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Original Paper

# Isoflavonoid production by adventitious-root cultures of *Iris germanica* (Iridaceae)

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**Abstract** We established the adventitious-root cultures of *Iris germanica*, a monocotyledonous plant known for isoflavonoid production. Irigenin (5,7,3′-trihydroxy-6,4′,5′-trimethoxyisoflavone) and iristectorigenin A (5,7,3′-trihydroxy-6,4′-dimethoxyisoflavone) along with their 7-*O*-$\beta$-D-glucosides, iridin and iristectorin A, respectively, were found as the major components in adventitious roots in the liquid medium, and the total isoflavone content was about 3.6 $\mu$mol per g fresh weight in 3-week-old cultures, which was much higher than the 0.7 $\mu$mol per g dry weight in the rhizome previously reported (Ali et al. 1983 *Phytochemistry* 22: 2061). Abiotic stress was applied by addition of 3 mM cupric chloride ($CuCl_2$) to the liquid medium. The isoflavone glucoside content was increased during the initial 6 h of $CuCl_2$ treatment, which was followed by a decrease; the aglycone level continued to increase throughout the 48 h of treatment. The decrease in glucoside content was negatively correlated with the increase in aglycone content between 6 and 48 h. The total isoflavone content (glucosides+aglycones) at 6 h after the start of $CuCl_2$ treatment was 1.4-fold the initial value, and nearly the same content was maintained for 48 h. Thus, the main effect of $CuCl_2$ treatment appeared to be the induction of hydrolysis of isoflavone glucosides. A cDNA of chalcone synthase was cloned, and the mRNA was expressed in the culture producing isoflavones.

**Key words:** Abiotic stress, adventitious-root cultures, chalcone synthase, *Iris germanica*, isoflavone biosynthesis.

Isoflavonoids constitute a large and distinctive subclass of flavonoids. There were about 770 isoflavonoid aglycones among the total of more than 6,000 flavonoids as of 1999 (Harborne 1999a, 1999b). Isoflavonoids have a rearranged structure compared to other flavonoids: the side phenyl (ring B) is attached to C-3 instead of C-2 in normal flavonoids. The pattern of isoflavonoid distribution in the plant kingdom is interesting. More than 90% of isoflavonoids are found in the Leguminosae (Dewick 1993; Tahara and Ibrahim 1995), but they are also found in a broad array of phylogenetically distant plant species, i.e., 18 families of angiosperms (13 families of dicotyledons and 5 families of monocotyledons), 2 families of gymnosperms and a family of bryophytes (Dewick 1993). In the Leguminosae, isoflavonoids are typical ecophysiologically-active compounds such as defense substances (for example, phytoalexins) and, in soybean, they are signal molecules in the early stages of symbiosis with rhizobial bacteria to form nitrogen-fixing root nodules (Dixon 1999; Aoki et al. 2000). They are biosynthesized in a branching pathway of flavonoid metabolism by the actions of a few specific enzymes including an isoflavonoid skeleton forming cytochrome P450 (P450) of CYP93C subfamily, 2-hydroxyisoflavanone synthase (IFS) (Akashi et al. 1999a; Steele et al. 1999). Recently, a complete identification of the enzymes/genes of the legume-specific isoflavone pathway has been accomplished (Akashi et al. 2003, 2005). In contrast, while some of the physiological functions of non-legume isoflavonoids have been suggested, biochemistry and molecular biology of their biosynthesis remain largely unexplored. Biosynthetic studies on non-leguminous isoflavonoids are important toward the understanding of the mechanism of molecular evolution of specific plant secondary metabolites with significant biological activities and characteristic distribution patterns.

In the family Iridaceae (monocotyledons), 18 *Iris* species have been reported to produce 46 isoflavonoid aglycones (Iwashina and Ootani 1998), perhaps the largest number of isoflavonoids in a single genus of non-leguminous plants. They accumulate mainly in the rhizomes, but the leaves of *Iris pseudacorus* treated with an abiotic stress agent, cupric chloride ($CuCl_2$), also

Abbreviations: BA, $N^6$-benzyladenine; CHS, chalcone synthase; EI, electron ionization; FAB, fast atom bombardment; HMBC, hetronuclear multiple bond coherence; IFS, 2-hydroxyisoflavanone synthase; MJ, methyl jasmonate; MS, Murashige-Skoog; NAA, $\alpha$-naphthylacetic acid; P450, cytochrome P450; RACE, rapid amplification of cDNA ends; Rt, retention time; RT, reverse transcription.

The nucleotide sequence of the CHS reported in this paper appears in the GenBank/EMBL/DDBJ nucleotide sequence database with the accession number AB219147.

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produce isoflavonoids (Hanawa et al. 1991a, 1999b). Thus, the plants of this genus can be nice materials to explore the mechanism of biosynthesis and the physiological functions of non-leguminous isoflavonoids. German iris, *Iris germanica* L., is known for the accumulation of a number of isoflavones (Dhar and Kalla 1972; Pailer and Franke 1973; Ali et al. 1983; Atta-ur-Rahman et al. 2002), and *in vitro* plant regeneration from embryogenic calli has also been reported (Jéhan et al. 1994; Shimizu et al. 1996). In the present study, we cultured adventitious roots of *I. germanica*, and analyzed the accumulation of free and conjugated isoflavones. We also examined the effects of $CuCl_2$ treatment of the adventitious roots on isoflavonoid production and cloned a chalcone synthase (CHS) cDNA. Interestingly, $CuCl_2$ appeared to mainly cause hydrolysis of isoflavone glucosides in *I. germanica* root cultures instead of the expected massive *de novo* production (elicitation) of isoflavonoids. The *I. germanica* adventitious-root cultures accumulating a high concentration of isoflavones may be interesting tools to examine the isoflavone metabolism in non-leguminous plants.

## Materials and Methods

### *Plant materials and culture conditions*

Calli generated from young leaves of *I. germanica* L. cv. Zebra (Sakata Seed, Kanagawa, Japan) were cultured on Murashige-Skoog (MS) medium (Murashige and Skoog 1962) containing 2 g $l^{-1}$ gellan gum, 30 g $l^{-1}$ sucrose, 250 mg $l^{-1}$ proline, 200 mg $l^{-1}$ triptone peptone, 2 mg $l^{-1}$ 2,4-dichlorophenoxyacetic acid and 0.1 mg $l^{-1}$ kinetin. We used the same medium except for the different concentration of gellan gum and the use of triptone peptone instead of casein hydrolysate for the callus induction from this species (Shimizu et al. 1996). Callus cultures were grown in the dark at 25°C with a 6-week culture cycle. Adventitious-root cultures were developed from the calli on MS medium containing 9 g $l^{-1}$ agar, 30 g $l^{-1}$ sucrose, 250 mg $l^{-1}$ proline, 200 mg $l^{-1}$ triptone peptone, 1 mg $l^{-1}$ $\alpha$-naphthylacetic acid (NAA) and 0.5 mg $l^{-1}$ $N^6$-benzyladenine (BA) in the dark at 25°C with a 3-week culture cycle. Liquid culture was started by inoculating 3-week-old adventitious roots (about 10 g) into 200 ml of the same medium without agar in a 500 ml Erlenmeyer flask. The cultures were grown in the dark at 25°C on a rotary shaker at 125 rpm for 3 weeks.

### *HPLC analysis of isoflavones in the adventitious roots*

Aqueous 3 mM $CuCl_2$ solution (1 ml) (Hanawa et al. 1991a, 1991b) or an equal volume of water sterilized with a membrane filter (Millex-GV; Millipore, Bedford, MA, USA) was added to a 2 ml Eppendorf tube containing 3-week-old adventitious roots (500 mg) grown on the solid medium. After incubation for 48 h in the dark, the adventitious roots were collected by suction filtration and homogenized in MeOH. The slurry was centrifuged (3,000 *g* for 5 min), and the MeOH extract was subjected to HPLC. HPLC was performed using a CAPCELL PAK C18 MG column (4.6×150 mm; Shiseido, Tokyo, Japan) at 40°C with a flow rate of 1 ml $min^{-1}$ and a linear gradient elution for 40 min from 40% to 70% (v/v) MeOH in water. The eluate was monitored by a multiwavelength detector (MD-2010, JASCO, Tokyo, Japan). Adventitious roots cultured in the liquid medium (200 ml) for 3 weeks as described above were treated with 6 ml of filter-sterilized 100 mM $CuCl_2$ solution (final concentration 3 mM) or water. Portions of the culture (10 ml) containing about 1.0 g (fresh weight) of tissues were periodically collected and extracted with EtOAc (10 ml) using a Polytron homogenizer. After centrifugation (3,000 *g* for 5 min), the EtOAc extracts were collected and analyzed by HPLC. Contents of isoflavones were determined from the peak areas of the compounds calibrated with those of known concentrations of the samples.

### *Purification and identification of isoflavones*

Adventitious roots (3-week-old, 400 g) on the solid medium were collected and extracted with MeOH (2 l) three times. Combined MeOH extracts were condensed and partitioned between water and EtOAc. Compounds (1a and 1b) giving the peak 1 [retention time (Rt) 6.6 min] on HPLC were partitioned into the EtOAc layer, and the EtOAc extract (2.2 g) was subjected to silica-gel (Wako gel C-200, Wako Pure Chemical Industries, Osaka, Japan; 200 g) column chromatography with stepwise gradients of $CHCl_3$ : MeOH = 9 : 1, 8 : 2 and 7 : 3 (v/v, 500 ml each). A fraction (430 mg) containing 1a and 1b was then applied onto the second silica-gel (100 g) column with $CHCl_3$ : MeOH = 7 : 3 (v/v) as the eluting solvent. A portion (40 mg) of a fraction (330 mg) containing 1a and 1b was further applied onto a cellulose (Funacel, Funakoshi, Tokyo, Japan; 60 g) column with 8% (v/v) $CH_3COOH$ in water to obtain compounds 1a (20 mg) and 1b (5 mg).

Adventitious roots (3-week-old, 500 g) on the solid medium were collected and soaked in filter-sterilized 1 mM $CuCl_2$ solution (2 l) for 48 h. EtOAc extract (2.5 g) was prepared and subjected to silica-gel column chromatography with stepwise gradients of $CHCl_3$ and MeOH as above. A fraction (1.1 g) containing compounds (2a and 2b) giving the peak 2 (Rt 18.0 min) was then applied onto a silica-gel column with hexane : EtOAc = 6 : 4 (v/v) as the eluting solvent. A portion (50 mg) of a fraction (650 mg) containing 2a and 2b was further subjected to cellulose column chromatography and eluted with 15% (v/v) $CH_3COOH$ in water to obtain 2a (30 mg) and 2b (5 mg). NMR spectra were recorded

on a JMN ECA-500 system (JEOL, Tokyo, Japan) in DMSO-$d_6$. Electron ionization (EI; ionization voltage, 70 eV) and fast atom bombardment (FAB) mass spectra were recorded on a JEOL SX-102A mass spectrometer.

### Cloning of CHS cDNA

Total RNAs were isolated from the rhizome of *I. germanica* using Straight A's mRNA isolation system (Novagen, Madison, WI, USA). cDNAs were synthesized by GeneRacer Kit (Invitrogen, Carlsbad, CA, USA). Degenerate primers, CHS/S1 (5′-TAYATGATGTAYCARCARGG-3′) and CHS/AS1 (5′-RTGDGCDATCCARAADAT-3′), were designed from conserved amino acid sequences of CHS. Reverse transcription (RT)-PCR was carried out using *ExTaq* DNA polymerase (Takara, Shiga, Japan) with the cDNAs as templates with 30 cycles of 95°C for 1 min, 45°C for 1 min and 72°C for 1 min, and a final extension step at 72°C for 3 min. PCR products were cloned into pT7Blue T-vector (Novagen) and sequenced. Specific primers, CHS1-5′ (5′-GAGTTCCAATCTGCGATCCCCAGC-3′) and CHS1-3′ (5′-CCAAGGACCTCGCCGAGAACAACC-3′), were designed from the partial CHS sequence, and 3′- and 5′-rapid amplification of cDNA ends (RACE) were performed according to the manufacturer's protocol. A set of specific primers, IgCHS1-F (5′-AAAAGCAAGCTTTTTAAGAGGAAG-3′) and IgCHS1-R 5′-CGTTTGTTGACAAGCTGTAGAGAAT-3′), were designed to amplify the full-length cDNA. PCR was carried out using KOD DNA polymerase (Toyobo, Tokyo, Japan) with 30 cycles of 94°C for 15 s, 60°C for 10 s and 72°C for 1 min, and a final extension step at 72°C for 2 min. The product was ligated to pT7Blue T-vector, and the nucleotide sequence of the cDNA (*IgCHS1*) was determined.

### Expression of the recombinant CHS protein, preparation of enzyme solution and assay

*Nde*I and *BamH*I sites were introduced into upstream and downstream regions of the initiation and stop codons, respectively, of *IgCHS1* by PCR using KOD polymerase with the primers CHS1-N [(5′-AA**CATATG**GCTAGTGTAGCGGAAAT-3′); *Nde*I site shown in bold] and CHS1-B [(5′-T**GGATCC**-CGCACAGACAAACAGTTA-3′; *BamH*I site shown in bold)] and IgCHS1 cDNA as the template. The *Nde*I-*BamH*I fragment of the PCR product was cloned into pET30a (Novagen). The recombinant protein was expressed in *Escherichia coli* BL21(DE3) (Novagen), and the crude extract (10,000 *g* supernatant, about 600 $\mu$g protein ml$^{-1}$) was prepared as described (Akashi et al. 2003).

Crude extract of *E. coli* (100 $\mu$l) was incubated with 150 $\mu$mol malonyl-CoA and 40 $\mu$mol 4-coumaroyl-CoA in a total volume of 130 $\mu$l at 30°C for 1 h. After the termination of reaction with 10 $\mu$l of 3M HCl, the EtOAc extract of the reaction mixture was analyzed by HPLC using 45% (v/v) MeOH in water as the solvent.

### RT-PCR analysis

Total RNAs were isolated from the adventitious roots using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). cDNAs were synthesized by Ready-To-Go T-Primed First Strand Kit (Amersham Biosciences, Buckinghamshire, England). RT-PCR was carried out using *ExTaq* DNA polymerase and specific primers (IgCHS1-F and IgCHS1-R). The reaction was initiated with denaturation at 94°C for 1 min, followed by 20 or 25 cycles of 3-step incubation (94°C, 1 min; 55°C, 1 min; 72°C, 1 min). The products after the electrophoresis on 1.2% (w/v) agarose gel were stained with ethidium bromide. The concentration of each template for PCR was adjusted to give equal amplification of actin cDNA.

## Results

### Culture of adventitious roots of I. germanica

Hard yellow calli were generated from *I. germanica* leaves on the MS medium containing 2,4-dichlorophenoxyacetic acid and kinetin in the dark (Figure 1A). The calli grew only slowly on the medium: the increase in fresh weight of the tissues after a 3-week culture was about 1.2-fold. The calli were then transferred to the medium containing various concentrations of NAA and BA. Adventitious roots were formed on the medium containing 1 mg l$^{-1}$ NAA and 0.5 mg l$^{-1}$ BA, and the roots were repeatedly isolated and cultured on the same medium for about 10 months (Figure 1B). The established adventitious-root cultures grew about 3-fold on a fresh weight basis in 3 weeks. In this study, we used these cultured adventitious roots for phytochemical and molecular biological analyses.

### Isoflavone production in the adventitious-root cultures and the effect of $CuCl_2$ treatment

As a pilot experiment, constituents in the root tissues with and without treatment with 3 mM $CuCl_2$ solution (Hanawa et al. 1991a, 1999b) for 48 h were analyzed by HPLC. MeOH extracts of both intact and water-treated (the control for $CuCl_2$ treatment) tissues gave a major HPLC peak (peak 1) at Rt 6.6 min (Figure 2A). In the HPLC of the extract of $CuCl_2$-treated tissues, peak 1 disappeared and a new peak 2 (Rt 18.0 min) appeared (Figure 2B). Ultraviolet spectra of compounds yielding peaks 1 ($\lambda_{max}$ 263 nm) and 2 ($\lambda_{max}$ 262 nm) analyzed by a multiwavelength detector were identical with the reported spectra of isoflavones ($\lambda_{max}$ about 266 nm) (Williams and Harborne 1997).

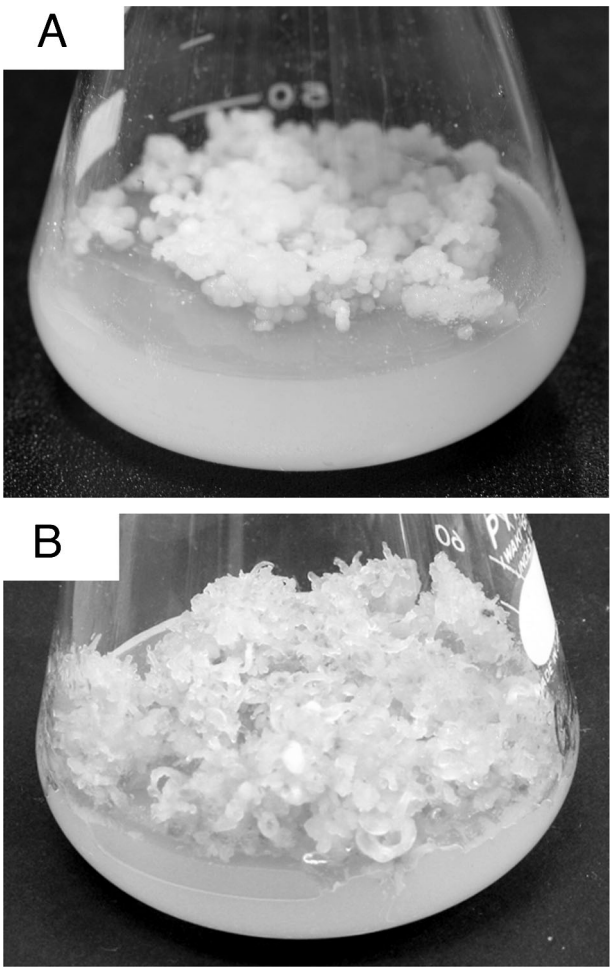



Figure 1. Callus (A; 6-week-old) and adventitious root (B; 3-week-old) cultures of *I. germanica*.

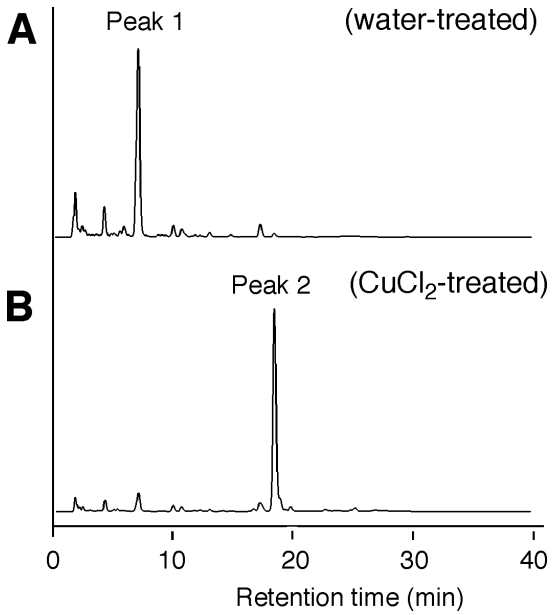



Figure 2. HPLC profiles of MeOH extracts of the adventitious-root cultures treated with water (A) and $CuCl_2$ (B). HPLC was monitored at 260 nm.

### *Identification of isoflavones*

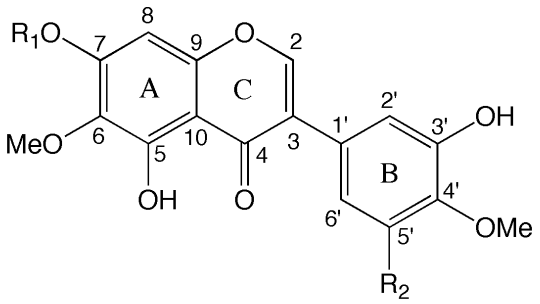
Compounds giving the HPLC peaks 1 and 2 were partially purified from large-scale cultures of the intact (400 g) and $CuCl_2$-treated (500 g) roots by silica-gel column chromatography. Preliminary $^1H$ NMR analysis suggested that both peaks represented the mixtures of two components. The materials were further subjected to cellulose column chromatography, which could separate 1a and 1b from the control tissues and 2a and 2b from the $CuCl_2$-treated tissues (Figure 3).

EI mass spectra of 2a ($[M]^+$ m/z 360, $C_{18}H_{16}O_8$) and 2b ($[M]^+$ m/z 330, $C_{17}H_{14}O_7$) as well as their $^1H$ and $^{13}C$ NMR spectra were identical with those reported for irigenin (5,7,3′-trihydroxy-6,4′,5′-trimethoxyisoflavone) (Ali et al. 1983) and iristectorigenin A (5,7,3′-trihydroxy-6,4′-dimethoxyisoflavone) (Hanawa et al. 1991a), respectively. The validity of the structures was further granted by 2D NMR spectra; in particular, the heteronuclear multiple bond coherence (HMBC) spectra. The 7-hydroxy-6-methoxy substitution of the ring A in both 2a and 2b was revealed from a singlet signal of the methoxyl protons showing a long-range coupling with C-6 and another singlet of H-8 with C-6, C-7, C-9 and C-10. The 3′-hydroxy-4′,5′-dimethoxy substitution of the ring B of 2a was displayed by correlation peaks with H-2′ (*d*, *J*=1.7 Hz) to C-3, C-3′, C-4′ and C-6′, H-6′ (*d*, *J*=1.7 Hz) to C-3, C-2′, C-4′ and C-5′, and two methoxyl singlets to C-4′ and C-5′. In contrast, the HMBC spectrum of 2b displayed correlation peaks with H-2′ (*d*, *J*=1.7 Hz) to C-3, C-3′, C-4′ and C-6′, H-5′ (*d*, *J*=8.6 Hz) to C-1′ and C-3′, H-6′ (*dd*, *J*=1.7 and 8.6 Hz) to C-3, C-2′ and C-4′, and the methoxyl protons to C-4′, indicating the 3′-hydroxy-4′-methoxy substituted ring B.

FAB mass spectra of 1a ($[M+H]^+$ m/z 523, $C_{24}H_{26}O_{13}$) and 1b ($[M+H]^+$ m/z 493, $C_{23}H_{24}O_{12}$) and their $^1H$ and $^{13}C$ NMR were also identical with the reported spectra of irigenin 7-*O*-*β*-D-glucopyranoside (iridin) (Atta-ur-Rahman et al. 2002) and iristectorigenin A 7-*O*-*β*-D-glucopyranoside (iristectorin A) (Morita et al. 1972; Shawl et al. 1984). The 2D NMR measurement further verified the structures. In the HMBC spectrum of 1a, a correlation peak was observed from the anomeric proton of the sugar (H-1″) to C-7. In addition, nuclear overhauser effect spectroscopy spectra of 1a and 1b showed a clear correlation signal between H-1″ and H-8, suggesting that the position of glucosyl linkage is 7-OH of the aglycones.

Irigenin (Pailer and Franke 1973; Ali et al. 1983), iristectorigenin A (Pailer and Franke 1973) and iridin (Dhar and Kalla 1972; Atta-ur-Rahman et al. 2002) are constituents of the rhizome of *I. germanica*. Iristectorin A has been reported from *Iris tectorum* (Morita et al. 1972), *Iris spuria* (Shawl et al. 1984) and *Iris kumaonensis* (Mahmood et al. 2002).

Ratios of the 5′-methoxyisoflavones to isoflavones




| | $R_1$ | $R_2$ |
|---|---|---|
| **1a**: Iridin | β-D-glucopyranose | OMe |
| **2a**: Irigenin | H | OMe |
| **1b**: Iristectorin A | β-D-glucopyranose | H |
| **2b**: Iristectorigenin A | H | H |

Figure 3. Isoflavones contained in the adventitious-root cultures.

without 5′-methoxyl in the partially purified compounds giving HPLC peaks 1 and 2 (Figure 2) were deduced from the integration values of the H-2 signals in their $^1$H NMR spectra, and both 1a : 1b and 2a : 2b were determined as 75 : 25. Adventitious roots cultured on the solid medium for 3 weeks contained a total of 2.3 $\mu$mol $g^{-1}$ fresh weight isoflavone glucosides (1a and 1b) and 0.03 $\mu$mol $g^{-1}$ fresh weight aglycones (2a and 2b).

### *Time course of the conversion of isoflavone glucosides into aglycones by $CuCl_2$ treatment*

The adventitious roots in the liquid medium were grown for 3 weeks until the late logarithmic or early stationary phase (average tissue weight, about 20 g in 200 ml), and the levels of isoflavone glucosides and aglycones were analyzed. In the liquid medium, the tissues accumulated 233±20 nmol $ml^{-1}$glucosides and 133±20 nmol $ml^{-1}$ aglycones (see Figure 4A, time 0), in contrast to the very low aglycone levels in the tissues on the solid medium. While the total isoflavonoid levels in plant tissues and in the medium were measured routinely in the EtOAc extracts of the culture, the isoflavonoids accumulated exclusively in the plant tissues and the amount detected in the medium was negligible (data not shown).

The adventitious-root cultures were treated with 3 mM $CuCl_2$, and the levels of isoflavone glucosides and aglycones were then analyzed periodically. This concentration of $CuCl_2$ was chosen from preliminary experiments which showed that the effects of a 48-h treatment with $CuCl_2$ were dose-dependent up to 3 mM and saturated at 5 mM (data not shown). As shown in Figure 4A, total isoflavone content increased to 1.4-fold of its initial level (367±20 nmol $ml^{-1}$) after a 6-h $CuCl_2$ treatment (517±25 nmol $ml^{-1}$), and its maximum

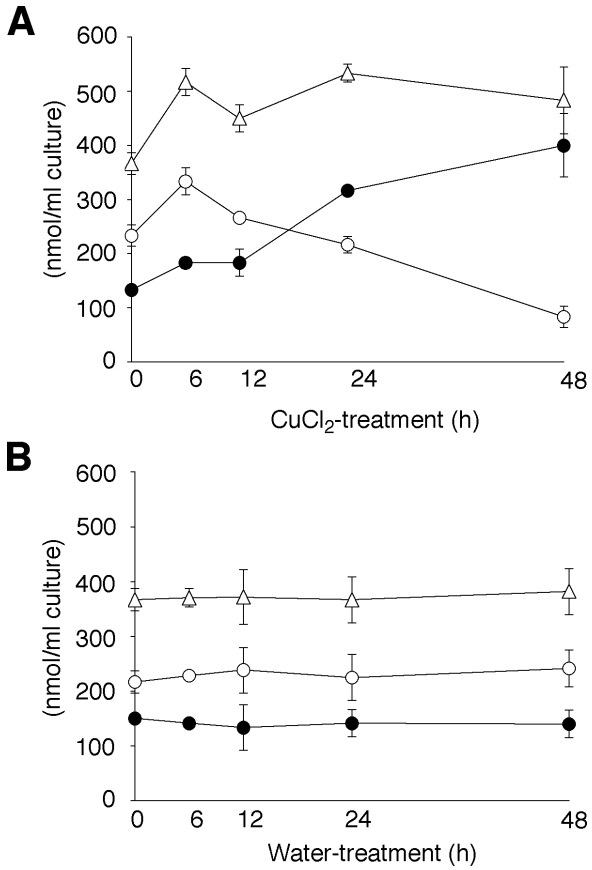



Figure 4. Time course of isoflavone levels in the adventitious-root cultures treated with $CuCl_2$ (A) and water (B). Open and closed circles indicate levels of isoflavone glucosides (1a plus 1b) and aglycones (2a plus 2b), respectively. Open triangle indicates the level of total isoflavones (the sum of 1a, 1b, 2a and 2b). The data are average of three experiments, and bars represent standard error.

content was maintained for 48 h under $CuCl_2$ treatment. The glucoside content was elevated after 6-h $CuCl_2$ treatment (333±25 nmol $ml^{-1}$) and then decreased to 1/3 of its initial level after 48-h (83±20 nmol $ml^{-1}$). On the other hand, the aglycone concentration gradually increased during 48-h treatment with $CuCl_2$ and reached to 400±58 nmol $ml^{-1}$. The amount of glucosides decreased (about 250 nmol $ml^{-1}$) and that of aglycones increased (about 220 nmol $ml^{-1}$) during the period from the 6th to 48th h of the $CuCl_2$ treatment were nearly the same, suggesting that the hydrolysis of the glucosides yielded the aglycones. Water had no effect on the conversion of the glucosides into aglycones (Figure 4B).

### *Effects of biotic stress agents*

Methyl jasmonate (MJ), yeast extract and $H_2O_2$ are known as biotic elicitors in many plants (Wasternack and Parthier 1997; Radman et al. 2003; Cheong and Choi 2003). The adventitious root cultures were treated with these stress agents at usual doses (Zhao and Sakai 2003) for 48 h, and the production of isoflavones was analyzed. When the cultures were treated with 200 $\mu$M MJ, the

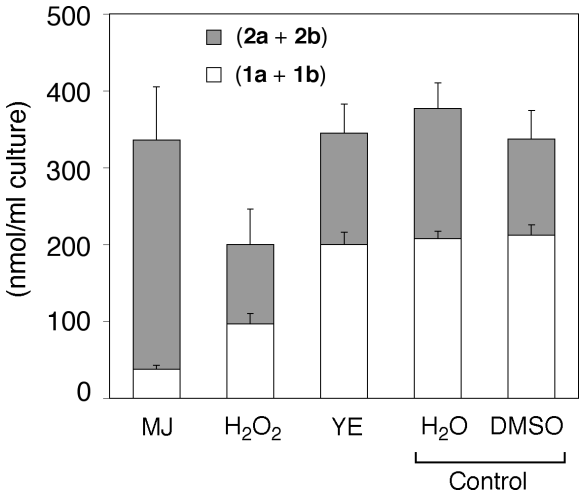



Figure 5. Effect of the biotic stress agents on isoflavone productions. The stress agents were added to the adventitious-root cultures in the liquid medium. After 48-h, EtOAc extracts of the tissues were analyzed by HPLC. As the control of methyl jasmonate, DMSO, and as the control of yeast extract and $H_2O_2$, water was added. The data are average of three experiments, and bars represent standard error. Abbreviations used are: DMSO, dimethyl sulfoxide; MJ, methyl jasmonate; YE, yeast extract.

content of isoflavone glucosides decreased (40±5 nmol $ml^{-1}$), while that of the aglycones increased (298± 64 nmol $ml^{-1}$) (Figure 5). The isoflavone contents in MJ-treated roots were the same as that in the $CuCl_2$-treated roots. Both isoflavone aglycone and glucoside contents decreased by 20 mM $H_2O_2$ treatment. Yeast extract [0.2% (w/v)] had no effect for the accumulation of isoflavones.

### *Cloning of CHS cDNA*

In order to confirm the presence of the flavonoid pathway in *I. germanica* and to examine the expression of genes encoding the biosynthetic enzymes in $CuCl_2$-treated adventitious roots, we cloned the CHS cDNA and analyzed its expression. CHS is the first committed enzyme of the flavonoid metabolism and produces naringenin chalcone from 4-coumaroyl-CoA and three molecules of malonyl-CoA. Degenerate primers were designed from the conserved amino acid regions of CHS. Primer-specific products were obtained from cDNAs prepared from mRNAs of rhizome of *I. germanica* by PCR. Nucleotide sequence analysis of twenty cloned cDNA fragments identified two partial CHS cDNAs (*IgCHS1* and *IgCHS2*). cDNAs of 5′- and 3′- ends were amplified by RACE, and the cDNA containing the full open reading frame of *IgCHS1* was isolated. *IgCHS2* cDNA with the full open reading frame was not obtained by 5′- and 3′-RACE. *IgCHS1* cDNA contained 1,409 bp nucleotides and encoded a polypeptide of 390 amino acids. At the amino acid level, the IgCHS1 is 87% identical with CHS sequences of both *Camellia sinensis* (accession no. D26593) (Takeuchi et al. 1994) and walnut (accession no. X94995) (Claudot et al. 1999).

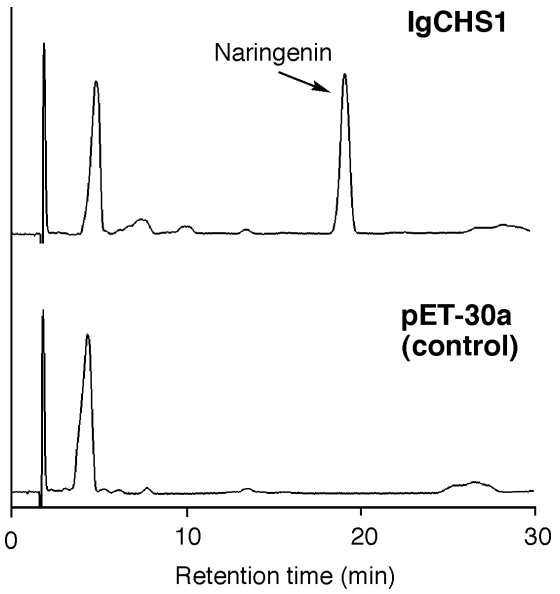



Figure 6. HPLC chromatogram of the product from the reaction of 4-coumaroyl-CoA and malonyl-CoA with crude extract of *E. coli* expressing IgCHS1. HPLC was monitored at 300 nm. For control, crude extract of *E. coli* transformed with pET-30a was incubated with the same substrates.

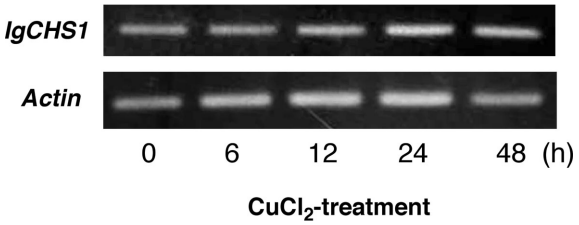



Figure 7. Expression of *IgCHS1* mRNA in adventitious-root cultures. RT-PCRs were performed with 20 or 25 cycles, and results from the semi-quantitative representative (25 cycles) are shown. Results obtained with 20 cycles were substantially the same.

CHS activity was demonstrated using the extract of the recombinant *E. coli* expressing *IgCHS1* cDNA. As shown in Figure 6, a flavanone naringenin, the spontaneous isomerization product of naringenin chalcone, was detected on HPLC of the extract from the reaction mixture of 4-coumaroyl-CoA and malonyl-CoA with the crude extract of *E. coli* expressing *IgCHS1*.

The accumulation of *IgCHS1* mRNA in the adventitious-root cultures was examined by RT-PCR analysis (Figure 7). While total isoflavonoid levels increased 6 h after $CuCl_2$ treatment (Figure 4A), no elevation in *IgCHS1* mRNA was observed, and the expression level did not change drastically during the experimental period (48 h). Thus, the constitutive accumulation of *IgCHS1* mRNA in the cultures with or without $CuCl_2$ treatment was demonstrated.

## Discussion

In this study, adventitious-root cultures of *I. germanica* were established. The adventitious-root cultures on the solid medium produced isoflavones almost exclusively in glucoside forms, whereas the culture in the liquid medium accumulated both isoflavone aglycones and glucosides in large amounts. The isoflavone contents in 3-week-old liquid cultures (1.2±0.1 mg iridin, 0.5±0.1 mg irigenin and 0.12±0.02 mg iristectorigenin A per about 1 g fresh weight tissues in 10 ml) were higher than those in rhizomes of the original plant (0.3 mg iridin, 0.1 mg irigenin and 0.01 mg iristectorigenin A per g dry weight) (Ali et al. 1983; Atta-ur-Rahman et al. 2003), and also within the same order of isoflavone levels in several cultivars of soybean seeds (about 3-8 mg isoflavones/g dry weight) (Wang and Murphy 1994; Aussenac et al. 1998).

In leguminous plants, the isoflavonoid pathway is elicited by several stress agents such as $CuCl_2$, yeast extract and MJ, with the induction of enzymes involved in the pathway (Barz and Welle 1992; Dixon et al. 1992; Dixon and Paiva 1995; Graham and Graham 1996; Akashi et al. 1997, 2005). Therefore, $CuCl_2$ treatment of *I. germanica* cultures was expected to cause massive *de novo* production of isoflavonoid defense substances, but only a small increase of total isoflavonoid contents (about 1.4-fold in molar basis) after a 6-h $CuCl_2$ treatment was observed (Figure 4A). The CHS transcript level was not increased by $CuCl_2$ treatment under this experimental condition (Figure 7). More rapid and transient induction of the isoflavonoid might have occurred, and further studies on the initial tissue response of the root to different concentrations of $CuCl_2$ may be needed. However, considering that the maximum product accumulation is usually detected 1 to 2 days after the elicitation of leguminous cell cultures (Nakamura et al. 1999), it is not likely that isoflavonoids are rapidly produced and degraded in $CuCl_2$-treated *Iris* cultures. Instead, apparent conversion of isoflavone glucosides into isoflavones in $CuCl_2$-treated *I. germanica* root cultures was demonstrated. A similar effect was observed by the addition of MJ to the adventitious-root cultures (Figure 5), a signal molecule which mediates the defense responses against wounding and pathogen attacks (Cheong and Choi 2003). Hydrolysis of glycoside linkages in preexisting chemicals such as glucosinolates and cyanogenic glycosides to release toxic substances is a mechanism of plant defense against herbivory (Harborne 1997) which could be mimicked by abiotic ($CuCl_2$) stress. Also, recent studies repeatedly describe the defensive functions of $\beta$-glucosidases and, in particular, family 1 glycoside hydrolases (Mizutani et al. 2002; Xu et al. 2004; Kawano et al. 2004). Furthermore, in chickpea cells and soybean roots, isoflavone glycosides are hypothesized to serve as pools for the release of aglycones to be incorporated into the phytoalexins (Barz and Welle 1992), and specific $\beta$-glucosidases of isoflavone conjugates have been purified (Hösel and Barz 1975; Hsieh and Graham 2001). It will be interesting to characterize the enzyme(s) responsible for the hydrolysis of isoflavone glucosides in *I. germanica* and compare the properties with the leguminous enzyme as well as family 1 glycoside hydrolases.

Particularly interesting is the enzyme/gene for the construction of isoflavonoid skeleton in *Iris* plants. A functional CHS cDNA was cloned from *I. germanica* in this study, and the mRNA was found to be expressed in the root cultures producing isoflavones as the major components, suggesting that the isoflavones are biosynthesized via phenylpropanoid/flavonoid pathway. In leguminous plants, the substrate of IFS (CYP93C) to construct the isoflavonoid skeleton is a flavanone, which is the common intermediate of biosynthesis of other flavonoid classes including flavones. IFS has been suggested to have evolved from an ancestral P450 which is also the ancestor of flavone synthase II (CYP93B) (Sawada et al. 2002; Sawada and Ayabe 2005). Regarding the biosynthesis of *Iris* isoflavonoids, *I. pseudacorus* leaves have been reported to contain a flavanone with a 2′-hydroxyl which could be a precursor of a particular isoflavone with the same substitution also contained in the same source (Hanawa et al. 1991a, 1991b). In the leguminous isoflavonoid biosynthesis, 2′-hydroxyl is introduced through the action of a P450 of CYP81E subfamily after the isoflavone structure is constructed (Akashi et al. 1998; Shimada et al. 2000; Liu et al. 2003). Thus, in the *Iris* genus, the mechanism of 2′-hydroxy-isoflavonoid biosynthesis could be slightly different from that in legumes, but flavanones could be the precursor of isoflavonoids.

As a preliminary trial, we performed PCR using several CYP93C-specific degenerate primers with the templates of *I. germanica* origin. However, no fragments were amplified by RT-PCR with the cDNA from $H_2O$-treated (control) or $CuCl_2$-treated tissues, and genome-PCR also failed to show signals (data not shown). It is conceivable that, if the mechanism of construction of isoflavonoid skeleton in *I. germanica* is similar to that in the Leguminosae, i.e., P450-dependent aryl migration, P450s of other (sub)families are responsible for the reaction. In such a case, there is a possibility of convergent evolution of similar catalytic functions from different ancestors in distant plants. Another possibility is that a mechanism completely different from the P450-catalyzed one of isoflavonoid construction operates in this species, reminiscent of 2-oxoglutarate-dependent flavone synthase I in parsley (Martens et al. 2001) instead of P450 flavone synthase II in all other species so

far reported (Akashi et al. 1999b; Martens and Forkmann 1999; Kitada et al. 2001). The adventitious-root cultures used in this study are expected to be a valuable tool for analyzing the mechanism of isoflavonoid biosynthesis in *Iris*, and we are pursuing biochemical studies on the isoflavonoid synthase activity in the adventitious-root cultures.

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