Mol Gen Genet (1989) 220:12–16

MGG

Tissue-specific expression of the *rolC* promoter of the Ri plasmid in transgenic rice plants

Rikyu Matsuki, Haruko Onodera, Taeko Yamauchi, and Hirofumi Uchimiya
Institute of Biological Sciences, University of Tsukuba, Tsukuba, Ibaraki 305, Japan

Summary. Transgenic rice plants were obtained from protoplasts treated with two plasmids by electroporation. Primary transformants were selected on the basis of resistance to hygromycin, conferred by one of the co-transferred plasmids. Out of 26 hygromycin-resistant plants 2 showed the reporter gene activity due to another plasmid possessing a chimeric gene consisting of the promoter (about 900 bp upstream non-coding region) of the ORF12 gene (*rolC*) of the Ri plasmid and the coding region for β-glucuronidase (GUS). Using a colorimetric reaction, the GUS enzyme was found to be localized in vascular tissues, demonstrating the similar expression of the *rolC* gene promoter in monocots and dicots (Sugaya et al. 1989; Schmülling et al. 1989).

Key words: *Oryza sativa* L. – Ri plasmid – *rolC* promoter – Tissue-specific expression

Introduction

Most dicotyledonous plants infected with *Agrobacterium rhizogenes* harboring the Ri plasmid produce adventitious roots (Moore et al. 1979; White and Nester 1980). Whole plants can also be regenerated from hairy root cultures carrying the T-DNA of the Ri plasmid (David et al. 1984; Tepfer 1984). Two T-DNA regions, designated TR- and TL-DNA of Ri plasmid A4 have been determined (Huffman et al. 1984; Jouanin 1984; De Paolis et al. 1985). The entire nucleotide sequence of TL-DNA indicates the presence of 18 open reading frames (ORFs) (Slightom et al. 1986). In transgenic tobacco plants, the organ-specific expression of the *rolC* (ORF12) gene of TL-DNA of the Ri plasmid has been reported (Nakamura et al. 1988; Schmülling et al. 1988). We have also demonstrated the tissue-specific expression of a chimeric gene consisting of the promoter region of *rolC* and the bacterial β-glucuronidase (GUS) structural gene in transgenic tobacco plants (Sugaya et al. 1989). Here we present evidence of expression of a reporter gene under the control of the 5′-non-coding sequence of the Ri plasmid *rolC* gene in rice, a graminaceous monocot.

Materials and methods

Plant material. Suspension cells derived from callus of mature rice embryo (*Oryza sativa* L. var. Yamahoushi) were maintained in liquid AA medium (Toriyama and Hinata 1985). The culture was kept on a gyratory shaker (100 rpm) at 25° C and subcultured every 2 weeks.

Plasmids. A 3.1 kb *Eco*RI-*Hin*dIII fragment containing the promoter region (5′ upstream non-coding DNA) of ORF12 (*rolC*) of the Ri plasmid, the β-glucuronidase structural gene (GUS) (Jefferson et al. 1987) and the terminator of the nopaline synthase gene was isolated from pBI101-O12-p1 (Sugaya et al. 1989). The same fragment was inserted into the *Eco*RI/*Hin*dIII sites of pUC12. The resulting plasmid was named pUC12-O12-GUS (Fig. 1). Plasmid pCH was constructed from pUC12 and a chimeric gene comprising the cauliflower mosaic virus (CaMV) 35 S promoter, the coding region of hygromycin phosphotransferase (hph, Gritz and Davies 1983) and the terminator of the nopaline synthase gene (Fig. 1).

Electroporation and selection of transformants. Suspension cultured cells (4–5 days old) were incubated in an enzyme solution containing 2.0% Cellulase R10, 0.2% Macerozyme R10, 0.1% $CaCl_2 \cdot 2H_2O$, 6.5% mannitol, pH 5.8 for 2 h (Toriyama and Hinata 1985). Protoplasts were filtered through nylon mesh (30 μm), and centrifuged at 100 *g* for 5 min. Pellets were suspended in buffer A consisting of

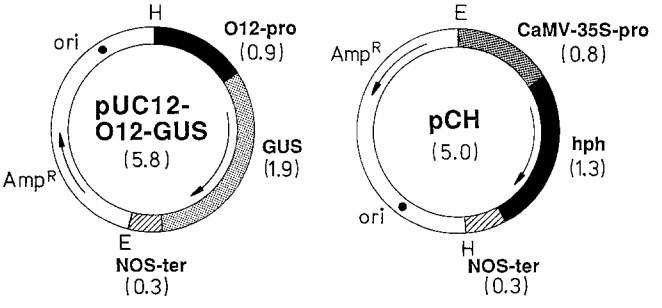

Fig. 1. Physical map of pUC12-O12-GUS and pCH. O12-pro, upstream non-coding region of ORF12 (*rolC*; Slightom et al. 1986). GUS, structural gene of bacterial β-glucuronidase (Jefferson et al. 1987); NOS-ter, terminator of the nopaline synthase gene (Bevan 1984). CaMV-35 S-pro: 35 S promoter of Cauliflower Mosaic Virus (Gardner et al. 1981); hph, the coding region of hygromycin B phosphotransferase (Gritz and Davies 1983). Amp^R, ampicillin resistant gene. E, *Eco*RI; H, *Hin*dIII. *Numerals* in *parentheses* indicate kb

Offprint requests to: H. Uchimiya

Table 1. Summary of DNA transformation experiments, resulting in the production of transgenic rice plants

Experiments	No. of Hm^r clones	No. of Hm^r clones producing plants	No. of Hm^r plants expressing GUS
1	495	22	1
2	310	4	1

In each experiment, 2.5×10^6 protoplasts were used. Hm^r, resistant to 30 μg/ml hygromycin. GUS, β-glucuronidase

Table 2. GUS activity in transgenic rice plants

Plants	Activity (4-MU pmol/min per mg protein)	
	Leaves	Roots
Control	3.4	7.9
Transformant 1	458.8	387.7
Transformant 2	1866.0	188.0

4-methyl-umbelliferyl-β-D-glucuronide was used as a substrate. 4-MU, 4-methyl-umbelliferon

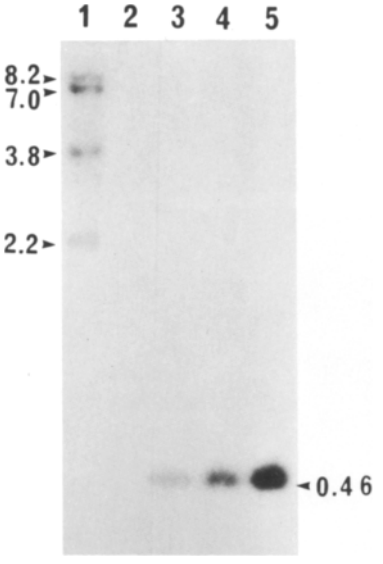

Fig. 2. An autoradiogram of DNA prepared from leaves of normal and transgenic rice plants. A 460 bp DNA fragment containing the 5′ upstream non-coding region of *rolC* was used as a probe. Lane 1, a transformant; Lane 2, a control plant; Lanes 3–5, ×1, ×2, and ×5 copy reconstruction of the *Eco*RI – *Hin*dIII fragment of pUC12-O12-p420 (details in materials and methods). Sizes of fragments indicate in kb

0.5 mM MES, pH 5.8, 7 mM KCl, 4 mM $CaCl_2 \cdot 2H_2O$, 0.35 M mannitol, and re-centrifuged. Protoplasts (1×10^6/ml) and the two plasmid DNAs (1 μg/ml each) were mixed in buffer A. *Hin*dIII-digested DNA (linear form) was used for experiments. A mixture of DNA and protoplasts in a plastic cuvette (inter-electrode distance, 0.4 cm) was subjected to an electrical pulse from a 220 μF capacitor charged at 375 V/cm (hand-made product; Toriyama et al. 1988). After electroporation, the same cuvette was kept on ice for 10 min. Protoplasts were then embedded in 1.2% agarose medium containing B5 salts (Gamborg et al. 1968), 2% sucrose, 5% glucose, 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D). After 2 weeks, agarose beads were transferred to liquid medium containing B5 salts, 2% sucrose, 2 mg/l 2,4-D, 50 μg/ml hygromycin B. After another month, growing calli were placed on medium containing N6 salts (Chu et al. 1975), 3% sucrose, 1 mg/l kinetin, 1.2% agarose to regenerate whole plants.

Analysis of DNA. DNA was prepared from leaves of rice plants according to Shure et al. (1983). DNA (5 μg) digested with restriction enzymes was run in a 1.0% agarose gel. After treatment of DNA with 0.4 N NaOH, denatured DNA was blotted onto a nitrocellulose filter, followed by hybridization with probe DNA which had been labeled with [^{32}P]dCTP using the Multiprime DNA Labeling System (Amersham). Probe DNA was prepared as follows: a 420 bp *Pvu*II-*Hpa*I fragment of pUC12-O12-GUS carrying the 5′ upstream non-coding region of the *rolC* gene (Slightom et al. 1986; Fig. 1) was cloned into the *Sma*I site of pUC12, giving pUC12-O12-p420. A 460 bp *Eco*RI – *Hin*dIII fragment of pUC12-O12-p420 was used as a probe for hybridization.

GUS assay. Spectrophotometric and histochemical analysis of GUS was done according to the method of Jefferson et al. (1987).

Results and discussion

Establishment of transformants

We attempted to transform rice protoplasts with two plasmids, pCH and pUC12-O12-GUS. We were able to obtain several hundred hygromycin-resistant calli, from which 26 plants were regenerated (Table 1). All plants thus obtained appeared to be albino, and were able to grow in medium containing 50 μg/ml hygromycin sulfate for successive selections.

Fluorometric analysis of GUS activity in transgenic plants revealed that two plants expressed foreign gene products. Shimamoto et al. (1989) have also reported co-transformation of rice protoplasts with the hygromycin B resistance gene and a CaMV 35 S promoter-GUS fusion gene.

Presence of foreign DNA

The DNA of the leaf tissues of two transformants was analyzed. Total DNA was digested with *Eco*RI + *Hin*dIII. As seen in Fig. 2, several bands (2.2, 3.8, 7.0, and 8.2 kb), which hybridized to a probe containing 420 bp of the 5′ upstream non-coding region of the *rolC* gene, were obvious in one of the transgenic rice plants, suggesting random integration of the foreign gene. A similar DNA blot pattern was observed in another transformant (data not shown).

Expression of a foreign gene

GUS activity directed by the 5′ non-coding signal of the *rolC* gene in leaves and roots of rice transformants was

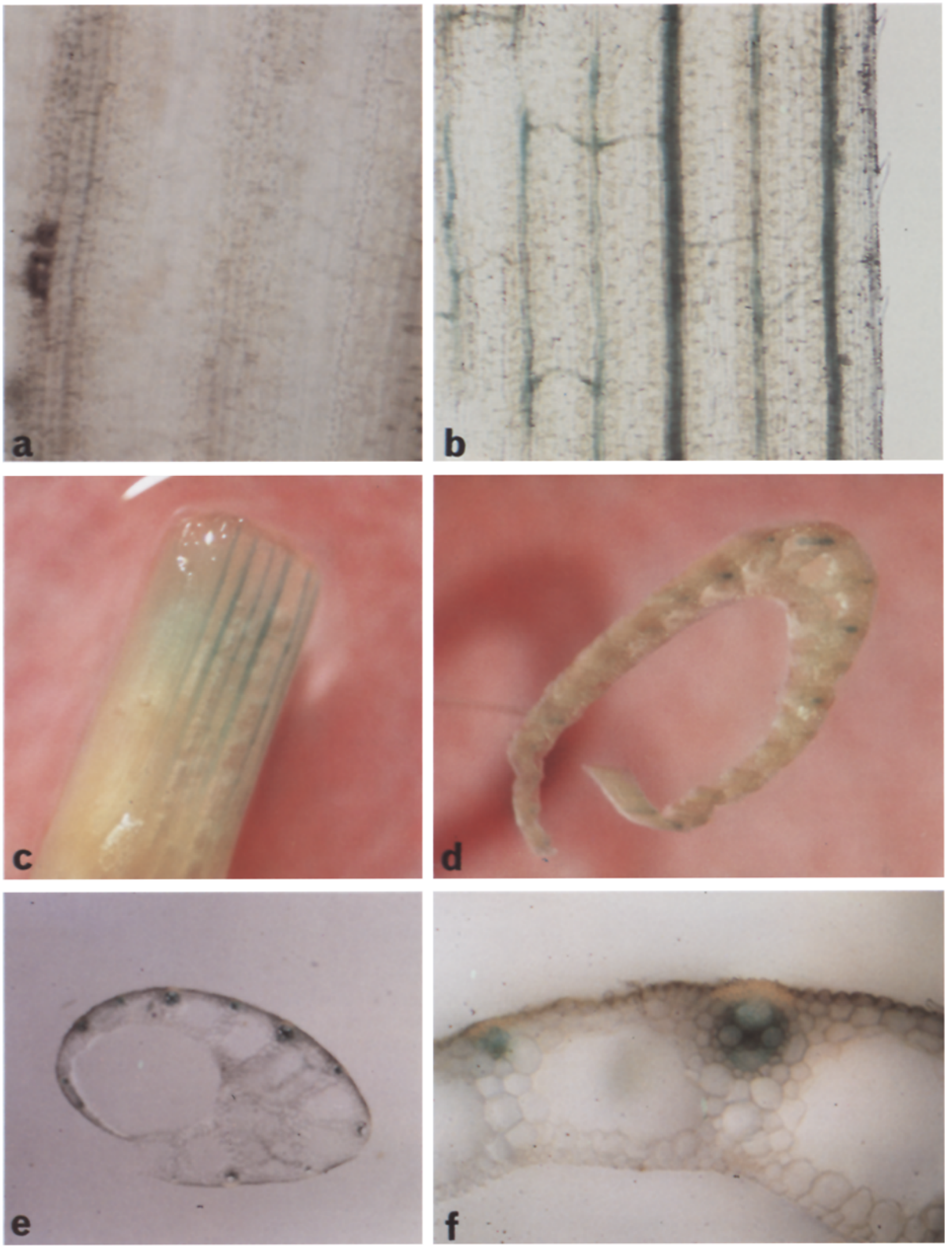

Fig. 3a–f. Histochemical observation of GUS gene expression in leaf and leaf sheath of rice plants. **a** A leaf of untransformed rice; **b** a leaf of a transgenic rice plant; **c** dissected leaf sheath of transformed rice; **d**, **e** and **f** cross-sections of leaf sheath of transgenic rice. Blue coloration indicates the sites (phloem cells) where GUS is present. 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid was used as a substrate

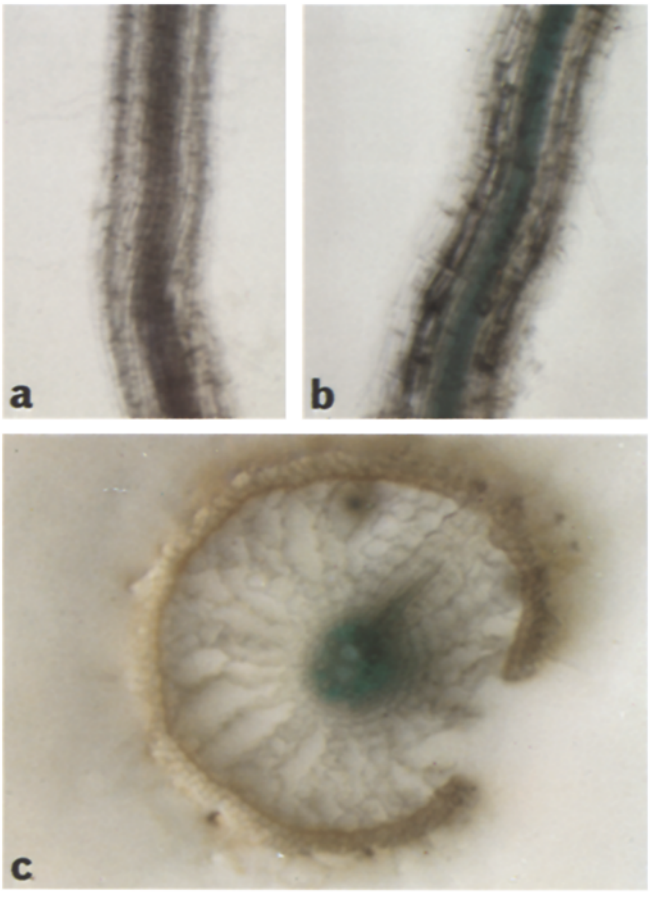

Fig. 4a–c. Expression of the GUS gene in roots of rice plants. **a** A control plant; **b** a transformant; **c** a cross-section of a transformant. Details can be seen in the legend to Fig. 3

significantly higher than in the control (Table 2). Unlike transgenic tobacco plants carrying the same chimeric gene, organ-related activation of GUS genes (i.e. roots > leaves) (Sugaya et al. 1989) was not observed in rice. This may be due to structural differences in vascular development in monocots and dicots.

In order to determine the sites of gene expression directed by the *rolC* promoter, 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid was fed to tissues of transgenic rice. In leaves GUS was found to be located only in vascular tissues (Fig. 3b). Such expression was not seen in control plants (Fig. 3a). The leaf sheath was then dissected to evaluate GUS expression at the cellular level (Fig. 3c, d). Blue coloration was limited only to the region where phloem cells were organized. A similar analysis was also carried out using roots (Fig. 4) and the cellular localization of GUS expression was found to be the same as in the leaf sheath. Using transgenic tobacco carrying the same promoter-GUS fusion gene, Sugaya et al. (1989) have reported phloem cell specific expression of the *rolC* promoter of the Ri plasmid. Therefore tissue-specific expression of the *rolC* promoter was proven to be the same in both monocotyledonous and dicotyledonous plants. It would be worthwhile to investigate common factors (both *cis*- and *trans*-acting), regulating the *rolC* gene promoter in higher plants.

Acknowledgements. We thank Dr. C.S. Loh for critical reading of this manuscript. This research was supported in part by Grants-in-Aid for Science Research from the Ministry of Education, Science and Culture, Japan, and a grant from the Rockefeller Foundation.

References

Bevan M (1984) Binary *Agrobacterium* vectors for plant transformation. Nucleic Acids Res 12:8711–8721

Chu CC, Wang CC, Sun CS, Hsu C, Yin KC, 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

David C, Chilton MD, Tempe J (1984) Conservation of T-DNA in plants regenerated from hairy root cultures. Bio/Technology 2:73–76

De Paolis A, Mauro ML, Pomponi M, Cardarelli M, Spanò L, Costantino P (1985) Localization of agropaine synthesizing functions in the TR region of the root-inducing plasmid of *Agrobacterium rhiogenes* 1855. Plasmid 13:1–7

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

Gardner RC, Howarth AJ, Hahn P, Brown-Luedi M, Shepherd RJ, Messing J (1981) The complete nucleotide sequence of an infectious clone of cauliflower mosaic virus by M13mp7 shotgun sequencing. Nucleic Acids Res 9:2871–2888

Gritz L, Davies J (1983) Plasmid-encoded hygromycin-B resistance – the sequence of hygromycin-B phosphotransferase gene and its expression in *Escherichia coli* and *Saccharomyces cerevisiae*. Gene 25:179–188

Huffman GA, White FF, Gordon MP, Nester EW (1984) Hairy-root-inducing plasmid: physical map and homology to tumor inducing plasmids. J Bacteriol 157:269–276

Jefferson RA, Kavanagh TA, Bevan M (1987) GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6:3901–3907

Jouanin L (1984) Restriction map of an agropaine-type Ri plasmid and its homologies with Ti plasmids. Plasmid 12:91–102

Moore L, Warner G, Strobel G (1979) Involvement of plasmid in the hairy root disease of plants caused by *Agrobacterium rhizogenes*. Plasmid 2:617–626

Nakamura T, Handa T, Oono Y, Kanaya K, Michikawa M, Uchimiya H (1988) Organ-specific mRNA in transgenic tobacco plants possessing T-DNA of Ri plasmids. Plant Sci 56:213–218

Schmülling T, Schell J, Spena A (1988) Single genes from *Agrobacterium rhizogenes* influence plant development. EMBO J 7:2621–2692

Schmülling T, Schell J, Spena A (1989) Promoters of the *rolA*, *B*, and *C* genes of *Agrobacterium rhizogenes* are differentially regulated in transgenic plants. The Plant Cell 1:665–670

Shimamoto K, Terada R, Izawa T, Fujimoto H (1989) Fertile transgenic rice plants regenerated from transformed protoplasts. Nature 338:274–276

Shure M, Wessler S, Fedoroff N (1983) Molecular identification and isolation of the *waxy* locus in maize. Cell 35:225–233

Slightom JL, Durand-Tardif M, Jouanin L, Tepfer D (1986) Nucleotide sequence analysis of TL-DNA of *Agrobacterium rhizogenes* agropine type plasmid: identification of open-reading frames. J Biol Chem 261:108–121

Sugaya S, Hayakawa K, Handa K, Uchimiya H (1989) Cell-specific

expression of the *rolC* gene of the TL-DNA of Ri plasmid in transgenic tobacco plants. Plant Cell Physiol 30:649–653

Tepfer D (1984) Transformation of several species of higher plants by *Agrobacterium rhizogenes:* Sexual transformation of the transformed genotype and phenotype. Cell 37:959–967

Toriyama K, Hinata K (1985) Cell suspension and protoplast culture in rice. Plant Sci 41:179–183

Toriyama K, Arimoto Y, Uchimiya H, Hinata K (1988) Transgenic rice plants after direct gene transfer into protoplasts. Bio/Technology 6:1072–1074

White FF, Nester EW (1980) Hairy root: plasmid encodes virulence traits in *Agrobacterium rhizogenes*. J Bacteriol 141:1134–1141

Communicated by J. Schell

Received June 9, 1989