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Note

Transformation and isoflavonoid analyses of suspension-cultured cells of soybean [*Glycine max* (L.) Merr. cv. Enrei]

Toshiyuki Waki, Seiji Takahashi, Toru Nakayama*

Department of Biomolecular Engineering, Graduate School of Engineering, Tohoku University, Sendai, Miyagi 980-8579, Japan
*E-mail: nakayama@seika.che.tohoku.ac.jp Tel & Fax: +81-22-795-7270

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Abstract We established cell suspension cultures of soybean [*Glycine max* (L.) Merr.] (cv. Enrei). Calli were induced from cotyledon explants on callus induction medium containing 1 mg l^{-1} kinetin and 0.1 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and then successfully suspension-cultured in Murashige and Skoog basal medium containing 1 mg l^{-1} kinetin and 0.05 mg l^{-1} 2,4-D. The resulting suspension-cultured cell line, designated as ECW1, has been maintained for 3 years by subculturing at 8-day intervals. Flavonoids that accumulated in ECW1 cells during cultivation were analyzed by high performance liquid chromatography. These analyses showed that 6″-*O*-malonyldaidzin was the most abundant flavonoid until day 8 of cultivation, followed by 6″-*O*-malonylgenistin, daidzin, and genistin. Trace amounts of isoflavone aglycons, glyceollin, and other flavonoids were detected during cell cultivation. We established an *Agrobacterium*-mediated method to transform suspension-cultured soybean cells using the ECW1 cell line as the host. A gene encoding green fluorescent protein was heterologously expressed under the control of the CaMV 35S promoter in ECW1 cells. The recombinant cells were stably maintained for 2.5 years via repeated subculturing. This is the first example of the stable transformation of suspension-cultured soybean cells using the *Agrobacterium* method. The cell line ECW1 is a promising tool for research on the biochemistry, molecular biology, and physiology of soybean.

Key words: *Glycine max* (L.) Merr. cv. Enrei, soybean, stable transformation, isoflavones, suspension-cultured cells.

Soybean [*Glycine max* (L.) Merr.] produces isoflavones, which are a legume-specific class of flavonoids. The major forms isoflavones produced by soybean plants are the 7-*O*-(6″-*O*-malonyl)-glucosides of daidzein and genistein (i.e., 6″-*O*-malonyldaidzin and 6″-*O*-malonylgenistin), which mainly accumulate in the roots, leaves, and seeds (Funaki et al. 2015; Graham 1991; Yoo et al. 2013). In soybean, isoflavones play very important roles in symbiotic relationships and defense mechanisms (Gould and Lister 2006). For example, endogenous isoflavones serve as *nod* gene inducers for bacterial production of Nod factors, which are essential for nodulation in soybean roots (Subramanian et al. 2007). Daidzein is exuded from the roots and serves as a chemo-attractant for nodule-inducing symbiotic soil microorganisms (Barz and Welle 1992; Graham 1991). Recently, Sugiyama et al. (2016) showed that, during the vegetative stage of soybean growth, isoflavones are secreted from the roots via the hydrolysis of their (malonyl)glucosides by a β-glucosidase localized in the root apoplast. Daidzein is also a precursor of glyceollin phytoalexins, which play a role in protection against pathogen infection (Ebel 1986; Graham and Graham 2000). Genistein displays antibiotic activity against fungal pathogens (Kramer et al. 1984; Rivera-Vargas et al. 1993) and is also involved in defense potentiation against pathogen infection and in establishing competence for elicitation in the glyceollin response (Graham and Graham 2000; Graham and Graham 1999; Park et al. 2002). In addition, there is growing interest in isoflavones in terms of human nutrition and medicine (Wiseman 2006). It has been shown that dietary intake of soybean isoflavones is related to decreased risks of various diseases, such as hormone-dependent cancers, cardiovascular disease, osteoporosis, and menopausal symptoms.

Cultured cells can be grown relatively quickly under strictly controlled conditions. As such, their growth is not affected by geographical and seasonal variations. Thus, plant cell cultures serve as an attractive alternative to whole plants as materials for the production of useful secondary metabolites and to study cellular physiology, metabolism, and their regulation in plants (Moscatiello et al. 2013; Ramachandra Rao and Ravishankar 2002).

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; GFP, green fluorescent protein; HPLC, high-performance liquid chromatography; MS, Murashige and Skoog.

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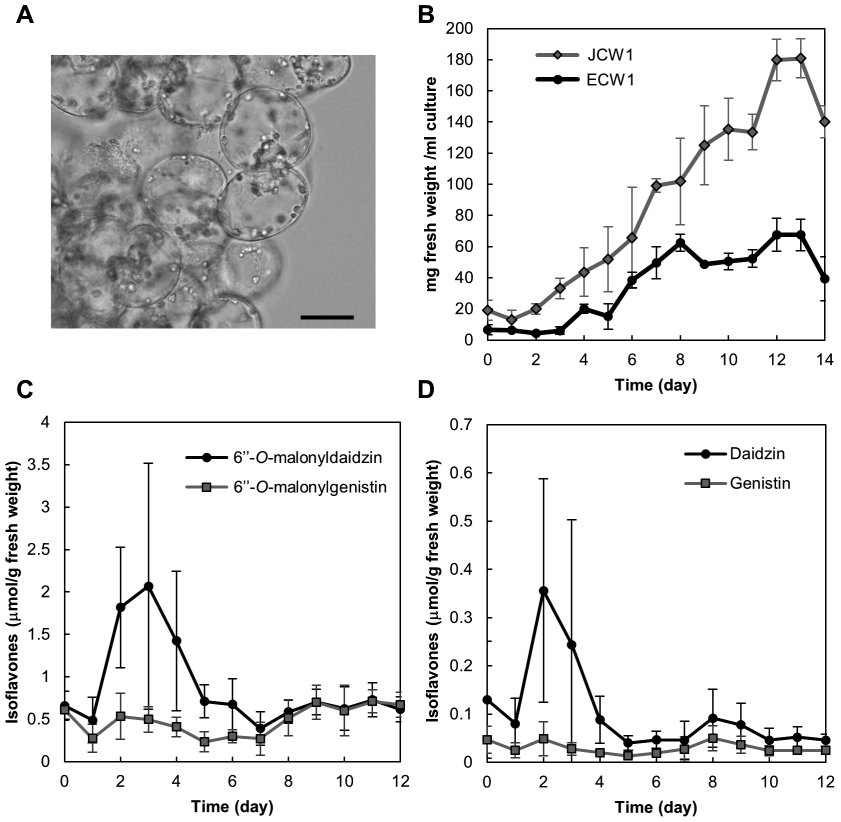

Figure 1. Suspension-cultured cell lines of soybean. (A) Suspension-cultured ECW1 cells grown in MS basal liquid medium containing 1 mg l^{-1} kinetin and 0.05 mg l^{-1} 2,4-D for 10 days in darkness. A bar, 30 μm. (B) Growth of ECW1 cell line (*closed circles*) and JCW cell line (*diamonds*) as determined by fresh weight of cells after removing liquid medium by centrifugation. *Error bars* show ±SDs of three independent determinations. (C) and (D) Changes in the flavonoid composition in ECW1 cells during cultivation [(C), 6″-*O*-malonyldaidzin (*closed circles*) and 6″-*O*-malonylgenistin (*squares*); (E), daidzin (*closed circles*) and genistin (*squares*)]. *Error bars* show ±SDs of four independent determinations.

Many suspension-cultured cell lines of soybean have been derived from different cultivars (Gamborg et al. 1968), and their isoflavonoid contents have been analyzed (Ames and Worden 1997; Federici et al. 2003; Ikeda et al. 1979; Sansanelli et al. 2014). Moreover, several studies have focused on the effects of biotic and abiotic stresses on isoflavonoid biosynthesis in soybean suspension-cultured cells (Akashi et al. 2009; Fett and Zacharius 1982; Hille et al. 1982; Kochs et al. 1987; Zacharius and Kalan 1990). Genetic engineering of soybean suspension-cultured cells could be a promising strategy to generate cells that are capable of producing isoflavonoids efficiently. However, there has been limited success in the genetic transformation of soybean suspension-cultured cells (Li et al. 1997). In this Note, we report on the establishment of a suspension-cultured cell line of soybean cv. Enrei, the flavonoids produced by this cell line, as well as its successful stable transformation via an *Agrobacterium*-mediated method.

To induce somatic callus formation, cotyledon explants (3-mm width) taken from 3-day-old soybean seedlings (cv. Enrei and cv. Jack) were placed onto callus-induction agar medium with the cut edge of the explants in contact with the medium. The callus-induction agar medium (pH 5.8), per liter, consisted of 4.6 g Murashige and Skoog (MS) plant salt mixture (Nihon Pharmaceutical, Tokyo, Japan), 1 ml of 1000-fold concentrate of Gamborg's vitamins (Sigma-Aldrich, St. Louis, MO, USA), 30 g sucrose, 1 mg kinetin, 0.1 mg 2,4-dichlorophenoxyacetic acid (2,4-D), and 6 g agar. After incubation for 2 weeks in the dark at 25°C, callus had formed from the cut edges of the explants. These calli were maintained under the same conditions as those used for callus induction by subculturing onto fresh medium every 3 weeks. To establish suspension-cultured cell lines, calli (1–2 cm) were divided into small pieces, each of which was transferred into 10 ml MS liquid medium (pH 5.8) containing varying concentrations of kinetin (0.1–10 mg l^{-1}) and 2,4-D (0.01–1 mg l^{-1}). The cultures were incubated at 25°C in the dark with shaking at 120 rpm. We established that the optimal phytohormone concentrations for suspension-cultured soybean cells (cv. Enrei and cv. Jack) were 1 mg l^{-1} kinetin and 0.05 mg l^{-1} 2,4-D. These concentrations resulted in the fastest cell proliferation rate. The suspension-cultured cells were subcultured at 8-day intervals by transferring 1 ml culture solution into 20 ml fresh medium. After subculturing several times, stably growing cultured cell

lines were established. The cultured cells were spherical or oval (Figure 1A). Figure 1B shows the typical growth curves of suspension-cultured cells of cv. Enrei and cv. Jack, designated as ECW1 and JCW1, respectively. The ECW1 cells proliferated very slowly during the first 3 days, but more rapidly during the following 5 days, as shown by the increase in fresh weight, followed by a stationary phase for a further 5 days. The maximum fresh weight was recorded at day 12. At this point, the fresh weight was more than 10-times greater than the initial fresh weight. During cultivation, cells were well separated from each other, although a small number of cell aggregates were present. Slight browning of the cultured cells was observed at day 10 or thereafter (not shown). The JCW1 cells proliferated during the first 12 days after inoculation, and then remained in the stationary phase for a further 5 days. The maximum fresh weight of the JCW1 cells (180 mg fresh weight/ml culture) was greater than that of the ECW1 cells (67.5 mg fresh weight/ml culture). Stably growing suspension-cultured cell lines of ECW1 and JCW1 were thus established and have been maintained for 3 years by subculturing at 8-day intervals.

The flavonoids that accumulated in the ECW1 cells were analyzed by reversed-phase high-performance liquid chromatography (HPLC), as described previously (Yoo et al. 2013). Specifically, weighed quantities of frozen cells were pulverized in liquid nitrogen with a mortar and a pestle. The flavonoids were extracted from the pulverized material with ethanol (10.0 ml per g material) at 4°C for 12 h followed by centrifugation and filtration (pore size, 0.22 μm) before HPLC analysis. The results are shown in Figures 1C and D. In the cultured cells, up to day 8 of cultivation, 6″-*O*-malonyldaidzin was the most abundant isoflavonoid, followed by 6″-*O*-malonylgenistin, daidzin, and genistin. Only trace amounts (less than 0.01 μmol g^{-1} fresh weight) of isoflavone aglycons (i.e., daidzein and genistein), glyceollin, and other flavonoids were detected during cell cultivation. The maximum amount of 6″-*O*-malonyldaidzin (4 times greater than that of 6″-*O*-malonylgenistin) was on day 3, and then the 6″-*O*-malonyldaidzin concentration decreased to a level comparable to that of 6″-*O*-malonylgenistin (Figure 1C). The amount of 6″-*O*-malonylgenistin did not significantly change (0.3–0.7 μmol g^{-1} fresh weight) throughout cultivation. Until day 8, the total amount of daidzein-related isoflavonoids was greater than that of genistein-related isoflavonoids. This isoflavonoid composition is similar to that reported for the roots of soybean seedlings, rather than that of their aerial parts (cotyledon and hypocotyls), despite the fact that the ECW1 cell line was derived from the cotyledons. For comparison, the concentrations, per g fresh weight, of 6″-*O*-malonyldaidzin, daidzein, and 6″-*O*-malonylgenistin in the lateral roots of seedlings of soybean cv. Enrei were reported to be approximately 0.52 μmol, 0.21 μmol, and 0.03 μmol, respectively (Yoo et al. 2013).

Next, we attempted to transform the established soybean suspension-cultured cells (ECW1 and JCW1; see above) using the *Agrobacterium* method. The cultured cells were co-cultivated with *Agrobacterium tumefaciens* cells which harbored the binary vector pBE2113Not containing the *sGFP(S65T)* gene. The *A. tumefaciens* strains examined for transformation were GV3101(pMP90), LBA4404(*vir*GN54D) (van der Fits et al. 2000), and EHA105. The *A. tumefaciens* transformant cells in the stationary-phase culture were washed, re-suspended in buffer [10 mM MES-KOH (pH 5.7) containing 10 mM $MgCl_2$ and 200 μM acetosyringone] to an optical turbidity of 0.5 at 600 nm. The *Agrobacterium* cell suspension was then allowed to stand at room temperature for 6 h. The soybean cells were grown at 25°C for 4–6 days in MS liquid medium in the dark with shaking at 120 rpm. To 5 ml culture, 50 μl *Agrobacterium* cell suspension (see above) and 50 μl 100 μM acetosyringone were added and the soybean cells were further grown at 25°C for 2 days in the dark with shaking at 120 rpm. The culture was allowed to stand for 10 min at room temperature before removing the supernatant. The co-cultivated soybean cells were washed three times with 5 ml MS liquid medium supplemented with 200 μg ml^{-1} Claforan (Hoechst Marion Roussel, Tokyo, Japan), suspended in 10 ml of the same medium, and then cultured at 25°C for 4 days in the dark with shaking at 120 rpm. The cultured cells were collected, suspended in 3 ml of the same medium, and plated on MS agar medium supplemented with 200 μg ml^{-1} Claforan and 50 μg ml^{-1} kanamycin. The cultured cells were then further grown at 25°C for 5–6 weeks in the dark until the newly formed calli reached 1–2 cm in size. A small cell aggregate from a callus was transferred to 20 ml MS liquid medium containing 200 μg ml^{-1} Claforan and 50 μg ml^{-1} kanamycin and the cultured cells were grown at 25°C in the dark with shaking at 120 rpm. After 7–10 days, an aliquot of the cell suspension was transferred to fresh medium containing 200 μg ml^{-1} Claforan and 50 μg ml^{-1} kanamycin. These cells were maintained as an independent cell line. Transformation of the cultured cells was confirmed by observation of cellular green fluorescence due to expression of green fluorescent protein (GFP) using a confocal laser microscope (excitation, 488 nm; observation, 495–555 nm). Among the *Agrobacterium* strains (see above) tested for transformation, EHA105 was the most successful strain for transforming cultured soybean cells. In the case of JCW1 cells (see above), kanamycin at concentrations of 10 μg ml^{-1} or higher resulted in severe browning of the cultured cells and their transformation was unsuccessful under the conditions described above. In the case of ECW1 cells, however, kanamycin even at a concentration

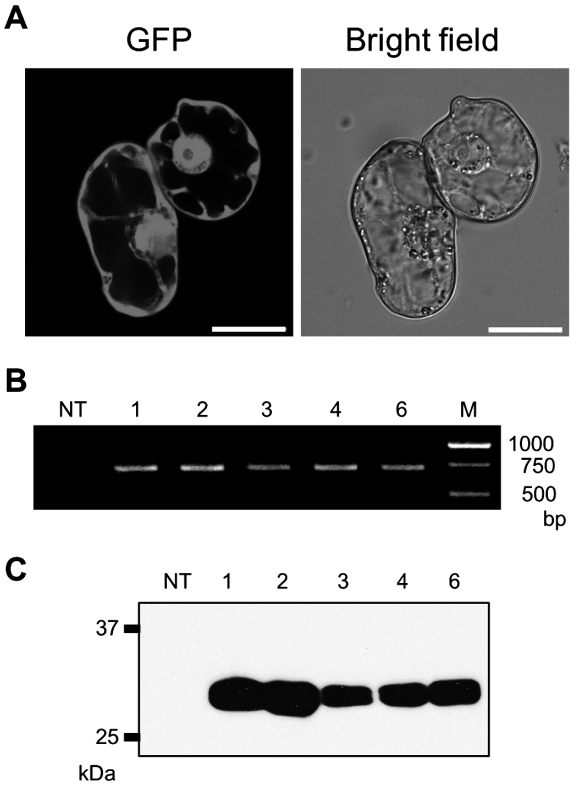

Figure 2. Heterologous expression of GFP by stable transformation of ECW1 cells. (A) Fluorescence imaging analyses of GFP-expressing recombinant cultured cells at day 6 of subculture. (*Left*) Fluorescence in cells was observed by laser scanning confocal microscopy (Leica TCS-SP8; Mannheim, Germany). For detection of GFP fluorescence, excitation wavelength was 488-nm white light laser pulse and signals at 495–555 nm were recorded using a HyD detector. (*Right*) Transmission image was recorded using a photomultiplier tube-type detector. Each image was collected in 'between lines' sequential scanning mode. Bars, 30 μm. (B) Full-length GFP DNA amplified by PCR from genomic DNAs of five different transgenic cell lines of ECW1 (1–4, 6) using *GFP*-specific primers. (C) western blotting analysis of GFP heterologously expressed in cells of five different transgenic lines of ECW1. NT, non-transgenic ECW1 cells.

of 50 μg ml^{-1} did not result in cell browning, and this permitted efficient transformation. Figure 2A shows confocal laser microscope images of the GFP-expressing recombinant ECW1 cells at day 6 of subculture. The recombinant cultured cells have been maintained for 2.5 years by subculturing at 8-day intervals into MS medium containing 200 μg ml^{-1} Claforan and 50 μg/ml kanamycin. After maintaining the recombinant cells for 2.5 years, the stability of transformation was examined by observing GFP fluorescence and by genomic PCR and western blotting analyses, as follows. Genomic DNA was extracted from five different recombinant cell lines using an innuPREP Plant DNA Kit (Analytik Jena, Jena, Germany). Using these DNAs as templates, PCR amplification was completed using GFP-specific primers (GFP-Fw, 5′-ATG GTG AGC AAG GGC G-3′; GFP-Rv, 5′-TTA CTT GTA CAG CTC GTC CAT G-3′) under the following thermal cycling conditions: 94°C for 2 min followed by 30 cycles of 94°C for 15 s, 55°C for 15 s, and 72°C for 1 min. A full-length GFP gene of the expected size (720 bp) was specifically amplified from each line (Figure 2B), confirming that these cell lines still harbored the *GFP* gene in their genomes even after 2.5 years of subculturing. Crude extracts of these recombinant cells were prepared by pulverizing cell material in extraction buffer [100 mM Tris-HCl (pH 8.0) containing 5 mM DTT]. The crude extracts were subjected to a western blotting analysis using peroxidase-conjugated mouse anti-GFP antibodies (Nacalai Tesque, Kyoto, Japan). The results showed that the GFP protein was present in all cell lines examined (Figure 2C), consistent with their cellular green fluorescence signals. All of these results confirmed the establishment of stable transformants. To the best of our knowledge, this is the first example of the stable transformation of suspension-cultured soybean cells using the *Agrobacterium* method.

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