BIOLOGIA PLANTARUM 52 (3): 513-516, 2008

BRIEF COMMUNICATION

Stability of β-glucuronidase gene expression in transgenic *Tricyrtis hirta* plants after two years of cultivation

S. MORI[1]*, E. OKA[1], H. UMEHARA[1], H. KOBAYASHI[2], Y. HOSHI[2], M. KONDO[2], K. OGATA[2] and M. NAKANO[1]

Faculty of Agriculture, Niigata University, 2-8050 Igarashi, Niigata 950-2181, Japan[1]
Niigata Agricultural Research Institute, 857 Nagakura, Nagaoka 940-0826, Japan[2]

Abstract

Transgenic plants of *Tricyrtis hirta* carrying the intron-containing β-glucuronidase (GUS) gene under the control of the CaMV35S promoter have been cultivated for two years. Four independent transgenic plants produced flowers 1 - 2 years after acclimatization, and all of them contained one copy of the transgene as indicated by inverse polymerase chain reaction (PCR) analysis. All the four transgenic plants showed stable expression of the *gus* gene in leaves, stems, roots, tepals, stamens and pistils as indicated by histochemical and fluorometric GUS assays, although differences in the GUS activity were observed among different organs of each transgenic plant. No apparent *gus* gene silencing was observed in transgenic *T. hirta* plants even after two years of cultivation.

Additional key words: GUS expression in different ornans, Japanese toad lily, PCR, transgene stability.

Tricyrtis hirta (Thumb.) Hook is an autumn-flowering, perennial plant native to Japan. The plants have attractive arching stems with exotic starry flowers. *T. hirta* is sometimes called 'Japanese toad lily' and has recently become popular as an ornamental for pot and garden uses. We had recently developed an *Agrobacterium*-mediated transformation system in *T. hirta* (Adachi *et al*. 2005). The transformation efficiency of *T. hirta* was much higher than that of other Liliaceous ornamentals, such as *Agapanthus praecox* ssp. *orientalis* (Suzuki *et al*. 2001), *Muscari armeniacum* (Suzuki and Nakano 2002) and *Lilium* sp. (Hoshi *et al*. 2004). In addition, *T. hirta* requires only one year from *in vitro* regeneration to flowering, different from most Liliaceous ornamentals. Therefore, this species seems to be suitable as a model for molecular breeding as well as molecular biological studies in Liliaceous ornamentals. For perennial and vegetatively propagated crops like *T. hirta*, long-term and stable expression of transgene(s) is indispensable for successful application of molecular breeding. Therefore, in the present study, we examined transgene (GUS reporter gene) expression in various organs of transgenic *T. hirta* plants after two years of cultivation for evaluating the validity of genetic transformation in the breeding programs of *T. hirta*.

One control, non-transgenic plant (C1) and five independent transgenic plants (T1 - T5) of *T. hirta* were cultivated for two years in a growth changer following acclimatization in 2003. These transgenic plants were produced by co-cultivation of embryogenic calluses with *Agrobacterium tumefaciens* strain EHA101/pIG121Hm (Adachi *et al*. 2005). This *Agrobacterium* strain contained the NPTII gene under the control of the NOS promoter, the intron-containing GUS gene under the control of the CaMV35S promoter and the HTP gene under the control of the CaMV35S promoter in the T-DNA region of the binary vector (Ohta *et al*. 1990). The non-transgenic plant C1 was a regenerant from embryogenic callus cultures, which were used as a target material for transformation. Among the five transgenic plants, three (T1, T2 and T3) and four (T1, T2, T3 and T4) produced flowers in 2004

Received 2 June 2006, *accepted* 15 March 2007.

Abbreviations: CaMV - cauliflower mosaic virus; GUS - β-glucuronidase; HPT - hygromycin phosphotransferase; NOS - nopaline synthase; NPTII - neomycin phosphotransferase II; PCR - polymerase chain reaction.

Acknowledgement: We would like to thank Dr. N. Ohtake, Faculty of Agriculture, Niigata University, for his valuable suggestions. This work was supported in part by a Grant-in-Aid for Scientific Research (No. 16380024) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

* Author for correspondence present address: Department of Agro-Environmental Science, Takushoku University Hokkaido Junior College, 4558 Menu, Fukagawa 074-8585, Japan; fax: (+81) 164 23 4411, e-mail: mori@takushoku-hc.ac.jp

and 2005, respectively. C1 produced flowers in both years. The five plants that produced flowers (C1, T1, T2, T3 and T4) were used in the present study. Although these five plants were still small and produced only a few flowers in 2005, they showed no apparent morphological variations.

To determine the transgene copy number in the transgenic plants, inverse PCR analysis was carried out according to Hoshi *et al*. (2004). Total genomic DNA was isolated from leaves by using the *Isoplant II* (*Nippon Gene*, Tokyo, Japan). The genomic DNA was digested with *Eco*RI, which cleaves at a site within the HPT gene, and joined to generate circular molecules. The first PCR was performed using the generated circular molecules as a template, the primer set, 5'-GGC CGT CTG GAC CGA TGG CTG TGT AGA AGT ACT CG-3' (P1) and 5'-TGC AGA ACA GCG GGC AGT TCG GTT TCA GGC AGG TC-3' (P2), and the *TaKaRa LA* kit (*Takara Bio*, Otsu, Japan). To amplify the fragment of the junctions of inserted DNA and plant genomic DNA, 30 cycles were performed using a programmed temperature control system *(2400-R*, *Perkin-Elmer*, Wellesley, USA) under the following conditions: 10 s at 98 °C and 10 min at 68 °C. The second PCR was performed under the same conditions as the first PCR except for the template and primer set. In order to increase the specificity, the second PCR performed using the first PCR product as a template and the primer set, 5'-GAC GCC CCA GCA CTC GTC CGA GGG CAA AGG AAT AG-3' (P3) and 5'-ACA CCC TGT GCA CGG CGG GAG ATG CAA TAG GTC AG-3' (P4). Amplified products were analyzed by electrophoresis in a 1.2 % agarose gel. The distance between the LB and the *Eco*RI site of plant genomic DNA depends on the position where the transgene was inserted into the plant genome. Therefore, bands at different positions and of different numbers are expected to reflect the integration of the transgene(s) into different positions and to provide an estimate of the copy number in the genome of transformants. All the four transgenic lines showed a single band at different positions, indicating that these lines were independent and each had one copy of the transgene (data not shown). The control, non-transgenic plant C1 yielded no amplified fragments.

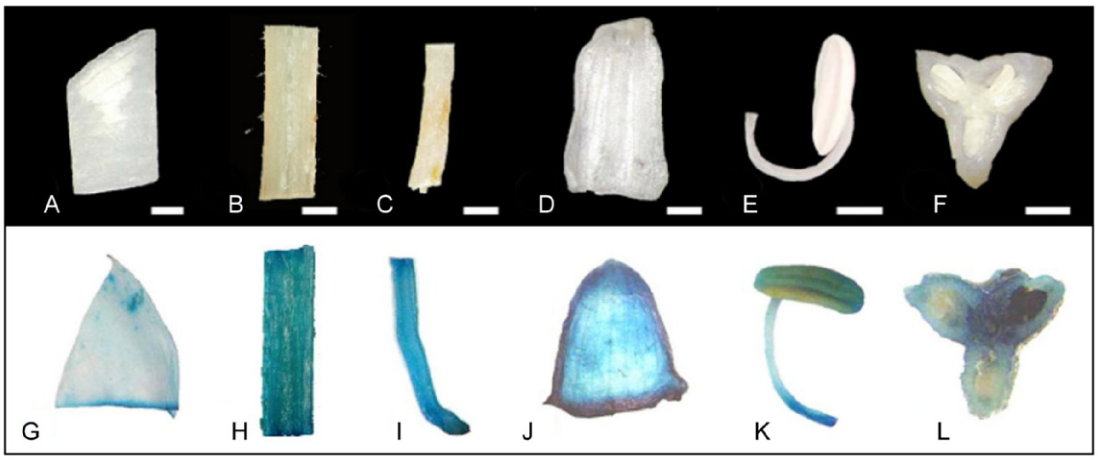

Fig. 1. GUS histochemical assay of leaves (*A*, *G*), stems (*B*, *H*), roots (*C*, *I*), tepals (*D*, *J*), stamens (*E*, *K*) and pistils (cross sections) (*F*, *L*) of the control, non transgenic plant C1 (*upper*; *A* - *F*) and the transgenic plant T1 (*lower*; *G* - *L*) of *Tricyrtis hirta* (*bars* = 2 mm). The investigation was performed in 2005.

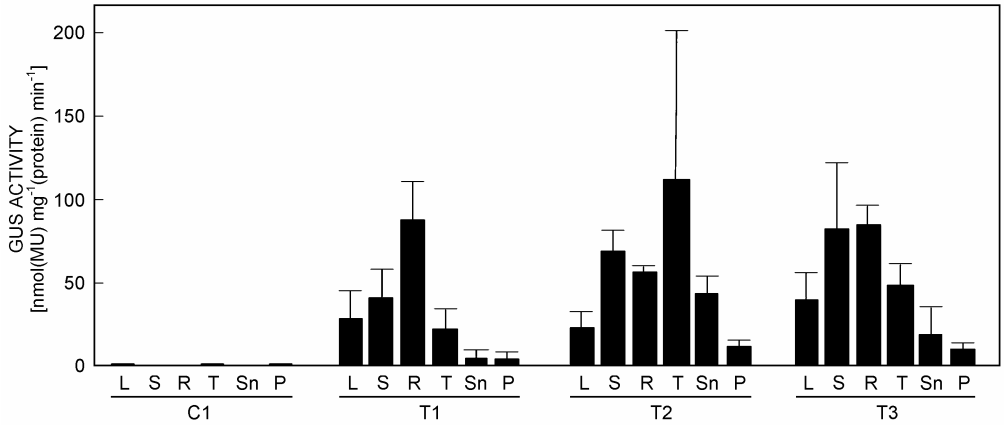

Fig. 2. Fluorometric quantification of GUS activity in various organs of the control, non-transgenic plant C1 and the transgenic lines T1, T2 and T3 of *Tricyrtis hirta*. The investigation was performed in 2005. Values represent the means ± SE, *n* = 3. L - leaves, S - stems, R - roots, T - tepals, Sn - stamens, P - pistils.

In order to examine the stability of transgene expression in transgenic *T. hirta* plants, leaves, stems, roots, tepals, stamens and pistils of non-transgenic and transgenic plants were subjected to histochemical and fluorometric GUS assays. Histochemical GUS assay was performed in 2004 and 2005 according to Jefferson (1987) with several modifications (Suzuki *et al*. 2001). Fluorometric GUS assay was carried out in 2005 according to Jefferson *et al*. (1987). No GUS activity was detected in any organs of C1. On the other hand, all the organs of T1 turned clear-blue following the assay, indicating that this plant expressed the GUS gene stably in these organs (Fig. 1). Moreover, the other three transgenic plants (T2, T3 and T4) showed GUS activity in all the organs examined (data not shown). Although most of the samples turned clear-blue following the assay, only a pale-blue staining was observed in stamens of the transgenic plants T1, T2 and T3 assayed in 2004, and pistils and stamens of the transgenic plant T4 assayed in 2005. For most organs, GUS activity was more strong in 2005 than in 2004. Using fluorometric GUS assay, GUS activity was scarcely detected in all the organs examined in C1 plants. On the other hand, apparent GUS activities were detected in all the three transgenic plants. However, marked differences in the GUS activity level were observed among different organs in each transgenic plant (Fig. 2). Generally, stems, roots and tepals showed higher GUS activity levels compared with the other organs in all the transgenic plants. No apparent differences in the GUS activities were observed among different transgenic plants (Fig. 2).

Although several papers have so far appeared on the production of transgenic perennial flower crops (Robinson and Firoozabady 1993, Kamo *et al*. 1995, Watad *et al*. 1998, Suzuki *et al*. 2001, Suzuki and Nakano 2002, Hoshi *et al*. 2004, Adachi *et al*. 2005, Suwanaketchanatit *et al*. 2007), stability of transgene expression after long-term cultivation of transgenic plants has been studied only for *Gladiolus* (Kamo 2003). In *Gladiolus*, expression of the GUS reporter gene under the control of several different promoters was detected in shoots and roots following three seasons of dormancy. Similarly, stable GUS gene expression could be detected in leaves, stems, roots, tepals, stamens and pistils of all the four transgenic plants of *T. hirta* following two years of cultivation. Although frequent occurrence of transgene silencing has been reported for various monocot species (Iyer *et al*. 2000), transgene silencing may not be a common occurrence in transgenic *T. hirta* containing the GUS gene under the control of the CaMV35S promoter. Generally, transgene copy number has been demonstrated to influence greatly the transgene expression level (Yang *et al*. 2005). There is a tendency that single copies of a transgene are more stably expressed than multiple ones in various species (Bavage *et al*. 2002, Cervera *et al*. 2000). In the present study, all the four transgenic plants of *T. hirta* had one copy of the transgene, which may be one of the causes of stable expression of the transgene following long-term cultivation.

In conclusion, transgenic plants of *T. hirta* showed stable expression of the transgene (GUS reporter gene) after two years of cultivation. This is essential for demonstrating the validity of genetic transformation for the improvement of *T. hirta*. We are now producing transgenic plants containing horticulturally valuable genes, such as genes for the anthocyanin biosynthetic pathway for flower color alteration, MADS-box genes for flower form alteration and genes for the gibberellin biosynthetic pathway for plant form (height) alteration.

References

Adachi, Y., Mori, S., Nakano, M.: *Agrobacterium*-mediated production of transgenic plants in *Tricyrtis hirta* (Liliaceae). - Acta Hort. **673**: 415-419, 2005.

Bavage, A.D., Buck, E., Dale, P., Moyes, C., Senior, I.: Analysis of a backcross population from a multi-copy transgenic *Brassica napus* line. - Euphytica **124**: 333-340, 2002.

Cervera, M., Pina, J.A., Juarez, J., Navarro, L., Pena, L.: A broad exploration of a transgenic population of citrus: stability of gene expression and phenotype. - Theor. appl. Genet. **100**: 670-677, 2000.

Hoshi, Y., Kondo, M., Mori, S., Adachi, Y., Nakano, M., Kobayashi, H.: Production of transgenic lily plants by *Agrobacterium*-mediated transformation. - Plant Cell Rep. **22**: 359-364, 2004.

Iyer, L.M., Kumpatla, S.P., Chandrasekharan, M.B., Hall, T.C.: Transgene silencing in monocots. - Plant mol. Biol. **43**: 323-346, 2000.

Jefferson, R.A.: Assaying chimeric genes in plant: the GUS gene fusion system. - Plant mol. Biol. Rep. **5**: 387-405, 1987.

Jefferson, R.A., Kavanagh, T.A., Bevan, M.W.: GUS-fusions: β-glucuronidase as a sensitive and versatile fusion marker in higher plants. - EMBO J. **6**: 3901-3907, 1987.

Kamo, K., Blowers, A., Smith, F., Van Eck, J., Lawson, R.: Stable transformation of *Gladiolus* using suspension cells and callus. - J. amer. Soc. hort. Sci. **120**: 347-352, 1995.

Kamo, K.K.: Long-term expression of the *uidA* gene in *Gladiolus* plants under control of either the ubiquitin, *rolD*, mannopine synthase, or cauliflower mosaic virus promoters following three seasons of dormancy. - Plant Cell Rep. **21**: 797-803, 2003.

Ohta, S., Mita, S., Hattori, T., Nakamura, K.: Construction and expression in tobacco of a small beta, Greek-glucuronidase (GUS) reporter gene containing an intron within the cording sequence. - Plant Cell Physiol. **31**: 805-813, 1990.

Robinson, K.E.P., Firoozabady, E.: Transformation of floriculture crops. - Sci. Hort. **55**: 83-99, 1993.

Suwanaketchanatit, C., Piluek, J., Peyachoknagul, S., Huene, P.S.: High efficiency of stable genetic transformation in *Dendrobium via* microprojectile bombardment. - Biol. Plant. **51**: 720-727, 2007.Suzuki, S., Nakano, M.: *Agrobacterium*-mediated production of transgenic plants of *Muscari armeniacum* Leichtl. ex Bak. - Plant Cell Rep. **20**: 835-841, 2002.

Suzuki, S., Supaibulwatana, K., Mii, M., Nakano, M.: Production

of transgenic plants of Liliaceous ornamental plant *Agapanthus praecox* ssp. *orientalis* (Leighton) Leighton via *Agrobacterium*-mediated transformation of embryogenic calli. - Plant Sci. **161**: 89-97, 2001.

Watad, A.A., Yun, D.-J., Matsumoto, T., Niu, X., Wu, Y., Kononowicz, A.K., Bressan, R.A., Hasegawa, P.M.: Microprojectile bombardment-mediated transformation of *Lilium longiflorum*. - Plant Cell Rep. **17**: 262-267, 1998.

Yang, L., Ding, J., Zhang, C., Jia, J., Weng, H., Liu, W., Zhang, D.: Estimating the copy number of transgenes in transformed rice by real-time quantitative PCR. - Plant Cell Rep. **23**: 759-763, 2005.