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

Tapetum-specific expression of the OsG6B promoter-β-glucuronidase gene in transgenic rice

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-glucuronidase (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.

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 *BamHI* or *EcoRI*. After electrophoresis, the DNA was transferred onto a nylon membrane (Nytran NY13N; Schleicher & Schuell). An *EcoRI* 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-
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).

Spikelets' tips were removed with scissors for histochemical GUS assay of spikelets and incubated in a solution containing 1 mM 5-bromo-4-chloro-3-indoly-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 transformation 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

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

<table>
<thead>
<tr>
<th>Medium</th>
<th>Composition</th>
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<tbody>
<tr>
<td>AB4</td>
<td>3 g/l K2HPO4, 1 g/l NaH2PO4, 1 g/l NH4Cl, 0.3 g/l MgSO4·7H2O, 0.15 g/l KCl, 0.01 g/l CaCl2, 2.5 mg/l FeSO4·7H2O, 5 g/l glucose, 15 g/l agar, pH 7.2</td>
</tr>
<tr>
<td>AA suspension</td>
<td>AA salts and amino acids, B5 vitamins, 20 g/l sucrose, 2 mg/l 2,4-D, 0.2 mg/l kinetin, 10 mg/l acetosyringone, pH 5.8</td>
</tr>
<tr>
<td>N6 callus induction (N6CI)</td>
<td>N6 salts and vitamins, 30 g/l sucrose, 2 mg/l 2,4-D, 2 g/l gelrite, pH 5.8</td>
</tr>
<tr>
<td>N6 coculture (N6CO)</td>
<td>N6 salts and vitamins, 30 g/l sucrose, 10 mg/l 2,4-D, 10 mg/l acetosyringone, 2 g/l gelrite, pH 5.2</td>
</tr>
<tr>
<td>N6 selection (N6SE)</td>
<td>N6 salts and vitamins, 30 g/l sucrose, 2 mg/l 2,4-D, 2 g/l gelrite, 50 mg/l carbenicillin, 50 mg/l hygromycin, pH 5.8</td>
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<tr>
<td>MS regeneration (MR기에)</td>
<td>MS 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</td>
</tr>
<tr>
<td>MS hormone free (MSHФ)</td>
<td>MS salts and vitamins, 30 g/l sucrose, 50 mg/l hygromycin, 8 g/l agar, pH 5.8</td>
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</table>

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)