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ORIGINAL ARTICLE

Increase of isoflavones in soybean callus by *Agrobacterium*-mediated transformation

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Abstract Plant secondary metabolites have always been a focus of study due to their important roles in human medicine and nutrition. We transferred the isoflavone synthase (IFS) gene into soybean [*Glycine max* (L.) Merr.] using the *Agrobacterium*-mediated transformation method in an attempt to produce transformed soybean plants which produced increased levels of the secondary metabolite, isoflavone. Although the trial to produce transgenic plant failed due to unestablished hygromycin selection, transformed callus cell lines were obtained. The induction rate and degree of callus were similar among the three cultivars tested, but light illumination positively influenced the frequency of callus formation, resulting in a callus induction rate of 74% for Kwangan, 67% for Sojin, and 73% for Duyou. Following seven to eight subcultures on selection media, the isoflavone content of the transformed callus lines were analyzed by high-performance liquid chromatography. The total amount of isoflavone in the transformed callus cell lines was three- to sixfold higher than that in control callus or seeds. Given the many positive effects of isoflavone on human health, it may be possible to adapt our transformed callus lines for industrialization through an alternative cell culture system to produce high concentrations of isoflavones.

Keywords Soybean callus · Isoflavonoids · *Agrobacterium*-mediated transformation · HPLC

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Introduction

Soybean [*Glycine max* (L.) Merr.], a well-known dicotyledonous crop grown worldwide, is famous for its content of plant proteins and secondary metabolites. Of the latter, the isoflavonoids have been receiving increasing attention for their multiple potential benefits in both plants and humans (Humphreys and Chapple 2000). In legumes, isoflavones function as signaling molecules for the symbiotic relationship between plant and rhizobial bacteria, as phytoalexins protecting plants from infection by microorganisms and fungus, and as floral pigments for attracting pollinators (Dixon and Steele 1999; Humphreys and Chapple 2000). Isoflavones share a similar chemical structure with human endogenous estradiol and can function as estrogen mimics, resulting in their name, phytoestrogens. Phytoestrogens play an important role in the prevention of breast and prostate cancer, cardiovascular disease, and osteoporosis (Cornwell et al. 2004; Cos et al. 2003; Dixon and Ferreira 2002; Huntley and Ernst 2004; Ososki and Kennelly 2003).

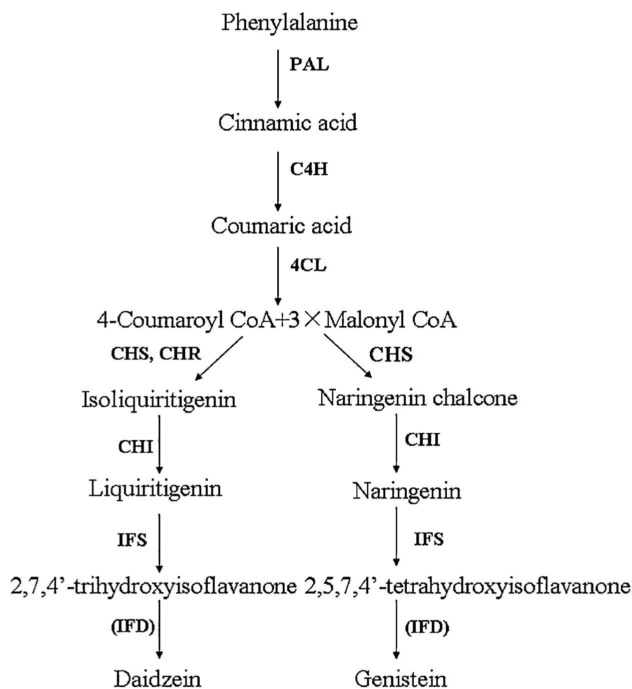

Fig. 1 Diagram of the phenylpropanoid pathway which produces the isoflavones. *PAL* Phenylalanine ammonia lyase, *C4H* cinnamate 4-hydroxylase, *4CL* 4 coumarate CoA ligase, *CHS* chalcone synthase, *CHR* chalcone reductase, *CHI* chalcone isomerase, *IFS* isoflavone synthase, *IFD* isoflavone dehydratase

Soybean isoflavones are produced from the phenylpropanoid pathway in which three main compounds (Fig. 1), existing in four chemical forms, are involved: the aglycone forms (daidzein, genistein, and glycitein); the glucoside forms (daidzin, genistin, and glycitin); their malonyl-glucoside and acetyl-glucoside forms (Reinli and Block 1996; Wang and Murphy 1994). The isoflavone synthase (IFS), a cytochrome P450 oxygenase, catalyzes the key step of isoflavone biosynthesis, resulting initially in the production of 2-hydroxyisoflavanone from the ubiquitous intermediates naringenin and liquirigenin; the 2-hydroxyisoflavanone may then go through a dehydration reaction catalyzed by 2-hydroxyisoflavanone dehydratase (HID) or it may also spontaneously dehydrate (Humphreys and Chapple 2000; Liu et al. 2007).

The successful cloning of the IFS gene (Akashi et al. 1999; Jung et al. 2000; Steele et al. 1999) has armed researchers with a tool by which to study the biosynthesis and metabolic engineering of isoflavones production by introducing the recombinant IFS gene into many plants or cells, such as Arabidopsis (Liu et al. 2002), alfalfa (Deavours and Dixon 2005), maize cells, tobacco, lettuce, and petunia (Liu et al. 2007; Yu et al. 2000). By combining gene activation using the transcription factor CRC and blocking a competing branch pathway, Yu et al. (2003) were able to engineer a fourfold increase in isoflavone synthesis. A study involving the IFS/CHI (chalcone isomerase) bifunctional enzyme in yeast and tobacco by in-frame gene fusion proved that IFS/CHI bifunctional enzyme worked more efficiently than each of these enzymes individually to produce isoflavones (Tian and Dixon 2006).

In the study reported here, we transferred the IFS gene into the soybean cotyledon and induced transformed callus as well as plant regeneration by the *Agrobacterium*-mediated soybean transformation method using mature cotyledonary-node as explants (Olhoft et al. 2003; Paz et al. 2004). Although regenerated plants were not obtained due to unestablished hygromycin selection, transformed callus was successfully obtained and subsequent gene introduction and increased isoflavone production were confirmed.

Materials and methods

Seed materials and preparation

Mature soybean seeds of three elite Korean cultivars, namely, Sojin, Kwangan, and Duyou were used in this experiment. All mature soybean seeds were kindly supplied by the soybean breeding team of Korean Rural Department of Agriculture. Dry seeds were surface sterilized (100–200 seeds/90 × 15-mm SPL petri dish) for about 16 h using chlorine gas produced by mixing 5 ml of 12 N HCl and 100 ml commercial bleach in a tightly sealed desiccator (Paz et al. 2006). The seeds were then stored in a refrigerator at −20°C until used. The day prior to inoculation, seeds were soaked in 1% sodium hypochlorite solution mixed with 3 drops of Tween 20 (Yakuri, Japan), shaken for 10 min on mini-3-D shaker, washed three times with sterilized, and held at room temperature overnight for inoculation. Several media were prepare for the experiments (Table 1).

Vector construction and *Agrobacterium* strain

A gene cassette, denoted the CIP cassette, was constructed by inserting the IFS gene provided by Dr. W.S. Chung (Konkuk University) between the seed-specific conglycinin promoter and the phaseolin 3′ terminator. The CIP cassette was then integrated into the T-DNA region of binary vector pCMABIA-1301 (CAMBIA, Australia), which carried the plant selection marker hygromycin, bacterial selection marker kanamycin, and the β-glucuronidase (GUS) gene as a reporter controlled by the cauliflower mosaic virus (CaMV) 35S promoter [Supplementary Electronic Material (ESM) Fig. S1]. The plasmid pCAMBIA1301-CIP was transferred into *Agrobacterium* strain EHA-105 by electronic shock and subsequent culture on the YEP media containing 100 mg l^{-1} kanamycin and 50 mg l^{-1} rifampicin, at 28°C for 2 days. Single colonies were obtained and tested by PCR to verify the vector transformation in

Table 1 List of media used in this experiment

Media	Composition	
Liquid YEP	NaCl 5 g l^{-1}, peptone 10 g l^{-1}, yeast extract powder 10 g l^{-1}, kanamycin 50 mg l^{-1}, rifampicin 25 mg l^{-1}, pH 7.0	
CCM (co-cocultivation media)	MES 4.26 g l^{-1}, Gamborg B5 (including vitamins) 0.32 g l^{-1}, sucrose 30 g l^{-1}, 6-BA 1.67 mg l^{-1}, gibberellic acid 0.25 mg l^{-1}, acetosyringone 0.2 mM, L-cysteine 3.3 mM, DTT, 1.0 mM, sodium thiosulfate 1.0 mM, Sigma agar 6.8 g l^{-1}, pH 5.4	
CIM (callus induction media)	MES 0.6 g l^{-1}, B5 3.2 g l^{-1}, sucrose 30 g l^{-1}, 2,4-D 2 mg l^{-1}, cefotaxine 500 mg l^{-1}, Sigma agar 7.6 g l^{-1}, pH 5.6	
	Hygromycin	10 mg l^{-1}
	6-BA	1 mg l^{-1}
Liquid media	CIM	Same as solid media but without agar

BA 6-Benzylaminopurine, *MES* 2-(*N*-morpholino)ethanesulfonic acid, *DTT* dithiothreitol

Agrobacterium. PCR-positive colonies were chosen, and competent cells were prepared by mixing actively growing *Agrobacterium* (0.8–1.0 at OD_{600}) with equal volumes of 30% glycerol. Aliquots of competent cells were frozen and kept them at −70°C until used.

Preparation of *Agrobacterium* cultures for inoculation

Before inoculation, 1 or 2 ml of *Agrobacterium* stock was mixed in 200 ml liquid YEP media with antibiotics (25 mg l^{-1} rifampicin, 50 mg l^{-1} kanamycin) and cultured in a 500-ml flask for nearly 20 h until the OD_{600} reached between 0.8 and 1.0. Thereafter, approximately 50 ml of *Agrobacterium* culture was centrifuged (7000 rpm, 20°C, 15 min) and the cell pellet thoroughly re-suspended in 15 ml liquid co-cultivation medium (CCM) for inoculation.

Inoculation and co-cultivation of soybean

The soaked seeds were inoculated with *Agrobacterium.* A longitudinal cut between the two cotyledons generated two identical explants; the seed coat and primary shoots were then removed and the hypocotyl also cut off using a No. 11 scalpel blade (Feather, Japan). The junction region between the cotyledon and hypocotyl was wounded by using the blade of the scalpel and making ten cuts perpendicular to the hypocotyl. All of the inoculated explants were immersed in 15 ml of the *Agrobacterium* suspension, treated with sonication for 20 s and vacuum for 30 s, and then placed at room temperature for 30 min. After inoculation, each ten cotyledons were blotted dry on sterilized filter paper and placed in a 90 × 15-mm petri dish containing approximately 30 ml of solid CCM. The CCM was overlaid with a piece of filter paper (Acvantec, Japan) to prevent overgrowth of *Agrobacterium.* The co-cultivation continued for 5 days at 24°C under a 18/6-h light/dark photoperiod.

Callus induction and subculture

After 5 days co-cultivation, the hypocotyl of the explants was cut off, and the explants were briefly washed to remove any remaining *Agrobacterium* and then blotted dry on sterilized filter paper. At the beginning of experiment, callus was naturally derived from the wound region of cotyledon. In an experiment to compare the efficiency of callus induction, each of the explants was embedded in solid CI (callus induction) medium in a petri dish and cultured in total darkness or under an 18/6-h light/dark photoperiod to observe the callus induction response among the three cultivars. The CI media used for first 2 weeks of the culture period did not contain hygromycin B, and the callus used as a control was induced under the same conditions as the experimental callus without any *Agrobacterium* inoculation. The induction rate was calculated as the number of cotyledons that showed callus formation/total number of cotyledons × 100 (%). The quality of the callus formation was estimated at three levels of callus induction: good (+++), average (++), or poor (+) callus formation. The percentage for the quality degree was calculated as the number of calli with the defined degree/total number of cotyledons showing callus formation × 100 (%).

Following transformation, two different subculture methods were tested for efficient callus induction: (1) liquid culture followed by solid culture; (2) solid culture only. Samples of randomly selected callus (diameter 0.5–1 cm) were carefully transferred onto the solid CI media or liquid CI media (both containing 10 mg l^{-1} hygromycin) to compare the selection and culture efficiency. The media was also supplemented with 1 mg l^{-1} 6-benzylaminopurine (6-BA) to compare the effect of 6-BA on the growth of the callus. The callus was to solid culture after 2 weeks of culture in liquid medium later. The survival rate was calculated as the number of calli showing active growth/number of total original calli placed in medium × 100%.

Histochemical assay of GUS activity

Calli were assessed for GUS activity (Jefferson et al. 1987) by randomly taking callus samples from each petri dish during different stages of subculture and placing them in GUS staining solution which contained a 5-bromo-4-chloro-3-indolyl glucuronide (X-Glu). The positive blue spots were visually assessed.

PCR and reverse transcription-PCR

Genomic DNA was extracted from both transformed callus cell lines subcultured for more than 3 months and from the control callus. The 1,000-bp *hpt* (encoding hygromycin B phosphotransferase) coding region was amplified using the 27-bp forward primer (5′-ATGAAAAAGCCTGAACT CACCGCGACG-3′) and 26-bp reverse primer (5′-CTAT TTCTTGCCCTCGGACGAGTGCT-3′). The PCR assay was performed using a Bioneer PCR kit and thermal cycler (PTC-100; MJ Reseach, Waltham, MA) under the following conditions: one cycle of 94°C for 10 min, 35 cycles of 94°C for 1 min, 55°C for 45 s, and 72°C for 1 min, with a final extension at 72°C for 10 min. The product was separated by electrophoresis on a 1% agarose gel with ethidium bromide.

Total RNA was also isolated from callus using the Invitrogen Plant RNA Purification Reagent kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The reverse transcription (RT)-PCR was performed using the Invitrogen One-Step RT-PCR kit with platinum *Taq* according to the manufacturer's instructions. Two primers were designed for the sequence of the IFS gene: a 23-bp forward primer (5′-CCCACGAGGCAACTTCCTT CAAC-3′) and a 24-bp reverse primer (5′-GTGGGCA AAGATAGACTCGTTGAC-3′).

General extraction and high-performance liquid chromatography analysis of isoflavones

Callus and seeds of the three cultivars were freeze dried (0.5 g), ground to powder, and then mixed with 50% MeOH with shaking for 12 h at room temperature. The extracts were then filtered through No. 2 Whatman filter paper and then filtered again through a 0.45-µm filter for the high-performance liquid chromatography (HPLC) analysis.

Isoflavone contents in all samples were analyzed using a reverse-phase C_{18} LiChro CART 5-µm column on an Agilent 1100 series liquid chromatograph (including the pumping system, vacuum degasser, autosampler, and UV-DAD detector) (Agilent Technologies, Santa Clara, CA). Sample injection volume was 20 µl, and the mobile phase was water with 1% acetic acid (A) and CH_3CN (B). A linear gradient elution was applied from 10 to 40% B, starting from 0 to 50 min, at a flow rate of 1.0 ml min^{-1}. The column temperature was 30°C, and the detection wavelength was set at 260 nm. Isoflavones were identified by comparing the sample retention times with those of authentic purified standards. The content of isoflavones was calculated by the standard regression equation of each compound.

Results and discussion

Callus induction of three Korean soybean cultivars

The IFS gene was transformed into Korean soybean cv. Sojin using the *Agrobacterium*-mediated transformation method in an attempt to produce transgenic soybean plants which intrinsically produced higher levels of the secondary metabolite isoflavone. Regeneration of the transgenic plant failed, but transformed callus was successfully obtained during hygromycin selection. Following preliminary experiments, we subsequently focused on developing an efficient callus induction system.

Three Korean soybean cultivars, Sojin, Kwangan, and Duyou, were used for the callus induction experiments due to their higher tissue culture response in the preliminary studies (data not shown). Following the transformation of soybean explants with the IFS gene, the explants were placed on callus induction media for 2 weeks without hygromycin selection pressure. Callus was formed from the wounded junction between the cotyledon and hypocotyl. Callus induction was rated and compared under two different lighting conditions, an 18/8-h photoperiod or total darkness (Fig. 2). The induction rate varied in the explants of the three genotypes when cultured under total darkness, with induction rates of 70, 79, and 69% for cvs. Kwangan, Sojin, and Duyou, respectively. In contrast, the induction rate was stable under the long-day (light) condition, with induction rates of 84, 84, and 84% for cvs. Kwangan, Sojin, and Duyou, respectively (Fig. 2a). These results suggest that callus induction was influenced positively by light illumination and that the plant genotype influenced the response. Given the fact that these three cultivars had been chosen for their high response in tissue culture, based on the results of the preliminary tests, we had expected a higher callus induction rate. The degree of callus formation was also investigated as an estimate of the qualitative characteristic of callus induction (Fig. 2b). It is important to achieve the initial induction of callus within a short period of time in order to obtain healthy and fast-growing callus at an early stage. The degree of the callus induction was evaluated visually as good (+++), average (++) or poor (+). The formation of a good degree of callus was

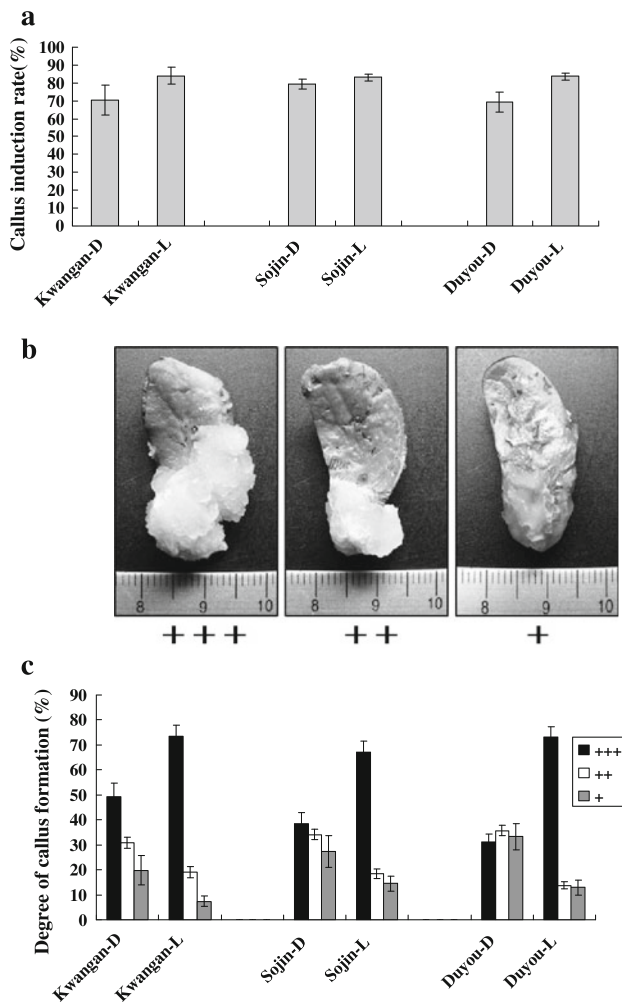

Fig. 2 Callus induction rate of three Korean soybean cultivars, Sojin, Kwangan, and Duyou (**a**), the defined degree of callus for visual evaluation (**b**), and the degree percentage of callus formation (**c**). *D* darkness, *L* 18/6-h light/dark photoperiod. The induction rate was calculated as the number of cotyledons that shows callus formation/ total number of cotyledons inoculated × 100%. The degree percentage was calculated as the number of callus with the defined degree/ total number of cotyledons that show callus formation × 100%. Data were obtained from three replications of experiment. +++ Good, ++ average, + poor. *Bar* Mean ± standard deviation (SD)

49% for Kwangan, 38% for Sojin, and 31% for Duyou in total darkness, which was much lower than the percentages in the light condition. Light illumination positively influenced the formation of a good degree of callus, with 74% for Kwangan, 67% for Sojin, and 73% for Duyou (Fig. 2c). This result indicates that light is an important factor in soybean callus induction. The morphology of callus from each of the three cultivars was different, with cvs. Kwangan and Sojin forming white or light-yellow, compact callus and Duyou forming liquid soft callus that could barely be handled with a blade or forceps. Although the texture of callus can be simply changed by modifying the media, such as by increasing the hardness of the media or

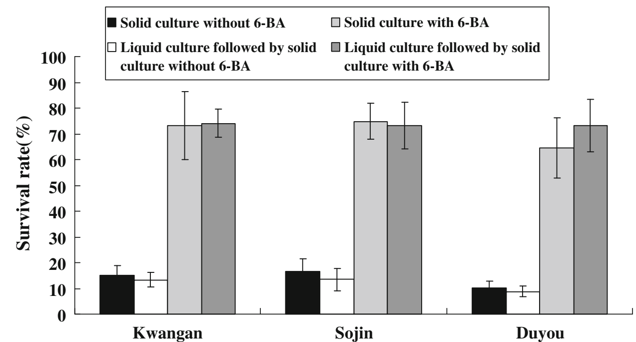

Fig. 3 The survival rate of different culture methods, with or without 6-benzylaminopurine (*6-BA*). Survival rate = number of cotyledons that show continuous callus growth/number of total cotyledons × 100%. Data were obtained from three replications of the experiment. *Bar* Mean ± SD

changing the hormone concentration, researchers should always be aware of which type of callus would be appropriate for a certain type of culture system before entering upon the mass production of that callus.

Comparison of different subculture methods and effect of 6-BA

Two different subculture methods, with or without 6-BA, were tested for callus induction on selection media: (1) liquid culture followed by solid culture and (2) solid culture only (Fig. 3). Liquid culture in the early stage of callus development was tested as a possible approach to enabling transformed callus to be selected as early as possible. Each subculture method was tested with or without 1 mg l^{-1} 6-BA to compare the effect of 6-BA in growing transformed callus under hygromycin pressure. In the absence of 6-BA, very low survival rates (around 10%) were observed for the transformed callus of all three cultivars, and there was little difference in survival rate between callus grown in liquid medium and that grown on solid medium. In comparison, callus showed a survival rate of approximately 70% when grown in/on medium supplemented with 6-BA. This result reveals that cytokinin 6-BA is an essential first requirement for efficient callus multiplication and survival. The age of the callus during testing was about 2 months. Our result indicates that the presence of both auxin and cytokine is essential during the early stages of callus growth and for survival under selection. To date, few studies have been reported on callus induction or the genetic transformation of callus of soybean.

Confirmation of the gene transformation and expression

The initial confirmation of gene introduction was determined using the GUS assay after 5 days of co-cultivation

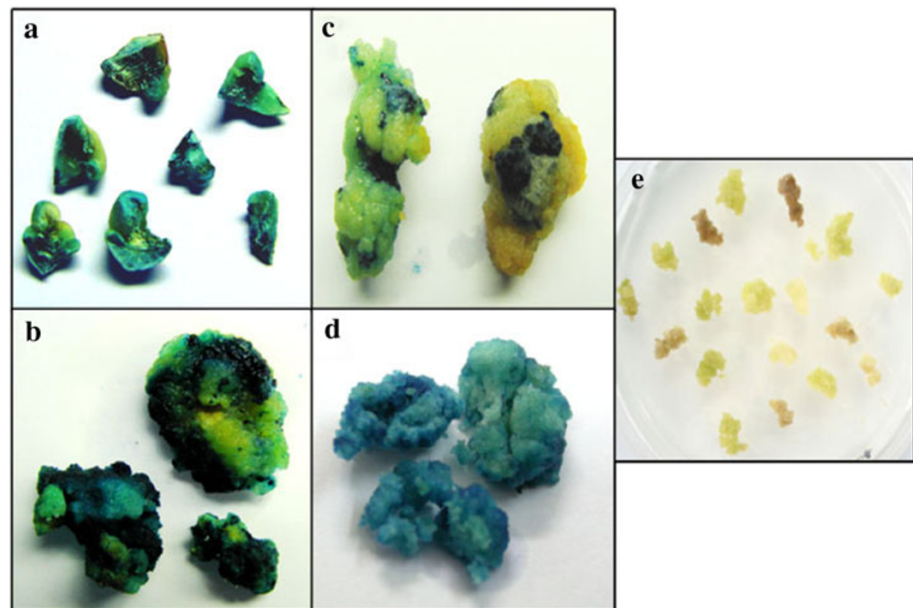

Fig. 4 β-Glucuronidase (GUS) analysis of transformed cotyledons and induced callus. GUS staining of cotyledons after 5 days of co-cultivation (**a**) and 1–4 weeks after transformation (**b**–**d**). Newly grown callus on the selection media was subcultured to fresh media at 2-week intervals (**e**)

(Fig. 4a). Distinctive GUS expression was detected from the co-cultivated cotyledons of soybean. All three cultivars showed similar levels of infection by *Agrobacterium*. In order to confirm that transformed cells continue to grow and multiply in callus induction media under hygromycin selection, GUS analyses were repeated during during the first 4 weeks after transformation (Fig. 4b–d). After 4 weeks of culture in liquid or solid media, newly grown callus was separated from old callus and subcultured every 2 weeks to isolate transformed cell lines. Callus from Duyou changed into a friable and transparent state compared to callus from Kwangan and Sojin, both of which were compact and yellow or green. Due to the unhealthy condition of the callus originating from Duyou, only cell lines from Kwangan (K1–K6, 6 lines) and Sojin (S1–S5, 5 lines) were maintained for further subculture. Differences in cell viability were clearly visible during continuous subculture under hygromycin selection, making it possible to choose only the yellow or green parts of newly grown callus for subculture to fresh media every 2 weeks (Fig. 4e). Several cell lines were isolated and established after >3 months of subculture in selection media, and GUS analysis was performed to detect homogeneous transformed cell lines. Based on the result of the GUS analysis, six cell lines from Kwangan and five cell lines from Sojin were subcultured and maintained. PCR amplification of genomic DNA confirmed the successful introgression of the foreign gene in the transformed cell lines (Fig. 5a). PCR analysis of genomic DNA extracted from the six randomly selected cell lines of Kwangan showed clear amplification of the hygromycin resistance gene (*hpt*). RT-PCR indicated that expression of the exogenous IFS in

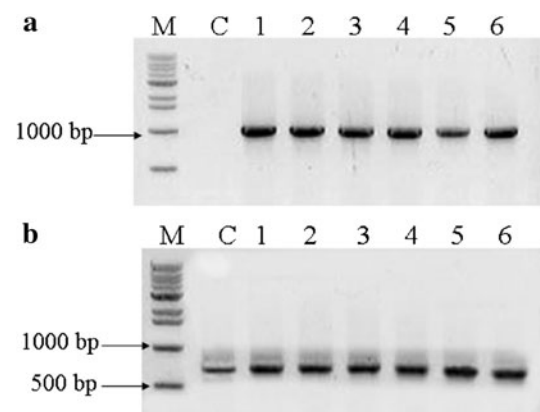

Fig. 5 Genomic DNA PCR with hygromycin primers (**a**) and reverse transcription (RT)-PCR with the isoflavone synthase (IFS) primers (**b**). *Lanes M* 1-kb marker, *C* non-transformed control callus, *1–5* transformed callus cell lines randomly picked from the petri dish

the transformed lines had increased the total IFS transcript amount in comparison to the control (Fig. 5b), although any incomplete homogeneity of the cell line tested may also have affected this result. The cell lines were maintained on selection media by subculture and used for HPLC analysis.

HPLC analysis of transformed cell lines

The five and six cell lines originating from cvs. Sojin and Kwangan, respectively, were analyzed for their isoflavone content relative to that of the non-transformed cell line and seed. The amounts of four isoflavones were checked: malonyl-genistin, malonyl-daidzin, genistein, and daidzein (Fig. 6a, c). There was relatively more malonyl-daidzin

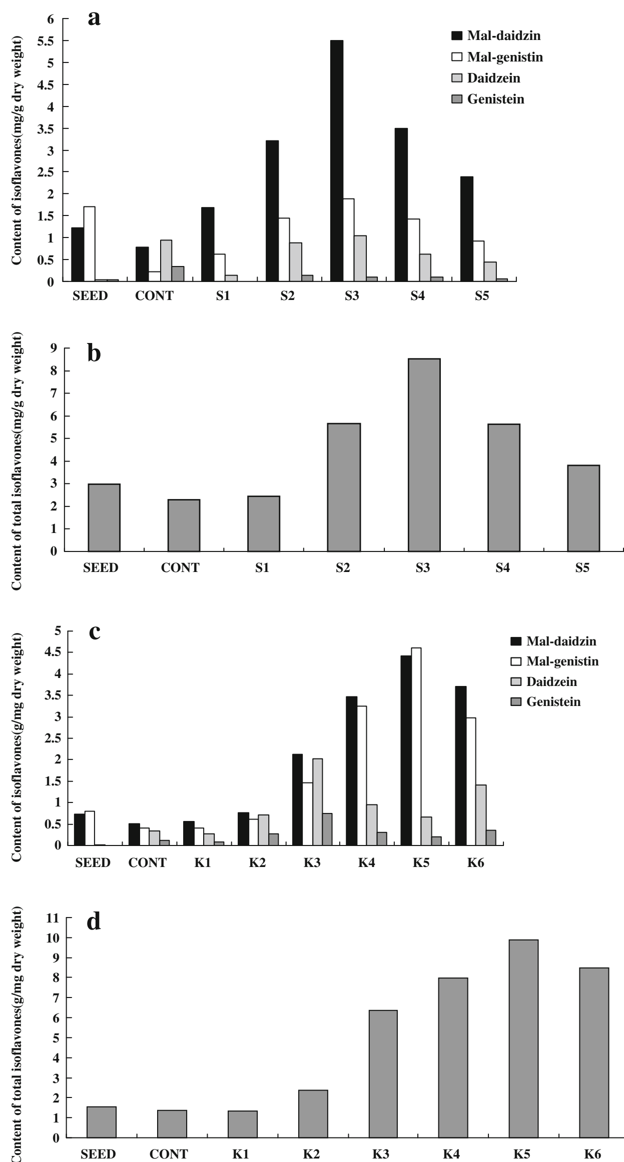

Fig. 6 The contents of four soy-isoflavones produced by soybean cvs. Sojin and Kwangan (**a**, **c**) and total amount of isoflavones (**b**, **d**) (mg g^{-1} dry weight). *S1–S5* Transformed cell lines of cv. Sojin, *K1–K6* transformed cell lines of cv. Kwangan, *CONT* control callus without transformation, *SEED* none-transformed seeds from the two cultivars

than of the other isoflavones in the transformed cell lines originating from cv. Sojin, although the amounts of the other isoflavones, malonyl-genistin, daidzein, and genistein, were similar to those found in the non-transformed cell line or seed. In comparison, the amounts of malonyl-daidzin and malonyl-genistin were similar in the transformed cell lines originating from cv. Kwangan and daidzein content slightly increased in the transformed cell lines of cv. Kwangan relative to the control line and seed. Even though the total amount of isoflavones was similarly increased in the transformed cell lines originating from both cultivars, the intrinsic composition of isoflavones was slightly different. The highest total content of isoflavone was 8.5 mg g^{-1} dry weight (S3 line) for cv. Sojin and 9.8 mg g^{-1} dry weight (K5 line) for cv. Kwangan; this is three to sixfold higher than that found in the seed or control cell line (Fig. 6b, d). To confirm the result from the cell lines which showed higher amounts of isoflavones, the contents of four soy-isoflavones for cell line S3 from cv. Sojin and K5 from cv. Kwangan and the total amount of isoflavones (mg g^{-1} dry weight) were measured after several subcultures to increase the volume of cells (Fig. 7a, b; ESM Fig. S2). Five samples of cells randomly selected from both cell lines, S3 and K5, were repeatedly analyzed by HPLC. Compared to the first HPLC analysis, overall isoflavone contents were elevated. Among the soy-isoflavones, malonyl-daidzin and malonyl-genistin were major forms of isoflavones, as in the first analysis. However, both transformed and control cell lines showed an increased level of isoflavone. Due to the limited scope of our experiments, we could not directly address the reason why two soybean transformed lines would show different compositions of isoflavone content after transformation with the same IFS gene. Nonetheless, it could be generally hypothesized that the different composition of isoflavones

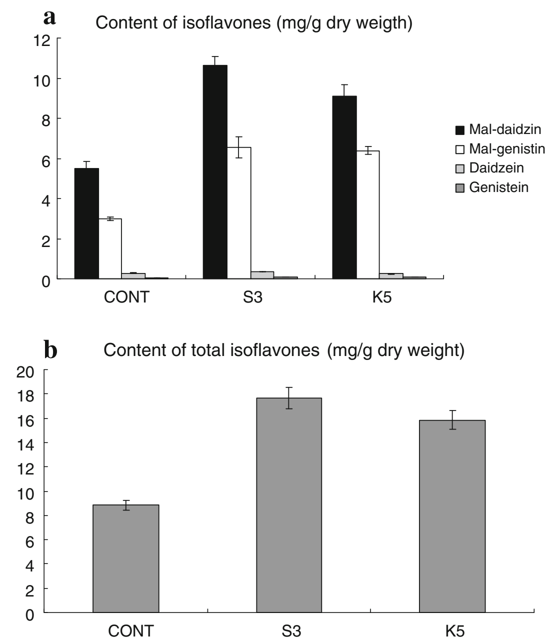

Fig. 7 Contents of four soy-isoflavones from the selected cell lines, S3 from cv. Sojin and K5 from cv. Kwangan (**a**) and the total amount of isoflavones (**b**) (mg g^{-1} dry weight). *S3* One of the transformed cell lines which shows the highest amount of isoflavone content among the callus lines originated from cv. Sojin, *K5* a callus cell line with the highest isoflavone content originating from cv. Kwangan, *CONT* control callus without transformation. Five replications were made with cells from five different areas of each line. *Bars* Standard deviation of five replications

may be an effect of a different genetic background, such as a different expression level of genes involved in the isoflavone pathway. The isoflavone pathway is one of the most complicated metabolic pathways involved in plant metabolism (Yu et al. 2000). The observed variation in isoflavone amount in the different cell lines may be due to a different homogeneity of each cell line or to a different level of ectopic IFS expression. It is important to realize that callus cell lines are easily exposed to somaclonal variation and that somaclonal variation is very difficult to prevent during continuous cell culture. Somaclonal variation could be one of the reasons for the variation in isoflavone level among cell lines (Federici et al. 2003).

The three- to sixfold increase in isoflavone content that was achieved through the introduction of a single IFS gene into soybean callus is a successful achievement when we consider the well-known health-promoting effects of isoflavone and its commercial value in the pharmaceutical industry. More effort should be brought into this research area to identify a way to manipulate the cell lines to produce even more isoflavone. To this end, the area upstream or downstream of the IFS gene can be manipulated to activate the phenylpropanoid pathway or reduce the competition of the substrate by knock-out of an alternative pathway, resulting in increased intermediate availability to IFS (Liu et al. 2007). Another possible approach is to manage the culturing conditions. The biosynthesis of isoflavone in soybean is known to be regulated by environmental conditions. The modification of some of the culture conditions, such as providing an essential substrate (i.e., phenylalanine or coumaric acid) or applying a relatively low temperature to cell culture, may turn out have a positive effect on isoflavone production.

To the best of our knowledge, this study is the first trial in which the IFS gene was transformed into soybean callus with the result of an increased production (three- to sixfold) of the total amount of isoflavones in the transformed callus cell lines relative to the control callus or the seeds. Future experiments by our group will focus on the transformation of a second set of genes with the aim of activating the flow of the metabolic pathway or reducing the competition of the substrate through knock-out of an alternative pathway.

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References

Akashi T, Aoki T, Ayabe S (1999) Cloning and functional expression of a cytochrome P450 cDNA encoding 2-hydroxyisoflavanone synthase involved in biosynthesis of the isoflavonoid skeleton in licorice. Plant Physiol 121:821–828

Cornwell T, Cohick W, Raskin I (2004) Dietary phytoestrogens and health. Phytochemistry 65:995–1016

Cos P, De Bruyne T, Apers S, Vanden Berghe D, Pieters L, Vlietinck AJ (2003) Phytoestrogens: recent developments. Planta Med 69:589–599

Deavours BE, Dixon RA (2005) Metabolic engineering of isoflavonoid biosynthesis in alfalfa. Plant Physiol 138:2245–2259

Dixon RA, Ferreira D (2002) Genistein. Phytochemistry 60:205–211

Dixon RA, Steele CL (1999) Flavonoids and isoflavonoids—a gold mine for metabolic engineering. Trends Plant Sci 4:394–400

Federici E, Touché A, Choquart S, Avanti O, Fay L, Offord E, Courtois D (2003) High isoflavone content and estrogenic activity of 25 year-old *Glycine max* tissue cultures. Phytochemistry 64(3):717–724

Humphreys JM, Chapple C (2000) Molecular ‘pharming’ with plant P450s. Trends Plant Sci 5:271–272

Huntley AL, Ernst E (2004) Soy for the treatment of perimenopausal symptoms: a systematic review. Maturitas 47:157–166

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6:3901–3907

Jung W, Yu O, Lau SM, O’Keefe DP, Odell J, Fader G, McGonigle B (2000) Identification and expression of isoflavone synthase, the key enzyme for biosynthesis of isoflavones in legumes. Nat Biotechnol 18:208–212

Liu CJ, Blount JW, Steele CL, Dixon RA (2002) Bottlenecks for metabolic engineering of isoflavone glycoconjugates in *Arabidopsis*. Proc Natl Acad Sci USA 99(22):14578–14583

Liu R, Hu Y, Li J, Lin Z (2007) Production of soybean isoflavone genistein in non-legume plants *via* genetically modified secondary metabolism pathway. Metab Eng 9(1):1–7

Olhoft PM, Flagel LE, Donovan CM, Somers DA (2003) Efficient soybean transformation using hygromycin B selection in the cotyledonary-node method. Planta 216:723–735

Ososki AL, Kennelly EJ (2003) Phytoestrogens: a review of present state of research. Phytother Res 17:845–869

Paz MM, Shou HX, Guo ZB, Zhang ZY, Banerjee AK, Wang K (2004) Assessment of conditions affecting *Agrobacterium*-mediated soybean transformation using cotyledonary node explant. Euphytica 136:167–179

Paz MM, Martinez JC, Kalvig AB, Fonger TM, Wang K (2006) Improved cotyledonary node method using an alternative explant derived from mature seed for efficient *Agrobacterium*-mediated soybean transformation. Plant Cell Rep 25:206–213

Reinli K, Block G (1996) Phytoestrogen content of foods-a compendium of literature values. Nutr Cancer 26(2):123–148

Steele CL, Gijzen M, Qutob D, Dixon RA (1999) Molecular characterization of the enzyme catalyzing the aryl migration reaction of isoflavonoid biosynthesis in soybean. Arch Biochem Biophys 367:146–150

Tian L, Dixon RA (2006) Engineering isoflavone metabolism with an artificial bifunctional enzyme. Planta 224:496–507

Wang H, Murphy PA (1994) Isoflavone composition of American and Japanese soybeans in Iowa: effects of variety, crop year, and location. J Agric Food Chem 42:1674–1677

Yu O, Jung W, Shi J, Croes RA, Fader GM, McGonigle B, Odell JT (2000) Production of the isoflavones genistein and daidzein in non-legume dicot and monocot tissues. Plant Physiol 124:781–794

Yu O, Shi J, Hession AO, Maxwell CA, McGonigle B, Odell JT (2003) Metabolic engineering to increase isoflavone biosynthesis in soybean seed. Phytochemistry 63:753–763