Metabolic Engineering of Lignan Biosynthesis in Forsythia Cell Culture

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Lignans are a large class of secondary metabolites in plants, with numerous biological effects in mammals, including antitumor and antioxidant activities. Sesamin, the most abundant furfuran-class lignan in sesame seeds (Sesamum plants), is produced by the cytochrome P450 enzyme CYP81Q1 from the precursor lignan, pinoresinol. In contrast, Forsythia plants produce dibenzylbutyrolactone-class lignans, such as matairesinol, from pinoresinol via the catalysis of pinoresinol/lariciresinol reductase (PLR) and secoisolariciresinol dehydrogenase. Here we present the engineering of lignan biosynthesis in Forsythia cell suspension cultures for the development of an efficient production method of beneficial lignans. A suspension cell culture prepared from leaves of Forsythia koreana produced lignans, mainly pinoresinol and matairesinol glucosides, at levels comparable with that obtained from the leaves. In an attempt to increase the pinoresinol content in Forsythia, we generated a transgenic cell line overexpressing an RNA interference (RNAi) construct of PLR (PLR-RNAi). Down-regulation of PLR expression led to a complete loss of matairesinol and an accumulation of approximately 20-fold pinoresinol in its glucoside form in comparison with the non-transformant. Moreover, the Forsythia transgenic cells co-expressing CYP81Q1 and PLR-RNAi exhibited production of sesamin as well as accumulation of pinoresinol glucoside. These data suggest Forsythia cell suspension to be a promising tool for the engineering of lignan production. To the best of our knowledge, this is the first report on transgenic production of an exogenous lignan in a plant species.

Keywords: Forsythia koreana • Lignan • Metabolic engineering • Sesamin • Sesamum indicum • Suspension cell culture.

Abbreviations: CaMV, cauliflower mosaic virus; DIR, dirigent protein; ESI IT-TOF MS, electrospray ionization ion-trap time-of-flight mass spectrometry; GFP, green fluorescence protein; LC-MS, liquid chromatography–mass spectrometry; MDB, methylenedioxy bridge; NPTII, neomycin phosphotransferase; PIP, pinoresinol-lariciresinol/isoflavone/phenylcoumaran benzylic ether reductase; PLR, pinoresinol/lariciresinol reductase; PNGT, putative pinoresinol glycosyltransferase; PSS, piperitol/sesamin synthase; RNAi, RNA interference; RT, retention time; RT–PCR, reverse transcription–PCR; SDG, secoisolariciresinol diglucoside; SIRD, secoisolariciresinol dehydrogenase; tNOS, nopaline synthase terminator.

Introduction

Lignans are naturally occurring phenylpropanoid dimers (C6–C3 unit; e.g. coniferyl alcohol), in which the phenylpropane units are linked by the central carbons of the side chains (Ayres and Loike 1990, Hearon and MacGregor 1995). Lignans are commonly included in the human diet, as they are widespread in the plant kingdom (Umezawa 2003, Milder et al. 2005, Peñalvo et al. 2008). Plant lignans are thought to play various important physiological and/or ecological roles in the interaction with insects, due to their antifeedant activity and effects on the endocrine system, although the precise mode of action remains to be elucidated (Harmatha and Dinan 2003, Schroeder et al. 2006). Intensive investigations have also been carried out on the beneficial biological effects on humans from the pharmacological and nutritional viewpoints (Ayres and Loike 1990). In the human digestive tract, plant lignans are metabolized by the intestinal microflora into enterodiol and enterolactone, which are known as

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Phytoestrogens (Heinonen et al. 2001). Phytoestrogens are believed to reduce the risk of several types of cancer and cardiovascular disease. Furthermore, there is a growing body of evidence on diverse biological effects in mammals, including antioxidative, antitumor and antiviral activities (Adlercreutz 1995, Fini et al. 2008, Yang et al. 2008). These bioactivities have attracted considerable attention to lignans as promising pharmaceutical agents as well as food supplements for human health. However, their biosynthetic pathways have yet to be completely elucidated (Apers et al. 2003, Suzuki and Umezawa 2007, Yang et al. 2008).

Sesame (Pedaliaceae, *Sesamum indicum*) is a major annual oilseed crop the seeds of which contain a large amount of lignans in the form of sesamin and sesaminol glucosides (Noguchi et al. 2008). Sesamin, classified as a furofuran lignan, is the most abundant water-insoluble lignan in sesame seeds [0.4–0.6% (w/w) of the seed oil] and is characterized by two methylenedioxy bridges (MDBs) (Umezawa 2003, Liu et al. 2006, Lee and Choe 2006). Sesamin exerts antioxidative activity through the metabolism of the MDB moieties, and an antihypertensive effect by inhibition of vascular superoxide production (Nakano et al. 2002, Nakai et al. 2003). Moreover, sesamin was shown to lessen damages to the liver caused by ethanol and lipid oxidation (Akimoto et al. 1993, Sirato-Yasumoto et al. 2001). Recently, we have identified a sesame cytochrome P450, CYP81Q1, as the first P450 enzyme responsible for lignan biosynthesis (Ono et al. 2006a). CYP81Q1 converts pinoresinol to sesamin via piperitol by catalyzing the formation of two MDB structures, and has been designated piperitol/sesamin synthase (PSS) (Fig. 1).

*Forsythia* (Oleaceae), commonly known as the golden bell flower, is a perennial plant and its leaves are used to make tea in eastern Asia. *Forsythia* spp. also produces large amounts of various lignans in leaves, stems, fruits and flowers (Kitagawa et al. 1984, Kitagawa et al. 1988, Nishibe et al. 1988, Guo et al. 2007, Piao et al. 2008). The major lignans of *Forsythia* are pinoresinol (furofuran), phillygenin (furofuran), secoisolariciresinol (dibenzylbutane) and matairesinol (dibenzylbutyrolactone) (Umezawa 2003) (Fig. 1). It should be noted that a large portion of these lignans are accumulated as O-glycosides, and their biosynthetic pathways have been mainly studied in lignan-rich plant species such as *Forsythia* and *Linum* (Linaceae) (Umezawa et al. 1991, Thompson et al. 1996, Berim et al. 2008). In *Forsythia*, lignans are derived from the initiating lignan, pinoresinol, as in the case of *Sesamum*. Pinoresinol is synthesized by the stereospecific coupling of two units of achiral E-coniferyl alcohol in the presence of a dirigent protein (DIR) and then further metabolized by certain biosynthetic enzymes, resulting in a high level of structural diversity (Davin et al. 1997, Davin and Lewis 2003). A pinoresinol-lariciresinol/isoavone/phenylcoumaran benzylic ether reductase (PIP) family enzyme,
pinoresinol-lariciresinol reductase (PLR), converts pinoresinol to secoisolariciresinol in an enantiomer-specific manner (Dinkova-Kostova et al. 1996, Gang et al. 1999), and subsequently secoisolariciresinol dehydrogenase (SIRD) converts secoisolariciresinol to matairesinol in Forsythia lignan biosynthesis (Xia et al. 2001) (Fig. 1). A Forsythia suspension cell culture (F. intermedia) was found to produce pinoresinol and matairesinol as major products, and the contents of pinoresinol were approximately 6-fold higher than that in the differentiated leaves (Schmitt and Petersen 2002). Such high and persistent production of lignans suggests that Forsythia culture cells are suitable hosts for the metabolic conversion of lignans.

Recently, engineering of various plant secondary metabolites, such as alkaloids and flavonoids, has resulted in increased amounts of target compounds or the generation of exogenous compounds with improved pharmacological properties both in planta and in vitro cell cultures (Ogita et al. 2003, Allen et al. 2004, Butelli et al. 2008, Badejo et al. 2009, Leonard et al. 2009). For example, the transgenic periwinkle (Catharanthus roseus) cell culture, expressing an alkaloid biosynthetic gene with re-engineered substrate specificity, acquired the capacity to produce various exogenous alkaloids when co-cultured with the substrates (Runguphan and O’Connor 2009). Moreover, novel flower colors were generated via metabolic engineering of the biosynthesis pathways in host plants by RNA interference (RNAi) of the biosynthetic enzymes for the endogenous flavonoid pigments, followed by the introduction of the flavonoid pigment-relevant genes of other plants (Ono et al. 2006b, Katsumoto et al. 2007). However, the production of exogenous lignans in transgenic plants has not been reported.

The goal of this study is the establishment of a custom-made lignan production platform in Forsythia cell culture by means of genetic engineering of lignan biosynthetic enzymes. The accumulation of the precursor pinoresinol was found to be remarkably increased in the down-regulated PLR transgenic cell lines, and sesamin was newly produced in the transgenic cell lines along with the co-expression of Sesamum CYP81Q1, demonstrating the potential of directed metabolic engineering in a Forsythia platform of lignan production.

Results

Growth and lignan accumulation in a Forsythia koreana suspension culture

Suspension culture cells prepared from leaves were maintained in Gamborg B5 medium supplemented with 5% sucrose and 2,4-D (0.2 mg l⁻¹) with repeated culture at 2-week intervals. Lignans were extracted from the wild-type F. koreana cells after a cultivation period of 15 d. The lignan mixtures were either treated with β-glucosidase or left untreated, and then subjected to HPLC analysis. In the wild-type cells, HPLC analysis detected no peaks corresponding to major lignans in the β-glucosidase-untreated lignan extract (Fig. 2A, B). In contrast, pinoresinol and matairesinol were exclusively observed when the lignan extract was treated with β-glucosidase (Fig. 2A, B). These results revealed that the major lignans in the wild-type F. koreana are pinoresinol and matairesinol, and that almost all (>99%) of the lignans were accumulated in their glucoside form, as seen in previous metabolic profiles in Forsythia (Kitagawa 1984, Nishibe 1988). The dry weight of the suspension culture reached its maximum on day 15 during the cultivation period of 30 d (0.79 g per flask with 50 ml of medium). The accumulation of pinoresinol and matairesinol was positively correlated with the cell suspension growth. Pinoresinol (0.74 mg g⁻¹) and matairesinol (1.31 mg g⁻¹) were extracted from the dry cells, and maintained for >30 d (Fig. 2A).

Transformation of suspension cells

The F. koreana suspension cells were transformed by an Agrobacterium-mediated method with a binary vector for constitutive expression of the green fluorescence protein (GFP) under the control of the 35S cauliflower mosaic virus (CaMV) promoter. After co-cultivation with Agrobacterium tumefaciens, transformed cells were selected by kanamycin. GFP fluorescence was observed in numerous 7-day-old cells, confirming the high level of transformation (~60%) of the F. koreana suspension cells (Fig. 3B).

To genetically modify the lignan biosynthetic pathway, suspension cells of F. koreana were transformed with A. tumefaciens containing the RNAi construct (PLR-RNAi) for knock-down of the endogenous PLR gene. More than 10 cell lines were initially selected and grown on B5 solid medium containing kanamycin and cefotaxime. The growth and morphology of the transgenic cell lines were apparently normal, compared with the control wild-type cell lines. Ultimately, two independent cell lines were selected and transferred to liquid B5 medium as suspension cultures. Similarly, two independent cell lines co-expressing the CYP81Q1 gene and PLR-RNAi were generated. We also repeatedly attempted to generate a transgenic cell line expressing CYP81Q1 alone, but no such cell line was obtained.

The absence of residual A. tumefaciens in all of the transgenic cell lines was confirmed by PCR of the virG gene (Fig. 3C). RT-PCR analysis of 7-day-old suspension cells revealed that the PLR gene was markedly reduced in the transgenic cells expressing PLR-RNAi (Fig. 3D). As expected, both expression of the exogenous CYP81Q1 gene and
suppression of PLR-RNAi were observed in the double transgenic cell lines (Fig. 3D).

Analysis of the lignans produced in the transgenic cell lines

The effect of knock-down of the expression of endogenous PLR on lignan biosynthesis was evaluated by HPLC analysis of pinoresinol and matairesinol, as performed in the wild type (Fig. 2). The content of matairesinol in both of the PLR-down-regulated cell lines (#1 and #2 in Fig. 3D) was reduced to non-detectable levels, and approximately 20-fold pinoresinol (18 mg g⁻¹) in its glucoside form was accumulated in comparison with the wild type (Fig. 4B, C).

The two PLR-RNAi and CYP81Q1 transgenic lines (#3 and #4 in Fig. 3D) not only had increased pinoresinol, as observed in the PLR-RNAi transgenic lines, but also accumulated a new product to a similar level. HPLC analysis indicated that this product exhibited a characteristic UV spectrum of absorption at 230 and 280 nm (not shown), and was eluted at a retention time (RT) of 16.9 min, which is identical to the RT of the authentic sesamin (Fig. 5A). Furthermore, liquid chromatography–mass spectrometry (LC-MS) analysis of the product detected an ion at m/z 337.109 [M+H−H₂O]⁺, which completely matched the calculated mass of authentic sesamin (Fig. 5B). Combined with the finding that the wild type did not produce sesamin (Fig. 5A), these data provide
evidence that the introduced Sesamum CYP81Q1 was functionally expressed in Forsythia and responsible for the production of the exogenous sesamin. Additionally, piperitol, the intermediate occurring in the biosynthesis of sesamin from pinoresinol by CYP81Q1 (Fig. 1), was not detected. This is consistent with the finding that almost no piperitol...
In this study, Forsythia transgenic culture cells competent in the high-level accumulation of pinoresinol and production of sesamin are presented. To date, lignan engineering has been accomplished by up- or down-regulation of the PLR gene. The production of (−)-hinokinin was almost completely suppressed in the induced hairy root line of Linum corymbulosum by transient RNAi against the plr-Lc1 gene, L. corymbulosum PLR (Hemmati et al. 2007, Bayindir et al. 2008). PLR-RNAi in a cell culture of L. perenne also stimulated a slight increase in the production of pinoresinol (Hemmati et al. 2007). In Arabidopsis thaliana, the double mutant lacking the cognate pinoresinol reductases AtPrR1 and AtPrR2 completely inhibited lariciresinol biosynthesis (Nakatsubo et al. 2008). Moreover, overexpression of the F. intermedia PLR gene in wheat results in an increase of an endogenous dibenzylbutane-class lignan, secoisolariciresinol diglucoside (SDG) (Ayella et al. 2007). In this study, we established transgenic F. koreana suspension cell lines in which the down-regulation of the endogenous PLR gene by RNAi effectively led to the complete loss of matairesinol and the accumulation of a large amount of pinoresinol (Figs. 3, 4). These findings also demonstrate that PLR (and probably SIRD also) is a key enzyme for dibenzylbutyrolactone-class lignan biosynthesis from pinoresinol. In keeping with this notion, Sesamum spp. is highly likely to have non-functional PLR genes, which is compatible with the fact that only furfuran-class lignans, such as sesamin and sesaminol, have been detected in sesame species (Umezawa 2003). The possibility that Sesamum spp. has no PLR gene cannot be entirely ruled out, given that no putative PLR homolog has been found in the expressed sequence tags (ESTs) prepared from sesame seeds (unpublished data). Otherwise, production of dibenzylbutane-class lignans could be limited to particular developmental stages and/or tissues. Further investigation of the sesame PLR genes is expected to provide important insight into the molecular evolution of the PIP family of enzymes.

Most of the pinoresinol was accumulated in its glycoside form in both the wild type and the transgenic cell lines expressing PLR-RNAi (Fig. 4). These results indicate that the glycosylating enzymes for pinoresinol are active in the suspension cell cultures as well as the Forsythia plants. Glycosylation of the free hydroxyl groups of phenolic compounds is generally considered to regulate the stabilization and sequestration of phytochemicals in plant cells. Glycosylation of pinoresinol by pinoresinol glycosyltransferases (PNGTs) also contributes to the approximately 20-fold accumulation of pinoresinol in its glycoside form (Fig. 4).

Relatively high yields of pinoresinol were achieved in the short term by the inhibition of PLR in Forsythia cell cultures (Fig. 4). In addition to the phytoestrogenic and antioxidant activities of pinoresinol (Okuyama et al. 1995), recent pharmacological and agricultural studies on pinoresinol derivatives have revealed other biological activities such as an antiviral effect against tobacco mosaic virus, inhibition of wheat coleoptile elongation, inhibition of the cell migration of mouse embryo fibroblasts and a synergistic antifungal effect with a coumarin, scopoletin (Macías et al. 2004, Carpinella et al. 2005, Ouyang et al. 2007, Do et al. 2009). These findings indicate the possibility that the present pinoresinol production system will contribute to the further
investigation of the in vivo effects of pinoresinol, and also the development of pinoresinol and its glucosylated form as novel supplemental foods and drugs.

Sesamin was successfully produced in the transgenic Forsythia cell lines co-expressing CYP81Q1 and PLR-RNAi (Fig. 5) This result indicates that pinoresinol was efficiently converted to sesamin by active CYP81Q1 exhibiting substrate specificity for pinoresinol (Ono et al. 2006a). To the best of our knowledge, this is the first report of the production of an exogenous lignan using transgenic plant cells. CYP81Q1 was shown to fail to utilize pinoresinol glucoside as its substrate (Ono et al. 2006a), suggesting that RNAi-based inhibition of pinoresinol-glycosylating activity leads to a dramatic improvement of sesamin production in the Forsythia transgenic cells. Identification of the PNGTs is currently in progress.

Lignan-containing plants are used for a great number of ailments in Chinese natural medicine as well as dietary foods (Milder et al. 2006, Thompson et al. 2006). The preparation of lignans with various pharmacological activities depends on the specific plant source. For example, podophyllotoxin (aryltetralinlactone) in Podophyllum roots and rhizomes has been exploited as a lead compound for an antibreast cancer drug, and also the clinical treatment of several cancers (Giri and Narasu 2000). However, such plant resources are frequently limited because of the high cost of plant hunting/collecting, poor cultivation systems, long growth phase and the low lignan content in planta. These disadvantages in host plants indicate that the establishment of an efficient and stable production system for lignans is of great interest. In vitro cell/tissue culture systems enable the simple manipulation of biosynthetic enzymes, alteration of the cultivation parameters, the addition of elicitors and the feeding of precursors (Petersen and Alfermann 2001, Ionkova 2007). Notably, suspension cell cultures occasionally possess a special advantage in productivity owing to the high growth rate and short cycle of proliferation under optimized conditions. In conclusion, the Forsythia cell culture system reported here is an efficient and promising platform for producing both endogenous and exogenous lignans by transgenic metabolic engineering.

Materials and Methods

Plant material and cell culture method

Forsythia koreana plants were kindly provided by Professor T. Umezawa (Research Institute for Sustainable Humanosphere, Kyoto University, Japan). For the induction of the callus cultures, discs of surface-sterilized leaves of F. koreana were placed on Gamborg BS medium (Duchefa Biochemie, Haarlem, The Netherlands) containing 3% sucrose, solidified with 0.9% Phyto-agar (Duchefa Biochemie) and supplemented with 2,4-D (0.2 mg l⁻¹), Nakarai, Kyoto, Japan) at 27°C under dark conditions for 6 weeks. Callus tissues were maintained by transferring them to the same medium at 4-week subculture intervals. Cell suspension cultures were initiated by transferring a fast growing callus to 50 ml of BS liquid medium in a 200 ml Erlenmeyer flask. All suspension cultures were agitated on a rotary shaker at 110 r.p.m. in the dark and subcultured every 2 weeks with an inoculum of 5 ml of old suspension cells.

Binary vector construction

To construct a binary vector for the RNAi of the PLR gene, two coding region fragments of the Forsythia PLR gene (accession No. AAC49608) were amplified by PCR using two primer sets containing restriction sites at the 5′ end (set 1, EcoRV-FiPLR-Fw1 (5′-gat atc ata ggc ctg aaa ttg ttg ata ttg) and BglII-FiPLR-Rv (5′-aga tct ctt aac ctc tgg atg aag ttt a); set 2, EcoRV-FiPLR-Fw2 (5′-gat atc cag tct ggt cga ggc tgt caa gct cgg a); and BglII-FiPLR-Rv). Template cDNA was synthesized by SuperScript II (Invitrogen, Carlsbad, CA, USA) from 1 µg of total RNA prepared from the leaves of F. koreana using the RNasy Plant Mini Kit (Qiagen, Valencia, CA, USA). The fragments amplified by primer set 1 (approximately size 800 bp) and set 2 (approximately size 700 bp) were digested by EcoRV and BglII, and EcoRV and SacI, respectively, and then ligated with each other, resulting in a chimeric PLR fragment which forms a hairpin–loop RNA structure designed to silence the endogenous PLR gene in Forsythia. This chimeric PLR for PLR-RNAi was driven by the CaMV 35S promoter in a binary vector pBINPlus (pSPB3103) (Fig. 3A) (Van Engelen et al. 1995). To construct further a binary vector co-expressing PLR-RNAi and CYP81Q1, a cassette of CYP81Q1 driven by the CaMV 35S promoter was inserted into the pBINPlus vector (pSPB3096). The plasmid was digested by PacI and then inserted into a PLR-RNAi cassette at the PacI site, resulting in the co-expressing vector (pSPB3104) (Fig. 3A). To construct a binary vector for GFP, the sGFP (S65T, Chiu et al. 1996) cDNA and the nopaline synthase terminator (tNOS) expressed under the CaMV 35S promoter were cloned into pUC18. The fragment of CaMV 35S-sGFP-tNOS was cut out with HindIII and EcoRI from the pUC18 plasmid and inserted into the HindIII and EcoRI sites of a pBI121 (Clontech, Mountain View, CA, USA) binary vector containing the neomycin phosphotransferase II (NPTII) gene for selection (Fig. 3A). GFP-expressing transgenic cells were imaged using Eclipse E400 fluorescence microscopy (Nikon, Tokyo, Japan), and digital images were analyzed using the Aqua-Lite computer program (Hamamatsu Photonics, Hamamatsu, Japan).

Transformation of suspension cells

A single colony of A. tumefaciens strain EHA105 with the binary vector was inoculated into LB (Luria–Bertani) liquid medium containing 50 mg ml⁻¹ kanamycin and grown at
28°C in a gyratory shaker (180 r.p.m.) to an OD₆₀₀ of 1. Agrobacterium tumefaciens cells (100 µl) were added to 10 ml of 4-day-old suspension cells in a 9 cm Petri dish. They were co-cultivated at 27°C for 2 d in the dark without agitation. The suspension cells were then washed four times with 10 ml of fresh liquid medium. To obtain stable and independent transformants, cells were plated onto agar medium containing 50 mg ml⁻¹ kanamycin and 100 mg ml⁻¹ cefotaxime. After several subcultivation steps, kanamycin-resistant calli were selected and transferred onto new agar medium containing only kanamycin, then resuspended in liquid medium to obtain suspensions of the stably transformed cells.

Genomic PCR analysis of Agrobacterium contamination

Total DNA was isolated from wild-type and transgenic cell lines using a DNeasy Plant Mini Kit (Qiagen). PCR was performed using Agrobacterium cells (2 µl) with the binary vectors and total DNA (100 ng) as a template. The following PCR primers were used to amplify DNA with ExTaq DNA polymerase (TAKARA BIO INC., Ohtsu, Japan) for 30 cycles of 94°C for 15 s, 55°C for 30 s, and 72°C for 1 min; S'-TTG GTT CGC ATT TTC GTC ATC CGC GG-3' and S'-CTC GAC TGG CAA TGA GAA GTT GCT CGC-3' for virG (accession No. X62885); S'-AAG ATG GAT TGC ACG CAG GT-3' and S'-CTG TCA AGA AGG CGA TAG AAG-3' for NPTII (accession No. AB48942); and S'-AGT TCG AGC CTG ATT ATC CC-3' and S'-GCA TGC CGC CAG CGT TCA TC-5' for Forsythia chloroplast DNA (ctDNA). The products were fractionated on a 1.5% agarose gel and visualized by ethidium bromide staining.

RT–PCR analyses

Total RNA was isolated from 7-day-old suspension cells by use of an RNaseasy Plant Mini Kit (Qiagen). First-strand cDNA synthesis was performed using 1 µg of total RNA with reverse transcriptase under the recommended conditions of SuperScript III (Invitrogen). The endogenous PLR (accession No. AAC49608) and rRNA (accession No. AJ236041) were amplified with the following primer sets: PLR-F (S'-ATG GGA AAA AGC AAA GTT TTG ATC ATT GG-3') and PLR-R (S'-CTC GAC TGA CAG CTT GAG GTA CTC TTC CAC-3') for PLR; and rRNA-F (S'-GAA ACC TGC AAA GCA GA-3') and rRNA-R (S'-CTG ACC TGG CGC GTG CTC CAG-3') for rRNA. To detect the introduced CYP81Q1 (accession No. AB194714) gene, a set of primers, S'-ATG GAA GCT GAA ATG CTA TAT TCA GCT-3'/S'-AAC GTT GGA AAG CTG ACG AAG ATT TTC TTG-3', was used. The PCR with ExTaq DNA polymerase (TAKARA BIO INC.) was run at 94°C for 1 min followed by 28 cycles at 94°C for 30 s, at 57°C for 30 s, at 72°C for 1 min, and a final extension at 72°C for 7 min (GeneAmp 2400, PerkinElmer, CA, USA). PCR products were visualized with 1.5% agarose gel electrophoresis by ethidium bromide staining.

Lignan extraction and HPLC analysis

The suspension cells were harvested by suction filtration, frozen in liquid nitrogen (LN₂) and lyophilized for 24 h. Freeze-dried cells (0.1 g) were ground in a mortar under cold conditions with LN₂, suspended in 1 ml of 80% ethanol and sonicated twice for 15 s in 4°C. The mixture was centrifuged and the supernatant was evaporated in vacuo to 100 µl. The remaining H₂O phase, containing the lignan glycoside, was digested at 40°C overnight with 6 U ml⁻¹ almond β-glucosidase (Sigma-Aldrich, St. Louis, MO, USA) in 0.1 M sodium phosphate buffer (pH 4.6). The resulting samples were adjusted with 50% acetonitrile and then centrifuged at 15,000 r.p.m. for 5 min. The supernatant was filtered through a Milllex-LH filter (0.45 µm 4 mm⁻¹; Millipore, Bedford, MA, USA) and then subjected to analysis by reverse-phase HPLC using a Develosil C30-UG-5 column (4.6×150 mm, Nomura Chemical, Aichi, Japan). The HPLC procedure was performed according to our previous study (Ono et al. 2006a) with an LC-2010A HT system (Shimadzu, Kyoto, Japan). Each sample was eluted with a linear gradient of 35–90% solvent B [90% acetonitrile containing 0.1% (v/v) trifluoroacetic acid] in solvent A [H₂O containing 0.1% (v/v) trifluoroacetic acid] for 20 min at a flow rate of 0.6 ml min⁻¹ and then was eluted with 90% solvent B for 7 min. Lignans were monitored by UV absorption at 280 nm, and identified by both RT and mass spectrum comparison with the standard sesamin.

MS analysis

MS data were acquired on an electrospray ion-trap time-of-flight mass spectrometer (ESI-IT-TOF MS) instrument with an LC-20AD (Shimadzu LC-MS-IT TOF, Kyoto, Japan). Standard sesamin (60 pmol) was applied to positive ion mode (+) LC-MS analysis using a Cadenza CD-C18 column (50×2 mm) with 3 µm particle size resin (Imtakt Co., Kyoto, Japan), and were eluted with an acetonitrile aqueous gradient (40–80% for 7 min and then isotropic elution at 80% for 4 min) containing 0.1% formic acid. Sesamin was detected as a dehydrated protonated molecule [M–H₂O+H]+ at m/z 337.110 (exact mass: 337.107) rather than a protonated molecule [M+H]+ (exact mass: 355.118), as previously reported (Yan et al. 2007). The purified samples from the transgenic cells obtained by HPLC were applied to LC-MS analysis, and sesamin was identified as the dehydrated protonated molecule.

Funding

The Ministry of Economy, Trade and Industry, Japan (Plant Factory project).
Acknowledgments

We thank to Professor T. Umezawa (Research Institute for Sustainable Humanosphere, Kyoto University) for providing a F. koreana plant and Dr. M. Horikawa (SUNBOR) for chemical synthesis of standard lignans. Pacific Edit reviewed the manuscript prior to submission.

References


Liu, Z., Saarinen, N.M. and Thompson, L.U. (2006) Sesamin is one of the major precursors of mammalian lignans in sesame seed (Sesamum indicum) as observed in vitro and in rats. J. Nutr. 136: 906–912.


(Received August 11, 2009; Accepted October 26, 2009)