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Transgenic Note

Production of transgenic plants and their early seed set in Japanese soybean variety, Kariyutaka

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Abstract A soybean variety, Kariyutaka was found to form much more precocious flower buds than those of the other six genotypes used in this study when *in vitro* cultured at 26°C with 16/8 h light/dark regime (fluorescent tubes at an intensity of about 100 μmols s^{-1} m^{-2}). In addition, it was a suitable genotype for soybean transformation because it was highly sensitive to *Agrobacterium* and it exhibited excellent shoot regeneration. Its transformation frequency (about 1%) was comparable to that of a variety Thorne, which is known as one of the suitable cultivars for *Agrobacterium*-mediated transformation. Because transgenic shoots of Kariyutaka formed *in vitro* precocious flower buds, T_1 seeds were obtained in only six months after co-cultivation with *Agrobacterium*. A few copy numbers of transgenes were inheritable to T_1 generations. T_1 plants also produced seed within 2–3 months when grown at the same regime as *in vitro* culture. Hence *Agrobacterium*-mediated transformation using Kariyutaka can be recommended as a model method for the rapid development of transgenic soybean lines.

Key words: *Agrobacterium*, early flowering formation, *Glycine max*, transformation.

Currently, soybean transformation can be obtained by direct gene transfer using particle bombardment or through the mediation of *Agrobacterium*. The former has a tendency to insert multiple copies of transgenes in the host genome or to cause fragmentation of transgenes that can sometimes cause the deactivation of transgenes by gene silencing (Reddy et al. 2003). In contrast, the plants transformed using *Agrobacterium* generally have low copy numbers of the transgenes. *Agrobacterium*-mediated transformation makes it easier to obtain stable expression of the gene of interest and this allows the rapid fixation of the inserted gene in transgenic plants.

In the circumstance for soybean transformation, the method mediated by *Agrobacterium* have limitations that several regular soybean varieties are used to generate transgenic plants and a suitable Japanese variety for transformation has not been found yet. In addition, it takes long period to develop transgenic soybean lines through genetic fixation of transgene. Transformation techniques in soybean have been often used for improvement of soybean seed contents such as accumulation of stearidonic acid (Eckert et al. 2006) or α-tocopherol (Eenennaam et al. 2003), or removal of the Gly m Bd 30 K protein which is a soybean allergen (Herman et al. 2003), or reduction of phytic acid (Shi et al. 2007; Nunes et al. 2006). Because these desired outcomes are usually evaluated in the genotype of homozygous transgenes, the initial heterozygous transformants are selfed to obtain homozygous plants. However, it takes 3–4 months to generate transgenic plants and it takes several more months to obtain transgenic T_1 seeds. Genetic fixation by self pollination can be accelerated if the periods needed to flower and set seeds are shortened. In a previous report, flowering was induced from *in vitro* shoots of soybean (Dickens and van Staden 1985). Therefore, establishment of transformation in genotypes forming early flower bud *in vitro* could lead to the rapid development of transgenic soybean lines.

In this study, we attempted to evaluate the ability of early flower bud formation *in vitro* among five Japanese soybean varieties. We found that a Japanese soybean variety, Kariyutaka formed flower buds *in vitro* much earlier than those of the other varieties at 26°C with 16/8 h light/dark regime. In addition, we generated transgenic Kariyutaka plants successfully.

Seven soybean varieties, Jack, Thorne, Fukuyutaka, Kariyutaka, Ohsuzu, Tachiyutaka, and Toyosuzu were used in this study. Methods of tissue culture and transformation were performed as described by Paz et al. (2006) with some modifications. We evaluated these varieties with respect to *in vitro* formation of flower buds

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and shoot regeneration from explants. Tissue culture was performed at 26°C with 16/8 h light/dark regime (white fluorescent tubes at an intensity of about 100 μmols s^{-1} m^{-2}). Soybean seeds were surface-sterilized for 6 h by exposure to chlorine gas generated by mixing 3.5 ml of 12 N HCl with 100 ml commercial bleach (5.25% sodium hypochlorite). The seeds were soaked in sterile distilled water for 16 h. A longitudinal cut along the hilum was made to separate the cotyledons, and the seed coat and the embryonic axis were removed. Moreover, the cotyledons were cut horizontally and sections containing cotyledonary node were prepared as explants in these experiments. The explants were embedded with their adaxial side up on shoot induction medium (SIM). After 2 weeks, the explants were transferred to fresh SIM. Developing shoots were cut off with a surgical scalpel during shoot induction. After 4 weeks of culture on SIM, explants were transferred to shoot elongation medium (SEM). Explants were subcultured on SEM every 2 weeks. After 4 weeks of culture on SEM, the number of explants forming flower buds was counted. Shoot regeneration from explants was evaluated in the seven varieties 2 months after culture on SEM.

The pIG121-Hm expression vector (Ohta et al. 1990; Hiei et al. 1994) was used for the assessment of sensitivity to infection by *Agrobacterium*. Explants were infected with *A. tumefaciens* strain EHA105 harboring pIG121-Hm to compare its infective ability on seven soybean varieties. The sensitivity to infection with *Agrobacterium* was assessed on the basis of the visual intensity level of a histochemical β-glucuronidase assay (GUS assay) in explants after 5 days of co-cultivation.

To evaluate the efficiency of soybean transformation, pMDC123-GFP vector was constructed by replacing the Gateway cloning site in pMDC123 (Curtis and Grossniklaus 2003) with sGFP (Niwa et al. 1999) under the control of cauliflower mosaic virus 35S promoter. The EHA105 harboring pMDC123-GFP was cultured in 250 ml YEP liquid medium and was grown until the OD_{650} reached 0.3 to 0.5 at 28°C . For co-cultivation, the cell density of bacteria was adjusted to OD_{650} from 0.7 to 0.8 by suspension with liquid co-cultivation medium (CCM) containing 400 mg l^{-1} cysteine and 154 mg l^{-1} dithiothreitol. About 50 explants were immersed in *Agrobacterium* suspension with liquid CCM. After inoculation, explants were placed by setting the adaxial side down on filter paper laid over CCM solidified with 0.425% agar in Petri dishes (90 mm diameter×20 mm depth). After 5 days of co-cultivation, explants inoculated with *Agrobacterium* were washed in liquid SIM containing 25 mg l^{-1} Meropen® (Dai Nippon Sumitomo Pharma). The explants were set with their adaxial side up on solid SIM containing 0.7% agar, and 25 mg l^{-1} Meropen® for 2 weeks, and then transferred on SIM containing 6 mg l^{-1} glufosinate-ammonium (Sigma-Aldrich). The early developing shoots were removed by a surgical scalpel during shoot induction. After a total 4 weeks of culture on SIM, explants were transferred to SEM containing 25 mg l^{-1} Meropen® and 6 mg l^{-1} glufosinate and were subcultured every 2 weeks. During culture of the elongating shoots, the presence of transgenic shoots was confirmed by observation of GFP fluorescence. Among transgenic shoots emitting GFP fluorescence, shoots elongating over 2 cm were obtained and were dipped in 1 mg l^{-1} indole butyric acid then transferred to rooting medium. After 2 weeks, rooting plantlets were transplanted to soil in pots. The T_0 plants were grown in a growth chamber at 26°C with 16/8 h light/dark regime (fluorescent tubes at an intensity of about 100 μmols s^{-1} m^{-2}).

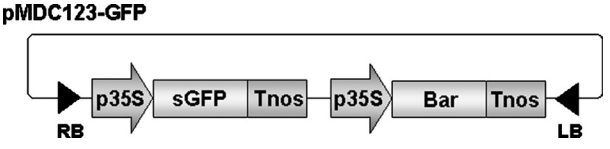

Figure 1. Structure of the expression plasmid, pMDC123-GFP. P35S, CaMV 35S promoter; Tnos, *A. tumefaciens* nopaline synthase terminator; sGFP, green florescent protein gene; Bar, phosphinothricin acetyltransferase gene; RB, right border; LB, left border. *Eco*RI is a unique restriction enzyme site in the T-DNA region of pMDC123-GFP.

Shoots were regenerated from explants in all 7 varieties after 4 weeks of culture on SIM. Varietal differences in the ability for precocious flower-bud formation were discerned in this study. Shoots of Kariyutaka showed a much higher ability to form *in vitro* flower buds than those of the other varieties (Figure 2A, B, Table 1). Transgenic Kariyutaka plants are expected to produce seeds earlier than the other varieties in this culture method. Although five Japanese soybean varieties showed nearly medium of maturity level in Japan, there was a large difference for the ability to form *in vitro* flower buds under the culture regime in this study. This fact indicates that the factor inducing *in vitro* flower buds is uncorrelated with their maturity levels. Culture conditions such as photoperiod, light source, concentration of cytokinins supplemented into medium, or some stresses might encourage the formation of flower bud from shoots of Kariyutaka. In fact, soybean under the cool white fluorescent light produced flowers early (Cober et al. 1996).

GUS assay revealed that Kariyutaka, Toyosuzu, Ohsuzu, Tachiyutaka, Jack and Thorne were similarly sensitive to infection with *Agrobacterium* (Table 2). Kariyutaka, which showed a high ability of forming flower buds during shoot elongation, had the highest ability for shoot regeneration from explants and the highest frequency of shoot elongation among five Japanese varieties used in this study (Table 2). The level of shoot regeneration in Kariyutaka was equivalent to that of Thorne (Table 2). Hence, Kariyutaka was the

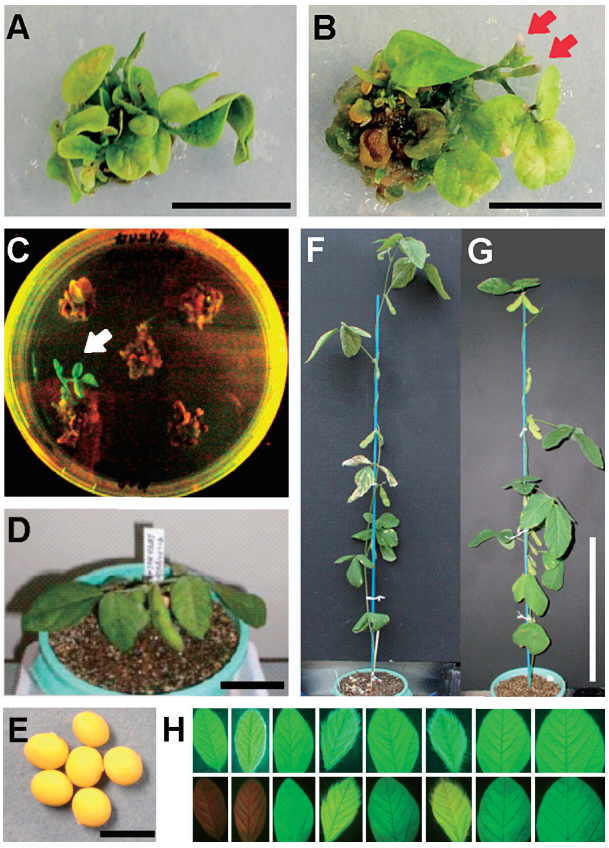

Figure 2. *In vitro* flower-bud formation in transgenic Kariyutaka. (A) Thorne explants after 4 weeks of culture on SEM, (B) Regeneration and flower bud formation from Kariyutaka explants after 4 weeks of culture on SEM. Red arrows indicate *in vitro* flower buds. Bars: 1.5 cm. (C) GFP-positive shoot of Kariyutaka. Explants grown in the 90 mm diameter plate were photographed under blue light at 490 nm wavelength. (D) Transgenic Kariyutaka plant after one month of potting. Bar: 5 cm. (E) Mature T_1 seeds of transgenic Kariyutaka. Bar: 10 mm. (F) Wild-type Kariyutaka after 2 months of sowing. (G) T_1 plants of Kariyutaka after 2 months of sowing. Bars: 30 cm. (H) Segregation of GFP in progenies of one transgenic Kariyutaka line (K1 line). Leaves were photographed under white (upper panels) or blue light (lower panels) at 490 nm wavelength.

most suitable variety for the rapid development of transgenic lines among genotypes used in this study.

We compared Kariyutaka to Jack and Thorne, which have been often used for *Agrobacterium*-mediated transformation (Yan et al 2000; Paz et al. 2004), for suitability to transformation using GFP expression analysis. Shoots expressing GFP (GFP-positive shoot) were regenerated from explants in all three varieties (Figure 2C). Total four GFP-positive plantlets were independently obtained from 522 explants of Kariyutaka (Table 3). The efficiency of transformation (about 1%) was comparable to that of Thorne (Table 3). All transgenic shoots of Kariyutaka formed flower buds early during cultivation on SEM. These transgenic Kariyutaka plants set pods after one month of potting (Figure 2D). T_1 seeds from transgenic Kariyutaka T_0 plants were obtained about 3 months earlier than from Thorne. This fact indicated that Kariyutaka may have switched from vegetative to the reproductive phase much earlier than Thorne under the culture regime in this study. Although only 5–10 of T_1 seeds were obtained from each transgenic T_0 Kariyutaka plant, they showed normal shape (Figure 2E). In short, T_1 seeds in transgenic Kariyutaka were obtained in only six months after co-cultivation with *Agrobacterium*.

Transgenic T_1 Kariyutaka plants showed normal growth similar to wild-type when cultured in a growth

Table 1. Ability of forming flower buds from cotyledon explants in seven soybean varieties.

Varieties	Number of explants	Number of explants forming flower buds	Efficiency (%)[1]
Jack	57	5	8.8
Thorne	47	0	0
Kariyutaka	52	23	44.2
Toyosuzu	47	5	10.6
Ohsuzu	43	2	4.7
Fukuyutaka	55	0	0
Tachiyutaka	49	3	6.1

[1] Percentage=(No. of explants forming flower bud/No. of explants used in this study) ×100.

Table 2. Levels of GUS expression after inoculation with *Agrobacterium* and ability of shoot regeneration from cotyledon explants in seven soybean varieties.

Variety	GUS expression		Shoot regeneration		
	Number of infected explants	Efficiency of infected (%)[1]	Number of explants	Regeneration rate (%)[2]	Number of shoots[3]
Jack	21	100	45	84.4	37 (0.82)
Thorne	19	89.5	40	75.0	36 (0.90)
Kariyutaka	18	94.4	44	70.5	28 (0.64)
Toyosuzu	16	87.5	39	61.5	15 (0.38)
Ohsuzu	21	95.2	38	60.5	8 (0.21)
Fukuyutaka	18	44.4	40	50.0	9 (0.23)
Tachiyutaka	19	84.2	40	30.0	5 (0.13)

[1] Percentage=(No. of GUS-positive explants with histochemical assay/No. of explants used in this study) ×100.
[2] Percentage=(No. of explants regenerating shoot/No. of explants used in this study) ×100.
[3] No. of shoots elongating over 5 mm. Numerical values in parentheses represent the No. of shoots/No. of explants.

Table 3. Efficiency of transformation in three soybean varieties.

Varieties	Number of infected explants	Number of explants expressing GFP	Number of T_0 plants expressing GFP	Transformation efficiency (%)[1]
Jack	399	3	0	0.0
Thorne	469	4	4	0.9
Kariyutaka	522	11	4	0.8

[1] Percentage=(No. of transgenic plants/No. of infected explants) ×100.

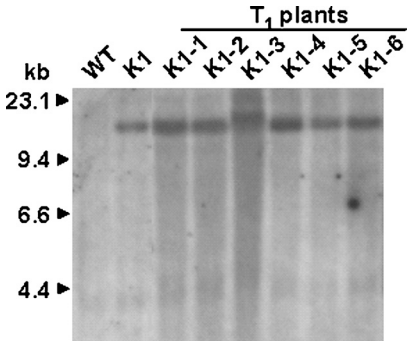

Figure 3. Southern blot analysis of transgenic Kariyutaka plants. WT and K indicate wild-type Kariyutaka and transgenic Kariyutaka lines, respectively. K1-1–K1-6 (GFP-positive plants shown in Figure 2H) were T_1 progenies derived from one transgenic T_0 Kariyutaka lines, K1 harboring a single copy of transgene. About 10 μg of genomic DNA was digested with restriction enzyme, *Eco*RI and subjected to electrophoresis on a 0.8% agarose gel.).

chamber at 26°C with 16 h photoperiod of fluorescent light (Figure 2F, G). The seeds of both T_1 Kariyutaka and wild-type plants were obtained only within 2–3 months after sowing in this artificial growth regime, whereas T_2 seeds of T_1 Thorne plants were obtained after 5 months. Although a few T_1 seeds were obtained in the T_0 Kariyutaka plants, the short generation cycle is expected to allow a more rapid fixation of the transgene and propagation of transgenic seeds by selfing. The T_1 generation showed a segregation pattern for the presence of GFP (Figure 2H). Southern blot analysis of GFP-positive T_1 generations by hybridization with a probe for *GFP* gene revealed that one (Figure 3) to several copies of transgenes were integrated into the T_1 plants. The low copy number of the transgene corresponded to previous reports of transformation mediated by *Agrobacterium* (Olhoft et al. 2003; Paz et al. 2006). This low copy number of the transgenes might assist an easy genetic fixation by self pollination.

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