Transgenic Forsythia plants expressing sesame cytochrome P450 produce beneficial lignans

Tomotsugu Koyama

Suntory Foundation for Life Sciences

Erika Matsumoto

Suntory Foundation for Life Sciences

Toshimi Okuda

Suntory Foundation for Life Sciences

Jun Murata

Suntory Foundation for Life Sciences

Manabu Horikawa

Suntory Foundation for Life Sciences

Naoki Hata

University of Shiga Prefecture

Atsushi Okazawa

Osaka Prefecture University

Eiichiro Ono

Suntory Global Innovation Center (SIC) Ltd.

HONOO SATAKE (✉ satake@sunbor.or.jp)

Suntory Foundation for Life Sciences

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Abstract

Lignans are widely distributed plant secondary metabolites that have received attention for their benefits to human health. Sesamin is a furofuran lignan that is conventionally extracted from *Sesamum* seeds and shows anti-oxidant and anti-inflammatory activities in the human liver. Sesamin is biosynthesized by the *Sesamum*-specific enzyme CYP81Q1, and the natural sources of sesamin are annual plants that are at risk from climate change. In contrast, *Forsythia* species are widely distributed perennial woody plants that highly accumulate the precursor lignan pinoresinol. To sustainably supply sesamin, we developed a transformation method for *Forsythia* leaf explants and generated transgenic *Forsythia* plants that heterologously expressed the *CYP81Q1* gene. High-performance liquid chromatography (HPLC) and LC-mass spectrometry analyses detected sesamin and its intermediate piperitol in the leaves of two independent transgenic lines of *F. intermedia* and *F. koreana*. We also detected the accumulation of sesamin and piperitol in their vegetatively propagated descendants, demonstrating the stable and efficient production of these lignans. These results indicate that CYP81Q1-transgenic *Forsythia* plants are promising platforms to produce diverse lignans and provide an important strategy for the cost-effective and scalable production of lignans.

Introduction

The aging of world populations highlights the importance of plant secondary metabolites such as alkaloids, flavonoids, terpenoids, and lignans with benefits for human health^{1,2}. Lignans are phenylpropanoid dimers with diverse functions, and dietary lignans have attracted attention as food nutrients^{3,4}. (+)-Sesamin is a furofuran lignan that is commercially available as a health-promoting supplement⁵. In mammals, (+)-Sesamin metabolites attenuate oxidation and inflammation for the protection of the liver^{6,7}. (+)-Sesamin also shows anti-cancer properties⁸. (+)-Sesamin is commercially available via extraction at low concentrations (0.4–0.6%, w/w) from *Sesamum indicum* (sesame) seed oil^{5,9,10}. Sesame plants, the strongest known synthesizers of (+)-sesamin, are annuals that are threatened by climate change^{11,12}. Thus, new plant sources are required for the efficient and stable production of (+)-sesamin.

In land plants, the lignan metabolic pathway branches from that of lignin at the coupling of monolignols such as coniferyl alcohol, which is synthesized from phenylalanine (Figure 1)^{13,14}. Dirigent protein metabolizes coniferyl alcohol and specifically synthesizes the precursor lignan pinoresinol (Figure 1)^{15–18}. The lignan metabolic pathway further diverges into structurally and functionally diverse lignans by plant species-specific enzymes (Figure 1)^{5,14,19}. In sesame plants, cytochrome P450 81Q1 (CYP81Q1) catalyzes the sequential conversion of (+)-pinoresinol to (+)-piperitol and (+)-sesamin (Figure 1)⁹.

Forsythia species such as *Forsythia intermedia* (Fi) and *F. koreana* (Fk) are widely distributed perennial woody plants. Extracts of *Forsythia* plants have been empirically used in traditional medicines²⁰. *Forsythia* species produce various lignans and other polyphenols^{21–23}, and their biosynthesis and

regulation have been analyzed^{18,20,21,24,25}. *Forsythia* leaves accumulate the precursor lignan (+)-pinoresinol at high levels but lack (+)-sesamin biosynthesis (Figure 1)²¹. To sustainably supply beneficial lignans including (+)-sesamin, heterologous expression of the lignan-biosynthetic enzyme genes in *Forsythia* plants is promising.

Previously, we demonstrated the ectopic accumulation of (+)-sesamin in cultured *CYP81Q1*-transgenic *Fk* cells^{26,27} and showed their ability to produce (+)-sesamin. However, the mass production of (+)-sesamin using transgenic cells is not practical in light of the cost of large-scale cell culture. In contrast, given the large biomass generated by *Forsythia* leaves, *CYP81Q*-transgenic *Forsythia* plants could efficiently and stably produce (+)-sesamin. In this study, we generated *CYP81Q1*-transgenic *Forsythia* plants that stably produce the intermediate (+)-piperitol and the product (+)-sesamin.

Results

Initially, we established a practical method for the transformation of *Forsythia* plants (Figure 2A). *Fi* leaf explants (n = 451) were soaked in a suspension of *Agrobacterium tumefaciens* cells harboring the *Pro35S:nGFP*²⁸ plasmid and regenerated shoots and roots during culture for two years (see Methods, Figure 2B to G, Table 1). Eight of ten independent kanamycin-resistant lines exhibited signals for *nGFP* presence and expression when examined by fluorescence microscopy (Figure 2H) and genomic (Figure 2I) and RT-PCR analyses (Figure 2J); no *nGFP* presence or expression was seen in wild-type (WT) plants. Two kanamycin-resistant plants did not show GFP presence or expression, probably due to somaclonal variation that eliminated the transgene (Figure 2I and 2J). Even after vegetative propagation for four years, newly generated *FiPro35S:nGFP* leaves maintained GFP fluorescence, demonstrating stable *Pro35S:nGFP* transformation of *Forsythia* plants.

Table 1
Summary on the generation of transgenic *Forsythia* plants

Plant species	Gene transformed	Leaf explants	Transgenic plants
<i>Forsythia intermedia</i>	<i>Pro35S:nGFP</i>	451	8 ¹
<i>Forsythia intermedia</i>	<i>Pro35S:CYP81Q1</i>	956	2 ²
<i>Forsythia koreana</i>	<i>Pro35S:CYP81Q1</i>	273	2 ²
1; Kanamycin-resistant plants presenting GFP fluorescence.			
2; Kanamycin-resistant plants presenting expression of the <i>CYP81Q1</i> gene in RT-PCR analysis.			

We also introduced the 35S promoter-regulated sesame *CYP81Q1* gene (*Pro35S:CYP81Q1*)²⁹ into *Fi* and *Fk*. After co-culture of 956 *Fi* and 273 *Fk* leaf explants with *Agrobacterium* cells harboring the *Pro35S:CYP81Q1* plasmid, the resulting transgenic *Forsythia* plants were propagated vegetatively in soil through repeated rounds of cutting and growth in our plant culture room—conditions under which

Forsythia plants continuously developed their leaves without flowering (Table 1). Eventually, two independent transgenic lines developed normally on soil (Figure 3A) and showed *CYP81Q1* gene presence (Figure 3B) and expression (Figure 3C).

To detect the accumulation of the products of CYP81Q1, we subjected the leaves of *FiPro35S:CYP81Q1*, *FkPro35S:CYP81Q1*, control *FiPro35S:nGFP*, and *FkWT* plants to high-performance liquid chromatography (HPLC) and LC-mass spectrometry (MS) analyses (Figure 4). HPLC and LC-MS analyses indicated that the heterologous expression of the *CYP81Q1* gene in *Forsythia* leaves resulted in the production of (+)-piperitol and (+)-sesamin (Figure 4), and that the leaves of the primary transformants *FiPro35S:CYP81Q1* and *FkPro35S:CYP81Q1* accumulated (+)-piperitol (14.23 and 39.45 µg/g DW, respectively) and (+)-sesamin (5.57 and 27.21 µg/g DW), while control *FiPro35S:nGFP* and *FkWT* plants did not (Table 2).

Table 2
The accumulation of (+)-piperitol and (+)-sesamin in *Pro35S:CYP81Q1* leaves.

Genotype	Piperitol (µg/gDW)	Sesamin (µg/gDW)	Piperitol + sesamin (µg/gDW)
<i>FinGFP</i> (n=1) primary transformant	n.d.	n.d.	n.d.
<i>FiCYP81Q1</i> (n=1) primary transformant	14.23	5.57	19.80
<i>FinGFP</i> (n=6) 2nd propagation	n.d.	n.d.	n.d.
<i>FiCYP81Q1</i> (n=6) 2nd propagation	39.57** (SD ± 25.26)	11.09* (SD ± 11.89)	50.66
<i>FinGFP</i> (n=5) 3rd propagation	n.d.	n.d.	n.d.
<i>FiCYP81Q1</i> (n=5) 3rd propagation	17.81* (SD ± 15.76)	1.69* (SD ± 1.60)	19.50
<i>FkWT</i> (n=1) primary plant	n.d.	n.d.	n.d.
<i>FkCYP81Q1</i> (n=1) primary transformant	39.45	27.21	66.65
<i>FkWT</i> (n=6) 2nd propagation	n.d.	n.d.	n.d.
<i>FkCYP81Q1</i> (n=6) 2nd propagation	34.39** (SD ± 14.24)	19.45** (SD ± 17.17)	53.84
<i>FkWT</i> (n=6) 3rd propagation	n.d.	n.d.	n.d.

n.d.; contents below detectable levels.

SD; standard deviation of six biological replicates.

*; P < 0.05, **; P < 0.01; mean values were significantly different from those of the control by two-tailed Student's *t* test.

Genotype	Piperitol (µg/gDW)	Sesamin (µg/gDW)	Piperitol + sesamin (µg/gDW)
<i>FkCYP81Q1</i> (n=6)	51.66**	21.50**	73.16
3rd propagation	(SD ± 16.48)	(SD ± 8.34)	
n.d.; contents below detectable levels.			
SD; standard deviation of six biological replicates.			
*; P < 0.05, **; P < 0.01; mean values were significantly different from those of the control by two-tailed Student's <i>t</i> test.			

We further examined whether the ability to biosynthesize piperitol and sesamin was inherited by descendant transgenic plants. After two and three rounds of vegetative propagation, descendant *Pro35S:CYP81Q1* plants produced similar amounts of (+)-piperitol and (+)-sesamin (Table 2). The total amounts of (+)-piperitol and (+)-sesamin (Table 2) exceeded those in *CYP81Q1-Fk* cells (10 µg/g DW)⁵. Altogether, we demonstrated that the *Pro35S:CYP81Q1* plants efficiently produced (+)-piperitol and (+)-sesamin.

Discussion

In this study, we provide evidence that transgenic *Forsythia* plants produce ectopic lignans, (+)-piperitol and (+)-sesamin. These results suggest that sesamol and sesaminol, which are antioxidant sesame lignans metabolized from (+)-sesamin by CYP92B14³⁰, is expected to be produced via additional introduction of the *CYP92B14* gene into *Pro35S:CYP81Q1* plants. Another lignan, podophyllotoxin, may also be produced in transgenic *Forsythia* plants. Podophyllotoxin is at present extracted from the rhizomes of *Podophyllum* species and clinically utilized in cancer therapy³¹. In *Podophyllum* podophyllotoxin biosynthesis, matairesinol, which *Forsythia* species also accumulate in the pathway downstream of pinresinol, is metabolized to pluviatolide by CYP719A23³². Additional enzymes (CYP71CU1, 2-oxoglutarate/Fe(II)-dependent dioxygenase, and *O*-methyltransferases) convert pluviatolide to the proposed precursor deoxypodophyllotoxin³³. Thus, methods for introducing multiple genes into *Forsythia* plants will pave the way for the generation of podophyllotoxin and its related compounds by transgenic plants. In a previous study, we generated triple-transgenic *Forsythia* cultured cells²⁶, suggesting the possibility of multigene transformation of *Forsythia* plants.

The production of various specialized plant metabolites has been attempted in transgenic and synthetic biology-based microorganisms^{1,35-40}. However, most microorganisms appear to lack (+)-sesamin and its precursor (+)-pinresinol^{9,41}. Moreover, tremendous bioinformatic and screening processes are often required for the generation of genetically-engineered microorganisms that produce plant lignans. Additionally, mass production using such engineered microorganisms could be limited by genetic instability, infectious contamination, unexpected product toxicity, and low fermentation performance^{38,40}.

In contrast, *Forsythia* species are perennial shrubs, and thus *Pro35S:CYP81Q1* plants are easily cultivated via explant, a marked advantage for stable growth and mass propagation in a plant factory.

Also of significance is the reproducible generation of transgenic *Forsythia* plants expressing the *CYP81Q1* gene. The lignan biosynthetic pathway has previously been modified in cultured plants, cells, and hairy roots of various species^{42–58}. For the scalable production of lignans, our transgenic *Forsythia* plant method provides an important strategy for future metabolic engineering of such lignan-producing plants. In conclusion, we have generated *Forsythia* plants as promising platforms for the efficient and sustainable heterologous production of beneficial lignans.

Materials And Methods

Plant growth conditions

F. intermedia and *F. koreana* plants were obtained from Niigata Botanical Garden and Dr. Toshiaki Umezawa (Kyoto University), respectively. The use of these plants for this study has been approved by the providers. All the experimental work on plant material described in this study complies with the relevant institutional, national, and international guidelines and legislation.

Both of *Forsythia* plants were maintained in pots filled with soil inside a plant culture room under a cycle of 16-h-light (photosynthetic photon flux density of 50 to 75 $\mu\text{mol m}^{-2} \text{sec}^{-1}$)/8-h-dark at 22°C unless otherwise indicated. After surface-sterilization using an ethanol and sodium hypochlorite solution, the *Forsythia* plants were maintained *in vitro* in a culturing box on modified MS medium (MS salts, MS vitamin, 30 g/L sucrose, 22.5 mg/L $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 5 g/L Agargel [Sigma-Aldrich, MO]) and transferred to fresh medium every two to three months.

Transformation and preparation of Agrobacterium cells

Agrobacterium tumefaciens GV3101 were individually transformed with *Pro35S:nGFP* (encoding nuclear-localized green fluorescent protein)²⁸ and *Pro35S:CYP81Q1*²⁹ plasmids using a Gene Pulser II electroporator (BioRad, CA). The resultant *Agrobacterium* cells were cultured in LB liquid medium at 27°C until the optical density at 600 nm (OD600) reached 1.5 to 2.0. The *Agrobacterium* cells were collected by centrifugation of the medium (HP25; Beckman, CA) at $6,000 \times g$ for 15 min at room temperature, resuspended in the transformation solution (Gamborg's B5 salts, MS vitamins, 30 g/L sucrose, 2.0 mg/L indole-3-acetic acid [IAA; Nacalai tesque, Japan], 0.5 mg/L 6-benzyladenopurine [BA; Nacalai tesque, Japan], 20 mg/L acetosyringone [Tokyo Chemical Industry, Japan], 0.02% 500W Additive [equivalent to Silwet L-77; DOW CORNING, MI]), and further diluted with additional transformation solution to a concentration of OD600 = 0.7.

Transformation and culture of Forsythia leaf explants

To prepare leaf explants, the third to sixth leaves from the top of one-month-cultured *Forsythia* plants were detached at their petioles and cut into 10 × 5 mm squares using a surgical knife. For the co-culture, the leaf explants were submerged in the *Agrobacterium* cell-suspended transformation solution for 2 min at room temperature, the transformation solution was wiped off using sterilized paper towels, and the explants were transferred onto sterilized filter papers (No.1 70 mm; ADVANTEC, Japan) over callus-inducing medium (CIM; Gamborg's B5 salts, B5 vitamin, 30 g/L sucrose, 2.0 mg/L IAA, 0.5 mg/L BA, 20 mg/L acetosyringone, 3 g/L gellan gum [Kanto Chemical, Japan]), and maintained at 22°C in the dark for three days. To suppress the overgrowth of the *Agrobacterium* cells, the leaf explants were transferred onto shoot-inducing medium (SIM; MS salts, MS vitamin, 30 g/L sucrose, 0.5 mg/L IAA, 2.0 mg/L BA, 3 g/L gellan gum) supplemented with 10 mg/L meropenem (Tokyo Chemical Industry, Japan) for four days in the dark. To isolate kanamycin-resistant plants, leaf explants were cultured on SIM supplemented with kanamycin (75 mg/L for *F. intermedia* and 50 mg/L for *F. koreana*; Nacalai tesque, Japan) and 10 mg/L meropenem under 16-h-light/8-h-dark conditions and transferred onto fresh medium every two weeks up to six months. Adventitious shoots regenerated at the periphery of the dedifferentiated explants were transferred onto shoot-elongating medium (MS salts, MS vitamin, 30 g/L sucrose, 22.5 mg/L CuSO₄·5H₂O, 2.0 mg/L BA, 5 g/L Agargel) supplemented with kanamycin and meropenem in sterilized glass tubes until the shoots elongated to 5 cm height. Shoots were transferred and maintained on hormone-free medium (MS salts, MS vitamin, 30 g/L sucrose, 22.5 mg/L CuSO₄·5H₂O, 5 g/L Agargel) until their rooting.

Microscopy

GFP fluorescence was observed using a M205 fluorescence stereomicroscope (Leica Microsystems, Germany) using the GFP3 filter and recorded by LAS AF software (Leica Microsystems, Germany).

Extraction of nucleotides and detection of genes

The genomic DNAs of *Forsythia* leaves were prepared using a Nucleon PhytoPure Genomic DNA Extraction kit (GE Healthcare, Sweden) according to the manufacturer's instruction. The total RNAs of *Forsythia* leaves were prepared using an RNAeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's instruction, cleared by precipitation in 4 M lithium chloride solution (final concentration), treated with DNase I (Qiagen, Germany), and further subjected to reverse transcription using SuperScript III (Thermo Fisher Scientific, MA) with an Oligo(dT) primer (Thermo Fisher Scientific, MA). For the detection of genes of interest, the genomic and complementary DNAs were individually subjected to PCR analysis using appropriate sets of primers (Supplementary Table S1), electrophoresed, and stained by ethidium bromide solution (Supplementary Figure S1).

Measurement of lignans

Cultured transgenic and control *Forsythia* plants were transferred into soil in pots, acclimatized for three weeks until rooting, and grown for two months until the plants reached 15 to 20 cm height. The third to fifth leaves from the top of each plant were pooled, frozen in liquid nitrogen, lyophilized to permit measurement of dry weight (DW) using an FDU-2110 device (EYELA, Japan), extracted with 50% methanol (v/v) containing 2.25 µM (final concentration) 2'-ethoxysesamin (Supplementary Figure S2 and

Table S2) as the internal standard, and processed as described previously²⁹. The leaf extracts were subjected into reverse-phase HPLC (Alliance 2960, Waters Corporation, MA) using a Develosil C30-UG-5 column (4.6 × 150 mm; Nomura Chemical, Japan) under conditions described previously²⁶. Lignans were monitored by UV absorption at 283 nm, and their concentrations were calculated by Empower2 software (Waters Corporation, MA) according to the areas of the peaks in the chromatograms while referencing standard curves of authentic piperitol and sesamin with technical duplicates or triplicates. For LC-MS analysis, the leaf extracts were subjected to LC-MS-IT-TOF (Shimazu, Japan) and analyzed as described previously³⁰. Lignans were detected using a photodiode array detector.

Statistical analysis

The lignan contents were measured using five or six biological replicates and statistically analyzed by two-tailed Student's *t* tests (Table 2).

Declarations

AUTHOR CONTRIBUTIONS

T.K., E.M., T.O., J.M., M.H., N.H., A.O., and E.O. performed experiments, and T.K. and H.S. designed the research and wrote the manuscript.

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COMPETING INTERESTS

The authors declare no competing interests.

DATA AVAILABILITY

All data generated or analyzed during this study are included in this published article and its supplementary information files.

References

1. Wurtzel, E. T. & Kutchan, T. M. Plant metabolism, the diverse chemistry set of the future. *Science* **353**, 1232–1236 (2016).
2. Jacobowitz, J. R. & Weng, J.-K. Exploring uncharted territories of plant specialized metabolism in the postgenomic era. *Ann. Rev. Plant Biol.* **71**, 631–658 (2020).

3. Durazzo, A. *et al.* Dietary lignans: Definition, description and research trends in databases development. *Molecules* **23**, 3251; (2018).
4. Rodríguez-García, C., Sánchez-Quesada, C., Toledo, E., Delgado-Rodríguez, M. & Gaforio, J. J. Naturally lignan-rich foods: A dietary tool for health promotion? *Molecules* **24**, 917; (2019).
5. Satake, H. *et al.* Essences in metabolic engineering of lignan biosynthesis. *Metabolites* **5**, 270–290; (2015).
6. Kabe, Y. *et al.* Annexin A1 accounts for an anti-inflammatory binding target of sesamin metabolites. *NPJ Sci. Food* **4**, 4; (2020).
7. Nakai, M. *et al.* Novel antioxidative metabolites in rat liver with ingested sesamin. *J. Agr. Food Chem.* **51**, 1666–1670 (2003).
8. Majdalawieh, A. F., Massri, M. & Nasrallah, G. K. A comprehensive review on the anti-cancer properties and mechanisms of action of sesamin, a lignan in sesame seeds (*Sesamum indicum*). *Eur. J. Pharm.* **815**, 512–521 (2017).
9. Ono, E. *et al.* Formation of two methylenedioxy bridges by a *Sesamum* CYP81Q protein yielding a furofuranlignan, (+)-sesamin. *Proc. Natl. Acad. Sci. USA* **103**, 10116–10121 (2006).
10. Andargie, M., Vinas, M., Rathgeb, A., Möller, E. & Karlovsky, P. Lignans of sesame (*Sesamum indicum*): A comprehensive review. *Molecules* **26**, 883; (2021).
11. Dossa, K. *et al.* The emerging oilseed crop *sesamum indicum* enters the “Omics” era. *Front. Plant Sci.* **8**, 1154; (2017).
12. Dossa, K. *et al.* The genetic basis of drought tolerance in the high oil crop *Sesamum indicum*. *Plant Biotechnol. J.* **17**, 1788–1803 (2019).
13. Davin, L. B. & Lewis, N. G. Dirigent proteins and dirigent sites explain the mystery of specificity of radical precursor coupling in lignan and lignin biosynthesis. *Plant Physiol.* **123**, 453–461 (2000).
14. Suzuki, S. & Umezawa, T. Biosynthesis of lignans and norlignans. *J. Wood Sci.* **53**, 273–284 (2007).
15. Kim, K. W. *et al.* Trimeric structure of (+)-pinoresinol-forming dirigent protein at 1.95 Å resolution with three isolated active sites. *J. Biol. Chem.* **290**, 1308–1318 (2015).
16. Yonekura-Sakakibara, K. *et al.* Seed-coat protective neolignans are produced by the dirigent protein AtDP1 and the laccase AtLAC5 in *Arabidopsis*. *Plant Cell* **33**, 129–152 (2021).
17. Gasper, R. *et al.* Dirigent protein mode of action revealed by the crystal structure of AtDIR6. *Plant Physiol.* **172**, 2165–2175 (2016).
18. Davin, L. B. *et al.* Stereoselective bimolecular phenoxy radical coupling by an auxiliary (dirigent) protein without an active center. *Science* **275**, 362–366 (1997).
19. Teponno, R. B., Kusari, S. & Spiteller, M. Recent advances in research on lignans and neolignans. *Nat. Prod. Rep.* **33**, 1044–1092 (2016).
20. Rosati, C, Cadic, A, Duron, M, & Simoneau, P. Forsythia. *Biotechnology in agriculture and forestry, Transgenic crops VI* (E.C. Pua & M.R. Davey eds.), 299-318 (2007).
21. Umezawa, T. Diversity in lignan biosynthesis. *Phytochem. Rev.* **2**, 371–390 (2003).

22. Guo, H., Liu, A. H., Ye, M., Yang, M. & Guo, D. A. Characterization of phenolic compounds in the fruits of *Forsythia suspensa* by high-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry. *Rap. Commun. Mass Spectrom.* **21**, 715–729 (2007).
23. Dong, Z. *et al.* *Forsythiae Fructus*: A review on its phytochemistry, quality control, pharmacology and pharmacokinetics. *Molecules* **22**, 1466; (2017).
24. Shiraishi, A. *et al.* De novo transcriptomes of *Forsythia koreana* using a novel assembly method: Insight into tissue- and species-specific expression of lignan biosynthesis-related gene. *PLoS ONE* **11**, e0164805; (2016).
25. Sun, L. *et al.* Comparative transcriptome analyses of three medicinal *Forsythia* species and prediction of candidate genes involved in secondary metabolisms. *J. Nat. Med.* **72**, 867–881 (2018).
26. Murata, J., Matsumoto, E., Morimoto, K., Koyama, T. & Satake, H. Generation of triple-transgenic *Forsythia* cell cultures as a platform for the efficient, stable, and sustainable production of lignans. *PLoS ONE* **10**, e0144519; (2015).
27. Kim, H. J. *et al.* Metabolic engineering of lignan biosynthesis in forsythia cell culture. *Plant Cell Physiol.* **50**, 2200–2209 (2009).
28. Koyama, T. *et al.* A regulatory cascade involving class II ETHYLENE RESPONSE FACTOR transcriptional repressors operates in the progression of leaf senescence. *Plant Physiol.* **162**, 991–1005 (2013).
29. Tera, M. *et al.* Identification of a binding protein for sesamin and characterization of its roles in plant growth. *Sci. Rep.* **9**, 8631; (2019).
30. Murata, J. *et al.* Oxidative rearrangement of (+)-sesamin by CYP92B14 co-generates twin dietary lignans in sesame. *Nature Commun.* **8**, 2155; (2017).
31. Gordaliza, M. Natural products as leads to anticancer drugs. *Clin. Transl. Oncol.* **9**, 767–776 (2007).
32. Marques, J. V. *et al.* Next generation sequencing in predicting gene function in podophyllotoxin biosynthesis. *J. Biol. Chem.* **288**, 466–479 (2013).
33. Lau, W. & Sattely, E. S. Six enzymes from mayapple that complete the biosynthetic pathway to the etoposide aglycone. *Science* **349**, 1224–1228 (2015).
34. Zhu Q. *et al.* Plant synthetic metabolic engineering for enhancing crop nutritional quality. *Plant Commun.* **1**, 100017 (2020).
35. Sato, F. & Kumagai, H. Microbial production of isoquinoline alkaloids as plant secondary metabolites based on metabolic engineering research. *Proc. Jpn. Acad. Ser. B: Phys. Biol. Sci.* **89**, 165–182 (2013).
36. Kotopka, B. J., Li, Y. & Smolke, C. D. Synthetic biology strategies toward heterologous phytochemical production. *Nat. Prod. Rep.* **35**, 902–920 (2018).
37. Pyne, M. E., Narcross, L. & Martin, V. J. J. Engineering plant secondary metabolism in microbial systems. *Plant Physiol.* **179**, 844–861 (2019).

38. Choi, K. R. *et al.* Systems metabolic engineering strategies: integrating systems and synthetic biology with metabolic engineering. *Trends Biotechnol.* **37**, 817–837 (2019).
39. Birchfield, A. S. & McIntosh, C. A. Metabolic engineering and synthetic biology of plant natural products – A minireview. *Curr. Plant Biol.* **24**, 100163; (2020).
40. Arya, S. S., Rookes, J. E., Cahill, D. M. & Lenka, S. K. Next-generation metabolic engineering approaches towards development of plant cell suspension cultures as specialized metabolite producing biofactories. *Biotech. Adv.* **45**, 107635; (2020).
41. Hano, C. F., Dinkova-Kostova, A. T., Davin, L. B., Cort, J. R. & Lewis, N. G. Lignans: Insights into their biosynthesis, metabolic engineering, analytical methods and health benefits. *Front. Plant Sci.* **11**, 630327; (2021).
42. Fuss E. Lignans in plant cell and organ cultures: An overview. *Phytochem. Rev.* **2**, 307–320 (2003).
43. Bayindir, Ü., Alfermann, A. W. & Fuss, E. Hinokinin biosynthesis in *Linum corymbulosum* Reichenb. *Plant J.* **55**, 810–820 (2008).
44. Ragamustari, S. K. *et al.* Substrate-enantiomer selectivity of matairesinol O-methyltransferases. *Plant Biotechnol.* **31**, 257–267 (2014).
45. Lalaleo, L. *et al.* Effect of in vitro morphogenesis on the production of podophyllotoxin derivatives in callus cultures of *Linum album*. *J. Plant Physiol.* **228**, 47–58 (2018).
46. Renouard, S. *et al.* Investigation of *Linum flavum* (L.) hairy root cultures for the production of anticancer aryltetralin lignans. *Int. J. Mol. Sci.* **19**, 990; (2018).
47. Bazaldúa, C. *et al.* Improving the production of podophyllotoxin in hairy roots of *Hyptis suaveolens* induced from regenerated plantlets. *PLoS ONE* **14**, e0222464; (2019).
48. Mikac, S. *et al.* Bioproduction of Anticancer Podophyllotoxin and Related Aryltetralin-Lignans in Hairy Root Cultures of *Linum flavum* L., *Reference series in phytochemistry, Plant cell and tissue differentiation and secondary metabolites*, (Ramawat K. G. *et al.* eds.), 503–540 (2021).
49. Markulin, L. *et al.* Pinoresinol–lariciresinol reductases, key to the lignan synthesis in plants. *Planta* **249**, 1695–1714 (2019).
50. Muranaka, T, Miyata, K, Ito, K, & Tachibana S. Production of podophyllotoxin in *Juniperus chinensis* callus cultures treated with oligosaccharides and a biogenetic precursor. *Phytochemistry* **49**, 491–496 (1998).
51. Esmaeilzadeh Bahabadi, S. *et al.* Time-course changes in fungal elicitor-induced lignan synthesis and expression of the relevant genes in cell cultures of *Linum album*. *J. Plant Physiol.* **169**, 487–491 (2012).
52. Bahabadi, S. E. *et al.* Increased lignan biosynthesis in the suspension cultures of *Linum album* by fungal extracts. *Plant Biotechnol. Rep.* **5**, 367–373 (2011).
53. Bahabadi, E. S. The effect of chitosan and chitin oligomers on gene expression and lignans production in *Linum album* cell cultures. *J. Med. Plants* **13**, 46–53 (2014).

54. Hazra, S., Bhattacharyya, D. & Chattopadhyay, S. Methyl jasmonate regulates podophyllotoxin accumulation in *Podophyllum hexandrum* by altering the ROS-responsive podophyllotoxin pathway gene expression additionally through the down regulation of few interfering miRNAs. *Front. Plant Sci.* **8**, 164 (2017).
55. Corbin, C. *et al.* Functional characterization of the pinoresinol–lariciresinol reductase-2 gene reveals its roles in yatein biosynthesis and flax defense response. *Planta* **246**, 405–420 (2017).
56. Yousefzadi, M. *et al.* The effect of light on gene expression and podophyllotoxin biosynthesis in *Linum album* cell culture. *Plant Physiol. Biochem.* **56**, 41–46 (2012).
57. Hata, N. *et al.* Differences in plant growth and leaf sesamin content of the lignan-rich sesame variety “Gomazou” under continuous light of different wavelengths. *Plant Biotechnol.* **30**, 1–8 (2013).
58. Morimoto, K. *et al.* Effects of light on production of endogenous and exogenous lignans by *Forsythia koreana* wildtype and transgenic cells. *Plant Biotechnol.* **28**, 331–337 (2011).

Figures

Figure 1

Biosynthesis of (+)-piperitol and (+)-sesamin in *Forsythia* plants by heterologous expression of the sesame *CYP81Q1* gene. Lignan biosynthesis is initiated from phenylalanine. Two coniferyl alcohols are coupled to the precursor lignan (+)-pinoresinol in land plants (green). In sesame plants, CYP81Q1 sequentially synthesizes (+)-piperitol and (+)-sesamin (red).

Figure 2

Generation of transgenic *Pro35S:nGFP* plants.

(A) A scheme of *Agrobacterium*-mediated transformation of *Forsythia* leaf explants followed by the regeneration of the whole plant body. CIM; callus-inducing medium, SIM; shoot-inducing medium.

(B) The *Pro35S:nGFP* plasmid. nptII; kanamycin resistance gene, 35S; cauliflower mosaic virus 35S promoter, NLS; nuclear localization signal, GFP; GREEN FLORESCENCE PROTEIN, nos; nos terminator.

(C) Leaf explants co-cultured with *Agrobacterium* cells.

(D) Leaf explants dedifferentiated into calluses and regenerating adventitious shoots.

(E) An adventitious shoot before separation from calluses.

(F) GFP fluorescence in a *FiPro35S:nGFP* shoot.

(G) A *FiPro35S:nGFP* plant transferred into soil.

(H) GFP fluorescence in a *FiPro35S:nGFP* leaf (*nGFP*, right) in contrast with the lack of fluorescence in a non-transformed wild-type (WT) leaf (left). Dotted white line marks the WT leaf margin.

(I) and (J) Genomic (I) and reverse transcription-polymerase chain reaction (RT-PCR) (J) analyses of *GFP* gene. *Cyt* (cytochrome) served as an internal control. The numbers above the gel images indicate the plant lines regenerated from independent calluses.

Scale bars = 1 cm in (C) to (H)

Figure 3

Generation of transgenic *Pro35S:CYP81Q1* plants.

(A) Control and *Pro35S:CYP81Q1* plants. Scale bars = 10 cm.

(B) and (C) Genomic (B) and reverse transcribed (RT)-PCR (C) analyses of *CYP81Q1* gene. *Cyt* (cytochrome) served as an internal control. The numbers above the gel images indicate the plant lines regenerated from independent calluses.

Figure 4

HPLC (A) and LC-MS (B) analysis of control and *Pro35S:CYP81Q1* plants.

Chromatograms showing specific accumulation of (+)-piperitol (P1) and (+)-sesamin (P2) in the *Pro35S:CYP81Q1* leaves. In (B), pinoresinol; m/z = 381.12, piperitol; m/z = 379.11, sesamin; m/z = 377.09, 3'-ethoxysesamin; m/z = 421.11, TIC; total ion current.

Supplementary Files

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