Note

Plant Biotechnology, **20** (3), 235-239 (2003)

Efficient *Agroinfiltration*-mediated Transient GUS Expression System for Assaying Different Promoters in Rice

Guo-qing SONG and Ken-ichi YAMAGUCHI[a]*

Life Science Laboratory, Mitsui Chemicals, INC. 1144 Togo, Mobara-shi, Chiba 297-0017, Japan
Present address: [a] Lab for Sustainable Horticulture, Minami Kyushu University, Hibarigaoka, Takanabe-cho, Miyazaki 884-0003, Japan
**Corresponding author* E-mail address: keny@nankyudai.ac.jp

Received 12 July 2002; accepted 28 January 2003

Abstract

An improved and efficient system for the expression of GUS in the shoots of 3-day-old rice seedlings was successfully developed using vacuum *Agroinfiltration*. Over 70% of inoculated shoots in the two *japonica* cultivars and one *indica* cultivar tested showed transient expression of GUS under the control of a CaMV 35S promoter interrupted by an intron. The GUS directed by a rice light-harvesting Chlorophyll-a/b-binding protein Cab promoter was also expressed in rice shoots *via* vacuum *Agroinfiltration*. Compared to particle bombardment method to test the efficiency of various promoters, transient GUS expression in rice *via Agroinfiltration* is more precise for assaying the inducible Cab promoter as well as the CaMV 35S constitutive promoter.

Key words: *Agrobacterium*, *Agroinfiltration*, GUS, light-harvesting Chl-a/b-binding protein Cab promoter, particle bombardment, rice (*Oryza sativa* L.), transient GUS expression.

Transient gene expression systems offer several advantages over stable transformation for studying gene expression and regulation, including being independent of regeneration from transformed cells. Although rice is a plant species that can be transformed, almost routinely (Christou, 1997; Hiei *et al.*, 1997), an efficient and reproducible transient expression system is still needed for the rapid analysis of foreign genes. Transient expression of β-glucuronidase gene (GUS) in rice can be achieved by particle bombardment of cell suspensions (Wang *et al.*, 1988; McElroy *et al.*, 1990). Using the common *Agrobacterium* infection method, the transient expression of GUS has been confirmed in various organs of rice seedlings (Li *et al.*, 1992) and rice Oc cells in the presence of acetosyringone (Yang H. *et al.*, 2000). However, the existing transient GUS expression systems in rice are not efficient enough for analyzing various promoters, including tissue- and stage-specific or other regulated promoters. *Agrobacterium* infiltration was recently reported to be another simple and efficient transient expression system for intact leaves of tobacco, *Phaselous acutifolus*, poplar and *Phaseolus vulgaris* (Kapila *et al.*, 1997; Yang Y. *et al.*, 2000). Transient GUS expression in rice based on vacuum infiltration with an *Agrobacterium* suspension has not been exploited. There has been little investigation of the reliability of transient expression systems in rice for the analysis of various promoters. The present report describes a simple and routine approach for the highly efficient transient expression of GUS in rice by vacuum infiltration of *Agrobacterium* suspension cells. Transient GUS expression systems which use common *Agrobacterium*-infection and particle bombardment were also compared. Transient GUS expression systems were assessed to test the efficiency of different plant promoters in rice, including the constitutive cauliflower mosaic virus 35S (CaMV 35S) promoter and the inducible light-harvesting Chl-a/b-binding protein (Cab) promoter.

Rice seeds (*Oryza sativa* L.) of one *indica* cultivar IR42 and two *japonica* cultivars (cv. Fujisaka and Koshihikari) were tested. Surface sterilization of dehulled seeds was performed according to Li *et al.* (1992). Seeds were germinated and grown on a medium containing 1/2 MS inorganic salts (Murashige and Skoog, 1962) and B5 vitamins supplemented with 3.2 g l^{-1} gellan gum and 30 g l^{-1} sucrose. Callus induction medium contains MS inorganic salts, B5, and 30 g l^{-1} sucrose supplem-

ented with 1 mg l^{-1} CH and 2.0 mg l^{-1} 2,4-D (2,4-dichlorophenoxyacetic acid). The pH of the media was adjusted to 5.6 prior to autoclaving. The seedlings were grown at 26-27 ℃.

Two binary vectors were employed (**Fig. 1**). The vector pIG121Hm contains a 5' CaMV 35S-intron-GUS-Tnos 3' GUS expression cassette interrupted by a modified intron of the castor bean catalase gene (Ohta *et al.*, 1990). This intron makes the GUS reporter expression cassette express detectable GUS activity in plant cells, but not in the cells of *Agrobacterium tumefaciens*. The CaMV 35S promoter (*Hin*dIII/*Xba*I fragment) in the GUS expression cassette of pIG121Hm was replaced by the Cab promoter of rice, and designated pCIG. Strong expression of the rice Cab promoter has ever been reported in transgenic rice (Tada *et al.*, 1991). The binary vectors were introduced into *Agrobacterium tumefaciens* strain EHA105 by electroporation.

A single colony of *Agrobacterium tumefaciens* strain EHA105 harboring individual constructs was grown in 2 ml of LB medium containing 50 mg l^{-1} of kanamycin and 30 mg l^{-1} of hygromycin at 30 ℃ for 48 h. A 20 μl aliquot of the culture was then inoculated into 20 ml of LB medium with the antibiotics and grown to an OD_{600} of 0.8-1.0. After centrifuging at 5000 rpm for 2 min, the bacterium pellet was resuspended in an equal volume of fresh LB medium supplemented with 100 μM acetosyringone, and incubated for 1 h at 30 ℃. Three-day-old shoots and seedlings were obtained from the seeds grown in the dark. Seven-day-old leaves were cut from the seeds cultured in the light. These explants were soaked in the bacterial suspension and evacuated in a chamber at 0.933 bar for 10 min. In a common *Agrobacterium* infection procedure, the explants were just infected with the bacterial suspension for 10 min. The explants were then placed and co-cultivated on three layers of wet filter paper, which was made by completely rinsing in callus induction medium (for pIG121Hm) or sterile water (for pCIG) containing 100 μM acetosyringone and then kept in a plastic Petri dish (90 mm x 20 mm). In a preliminary experiment, expression of pCab-GUS-Tnos was tested in various co-cultivation media, including MS, water, and callus induction medium with and without sucrose. The Petri dish was sealed with parafilm and kept at 22-24 ℃ under 16 h of fluorescent light per day for 6 days. Transient GUS expression was determined histochemically according to the procedure of Jefferson (1987). Visible blue spots were counted and served as a criterion for quantifying GUS activity.

The binary vector pIG121Hm was used to optimize the transformation conditions in rice using vacuum *Agroinfiltration*. After co-cultivation with *Agrobacterium* for 6 days in callus induction medium containing 100 μM acetosyringone following bacterium vacuum infiltration, numerous blue spots indicative of the expression of p-CaMV 35S-intron-GUS-Tnos were observed on various explants, including the shoots of 3-day-old and whole materials of 3-day-old seedlings, and the leaves of 7-day-old seedlings. **Fig. 2A-D** show representative results obtained from different experiments. Reproducible and efficient transient expression of GUS occurred in shoots from 3-day-old rice seedlings grown in the dark (**Fig. 2A**).

The results of transient expression of GUS directed by the CaMV 35S promoter in one *indica* variety (IR42) and two *japonica* varieties (cv. Fujisaka and Koshihikari) are shown in **Table 1**. Transient GUS expression was observed in more than 70% of inoculated 3-day-old shoots of the 3 rice cultivars tested based on the vacuum infiltration of *Agrobacterium tumefaciens* cells. An average of 7 visible blue spots were seen on each GUS-positive shoot. Moreover, no significant difference was observed in the expression of GUS between the *indica* cultivar IR42 and the two *japonica* cultivars (cv. Fujisaka and Koshihikari). In contrast, the frequency of transient GUS expression by common *Agrobacterium*-mediated infection without vacuum infiltration was no more than 20% (mean value), and similar to a previous report, the GUS expression varied in different rice cultivars (Li *et al.*, 1992).

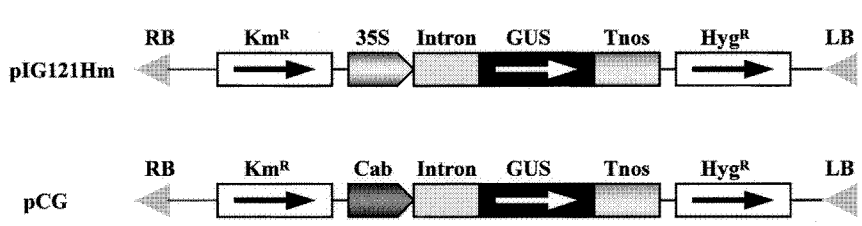

Fig. 1 Schematic representation of plant expression vectors. (A) pIG121Hm. (B) pCIG. RB and LB, right and left borders of T-DNA, respectively; *Km*^{R}, kanamycin-resistant gene; *35S*, cauliflower mosaic virus 35S promoter; Cab, the rice light-harvesting Chl-a/b-binding protein Cab promoter; Intron, a modified intron of the castor bean catalase gene; *Tnos*, the terminator of the nopaline synthase gene; *Hyg*^{R}, hygromycin-resistant gene.

With the vacuum infiltration method, the explants influenced the transient expression of GUS. The frequency of transient expression of GUS in the 7-day-old leaves was less than 10%. Compared with 3-day-old seedlings, higher efficiency of GUS expression was found in 3-day-old shoots, in terms of the percentage of GUS-positive explants (**Table 1**). Infiltration which leads to a very high number of bacteria entering the intercellular spaces of the plant cells has been shown to be the major reason for the improved transient expression of GUS or a higher efficiency in stable transformation (Kapila *et al.*, 1997; Kisaka *et al.*, 2000). As reported in other plant species (Kapila *et al.*, 1997; Yang H. *et al.*, 2000), our results demonstrated that transient GUS expression based on *Agroinfiltration* was applicable to monocotyledonous plant rice.

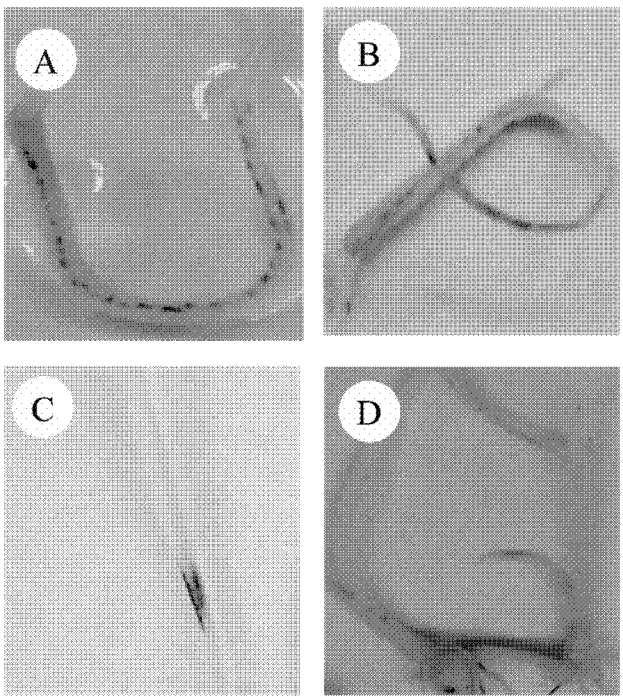

Fig. 2 Expression of GUS in rice (cv. Fujisaka) after inoculation with *A. tumefaciens* strain EHA105 harboring pIG121Hm and pCIG following vacuum infiltration. (A) 3-day-old shoot (pIG121Hm). (B) 3-day-old shoot (pCIG). (C) 3-day-old seedlings (pIG121Hm). (D) 7-day-old leaf (pIG121Hm).

Co-cultivation media appeared to affect the transient expression of GUS directed by the Cab promoter. As shown in **Fig. 2B**, 6 days after co-cultivation on sterile water-soaked filter paper, transient expression of the pCab-intron-GUS-Tnos occurred on 3-day-old shoots of rice (cv. Fujisaka) following introduction by vacuum *Agroinfiltration*. Visible blue spots appeared on 56.3% of the inoculated explants. An average of 5 blue spots were observed per GUS-positive explant. However, a lower efficiency of transient GUS expression was found in shoots co-cultivated on callus induction medium with or without sucrose (data not shown). When the efficiency of transient GUS expression by vacuum *Agroinfiltration* was compared using p-CaMV 35S and p-Cab, the former gave slightly better results, although the blue spots appeared the same in appearance.

We also tested transient expression of GUS in rice shoots and leaves by micro-particle bombardment. An IDERA <GIE-III> biolistic particle delivery device was used. The procedure for micro-particle bombardment was modified from the published protocols (Klein *et al.*, 1988; Tomes *et al.*, 1990). Recombinant plasmids used for particle bombardment were prepared using QIAGEN Tip 100. Eight micrograms of plasmid DNA for each construct were precipitated onto 2 mg tungsten particles (average diameter 1.0 μm). Leaves from 7-day-old seedlings grown in the light and 3-day old shoots from the seeds cultured in the dark were used as

Table 1 Transient GUS expression in rice by *Agroinfiltration* and common *Agrobacterium* infection

	Explants (3-day old age)	Cultivar	No. of explants	Gus$^+$ (%)[1)]
Common *Agrobacterium* infection	shoots	IR42	60	21.6
		Koshihikari	60	15.0
		Fujisaka	60	13.3
Agroinfiltration	seedlings	IR42	37	59.5
		Koshihikari	61	42.6
		Fujisaka	42	40.5
	shoots	IR42	80	72.5
		Koshihikari	56	73.2
		Fujisaka	20	75.0

[1)]Percentage of explants exhibiting one or more visible blue spots following staining with X-gluc.

explants. The leaves were cut into segments 4-5cm long. Thirty explants were arranged on 3 layers of filter paper soaked with callus induction medium (for plG121Hm) or sterile water (for pCIG) in the center of 90x20 mm Petri dishes. Prior to bombardment, microcarriers were suspended by sonication. Each Petri dish was bombarded twice with 20 μl aliquots containing 0.8 μg of target DNA at power level 6 blank (6 cm target distance). The acceleration pressure was 6 bar and the chamber was evacuated to 0.266 bar. The Petri dishes were then sealed and kept under continuous light at 26℃. Target tissues were tested 24 h after bombardment. The expression of p-CaMV 35S-intron-GUS appeared much more efficient than p-Cab-intron-GUS no matter what kinds of explants used. Seven-day-old leaves were found to be efficient in transient expression of both the CaMV 35S promoter and the Cab promoter after bombardment. An average of 26 blue spots, indicating the expression of p-CaMV 35S-intron-GUS, were observed on 90% of bombarded leaves, whereas only 4 (mean value) weak blue spots indicating expression of the GUS directed by the Cab promoter appeared on 48% of GUS-positive leaves under optimal conditions. When 3-day old shoots were used as explants, expression of the CaMV 35S promoter was observed in 85% of the bombarded shoots, and expression of the Cab promoter was detectable in 25% of the bombarded shoots, which is no more than half of the value *via Agroinfiltration* (56.3%). The results indicated that transient expression system *via Agroinfiltration* was more suitable for expression of the inducible Cab promoter.

The transient expression of GUS by particle bombardment has been successfully used to study the enhancing effects of intron in banana (Dugdale *et al.*, 2001). In our case, the results clearly indicated that the transient GUS expression under the direction of the CaMV 35S promoter was either higher (*via Agroinfiltration*) or much higher (by particle bombardment) than that using the Cab promoter. On the other hand, GUS under the control of the Cab promoter has been reported to be expressed at a much higher level than that under the control of the CaMV 35S promoter in the leaves of stable transformed rice (Tada *et al.*, 1991; Takeuchi *et al.*, 2000). These results indicated that neither of the transient expression approaches held promise when it was used to compare the inducible Cab promoter with the constitutive CaMV 35S promoter. Compared with the transient system by particle bombardment approach, transient expression *via Agroinfiltration* may have potential use for assaying inducible promoters.

Commonly, transient gene expression in plants can be achieved by direct gene-delivery methods including biolistic (Klein *et al.*, 1987; Luan and Bogorad 1992), electroporation (Lindsey and Jones 1987; Dekeyser *et al.*, 1990) and the polyethylene glycol-mediated transformation (McElroy *et al.*, 1990). Our results supported the previous reports that particle bombardment method was efficient in transient expression of some constitutive promoters (Klein *et al.*, 1987; Wang *et al.*, 1988). Compared with direct gene-delivery transient expression systems, transient expression systems mediated by common *Agrobacterium* infection or vacuum infiltration enhanced-infection have advantages for analyzing the virulence loci of *Agrobacterium* (Wilmink *et al.*, 1992), and can be conducted efficiently without requiring expensive equipment or complicated procedures (Kapila *et al.*, 1997; Yang H. *et al.*, 2000). Our results demonstrate that the *Agrobacterium* transient expression system *via Agroinfiltration* is a simple and efficient approach in rice for assaying the inducible Cab promoter as well as the constitutive CaMV35S promoter.

Acknowledgements

We would like to thank Prof. H. Shimada, Science University of Tokyo, for critical reading, and Dr. H. Honda for his helpful discussions. We would also like to thank Ms. M. Fujishige and Y. Suzuki for their expert technical assistance. This work was partially supported by the New Energy and Industrial Technology Development Organization (NEDO).

References

Christou, P., 1997. Rice transformation: bombardment. Plant Mol. Biol., **35**: 197-203.

Dekeyser, R. A., Claes, B., De Rycke, R. M. U., Habets, M. E., Van Montagu, M., Caplan, A. B., 1990. Transient gene expression in intact and organized rice tissues. Plant Cell, **2**: 591-602.

Dugdale, D., Becker, D. K., Harding, R. M., Dale, J. L., 2001. Intron-mediated enhancement of the banana bunchy top virus DNA-6 promoter in banana (*Musa* spp.) embryogenic cells and plants. Plant Cell Rep., **20**: 220-226.

Kisaka, E. H., Kanno, A., Kameya, T., 2000. Transformation of soybean by infecting embryogenic calli with *Agrobacterium tumefaciens* and that of soybean and kidney bean by injecting the bacteria into germinating seeds. Plant Biotechnol., **17**: 187-194.

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 T-DNA. Plant J., **6**: 271-282.

Jefferson, R. A., 1987. Assaying chimeric genes in plants:

the GUS gene fusion system. Plant Mol. Biol. Rep., **17**: 387-405.

Kapila, J., De Rycke, R., Van Montagu, M., Angenon, G., 1997. An Agrobacterium-mediated transient gene expression system for intact leaves. Plant Sci., **122**: 101-108.

Klein, T. M., Wolf, E. D., Wu, R., Sanford, J. C., 1987. High-velocity microprojectiles for delivering nucleic acids into living cells. Nature, **327**: 70-73.

Klein, T. M., Gradziel, T., Fromm, M. E., Stanford, J. C., 1988. Factors influencing gene delivery into *Zea mays* cells by high-velocity microprojectiles. Biotechnology, **6**: 559-563.

Li, X. Q., Liu, C. N., Ritchie, S. W., Peng, J. Y., Gelvin, S. B., Hodges, T. K., 1992. Factors influencing Agrobacterium-mediated transient expression of gusA in rice. Plant Mol. Biol., **20**: 1037-1048.

Lindsey, K., Jones, M. G. K., 1987. Transient gene expression in electroporated protoplasts and intact cells of sugar beet. Plant Mol. Biol., **10**: 43-52.

Luan, S., Bogorad, L., 1992. A rice gene promoter contains separate *cis*-acting elements that regulate expression in dicot and monocot plants. Plant Cell, **4**: 971-981.

McElroy, D., Zhang, W., Cao, J., Wu, R., 1990. Isolation of an efficient actin promoter for use in rice transformation. Plant Cell, **2**: 163-171.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth of and bioassays with tobacco tissue cultures. Physiol. Plant. **15**: 473-497.

Ohta, S., Mita, S., Hattori, T., Nakamura, K., 1990. Construction and expression in tobacco of a β-glucuronidase (Gus) reporter gene containing an intron with coding sequence. Plant Cell Physiol., **31**: 805-813.

Tada, Y., Sakamoto, M., Matsuoka, M., Fujimura, T., 1991. Expression of a monocot LHCP promoter in transgenic rice. EMBO J., **10**: 1803-1808.

Takeuchi, Y. Akagi, H., Kamasawa, N., Osumi, M., Honda, H., 2000. Aberrant chloroplasts in transgenic rice plants expressing a high level of maize NADP-dependent malic enzyme. Planta, **211**: 265-274.

Tomes, D. T., Ross, M., Higgens, A. G., Rao, A G., Staebell, M., Howard, J., 1990. Direct DNA transfer into intact plant cells and recovery of transgenic plants via microprojectile bombardment. In: Plant Molecular Biology Manual A13, pp. 1-22. Kluwer Academic Publishers, Belgium.

Wang, Y., Klein, T. M., Fromm, M., Cao, J., Sanford, J. C., Wu, R., 1988. Transient expression of foreign genes in rice, wheat and soybean cells following particle bombardment. Plant Mol. Biol., **11**: 433-439.

Wilmink, A., Van de Ven, B. C. E., Dons, J. J. M., 1992. Expression of the GUS-gene in the monocot tulip after induction by particle bombardment and *Agrobacterium*. Plant Cell Rep., **11**: 76-80.

Yang, H., Morita, A., Matsubayashi, Y., Nakamura, K., Sakagami, Y., 2000. A rapid and efficient system of Agrobacterium infection-mediated transient gene expression in rice Oc cells and its application for analysis of the expression and antisense suppression of prepro-phytosulfokine, a precursor of phytosulfokine-a, encoded by OsPSK gene. Plant Cell Physiol., **41**: 811-816.

Yang, Y., Li, R., Qi, M., 2000. *In vivo* analysis of plant promoters and transcription factors by agroinfiltration of tobacco leaves. Plant J., **22**: 543-551.