**Short Communication** Plant Biotechnology, **15**(4), 213-216(1998)

# Effects of Several Antibiotics and Bialaphos on the Growth and Organ Formation of *Lilium formosanum* Calli and Transient Expression of the *gus*A Gene after Co-cultivation with *Agrobacterium tumefaciens*

Sakae SUZUKI*, Yoshiji NIIMI*, Toshiaki SAKAKIBARA*,
Keizo HOSOKAWA**, Saburo YAMAMURA** and Masaru NAKANO*

* Faculty of Agriculture, Niigata University, 2-8050 Ikarashi, Niigata 950-2181, Japan
** Iwate Biotechnology Research Center, 22-174-4 Narita, Kitakami 024-0003, Japan

Received 24 April 1998; accepted 26 June 1998

Gene transfer techniques hold much promise for genetic improvement of ornamental as well as crop plants [1]. In the genus *Lilium*, which contains a number of important ornamental species and hybrids, however, few systems for producing transgenic plants have yet been established [2]. For establishing transformation systems, it is prerequisite to develop an efficient selection method of stably transformed cells and tissues. In the present study, therefore, we examined the effects of several selective agents as well as antibiotics for eliminating *Agrobacterium* on the growth and organ formation of *L. formosanum* calli. We also report here transient expression of the *gus*A gene after co-cultivation of the calli with an engineered strain of *Agrobacterium tumefaciens*.

Bulb scale-derived calli of *L. formosanum* [3] were used as a plant material. The calli were maintained for more than 2 years by subculturing monthly at 25°C in the dark on a callus proliferation medium (C medium), which consisted of MS basal medium [4], 1 mg$l^{-1}$ picloram, 30 g$l^{-1}$ sucrose and 2 g$l^{-1}$ gellan gum. For inducing organ formation, the calli were transferred to a regeneration medium (R medium), which consisted of MS basal medium, 30 g$l^{-1}$ sucrose and 2 g$l^{-1}$ gellan gum, and incubated at 25°C under continuous illumination (ca. 35 $\mu$mol $m^{-2}$ $sec^{-1}$) with fluorescent lamps. To examine the effect of various agents, 0.5 g fresh weight (FW) of the calli 2 weeks after subculture were inoculated on C or R medium, each of which was further supplemented with various concentrations (**Fig. 1**) of a selective agent (kanamycin, G418, hygromycin or bialaphos) or an antibiotic for eliminating *Agrobacterium* (carbenicillin or cefotaxime). For examining organ formation, the calli were subcultured every 4 weeks on the same fresh medium. Data of the growth and organ formation of the calli were recorded 4 and 12 weeks after inoculation, respectively.

**Fig. 1** shows the effects of various agents on the growth and organ formation of *L. formosanum* calli. Among the four selective agents examined, G418, hygromycin and bialaphos inhibited callus growth to various extents; notably, callus growth was completely inhibited by 50 mg$l^{-1}$ or more of hygromycin. Callus browning was observed on the media containing 25 mg$l^{-1}$ or more of hygromycin and 1 mg$l^{-1}$ or more of bialaphos. On the other hand, kanamycin had no effect on callus growth at all concentrations tested. Among two antibiotics for eliminating *Agrobacterium*, 300 mg$l^{-1}$ or more of cefotaxime slightly inhibited callus growth without callus browning, whereas carbenicillin rather stimulated callus growth at all concentrations tested. Organ formation from the calli was inhibited to various extents by all four selective agents; especially, it was completely inhibited by both hygromycin and bialaphos at all concentrations tested, and by 25 mg$l^{-1}$ or more of G418. On the other hand, both antibiotics for eliminating *Agrobacterium*, cefotaxime and carbenicillin, promoted shoot and root formation from the calli; notably more than two-fold increase of the number of both shoots and roots was obtained by 400 and 500 mg$l^{-1}$ of carbenicillin. These results indicate that hygromycin may be a suitable agent for selecting both transformed calli and organs of *L. formosanum*, and that G418 and bialaphos may also be suitable for selecting transformed organs from the calli. Generally, monocotyledonous plants show a high level of natural tolerance to kanamycin, and G418, hygromycin and bialaphos have been alternatively used as selective agents in several Gramineous plants [5-7]. Microprojectile bombardment-mediated transformants of *L. longiflorum* were also selected by bialaphos [2]. Both cefotaxime and carbenicillin seem to be suitable for eliminating *Agrobacterium* from callus cultures of *L. formosanum*. Although the mechanism of a stimulatory effect of these two betalactam antibiotics on the growth and organ for-

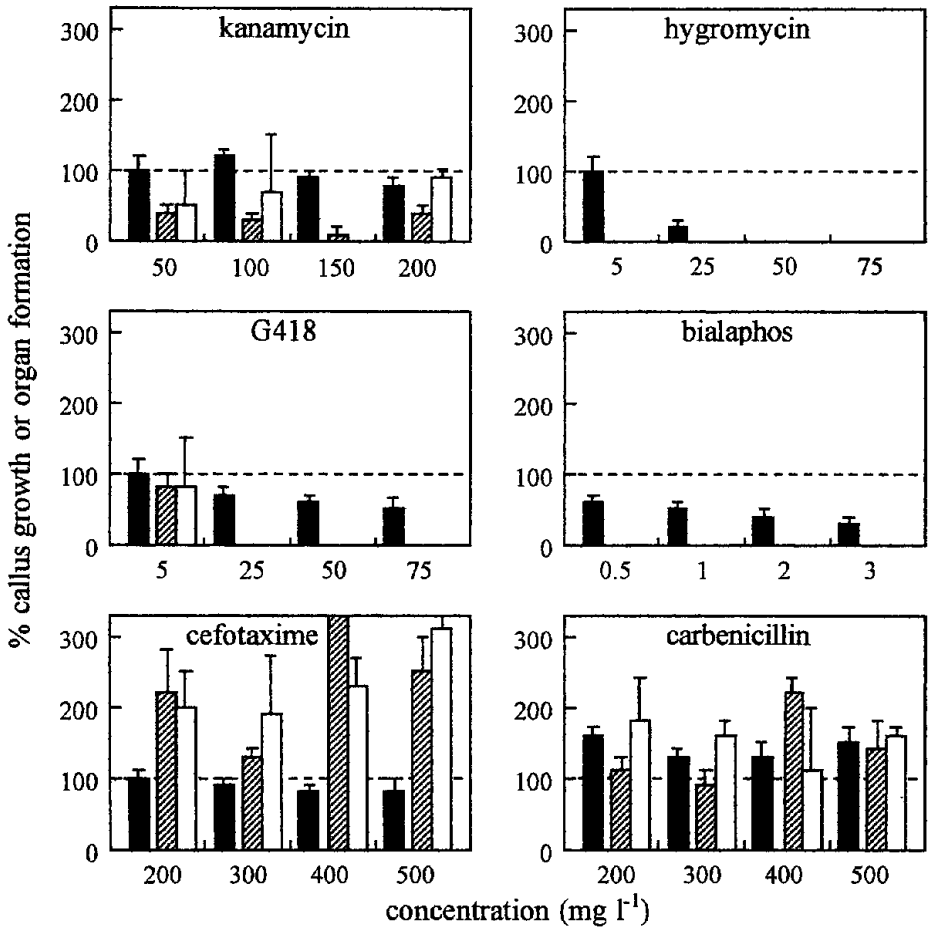


**Fig. 1** Growth and organ formation of *Lilium formosanum* calli on media containing various concentrations of antibiotics and bialaphos. The% callus growth and organ formation represent the% increase in callus fresh weight ■ and the number of shoots ▨ or roots □, respectively, as a percentage of the value of the control treatment (without both antibiotics and bialaphos). Data were recorded 4 and 12 weeks after incubation for callus growth and organ formation, respectively. Values represent the mean of three independent experiments.

mation of *L. formosanum* calli is not identified at present, similar observations have been reported for several plant species [8-10].

For co-cultivating *L. formosanum* calli with *Agrobacterium*, used was *A. tumefaciens* strain EHA101 [11] harboring the binary vector pIG121Hm [12] containing the β-glucuronidase (*gus*A) gene with an intron fused to cauliflower mosaic virus (CaMV) 35S promoter, the neomycin phosphotransferase II (*neo*) gene under the control of nopaline synthase promoter, and the hygromycin phosphotransferase (*hpt*) gene under the control of CaMV 35S promoter. Prior to co-cultivation, *Agrobacterium* was inoculated into 30 m*l* of liquid YEP medium [13] containing 50 mg$l^{-1}$ kanamycin, 50 mg$l^{-1}$ hygromycin and 20 mg$l^{-1}$ acetosyringone (AS) and incubated for more than 24 h at 28°C with reciprocal shaking (150 cycles $min^{-1}$). Bacterial cells collected by centrifugation (2000 g, 10 min) were resuspended in liquid MS medium containing 10 mM MES, 10 g$l^{-1}$ glucose and 0, 10, 20, 50, 100 or 200 mg$l^{-1}$ of AS, pH 5.2, to a final $OD_{600}$ of 0.5. *L. formosanum* calli 2 weeks after subculture were immersed into the bacterial suspension at 25°C for 1, 5, 30 or 60 min, and blotted on sterile filter papers. For co-cultivation with *Agrobacterium*, the calli were then placed onto C medium further supplemented with 10 mM MES, 10 g$l^{-1}$ glucose and 20 mg$l^{-1}$ of AS, pH5.2, and incubated at 25°C in the dark. Histochemical localization of the *gus*A gene expression was detected in the calli constantly during the co-cultivation period by the method of Jefferson [14]. In a preliminary experiment, no endogenous GUS expression was detected in the control, non-co-cultivated calli.

Although GUS histochemical assay was carried out every day for 5 days after the initiation of co-cultivation, GUS-positive cells were never observed on the calli from any co-cultivation treatment. However, when the co-cultivation period was extended to 15-30 days, some calli showed several blue spots resulted

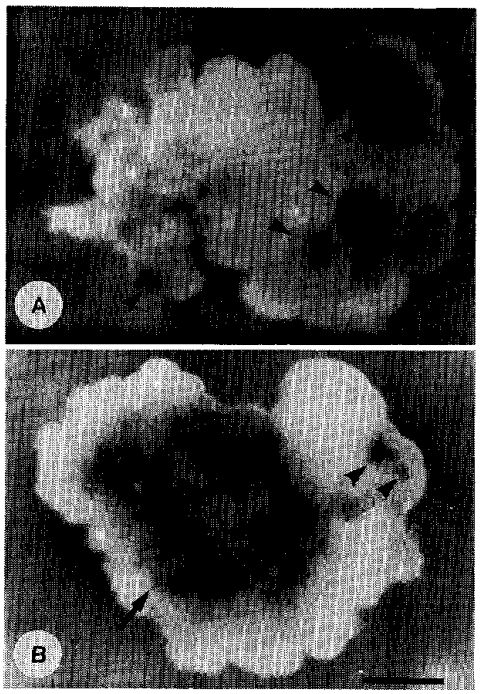


**Fig. 2** *Lilium formosanum* calli showing blue spots (arrow heads) following co-cultivation with *Agrobacterium tumefaciens* strain EHA101 containing the binary vector pIG121Hm. (A) A callus clump which was immersed into the bacterial suspension containing 50 mg$l^{-1}$ AS for 5 min and co-cultivated for 27 days. Bar=1 mm. (B) A partly-browned (arrow) callus clump which was immersed into the bacterial suspension containing 200 mg$l^{-1}$ AS for 60 min and co-cultivated for 30days. Bar=0.5 mm.

from transient or stable expression of the *gus*A gene (**Fig. 2-A**). Generally, 1 to 8 spots were observed on a callus clump of ca. 4 mm in diameter. Although no overgrowth of *Agrobacterium* was observed even 30 days after co-cultivation, the calli frequently browned with the increase of the duration of co-cultivation. However, blue spots were detected in the partly-browned calli (**Fig. 2-B**). There was no apparent relationship between the number and size of blue spots and various co-cultivation treatments tested in the present study.

For selecting stably transformed tissues, the calli co-cultivated for 15-30 days were transferred onto C medium with 50 mg$l^{-1}$ hygromycin and 500 mg$l^{-1}$ carbenicillin and incubated at 25°C in the dark. However, surviving calli showed no GUS expression 4 weeks after transfer. In addition, all of the calli browned and died by 6 weeks after transfer. This is probably due to little or no integration of T-DNA to the *L. formosanum* genome. Although, in the past, monocotyledonous plants had been considered to be outside the host range of *Agrobacterium*, production of transgenic plants using the *Agrobacterium*-mediated method has recently been reported for rice [15, 16], maize [17, 18] and asparagus [19, 20]. In the genus *Lilium*, no papers have appeared on the production of *Agrobacterium*-mediated transgenic plants, although Langeveld *et al.* [21] reported transient expression of the *gus*A gene after inoculating *Agrobacterium* into stem internodes of *L. longiflorum*. The present study provides the possibility of *Agrobacterium*-mediated transformation of a monocotyledonous ornamental, *L. formosanum*, although no stably transformed tissues or plants were obtained. Further study should be concentrated to optimize conditions of transformation as well as to select suitable bacterial strains.

## Acknowledgments

We are grateful to Dr. H. Anzai, Pharmaceutical Research Center, Meiji Seika Ltd., for kindly providing purified bialaphos. This research was supported in part by Grants-in-Aid from the Ministry of Education, Science and Culture, Japan (No. 09460017).

## References

[1] Woodson, W. R., 1991. HortSci., **26**: 1029-1033.
[2] Watad, A. A., Yun, D.-J., Matsumoto, T., Niu, X., Wu, Y., Kononowicz, A. K., Bressan, R. A., Hasegawa, P. M., 1998. Plant Cell Rep., **17**: 262-267.
[3] Sakakibara, T., Nakano, M., Niimi, Y., 1996. J. Japan. Soc. Hort. Sci., **65** (Suppl. 2): 622-623. (in Japanese)
[4] Murashige, T., Skoog, F., 1962. Physiol. Plant., **15**: 473-497.
[5] Hauptmann, R. M., Vasil, V., Ozias-Akina, P., Tabaeizadeh, Z., Rogers, S. G., Fraley, R. T., Horsch, R. B., Vasil, I. K., 1988. Plant Physiol., **86**: 602-606.
[6] Dekeyser, R., Claes, B., Marichal, M., Van Montagu, M., Caplan, A., 1989. Plant Physiol., **90**: 217-223.
[7] Rathore, K. S., Chowdhury, V. K., Hodges, T. K., 1993. Plant Mol. Biol., **21**: 871-884.
[8] Mathias, R. J., Mukasa, C., 1987. Plant Cell Rep., **6**: 454-457.
[9] Holford, P., Newbury, H. J., 1992. Plant Cell Rep., **11**: 93-96.
[10] Nakano, M., Mii, M., 1993. J. Plant Physiol., **141**: 721-725.
[11] Hood, E. E., Helmer, G. L., Fraley, R. T., Chilton, M.-D., 1986. J. Bacteriol., **168**: 1291-1301.
[12] Ohta, S., Mita, S., Hattori, T., Nakamura, K., 1990. Plant Cell Physiol., **31**: 805-813.

[13] An, G., Ebert, P. R., Mitra, A., Ha, S. B., 1988. In "Plant Molecular Biology Manual. A3." (eds. by Gelvin, S. B., *et al.*), p. 1-19, Kluwer Academic Publishers, Dordrecht.

[14] Jefferson, R. A., 1987. Plant Mol. Biol. Rep., **5**: 387-405.

[15] Chan, M.-T., Chang, H.-H., Ho, S.-L., Tong, W.-F., Yu, S-M., 1993. Plant Mol. Biol., **22**: 492-506.

[16] Hiei, Y., Ohta, S., Komori, T., Kumashiro, T., 1994. Plant. J., **6**: 271-282.

[17] Gould, J., Devery, M., Hasegawa, O., Ulian, E. C., Peterson, G., Smith, R. H., 1991. Plant Physiol., **95**: 426-434.

[18] Ishida, Y., Saito, H., Ohta, S., Hiei, Y., Komari, T., Kumashiro, T., 1996. Nature Biotech., **14**: 745-750.

[19] Bytebier, B., Deboeck, F., De Greve, H., Van Montagu, M., Hernalsteens, J. P., 1987. Proc. Natl. Acad. Sci. USA, **84**: 5345-5349.

[20] Delbreil, B., Guerche, P., Jullien, M., 1993. Plant Cell Rep., **12**: 129-132.

[21] Langeveld, S. A., Gerrits, M. M., Derks, A. F. L. M., Boonekamp, P. M., Bol, J. F 1995. Euphytica, **85**: 97-100.