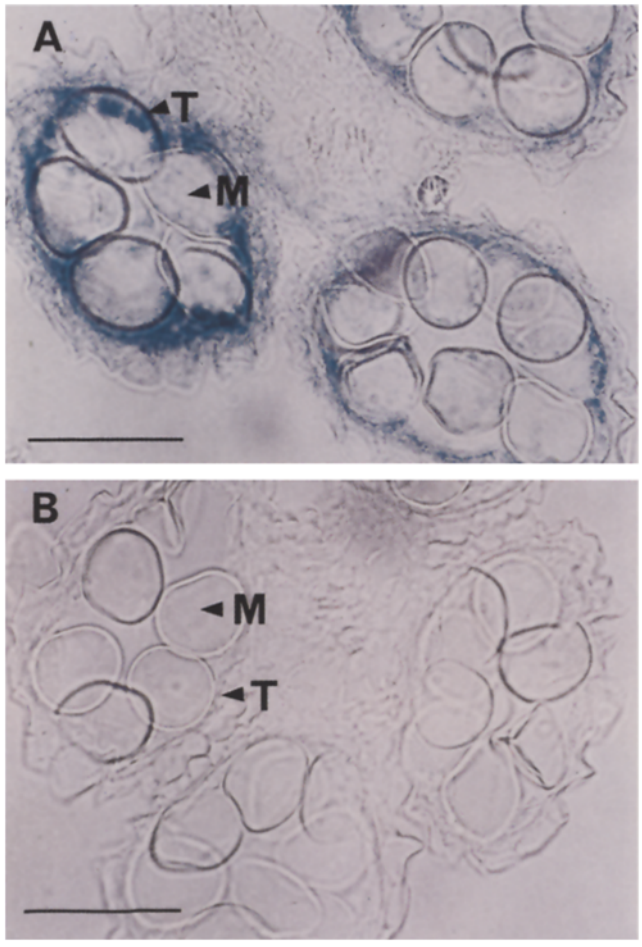

Fig. 3 Histochemical analysis of GUS activity in anthers at the middle bicellular pollen stage in transformed (**A**) and untransformed (**B**) rice. Blue staining indicates GUS activity (*M* microspore, *T* tapetum). *Bar*=50 μm

GUS activity of transgenic rice was not detected in anthers at the tetrad stage, while it was observed after microspore mitosis. Thus the expression pattern of the *GUS* gene in transgenic rice was different from that in transgenic tobacco. Such a different pattern of tissue-specific expression has been reported for other genes introduced into heterologous hosts (Sato et al. 1991; Thorsness et al. 1991; Toriyama et al. 1991; Xu et al. 1993). However, it is noteworthy that the 1.6-kb promoter region of the *Osg6B* gene is active in the tapetum of monocotyledonous species as well as in dicotyledonous species.

Some other anther-specific genes have been isolated from rice (Xu et al. 1993; Zou et al. 1994). To our knowledge, however, this is the first demonstration of an anther-specific promoter that directs tapetum-specific expression in rice. The *Osg6B* promoter has been successfully used to produce male-sterile tobacco, with the promoter fused to cDNA of endo-β-1,3-glucanase (Tsuchiya et al. 1995) or cDNA of phenylalanine ammonia-lyase (Matsuda et al. 1996). The *Osg6B* promoter might be useful for producing male-sterile rice and for studying male reproductive properties in rice.

Acknowledgements The authors wish to thank Dr. Hamid Rashid for the critical review of the manuscript and suggestions for improvement. This study was supported in part by Grants-in-Aid for Special Research on Priority Areas (no. 07281101, 07281102 and 07281103; Genetic Dissection of the Sexual Differentiation and Pollination Process in Higher Plants) from the Ministry of Education, Science, Culture and Sports, Japan, and by a grant pioneering research project in biotechnology from the Ministry of Agriculture, Forestry and Fisheries, Japan. S.Y. and T.T. are the recipients of JSPS Fellowships for Japanese Junior Scientists.

References

An G, Ebert PR, Mitra A, Ha SB (1988) Binary vectors. In: Gelvin SB, Schilpeoort RA (eds) Plant molecular biology manual. Kluwer, Dordrecht, pp 1–19

Chilton MD, Currier TC, Farrad SK, Bendich AJ, Gordon MP, Nester EW (1974) *Agrobacterium tumefaciens* DNA and PS8 bacteriophage DNA not detected in crown gall tumors. Proc Natl Acad Sci USA 71:3672–3676

Chu CC, Wang CC, Sun CS, Hsu C, Yin C, Chu CY, Bi FY (1975) Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. Sci Sin 18:659–668

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50: 151–158

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

Hood EE, Helmer GL, Fraly RT, Chilton MD (1986) The hypervirulence of *Agrobacterium tumefacience* A281 is encoded in a region of pTiBo542 outside of T-DNA. J Bacteriol 168:1291–1301

Jefferson RA (1987) Assaying chimeric genes in plants: the GUS gene fusion system. Plant Mol Biol Rep 5:387–405

Kano-Murakami Y, Yanai T, Tagiri A, Matsuoka M (1993) A rice homeotic gene, *OSH1*, causes unusual phenotypes in transgenic tobacco. FEBS Lett 334:365–368

Leisy DJ, Hnilo J, Zhao Y, Okita W (1989) Expression of a rice glutelin promoter in transgenic tobacco. Plant Mol Biol 14:41–50

Matsuda N, Tsuchiya T, Kishitani S, Tanaka Y, Toriyama K (1996) Partial male sterility in transgenic tobacco carrying antisense and sense *PAL* cDNA under the control of a tapetum-specific promoter. Plant Cell Physiol 37:215–222

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15: 473–497

Murray MG, Thompson WF (1980) Rapid isolation of high molecular weight plant DNA. Nucleic Acid Res 8:4321–4325

Sato T, Thorsness MK, Kandasamy MK, Nishio T, Hirai M, Nasrallah JB, Nasrallah ME (1991) Activity of an *S* locus gene promoter in pistils and anthers of transgenic *Brassica*. Plant Cell 3:867–876

Thorsness MK, Kandasamy MK, Nasrallah ME, Nasrallah JB (1991) A *Brassica S*-locus gene promoter targets toxic gene expression and cell death to the pistil and pollen of transgenic *Nicotiana*. Dev Biol 143:173–184

Toriyama K, Thorsness MK, Nasrallah JB, Nasrallah ME (1991) A *Brassica S*-locus gene promoter directs saprophytic expression in the anther tapetum of transgenic *Arabidopsis*. Dev Biol 143:427–431

Tsuchiya T, Toriyama K, Nasrallah ME, Ejiri S (1992) Isolation of genes abundantly expressed in rice anthers at the microspore stage. Plant Mol Biol 20:1189–1193

Tsuchiya T, Toriyama K, Ejiri S, Hinata K (1994) Molecular characterization of rice genes specifically expressed in the anther tapetum. Plant Mol Biol 26:1737–1746

Tsuchiya T, Toriyama K, Yoshikawa M, Ejiri S, Hinata K (1995) Tapetum-specific expression of the gene for an endo-β-1,3-glucanase causes male sterility in transgenic tobacco. Plant Cell Physiol 36:487–494

Xu H, Davis SP, Kwan BYH, O'Brien AP, Singh M, Knox RB (1993) Haploid and diploid expression of a *Brassica campestris* anther-specific gene promoter in Arabidopsis and tobacco. Mol Gen Genet 239:58–65

Yamaguchi-Shinozaki K, Mino M, Mundy J, Chua NH (1990) Analysis of an ABA-responsive rice gene promoter in transgenic tobacco. Plant Mol Biol 15:905–912

Zou JT, Zhan XY, Wu HM, Wang H, Cheung AY (1994) Characterization of a rice pollen-specific gene and its expression. Am J Bot 81:552–561