Transgenic Forsythia plants expressing sesame cytochrome P450 produce beneficial lignans

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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 prototypes to produce diverse lignans and provide an important strategy for the cost-effective and scalable production of lignans.

The aging of world populations highlights the importance of plant secondary metabolites such as alkaloids, flavonoids, terpenoids, and lignans with benefits for human health1,2. Lignans are phenylpropanoid dimers with diverse functions, and dietary lignans have attracted attention as food nutrients3,4. (+)-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 liver6,7. (+)-Sesamin also shows anti-cancer properties8. (+)-Sesamin is commercially available via extraction at concentrations (4–6 mg/g) from Sesamum indicum (sesame) seed oil5,9,10. Sesame plants, the strongest known synthesizers of (+)-sesamin, are annuals that are threatened by climate change11,12. 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 (Fig. 1)13,14. Dirigent protein metabolizes coniferyl alcohol and specifically synthesizes the precursor lignan pinoresinol (Fig. 1)15-18. The lignan metabolic pathway further diverges into structurally and functionally diverse lignans by plant species-specific enzymes (Fig. 1)5,14,19. In sesame plants, cytochrome P450 81Q1 (CYP81Q1) catalyzes the sequential conversion of (+)-pinoresinol to (+)-piperitol and (+)-sesamin (Fig. 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 polyphenols21-23, and their biosynthesis and regulation have been analyzed24,25,26,27,28,29. Forsythia leaves accumulate the precursor lignan (+)-pinoresinol at high levels but lack (+)-sesamin biosynthesis (Fig. 1)31. 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 cells25,26 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

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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 (Fig. 2A). Fi leaf explants (n = 451) were soaked in a suspension of *Agrobacterium tumefaciens* cells harboring the Pro35S:nGFP28 plasmid and regenerated shoots and roots during culture for two years (see “Materials and methods”, Fig. 2B–G, Table 1). Eight of ten independent kanamycin-resistant lines exhibited signals for nGFP presence and expression when examined by fluorescence microscopy (Fig. 2H) and genomic (Fig. 2I) and RT-PCR analyses (Fig. 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 (Fig. 2I,J). Even after vegetative propagation for four years, newly generated FiPro35S:nGFP leaves maintained GFP fluorescence, demonstrating stable Pro35S:nGFP transformation of *Forsythia* plants.

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 (Fig. 3A) and showed CYP81Q1 gene presence (Fig. 3B) and expression (Fig. 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 (Fig. 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 (Fig. 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).

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. In contrast, the LC–MS analysis detected pinocembrin (P3 in Fig. 4B) and the content of pinocembrin was not changed in the control and the transgenic Pro35S:CYP81Q1 plants [(FiPro35S:nGFP and FiPro35S:CYP81Q1 (9.34 ± 3.64 and 11.68 ± 5.44 mg/g DW), respectively).

**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 fluorescence 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, 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).

Table 1. Summary on the generation of transgenic Forsythia plants. *Kanamycin-resistant plants presenting GFP fluorescence. †Kanamycin-resistant plants presenting expression of the CYP81Q1 gene in RT-PCR analysis.

| Plant species       | Gene transformed | Leaf explants | Transgenic plants |
|---------------------|------------------|---------------|-------------------|
| Forsythia intermedia| Pro35S:nGFP      | 451           | 8                 |
| Forsythia intermedia| Pro35S:CYP81Q1    | 956           | 2†                |
| Forsythia koreana   | Pro35S:CYP81Q1    | 273           | 2†                |

Discussion

In this study, we provide evidence that transgenic Forsythia plants produce ectopic lignans, (+)-piperitol and (+)-sesamin. These results suggest that sesamolin and sesaminol, which are antioxidant sesame lignans metabolized from (+)-sesamin by CYP92B14†, 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 therapy\(^\text{31}\). In \textit{Podophyllum} podophyllotoxin biosynthesis, matairesinol, which also accumulate in the pathway downstream of pinoresinol, is metabolized to pluviatolide by CYP719A2\(^\text{32}\). Additional enzymes (CYP71CU1, 2-oxoglutarate/Fe(II)-dependent dioxygenase, and O-methyltransferases) convert pluviatolide to the proposed precursor deoxypodophyllotoxin\(^\text{33}\). Thus, methods for introducing multiple genes into \textit{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 \textit{Forsythia} cultured cells\(^\text{26}\), suggesting the possibility of multigene transformation of \textit{Forsythia} plants.

The production of various specialized plant metabolites has been attempted in transgenic and synthetic biology–based microorganisms\(^\text{9,41}\). However, most microorganisms appear to lack (+)-sesamin and its precursor (+)-pinoresinol\(^\text{9,41}\). 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\(^\text{9,41}\). In contrast, \textit{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. Moreover, a plant factory provides plasticity in place and time of the production of sesamin in plants\(^\text{42}\), unlike agricultural production that is limited by climates, seasons, and farmlands. Thus, we will be able to produce sesamin using our transgenic \textit{Forsythia} plants in a plant factory.

Also of significance is the reproducible generation of transgenic \textit{Forsythia} plants expressing the CYP81Q1 gene. The lignan biosynthetic pathway has previously been modified in cultured plants, cells, and hairy roots of \textit{Carthamus}, \textit{Linum}, \textit{Hyptis}, \textit{Juniperus}, \textit{Podophyllum}, \textit{Sesamum}, and \textit{Forsythia} species\(^\text{43–59}\), but the transgenic plants have not been reported. For the scalable production of lignans, our transgenic \textit{Forsythia} plant method provides an important strategy for future metabolic engineering of such lignan-producing plants.

The transgenic \textit{Forsythia} plants have limitation in the content of sesamin as compared with sesame seeds. To increase the content of sesamin in the transgenic \textit{Forsythia} plants, we will be able to apply multigene transformation strategy. Previously, triple-transgenic \textit{Forsythia} cells with the RNAi construct for endogenous pinoresinol-lariciresinol reductase, and the overexpression-construct of pinoresinol glucosylating enzyme, which increase the level of the precursor pinoresinol, as well as CYP81Q1 gene, produced higher level of sesamin than the single-transgenic CYP81Q1 cells\(^\text{39}\). The same strategy of multigene transformation of \textit{Forsythia} plants is expected to increase the content of sesamin. Moreover, overexpression of enzymes upstream of pinoresinol stimulated accumulation of podophyllotoxin-related lignans\(^\text{60}\). Thus, overexpression of the upstream enzymes may increase the content of sesamin in transgenic \textit{Forsythia} plants. Because the content of pinoresinol was not changed in the control and the Pro35S::CYP81Q1 plants (Supplemental Fig. S1), the moderate activity of CYP81Q1 seems...
to limit the rate of production of sesamin from pinoresinol. As many cytochrome P450 enzymes function in complex with their native oxidoreductases, co-expression of 
\[\text{CYP81Q1}\] and oxidoreductase genes in transgenic \textit{Forsythia} plants is a future option to strengthen the activity of \text{CYP81Q1} for higher production of sesamin. In addition, the red light condition increased the content of sesamin in the transgenic \textit{Forsythia} cells, suggesting that irradiation of red light to transgenic \textit{Forsythia} plant may increase the content of sesamin.

In conclusion, we have generated \textit{Forsythia} plants as promising prototypes for the efficient and sustainable heterologous production of beneficial lignans.

**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 (P3); \(m/z = 381.12\), piperitol; \(m/z = 379.11\), sesamin; \(m/z = 377.09\), 3′-ethoxysesamin; \(m/z = 421.11\), TIC total ion current.

**Table 2.** The accumulation of (+)-piperitol and (+)-sesamin in Pro35S:CYP81Q1 leaves. \textit{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) |
|--------------|--------------------|------------------|-----------------------------|
| FinGFP (n = 1) primary transformant | n.d. | n.d. | n.d. |
| FiCYP81Q1 (n = 1) primary transformant | 14.23 | 5.57 | 19.80 |
| FinGFP (n = 6) 2nd propagation | n.d. | n.d. | n.d. |
| FiCYP81Q1 (n = 6) 2nd propagation | 39.57** (SD ± 25.26) | 11.09* (SD ± 11.89) | 50.66 |
| FinGFP (n = 5) 3rd propagation | n.d. | n.d. | n.d. |
| FiCYP81Q1 (n = 5) 3rd propagation | 17.81* (SD ± 15.76) | 1.69* (SD ± 1.60) | 19.50 |
| FAWT (n = 1) primary plant | n.d. | n.d. | n.d. |
| FiCYP81Q1 (n = 1) primary transformant | 39.45 | 27.21 | 66.65 |
| FAWT (n = 6) 2nd propagation | n.d. | n.d. | n.d. |
| FiCYP81Q1 (n = 6) 2nd propagation | 34.39** (SD ± 14.24) | 19.45** (SD ± 17.17) | 53.84 |
| FAWT (n = 6) 3rd propagation | n.d. | n.d. | n.d. |
| FiCYP81Q1 (n = 6) 3rd propagation | 51.66** (SD ± 16.48) | 21.50** (SD ± 8.34) | 73.16 |
Materials and methods

Plant growth conditions. *F. intermedia* and *F. koreana* plants were obtained from Niigata Prefectural 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 µmol m⁻² s⁻¹)/8-h-dark at 22 °C unless otherwise indicated. After surface-sterilization of 10 cm length *Forsythia* shoots using 70% ethanol for 30 s and 1% sodium hypochlorite solution for 15 min, 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 CuSO₄·5H₂O, 5 g/L Agar gel [Sigma-Aldrich, MO]) and transferred to fresh medium every two to three months.

Transformation and preparation of *Agrobacterium* cells. For construction of Pro35S::nGFP, the NLS was inserted upstream of GFP and the resulting fusion NLS-GFP genes was inserted into pB1101. For construction of Pro35S::CYP81Q1, the coding sequence of CYP81Q1 (accession number AB194714) was inserted downstream of the Pro35S promoter in pBINplus. *Agrobacterium tumefaciens* GV3101 cells were individually transformed with Pro35S::nGFP and Pro35S::CYP81Q1 plasmids using a Gene Pulser II electroporator (Bio-Rad, 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 6000×g for 15 min at room temperature, resuspended in the transformation solution (Gamborg’s B5 salts, B5 vitamin, 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% 500 W 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; ADV ANTEC, 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 Agar gel) 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 Agar gel) 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 Fig. S2).

Measurement of lignans. Cultured transgenic and control *Forsythia* plants were transferred into soil in pots, acclimated 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 (EYL A, Japan), extracted with 50% methanol (v/v) containing 2.25 µM (final concentration) 2′-ethoxyxysemin (Supplementary Fig. S3 and Table S2) as the internal standard, and processed as described previously. 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 previously. Lignans were monitored by UV absorption at 283 nm, and their concentrations were calculated by Empower2 software (https://www.waters.com/waters/library.html?locale=en_US&lid=1529008,Waters Corporation, MA) according to the areas of the peaks in the
chroomatograms 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 previously. Lignans were detected using a photodiode array detector and analyzed by LabSolutions LCMS version 3.8.1 (https://www.an.shimadzu.co.jp/lcms/support/download/index.htm, Shimazu, Japan).

**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 and Supplemental Fig. S1).

Received: 19 January 2022; Accepted: 6 June 2022
Published online: 16 June 2022

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Acknowledgements
We thank the staff of the Niigata Prefectural Botanical Gardens and Dr. Umezawa for providing shoots of Forsythia plants. We thank Drs. Shinzo Tsuda, Yukihisa Katsumoto, and Yoshihiko Nanasato for helpful discussion and the Ministry of Economy, Trade and Industry for funding.

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.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-022-14401-9.

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