Metabolic Engineering of Lignan Biosynthesis Pathways for the Production of Transgenic Plant-Based Foods

15

Honoo Satake, Tomotsugu Koyama, Erika Matsumoto, Kinuyo Morimoto, Eiichiro Ono, and Jun Murata

Abstract

Lignans are major phytochemicals biosynthesized in several plants including *Sesamum*, *Linum*, *Forsythia*, and *Podophyllum* genus, and a great variety of lignans have received wide attentions as leading compounds of novel drugs for tumor treatment and healthy diets to reduce of the risks of lifestyle-related diseases. Recent genome and transcriptome studies have characterized multiple novel lignan-biosynthetic enzymes, and thus have opened new avenues to transgenic metabolic engineering of various nonmodel dietary or medicinal plants. *Forsythia* and *Linum* are the most useful and prevalent natural and agricultural sources for the development of both transgenic foods and medicinal compounds. Over the past few years, transiently gene-transfected or transgenic *Forsythia* and *Linum* plants or cell cultures have been shown to be promising platforms for the sustainable and efficient production of beneficial lignans. In this chapter, we present the essential knowledge and recent advances regarding metabolic engineering of lignans based on their biosynthetic pathways and biological activities and the perspectives in lignan production via metabolic engineering.

Keywords

Lignan • Biosynthesis • Metabolic engineering • Transgenic plant, *Forsythia*, *Linum*

H. Satake (✉) • T. Koyama (✉) • E. Matsumoto (✉) • K. Morimoto (✉) • J. Murata (✉)
Bioorganic Research Institute, Suntory Foundation for Life Sciences, Seika, Kyoto, Japan
e-mail: satake@sunbor.or.jp; koyama@sunbor.or.jp; matsumoto@sunbor.or.jp; sial_glc@yahoo.co.jp; murata-j@sunbor.or.jp

E. Ono (✉)
Research Institute, Suntory Global Innovation Center (SIC) Ltd., Seika, Kyoto, Japan
e-mail: Eiichiro_Ono@suntory.co.jp

S. Jha (ed.), *Transgenesis and Secondary Metabolism*, Reference Series in Phytochemistry, DOI 10.1007/978-3-319-28669-3_11

Abbreviations

CAD	Cinnamylalcohol dehydrogenase
CCR	Cinnamoyl-CoA reductase
DIR	Dirigent protein
ER	Estrogen receptor
MAPK	Mitogen-activated protein kinase
MeJA	Methyl jasmonate
MOMT	Matairesinol *O*-methyltransferase
PAL	Phenylalanine ammonialyase
PIP	Pinoresinol-lariciresinol/isoflavone/phenylcoumaran benzylic ether reductase
PLR	Pinoresinol-lariciresinol reductase
PTOX	Podophyllotoxin
RNAi	RNA interference
SA	Salicylic acid
SDG	Secoisolariciresinol diglucoside
SIRD	Secoisolariciresinol dehydrogenase

Contents

1 Introduction

Functional foods, dietary supplements, and drug compounds are largely derived from specialized metabolites, previously called secondary metabolites of plants, including alkaloids, flavonoids, isoflavonoids, and lignans. Recently, the rapid increase in the number of elderly individuals has required various medical costs, which may eventually cause a serious disruption in essential medical care systems and national financial burdens. To address these issues, the consistent and appropriate intake of dietary supplements and the efficient development of clinical drugs are the most promising and effective ways to increase the healthy life expectancy and prevent lifestyle-related diseases. In this context, intensive efforts should be made on the development of functional foods and supplements as well as of clinical agents.

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 (Fig. 1). These specialized metabolites are classified

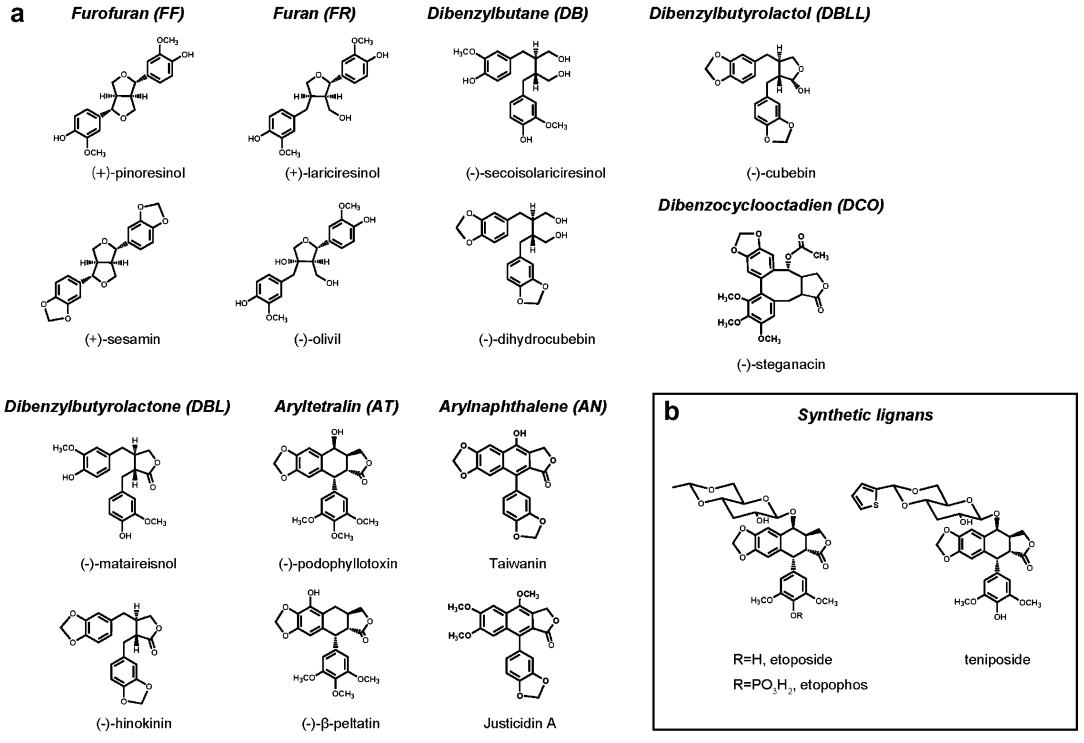

Fig. 1 Chemical structures of typical lignans in dietary and medicinal sources (**a**) and synthetic podophyllotoxin derivatives (**b**)

into eight groups based on their structural patterns, including their carbon skeletons, the way in which oxygen is incorporated into the skeletons, and the cyclization pattern: furofuran, furan, dibenzylbutane, dibenzylbutyrolactone, aryltetralin, arylnaphthalene, dibenzocyclooctadiene, and dibenzylbutyrolactol [1, 2]. Lignans have been shown to exhibit not only various pharmaceutical activities but also preventive or reductive effects on extensive life-related diseases (see the following section) [3–15], indicating the prominent potentials as functional foods and supplements. Indeed, a sesame lignan, sesamin, is already commercially available as functional supplements for antihypertension and protection of the liver based on reduction of lipid oxidation. Unfortunately, plant sources of lignans are frequently limited because of the high cost of plant hunting and collection, poor cultivation systems, long growth phase, and the low lignan content. For instance, sesamin is extracted from sesame seed oil, the most abundant source of this compound. Nevertheless, sesamin at most constitutes 0.4–0.6% (w/w) of sesame seed oil. Moreover, sesame seeds are cultivated only once per year, limiting the ability to obtain large amounts of this compound. Likewise, podophyllotoxin (PTOX), a precursor of semisynthetic antitumor drugs (Fig. 1), is isolated from the roots and rhizomes of *Podophyllum hexandrum*, which is distributed in very limited regions, and is now endangered due to overharvesting and environmental disruption [16]. Moreover, lignans and their precursors are not or faintly biosynthesized in prevalent model plants such as *Arabidopsis thaliana* and *Nicotiana tabacum*, given that lignan biosynthetic pathways for plant specialized metabolites involve multiple enzymatic steps that are absent in most plant species including these model plants. Thus, transformation of a whole set of the biosynthetic genes would be required for the generation of transgenic model plants that could produce phytochemicals, indicating the critical shortcomings of these plants as biological lignan producers [12, 13, 17]. Similarly, transgenic microbial or animal cells are also not suitable for any biological lignan producers. In addition, the complicated chemical structures of lignans and the related compounds (Fig. 1a) make stereoselective organic synthesis impractical and costly for lignan producing large supplies of these compounds. These drawbacks indicate the requirement for efficient, stable, and sustainable systems for lignan production using lignan-rich plants or its precursor compound-rich plants.

There has been a growing body of reports on the molecular characterization of the enzymes involved in the biosynthesis of lignans, lignan production using lignan-rich plants or cultured plant cells [18–23]. These recent findings have allowed us to attempt the metabolic engineering of lignan biosynthesis using transgenic lignin-rich plants such as *Linum* and *Forsythia*. In this chapter, we provide current knowledge of lignan production via metabolic engineering and perspectives in the development of metabolic engineering-based lignan production.

2 Lignan Biological Activity on Mammals

Lignans exhibit a wide variety of bioactivities on plants, insects, and mammals [12, 13, 24–29], but special attentions have been paid to their unique antitumor-associated activities and reduction of the risks of lifestyle-related diseases. The modes of actions of lignans on mammals are classified in two ways: the pharmacological actions of specific metabolites of lignans by intestinal microflora and those of intact lignans. Many of lignans and their glycosides, including pinoresiniol, sesamin, lariciresinol, secoisolariciresinol, and matairesinol, are metabolized by intestinal microflora to yield enterodiol and enterolactone, which are defined as enterolignans or mammalian lignans [30–32]. These lignan metabolites are believed to elicit the modest estrogen (mammalian female steroid hormone)-like activity in mammals. For example, enterolignans bind to the mammalian estrogen receptors (ER), ERα or ERβ, which are key regulatory nuclear receptors in the sexual maturation of genital organs [33, 34]. Consequently, enterolignans, combined with other intestinal flora generating metabolites of isoflavones and coumestans, are also called phytoestrogens.

Intact lignans have also been detected in the sera of mammals fed with lignan-rich diets, suggesting that nonmetabolized lignans are also taken up by the mammalian digestive system, and exhibit ER-independent activities in vivo and in vitro, including tumor growth suppression, angiogenesis inhibition, and reduction of diabetes [6, 35–40]. Furthermore, lignans have been shown to manifest positive effects on other lifestyle-related diseases. Administration of flaxseed lignan complexes improved hyperglycemia and reduced markers of type II diabetes in elderly patients and various animal models [41, 42]. In particular, secoisolariciresinol diglucosides (SDG), secoisolariciresinol, enterodiol, and enterolactone inhibited pancreatic α-amidase activity in a noncompetitive manner [43]. Sesamin and its metabolites also exhibited antihypertensive activities [44–46]. Moreover, the antioxidative activity of sesamin is believed to be involved in protecting the liver from oxidation by alcohols, lipids, and oxygen radicals [44, 47–49]. In human intestinal Caco 2 cells, pinoresinol suppressed expression of Cox-2, an inducible prostaglandin synthase that is responsible for the synthesis of prostaglandin H, a precursor of any other prostaglandins, leading to the decrease in the production of inflammatory factors, such as interleukin-6 and prostaglandin E2 [35]. In contrast, matairesinol increased levels of prostaglandin E2 [35]. These findings proved that pinoresinol and matairesinol have opposite activities in these cells [35].

Of the most prominent epidemiological significance is that intake of lignan-rich foods, such as flaxseeds and sesame seeds, has been shown to reduce breast cancer risk and to improve the breast cancer-specific survival of postmenopausal women [39, 50–55]. Moreover, serum enterolactone levels were positively correlated with

the improvement of prognosis in postmenopausal women with breast cancer [56]. These epidemiological studies suggest the unique and beneficial suppressive activity of lignans against breast cancer risks in elderly women.

Dietary lariciresinol was found to suppress tumor growth and angiogenesis in nude mice implanted with human MCF-7 breast cancer via the induction of apoptosis and the upregulation of ERβ expression [40]. SDG elicited potent inhibition of cell proliferation and induction of the apoptosis of breast cancer cells via the downregulation of ER- and growth factor-mediated gene expression in athymic mice [57]. Sesamin was found to reduce signaling downstream of mitogen-activated protein kinase (MAPK) [58]. Additionally, the inhibitory effect of sesamin on breast tumor growth is likely to be more potent than SDG [58]. These pharmacological effects, combined with the abundance of lignans in flax or sesame seeds and oils, confirm that the seeds and oils are promising functional diets for the prevention of breast cancer.

PTOX and its structurally related natural derivatives exhibit the suppressive activity on mitotic spindle assembly by binding to tubulin, resulting in cell cycle arrest at metaphase [22]. The PTOX semi-synthetic derivatives, etoposide, teniposide, and etopophos (Fig. 1b), are clinically utilized to treat certain types of cancers, including testicular/small-cell lung cancer, acute leukemia, Hodgkin's and non-Hodgkin's lymphoma [58, 59]. These PTOX-derived antitumor drugs induce apoptosis of tumor cells by binding to topoisimease II, a key enzyme for cell division [58, 59]. In addition, other new synthetic PTOX derivatives, including GP-11, NK-611, TOP-53, GL-331, and NPF, are undergoing phase I or II clinical trials as novel cancer drugs [22, 59]. Consistent with the difficulty in efficient chemical synthesis of PTOX due to its complicated structure, these findings reinforce the importance of PTOX as a natural seed material for the production of various anticancer drugs.

Altogether, these epidemiological and physiological studies demonstrate that lignans exert beneficial effects as dietary compounds or medicinal agents for the prevention of lifestyle-related diseases, such as cancer, hypertension, and diabetes. Of particular interest is that respective lignans exhibit specific bioactivities in mammals, strongly suggesting the requirements for the efficient, stable, and sustainable production of these compounds of interest. In other words, these findings not only endorse the high usefulness of lignin-rich sesame and flax seeds as unique functional foods but also shed light on the importance of the development of novel lignan production systems using transgenic lignan-rich plants.

3 Lignan Biosynthesis Pathways

Two major lignan biosynthesis pathways have thus far been identified. Both of the pathways originate from the coupling of achiral *E*-coniferyl alcohol, leading to the generation of pinoresinol, a basal lignan (Fig. 2). A pinoresinol synthase has yet to be identified. However, a dirigent protein (DIR) was shown to participate in the stereo-specific dimerization of *E*-coniferyl alcohol [60]. In several plants including *Sesamum*, pinoresinol is metabolized into piperitol, followed by further conversion

Fig. 2 Biosynthesis pathways of major lignans. Chemical conversions at each step are indicated in *red. Solid and broken lines* represent identified and unidentified enzyme-catalyzed reactions, respectively

into (+)-sesamin by a cytochrome P450 family enzyme, CYP81Q1, which is responsible for the formation of two methylenedioxy bridges [61]. The CYP81Q1 gene is expressed almost exclusively in sesame seeds, which is compatible with sesamin production at the highest level in sesame seeds [61]. Sesamin is anticipated to be further metabolized into sesaminol and sesamolin (Fig. 2), although the relevant enzymes remain to be characterized. Sesaminol is glucosylated at its 2-hydroxyl group by the homologous enzymes UGT71A8 (*S. radiatum*), 9 (*S. indicium*), and 10 (*S. alatum*) [62]. Moreover, the resultant sesaminol 2-*O*-monoglucoside is further glucosylated by UGT94D1, which is specific to the glucosylation of sesaminol 2-*O*-monoglucoside at 6-position of the conjugated glucose conjugated by UGT71A18 [62].

No genes homologous to CYP81Q1 have been detected in diverse lignan-rich plant species including *Forsythia*, *Linum*, or *Podophyllum* [63–69]. This is in good agreement to the findings that these plants fail to biosynthesize sesamin and its derivatives. Instead, pinoresinol is stepwisely reduced to lariciresinol and then secoisolariciresinol by pinoresinol-lariciresinol reductase (PLR), a member of the pinoresinol-lariciresinol/isoflavone/phenylcoumaran benzylic ether reductase (PIP) family in extensive plant species including *Forsythia*, *Linum*, and *Podophyllum* [70–75]. PLR converts pinoresinol to secoisolariciresinol via lariciresinol (Fig. 2). Pinoresinol also undergoes glucosylation by UGT71A18, a UDP-glucose-dependent glucosyltranferase [76]. Such glycosylation is highly likely to suppress the chemical reactivity of a phenolic hydroxyl group of pinoresinol and to potentiate high water solubility of pinoresinol aglycone, resulting in large and stable amounts of pinoresinol [1, 2, 11, 12]. Indeed, approximately 90% of pinoresinol is accumulated in its glucosylated form in *Forsythia* spp. [77, 78]. Thus, PLR-catalyzed metabolism and UGT71A18-directed glucosylation are reciprocally competitive pathways (Fig. 2), given that both of them share pinoresinol as a substrate. *PLR* shows opposite seasonal alteration in gene expression against *UGT71A18*; in *Forsythia* leaves in Japan, *PLR* gene is intensely expressed from April to August but poorly from September to November, whereas gene expression of *UGT71A18* is observed at high level from September to November but at faint or no level from April to August in Japan [78]. These findings indicate that PLR and UGT71A18 participate in the competitive regulation of lignan biosynthesis via pinoresinol metabolism. In *A. thaliana*, AtPrR1 and 2 are only responsible for the reduction of pinoresinol to lariciresinol [74], and lariciresinol and pinoresinol are glucosylated by another novel UDP-glucose-dependent glucosyltranferase, UGT71C1 [79], revealing the diversity of lignan metabolism among plant species.

Secoisolariciresinol, like pinoresinol and lariciresinol, undergoes two metabolic pathways (Fig. 2). First, Secoisolariciresinol is converted into matairesinol by secoisolariciresinol dehydrogenase (SIRD) [80]. Second, a novel UDP-glucose-dependent glucosyltranferase in *Linum*, UGT74S1, generates secoisolariciresinol monoglucoside and SDG [81]. Matairesinol is metabolized to arctigenin (Fig. 2) by matairesinol *O*-methytransferase (MOMT) via methylation of a phenolic hydroxyl group in various plants including *F. koreana*, *Carthamus tinctorius*, and *Anthriscus sylvestris* [82, 83]. Additionally, 70–90% of matairesinol is glucosylated throughout the year in the *Forsythia* leaves [78], although characterization of matairesinol-glucosylating enzymes awaits further study. As shown in Fig. 2, the biosynthetic pathways downstream of matairesinol are complexed and relatively species-specific. In *Linum*, *Anthriscus*, and *Podophyllum* plants, matairesinol is also converted into hinokinin, yatein, or PTOX via multiple biosynthetic pathways [1, 2, 12, 13, 60]. In *A. sylvestris*, AsTJOMT exclusively methylates the 5-hydroxyl group of thujaplicatin, an intermediate of the PTOX biosynthesis pathway [84].

The homologous enzymes, CYP719A23 (from *P. hexandrum*) and CYP719A24 (from *P. peltatum*) participate in the conversion of matairesinol into pluviatolide, a more downstream intermediate of PTOX (Fig. 2), via methylenedioxy bridge formation [63, 64]. Quite recently, six novel genes, which were also detected by

NGS-based transcriptome, have been characterized from *P. hexandrum* and shown to be responsible for the PTOX biosynthesis [23]. CYP71CU1 was found to hydroxydise (−)-5′-desmethoxy-yatein into (−)-5′-desmethyl-yatein followed by *O*–methylation by OMT1to (−)-yatein (Fig. 2). (−)- yatein is converted into (−)-deoxy-podophyllotoxin, which is demethylated to (−)-4′-desmethyl-deoxy-podophyllotoxin by CYP71BE54 (Fig. 2). CYP82D61 was shown to participate in the production of (−)-4′-desmethyl-epipodophyllotoxin via hydroxylation of (−)-4′-desmethyl-deoxy-podophyllotoxin (Fig. 2). Notably, (−)-4′-desmethyl-epipodophyllotoxin, which is an aglycone of an antitumor drug, etoposide, was detected in transgenic tobacco transected with these six genes [23]. Taken into account that (−)-4′-desmethyl-epipodophyllotoxin is synthesized from PTOX in the industrial production of etoposide, this study leads to the development of the novel procedure for the production etoposide using transgenic tobacco as well as explored total biosynthesis pathways of PTOX and its related lignans [23].

Over the past few years, the genomes or transcriptomes of lignan-rich plants including *Linum* [68, 85, 86], *Sesamum* [65–67], and *Podophyllum* [23, 63, 64, 69] have been documented, followed by *in silico* detection of functional genes. Particularly, next-generation sequencers (NGS)-based *de novo* transcriptome has been shown to be a powerful procedure for molecular characterization of lignan-biosynthetic genes at the first step, as described above. These findings are highly likely to remarkably enhance the molecular and functional characterization of unknown lignan biosynthetic enzymes. In addition, it is suggested that a *Podophyllum* endophyte may produce PTOX [87]. NGS analyses of the genome, metagenome, and transcriptome of *Podophyllum* and its endophytes are expected to provide crucial clues to understanding the PTOX biosynthesis pathways.

4 Metabolic Engineering of Lignan Biosynthesis

A growing body of studies has revealed that lignan biosynthesis is altered by genetic modification, light, and elicitors. This section presents an overview and discussion of recent progress in major lignan metabolic engineering using plants, plant cells, and organ cultures.

4.1 Metabolic Engineering of Transgenic Plants and Cells

Stable transfection or gene silencing, namely authentic transgenic metabolic engineering of a lignan biosynthetic enzyme gene is expected to directly alter the lignan production cascades in host plants, organs, and cells. To date, *Forsythia* and *Linum* cell, organ cultures, and plants are attempted to generate the transgenic plants among lignan-rich plants. *Agrobacterium*-based gene introduction was employed for transformation of both *Forsythia* and *Linum*, which is also essentially common to generation of transgenic model plants [17, 77, 88–94]. Figure 3 demonstrates the typical procedure for *Agrobacterium*-based transformation of *Forsythia*. In the

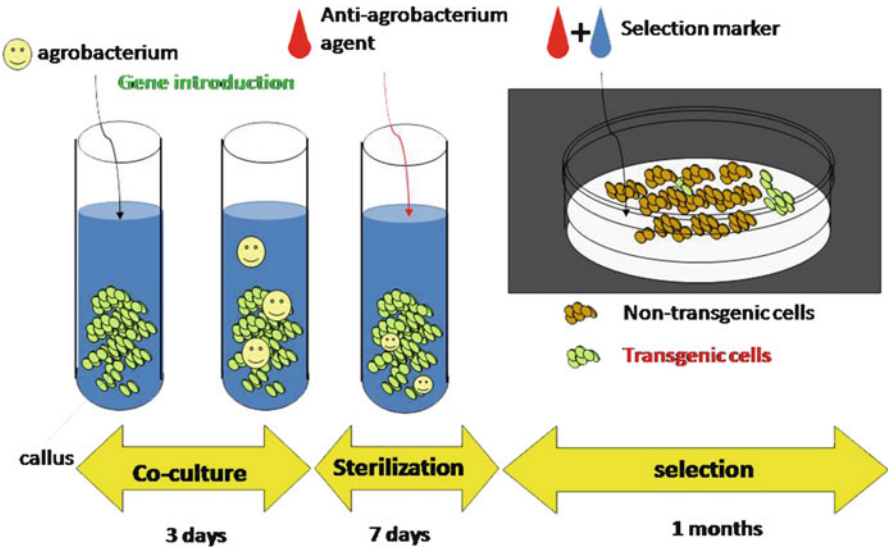

Fig. 3 Scheme for generation of transgenic *Forsythia* mediated by *Agrobacterium*. This process is common for the generation of transgenic *Forsythia* plants and suspension cultures

subsection, we present the recent progress in transformation of *Forsythia* and *Linum* and metabolic engineering of lignan biosynthetic pathways using these plants.

4.1.1 Transgenic *Forsythia* Cells

Forsythia is a perennial plant commonly known as the golden bell flower and is used for a variety of Chinese medicines and health diets [1, 2, 5, 7, 12, 13]. As shown in Fig. 2, *Forsythia* biosynthesizes pinoresinol, lariciresinol, secoisolariciresinol, matairesinol, and arctigenin, with >90% of pinoresinol, >80% of matairesinol, and 40–80% of arctigenin accumulated in glucosylated forms [12, 13, 17, 77, 78]. Seasonal changes in amounts of major *Forsythia* lignans and the relevant gene expression were also reported. All of the lignans in the leaf continuously increased from April to June, reached the maximal level in June, and then decreased [78]. *PLR* was stably expressed from April to August, whereas the *PLR* expression was not detected from September to November [78]. In contrast, the UGT71A18 expression was detected from August to November but not from April to July. The *SIRD* expression was prominent from April to May, not detected in June to July, and then increased again from September to November [78]. These expression profiles of the lignan-synthetic enzymes are essentially correlated with the alteration in lignan amounts.

Several transgenic *Forsythia* plants and cells have been documented for the past 5 years [17, 77, 89]. It is noteworthy that the regeneration efficiency of callus (shoot formation and rooting) and optimal condition for them differ among the variety of *Forsythia* species (*F. koreana*, *F. intermedia*, and *F. suspense*) (Fig. 4). For instance, *F. koreana*, *F. intermedia*, and *F. suspense* explants regenerated more than 100, 36, and 4 shoots per leaf, respectively [89]. Likewise, *F. intermedia* calli, unlike

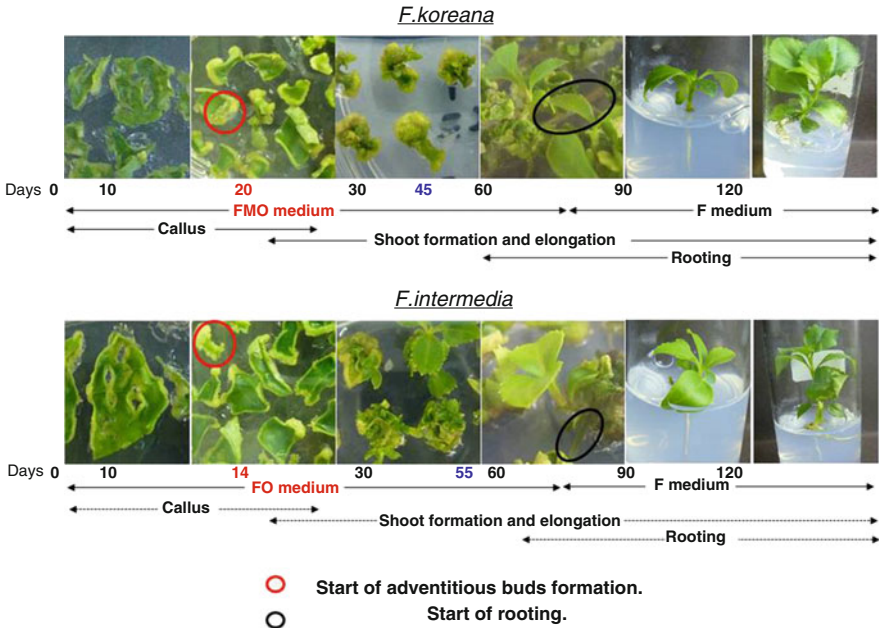

Fig. 4 Different conditions in regeneration between *Forsythia* varieties (*F. koreana* and *F. intermedia*). Note that different media is used for regeneration of the respective *Forsythia* species. Culturing periods also vary between these species

F. koreana, calli, were much more effectively regenerated on the F0 medium than on the FM0 medium [89]. Two transgenic *F. intermedia* and one transgenic *F. koreana* have acquired hygromycin resistance, but none of them have exhibited metabolic alteration in lignan biosynthesis [89]. Moreover, the greatest drawback in generation of transgenic *Forsythia* lies in extremely low transformation efficiencies. These findings strongly suggest the potential requirement for innovation of transgenic *Forsythia* plant generation. In other words, a high-efficient transgenic method for *Forsythia* is expected to remarkably enhance transgenic metabolic engineering-based lignan production using transgenic *Forsythia*.

The transgenic metabolic engineering of *Forsythia* culture cells was originally reported in 2009. *F. koreana* suspension cells stably transfected with a PLR-RNA interference (RNAi) sequence (*PLR*-RNAi) showed complete loss of matairesinol and an approximately 20-fold increase in total pinoresinol (pinoresinol aglycone and glucoside), compared with the wild type cells [77]. Furthermore, *Forsythia* transgenic cells CPi-Fk, which are stably double transfected with *PLR*-RNAi and the sesamin-producing enzyme *CYP81Q1* (Fig. 2), produced sesamin (0.01 mg/g dry weight of the cell [DW]) (Fig. 5), although sesamin is not biosynthesized in native *Forsythia* [77]. This is the first success in the metabolic engineering leading to an exogenous lignan using transgenic plant cells, demonstrating that the *Forsythia* cell culture system is an efficient and promising platform for producing both endogenous and exogenous lignans by transgenic metabolic engineering. A striking feature is

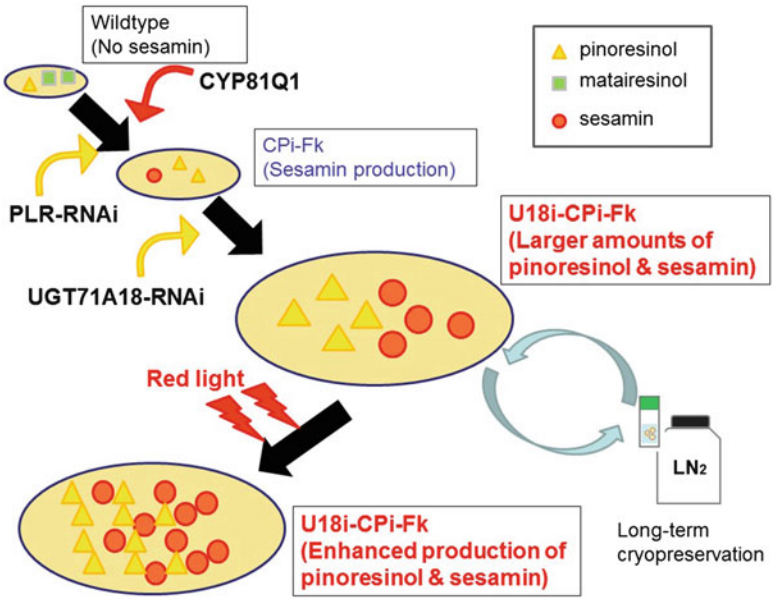

Fig. 5 Metabolic engineering of *Forsythia* suspension cell cultures. The double-transgenic *Forsythia* suspension cells, CPi-Fk, acquired the ability to produce sesamin by stable transfection of *PLR*-RNAi and an exogenous (*Sesamum*) *CYP81Q1* gene. The triple-transgenic cells, U18i-CPi-Fk, were generated by the introduction of UGT71A18-RNAi into CPi-Fk and exhibit higher productivity of pinoresinol and sesamin than CPI-Fk. The lignan productivity is approximately three- to fivefold upregulated under red LED. U18i-CPi-Fk can also be stocked in liquid nitrogen for a long period, and re-thawed U18i-CPi-Fk exhibit as high productivity of sesamin as noncryopreserved U18i-CPi-Fk

that light irradiation has been shown to improve the production of both endogenous and exogenous lignans by CPi-Fk cells. Irradiation of CPi-Fk cells for 2 weeks with white fluorescent, blue LED, and red LED light increased sesamin production 2.3-fold, 2.7-fold, and 1.6-fold, respectively, compared with cells cultured in the dark [94]. Likewise, irradiation of CPi-Fk cells increased pinoresinol (aglycone and glucoside) production 1.5- to 3.0-fold [94]. Intriguingly, expression of the pinoresinol-glucosylating enzyme UGT71A18 was also downregulated in CPi-Fk cells under blue LED or red LED light [94]. This reduction of the expression of UGT71A18 is also likely to contribute to the increase of sesamin production [94], given that pinoresinol glucoside is not metabolized into sesamin by CYP81Q1 [12, 75], and 90% of pinoresinol is glucosylated in *Forsythia* wildtype cells [12, 13, 17, 77, 78]. In other words, these findings suggested that suppression of UGT71A18 by RNAi might contribute to an increase in productivity of pinoresinol and sesamin in CPi-Fk. This presumption was substantiated in our subsequent study. Quite recently, we have created more efficient, stable, and sustainable sesamin production system using triple-transgenic *Forsythia koreana* cell suspension cultures, U18i-CPi-Fk, compared to CPi-Fk [17]. These transgenic cells were generated by stable

transfection of CPi-Fk with an RNAi sequence against the pinoresinol-glucosylating enzyme UGT71A18. *UGT71A18* expression was not detected in the triple-transgenic *Forsythia* cells [17]. Moreover, U18i-CPi-Fk accumulated approximately fivefold higher amounts of pinoresinol aglycone than CPi-Fk, and the ratio of pinoresinol aglycone to total pinoresinol in U18i-CPi-Fk is 81.81 ± 6.43%, which is approximately 6.5-fold greater than that in CPi-Fk (13.19 ± 2.35%). These results proved that UGT71A18-RNAi contributed a great deal to the increase in the ratio of pinoresinol aglycone to total pinoresinol. Notably, U18i-CPi-Fk has also been shown to display 1.4-fold higher production of sesamin than CPi-Fk [17], confirming that the suppression of UGT71A18 gene expression is effective for improvement of the sesamin production. Furthermore, pinoresinol aglycone was 3.4-fold and 2.8-fold greater produced under white fluorescent and red LED, respectively, than under the dark condition. Consistently, sesamin production in U18i-CPi-Fk was approximately threefold (31.02 ± 3.45 μg/g DW) upregulated specifically under red LED, whereas white fluorescent or blue light failed to affect sesamin production [17]. It should be noteworthy that the light types effective for the sesamin production differed between CPi-Fk and U18i-CPi-Fk; the sesamin production was potentiated exclusively by blue LED light in CPi-Fk [94], whereas red LED light upregulated the sesamin production in U18i-CPi-Fk [17]. The molecular mechanism underlying such different sensitivity of these transgenic *Forsythia* cells remains unclear, but the suppression of UGT71A18 gene is likely to alter other biosynthetic pathways than pinoresinol glucosylation, which ultimately may affect light sensitivity of the sesamin production. In addition, upregulation of lignan production by light was also observed in other natural plants or cells. In *Linum* species, suspension of *L. album* cells produced twofold more PTOX under red light than those in the dark [95]. Irradiation of *S. indicum* leaves 3–5 weeks after sowing with blue LED light increased sesamin content twofold, compared with white fluorescent light, whereas irradiation with red LED light reduced sesamin content twofold [9, 96]. In combination, these findings highlight the different specificity of light types to lignan production among lignan compounds and host plant species.

U18i-CPi-Fk has also been found to possess another prominent advantage over CPi-Fk, that is, long-term and reproducible storage [17]. Universal procedures for long-term stock of plant cell cultures, unlike those of seeds, bacteria, or animal cells, have not been well established, and cryopreservation procedures for a particular plant species are not always applicable to other plant cells [97–99]. Indeed, we failed to establish any procedure for long-term stock of CPi-Fk, and thus observed a decrease in the growth rate of CPi-Fk cells after 2 years of culture and, eventually, proliferation loss. Nevertheless, we have developed a procedure for sodium alginate-based long-term storage of U18i-CPi-Fk in liquid nitrogen [17]. Moreover, production of sesamin in U18i-CPi-Fk re-thawed after 6-month cryopreservation was equivalent to that of noncryopreserved U18i-CPi-Fk, proving the reproducible functionalities of U18i-CPi-Fk [17]. Altogether, the high lignan (pinoresinol and sesamin) productivity and establishment of the freeze stocks of U18i-CPi-Fk endorses the marked usefulness of U18i-CPi-Fk as a stable and sustainable platform of lignan production (Fig. 5).

4.1.2 Transgenic *Linum*

Linum spp. (flax, Linaceae) are annual flowering plants comprised of approximately 200 species. This genus has received pharmaceutical and medicinal attention due to the presence of various lignans, including PTOX and its related compounds, which are practically applied for the semisynthesis of antitumor drugs for breast and testicular cancers described above. Since *Linum* is also known to biosynthesize PTOX and its derivatives, and the procedures for tissue and cell culture are well established, optimal conditions and stimulating factors for production of various lignans, including (−)-podophyllotoxin, by *Linum* calli, suspension cell cultures, and roots have been extensively investigated as described in the followings. Recently, flax seeds and oils have also received attentions as functional foods due to the contents of lignans beneficial for human health [14, 15]. Accordingly, metabolic engineering of *Linum* is also highly likely to contribute a great deal to the development of novel lignan production and transgenic foods.

In various *Linum* species, the effects of RNAi on production of endogenous lignans via metabolic engineering were investigated. *PLR*-RNAi-transgenic plants of *L. usitatissimum* showed the high accumulation of pinoresinol diglucoside and loss of SDG in the seed coat [90]. Intriguingly, these *PLR*-RNAi-transgenic *L. usitatissimum* plants produced the 8–5′ linked neolignans, dehydrodiconifnyl alcohol and dihydro-dehydrodiconifnyl alcohol, while these neolignans were not biosynthesized in the wildtype plants [90]. These findings indicate that RNAi occasionally affects some biosynthetic pathways in an indirect fashion.

4.1.3 Transient Transformation of *Linum*

Hairy roots of *L. perenne* transiently transfected with *PLR*-RNAi reduced the production of the major endogenous lignan, justicidin B, to 25%, compared with the untreated hairy roots [72]. Likewise, transient transfection of *L. corymbulosum* hairy roots with PLR-RNAi resulted in a marked reduction of hinokinin [73]. Combined with the justicidin B and hinokinin biosynthetic pathways, in which PLR converts pinoresinol into secoisolariciresinol (Fig. 2), these findings indicate that PLR-directed conversion of pinoresinol into secoisolariciresinol is a rate-limiting step of justicidin B and hinokinin biosynthesis, at least in the hairy roots of *L. perenn and L. corymbulosum,* respectively. Therefore, identification and genetic manipulation of justicidin B and hinokinin synthase will contribute a great deal to the establishment of procedures for the direct metabolic engineering of these lignans. Taken together, these findings reinforce the potential of *Forsythia* and *Linum* transgenic or transiently gene-transfected cells and plants as the metabolic engineering-based platforms for on-demand production of both endogenous and exogenous lignans. The draft genome and transcriptome of *L. usitatissimum* [68, 85, 86] will also accelerate the identification of the enzymes involved in the biosynthesis of *Linum* lignans, leading to the efficient lignan production using gene-modified plant sources.

4.1.4 What Should Be Considered for Lignan Production via Metabolic Engineering Using Gene-Modified Plants?

To establish gene-modified plant platforms for lignan production, we should consider two crucial factors: the type of host and the use of transgenic or transiently transfected hosts. Host types can be classified into plants, organs, and cell cultures. For example, although the amount of sesamin produced by U18i-CPi-Fk cells is ~100-fold lower than that by native sesame seeds, U18i-CPi-Fk-based lignan metabolic engineering has several advantages. Furthermore, the *Forsythia* transgenic cells are propagated tenfold in 2 weeks in standard culture medium [17] and can be cultivated at all times and locations which is also endorsed by the fact that U18i-CPi-Fk can be stocked for a long time [17]. In contrast, sesame seeds are cultivated in limited regions only once a year. Moreover, the conditions used in the culturing of U18i-CPi-Fk cells, including temperature, light wavelength and intensity, and medium components, can be altered to optimize sesamin production [17, 77]. *Forsythia* plants have much greater biomass, with higher amount of lignans, than suspension cell cultures, and these plants can grow and propagate from small explants without flowering or seed formation [89]. However, efficient generation of transgenic *Forsythia* plants still requires further basic research due to the markedly low transformation efficiency by any known gene transfection methods [89]. In addition, as mentioned above, the optimal culturing and regeneration conditions were found to vary among *Forsythia* species [89]. In other words, the development of a procedure for efficient generation of transgenic *Forsythia* would surely enhance novel lignan production system.

The generation of both stable (namely transgenic) and transient transfectants of *Linum* species are well established [72, 73, 90, 93], and thus the amounts of precursors or intermediates of targeted lignans are major determinants for the employment of cell cultures, organ cultures, or plants as host platforms. Additionally, gene-modified host plants may fail to normally grow or to produce lignans of interest due to cytotoxicity of lignans, although the underlying molecular mechanisms have not fully been elucidated. Therefore, generation of lignan-producing plants using multiple plant species is occasionally required.

The second factor involves construction of either transgenic or transiently transfected hosts. Transgenic plants and cell cultures, once generated, are sustainably used for lignan production and readily upscaled, whereas generation of transgenic plants, in particular nonmodel plants, may be time- and cost-consuming. Moreover, cultivation of transgenic plants in general requires a closed facility for transgenic plants. Transiently transfected plants require repeated transfections, and transient transfection of multiple genes may dramatically decrease the transfection efficiency. Furthermore, massive transient transfection methods for industrial use remains to be fully developed [100]. Further research on lignan metabolic engineering, using transgenic or transiently gene-transfected plants, organ cultures, and cell cultures, is expected to lead to the establishment of both universal and molecular species-specific strategies for gene-regulated metabolic engineering of lignan biosynthesis pathways.

4.2 Metabolic Engineering by Elicitation

Plant defense systems are triggered upon injury or infection via signaling by the phytohormones, methyl jasmonate (MeJA) and salicylic acid (SA), and treatment with elicitors, including fungi, their extracts, and the glycan components, MeJA and SA, also mimic such activation. Moreover, lignans, at least in part, are likely to be implicated with host defense systems [12, 13, 22, 101]. In combination, elicitors are expected to enhance lignan biosynthesis [13, 22, 102]. As summarized in Table 1, the effects of various elicitors on lignan production have been examined in a wide variety of cell cultures and hairy roots of *Forsythia, Juniperus,* and *Podophyllum* (Table 1).

MeJA and SA were found to increase the production of PTOX and the structurally related lignan production or the gene expression of lignan biosynthetic enzymes responsible for biosynthesis of conifenyl alcohol, phenylalanine ammonialyase (PAL), cinnamoyl-CoA reductase (CCR), and cinnamylalcohol dehydrogenase (CAD) in cell suspension cultures of *L. album* [103, 104] and *L. nodiflorum* [103], *Podophyllum hexandrum* [105], and callus of *L. austriacum* callus culture [106]. These phytohormones also increased the PTOX production or the relevant gene expression in hairy roots of *L. tauricum* [107]. Additionally, an increase in production of pinoresinol and matairesinol by MeJA was observed in *Forsythia intermedia* cell suspension culture [108].

Chitosan, chitin oligomers, and other glycans also enhanced PTOX production or gene expression of lignan biosynthetic enzymes in *Juniperus chinensis* callus culture [109], *L. austriacum* callus culture [106], and *L. album* cell suspension culture and hairy roots [110–112]. In particular, comparisons of chitin tetramer, pentamer, and hexamer and chitosan tetramer and pentamer showed that treatment of *L. album* hairy roots with chitosan hexamer for 5 days most potently enhanced PTOX and lariciresinol production, as well as upregulating the expression of *PAL*, *CCR*, *CAD*, and *PLR* genes [112]. In summary, treatment with these elicitors resulted in two- to sevenfold increase in PTOX synthesis and expression of genes encoding enzymes involved in the early steps of lignan biosynthesis in various plant cells and hairy roots.

Fungal co-culturing, extracts, and filtrate exhibited unique effects on the metabolic engineering of lignan production (Table 1). *Botrytis cinerea*, *Phoma exigua*, and *Fusarium oxysporum* extracts triggered the accumulation of monolignols and enhanced PAL activity and gene expression of *PAL*, *CCR*, and *CAD* in *L. usitatissimum* cell suspension cultures [113]. Treatment of *L. album* cell cultures with *Fusarium graminearum* extract for 5 days increased PTOX sevenfold and PAL, CCR, and CAD mRNAs $>$ tenfold compared with untreated cells. These results confirmed that this extract is a more potent elicitor of PTOX production and PAL, CCR, and CAD expression than treatment with chitosan, chitin, or MeJA treatment for 3 days [110, 111, 114].

Rhizopus stolonifer and *Rhizoctonia solani* extract stimulated 8.8-fold and 6.7-fold greater accumulation of lariciresinol, instead of PTOX, in *L. album* cell cultures after 5-days treatment as compared with untreated cells, and the highest (6.5-fold)

Table 1 List of major elicitors and their effects on lignan biosynthesis

Elicitor	Target	Effect	Refs
Chito-oligosaccharides (1 mg)	*Juniperus chinensis* callus culture	Increased PTOX production	[104]
Methyl jasmonate (MeJA) (100 μM)	*Forsythia intermedia* cell suspension culture	Increased pinoresinol and matairesinol production	[103]
Mannan (0.1 mg mL^{-1}) β-1,3-glucan (0.1 mg mL^{-1}) Ancymidol (10^{-7} M)	*L. austriacum* callus culture	Enhanced activity of tyrosine ammonia-lyase (TAL), coumarate 3-hydroxylase (C3H), polyphenoloxidase (PPO) and PAL Increased PTOX, 6-MPTOX, dPTOX, α- and β-peltatins production Increased PTOX and α-peltatins production Increased PTOX, 6-MPTOX, dPTOX and α- peltatins production	[101]
Indanoyl-isoleucine (5–100 μM) Coronalon, (10–50 μM) MeJA(100 μM)	*L. nodiflorum* cell suspension culture	Increased deoxypodophyllotoxin production Enhanced activity of 6-hydroxylase and β-peltatin 6-*O*-methyltransferase, Increased 6-MPTOX and 5′-d-6-MPTOX production	[97]
MeJA (100 μM)	*L. album* cell suspension culture	Increased PTOX production	[98]
Botrytis cinerea extract (3% v/v) *Phoma exigua* extract (3% v/v) *Fusarium oxysporum* extract (3% v/v)	*L. usitatissimum* cell suspension culture	Rapid stimulation of the monolignol pathway, enhanced PAL activity, and expression of genes encoding PAL, CCR, and CAD	[108]
MeJA(50–200 μM)	*L. tauricum* hairy root culture	Increased 6MPTOX and 4′-DM6MPTOX production	[102]
Salicylic acid (SA) (10 μM)	*L. album* cell suspension culture	Enhanced *PAL*, *CCR*, and *CAD* gene expression and PTOX production	[99]
Chitin (100 mg l^{-1}) Chitosan (100–200 mg l^{-1}) MeJA (100–200 μM)	*L. album* cell suspension culture	Increased lariciresinol and/or PTOX production	[105]
Fusarium graminearum extract(1%v/v) *Sclerotinia sclerotiorum* extract (1%v/v) *Rhizopus stolonifer*	*L. album* cell suspension culture	Enhanced *PAL*, *CCR*, *CAD*, and *PLR* gene expression Increased PTOX and lariciresinol production	[105, 109]

(*continued*)

Table 1 (continued)

Elicitor	Target	Effect	Refs
extract(1% v/v) *Rhizoctonia solani* extract(1% v/v)			
MeJA(10–100 μM)	*Podophyllum hexandrum* cell suspension culture	Changes in cell proteome Increased PTOX production	[109]
Fusarium graminearum extract (1%v/v) *Sclerotinia sclerotiorum* extract (1%v/v) *Trichoderma viride* extract (1%v/v) Chitosan (100 mg l^{-1})	*L. album* hairy root culture	Enhanced *PAL*, *CCR*, *CAD*, and *PLR* gene expression Increased PTOX, 6MPTOX, and lariciresinol production	[106]
Chitosan and chitin oligomers (100 mg l^{-1})	*L. album* cell suspension culture	Enhanced *PAL*, *CCR*, *CAD*, and *PLR* gene expression Increased PTOX, 6MPTOX, and lariciresinol production	[107]
Fusarium graminearum culture filtrate (1% v/v)	*L. album* cell suspension culture	Increased phenolic compound, PTOX, and lariciresinol production Enhanced PAL activity	[110]

PLR gene induction was observed in *L. album* cell cultures treated with *Rhizopus stolonifer* extract for 2 days [114]. Similar data were obtained in *L. album* hairy roots with the same fungal extracts [111] or *L. album* cell suspension culture with *Fusarium graminearum* culture filtrate [115], but the latter manifested less lignan production. These studies revealed that fungal extract exhibited the species-specific effects on the lignan biosynthesis pathways, although investigation of the molecular basis awaits further study.

As described above, the regulation of gene expression has thus far been restricted to enzymes responsible for the upstream of lignan biosynthesis pathways. Therefore, the effects of these elicitors on lignans and the relevant biosynthetic genes downstream of PLR, such as SIRD or 719A23 (Fig. 2), would provide a clue to understanding the molecular mechanisms underlying upregulation of PTOX production and to identifying more effective elicitors for lignan production.

5 Conclusions

In this chapter, we have provided diverse recent advances in metabolic engineering for lignan production by plants, including: (i) the molecular characterization of novel genes encoding enzymes for biosynthesis pathways of dietary and medicinal lignans;

(ii) the production of both endogenous and exogenous lignans by transient or stable transfection of lignan biosynthetic genes into cultured cells, tissues, and plants; (iii) the long-term stock and following reproduction of the cell functionality of a transgenic *Forsythia* lignan producing cells, U18i-CPI-Fk; and (iv) the upregulation of productivity of lignans in cells and plants by exogenous stimuli such as light and elicitors in a plant species- and lignan-specific fashion. Taken together, combination of transgenesis, light, and elicitors will be a promising strategy for further improvement of the lignan productivity. For example, elicitation of U18i-CPi-Fk under red LED light is expected to increase the amounts of sesamin and/or pinoresinol. Moreover, bioinformatic integration of the aforementioned experimental data is likely to enable the systematic prediction of optimal lignan production strategy: hosts (cells, organ cultures, plants), light conditions, elicitor types, and transfection types. For example, three *Forsythia* varieties, *F. koreana*, *F. intermedia,* and *F. suspensa*, displayed differential growth and regeneration in a medium component-dependent fashion or selection marker antibiotics-dependent fashions [89], and *Linum* spp. showed genus-specific sensitivities to different elicitors (Table 1).

Public acceptance of transgenic dietary products is not yet sufficient all over the world. Nevertheless, it should be noted that lignans produced by transgenic hosts are chemically identical to natural ones and free from any recombinant genes or proteins. Thus, public acceptance for lignans produced by transgenic plants should also be more easily garnered than that for transgenic foods themselves. In this context, we will pay more attention to the establishment of scaling-up and following industrialization of the lignan production systems as well as the development of efficient generation of transgenic plants in the near future [116–118]. Large-scale lignan production by transgenic plants must be carried out in a closed cultivation system to prevent contamination of the environment by transgenic plants. Recently, various closed plant factories have been emerging, which completely shut off a gene flow into the outer environment and enables the transgenic plants-based molecular breeding of genes or compounds of interest under optimal and sterile conditions [116–118]. Such advances in the metabolic engineering of lignan biosynthesis will surely pave the way for the conversion of conventional agricultural lignan production to innovative industrial production of various beneficial lignans and, ultimately, contribute a great deal to the improvement of quality of life and national financial burdens for medical care via extension of our healthy life expectancy owing to the preventive effects of lignans on diverse diseases.

Acknowledgments This work was, in part, supported by the Plant Factory Project of the Ministry of Economy, Technology, and Industry of Japan.

References

1. Umezawa T (2003) Diversity in lignan biosynthesis. Phytochem Rev 2:371–390
2. Suzuki S, Umezawa T (2007) Biosynthesis of lignans and norlignans. J Wood Sci 53:273–284

3. Macías FA, López A, Varela RM, Torres A, Molinillo JMG (2004) Bioactive lignans from a cultivar of *Helianthus annuus*. J Agric Food Chem 52:6443–6447
4. Lee J, Choe E (2006) Extraction of lignan compounds from roasted sesame oil and their effects on the autoxidation of methyl linoleate. J Food Sci 71:C430–C436
5. Guo H, Liu A-H, Ye M, Yang M, Guo D-A (2007) Characterization of phenolic compounds in the fruits of *Forsythia suspense* by high-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry. Rapid Commun Mass Spectrom 21:715–729
6. Peñalvo JL, Adlercreutz H, Uehara M, Ristimaki A, Watanabe S (2008) Lignan content of selected foods from Japan. J Agric Food Chem 56:401–409
7. Piao X-L, Jang M-H, Cui J, Piao X (2008) Lignans from the fruits of *Forsythia suspensa*. Bioorg Med Chem Lett 18:1980–1984
8. Hata N, Hayashi Y, Okazawa A, Ono E, Satake H, Kobayashi A (2010) Comparison of sesamin contents and CYP81Q1 gene expressions in aboveground vegetative organs between two Japanese sesame (*Sesamum indicum* L.) varieties differing in seed sesamin contents. Plant Sci 178:510–516
9. Hata N, Hayashi Y, Ono E, Satake H, Kobayashi A, Muranaka T, Okazawa A (2013) 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
10. Okazawa A, Hori K, Okumura R, Izumi Y, Hata N, Bamba T, Fukusaki E, Ono E, Satake H, Kobayashi A (2011) Simultaneous quantification of lignans in *Arabidopsis thaliana* by highly sensitive capillary liquid chromatography-electrospray ionization-ion trap mass spectrometry. Plant Biotechnol 28:287–293
11. Schmidt TJ, Klaes M, Sendker J (2012) Lignans in seeds of *Linum* species. Phytochemistry 82:89–99
12. Satake H, Ono E, Murata J (2013) Recent advances in metabolic engineering of lignan biosynthesis pathways for the production of transgenic plant-based foods and supplements. J Agric Food Chem 61:11721–11729
13. Satake H, Koyama T, Bahabadi SE, Matsumoto E, Ono E, Murata J (2015) Essences inmetabolic engineering of lignan biosynthesis. Metabolites 5:270–290
14. Kajla P, Sharma A, Sood DR (2015) Flaxseed-a potential functional food source. J Food Sci Technol 52:1857–1871
15. Goyal A, Sharma V, Upadhyay N, Gill S, Sihag M (2014) Flax and flaxseed oil: an ancient medicine & modern functional food. J Food Sci Technol 51:1633–1653
16. Chaurasia OP, Ballabh B, Tayade A, Kumar R, Kumar GP, Singh SB (2012) *Podophyllum* L.: an endangered and anticancerous medicinal plant–an overview. Indian J Tradit Knowl 11:234–241
17. Murata J, Matsumoto E, Morimoto K, Koyama T, Satake H (2015) Generation of triple-transgenic *Forsythia* cell cultures as a platform for the efficient, stable, and sustainable production of lignans. PLoS One 10:e0144519
18. Ionkova I (2007) Biotechnological approaches for the production of lignans. Phcog Rev 1:57–68
19. Ionkova I, Antonova I, Momekov G, Fuss E (2010) Production of podophyllotoxin in *Linum linearifolium* in vitro cultures. Pharmacogn Mag 6:180–185
20. Ionkova I (2011) Anticancer lignans – from discovery to biotechnology. Mini Rev Med Chem 11:843–856
21. Lata H, Mizuno CS, Moraes RM (2009) The role of biotechnology in the production of the anticancer compound podophyllotoxin. Methods Mol Biol 547:387–402
22. Malik S, Biba O, Grúz J, Arroo RRJ, Strnad M (2014) Biotechnological approaches for producing aryltetralin lignans from *Linum* species. Phytochem Rev 13:893–913
23. Lau W, Sattely ES (2015) Six enzymes from mayapple that complete the biosynthetic pathway to the etoposide aglycone. Science 349:1224–1228

24. Oliva A, Moraes RA, Watson B, Duke SO, Dayan FE (2002) Aryltertralin lignans inhibit plant growth by affecting formation of mitotic microtubular organizing centers. Pestic Biochem Phys 72:45–54
25. Harmatha J, Dinan L (2003) Biological activities of lignans and stilbenoids associated with plant-insect chemical interaction. Phytochem Rev 2:321–330
26. Schroeder FC, del Campo ML, Grant JB, Weibel DB, Smedley SR, Bolton KL, Meinwald J, Eisner T (2006) Pinoresinol: a lignol of plant origin serving for defense in a caterpillar. Proc Natl Acad Sci U S A 103:15497–15501
27. Cutillo F, D'Abrosca B, DellaGreca M, Fiorentino A, Zarrelli A (2003) Lignans and neolignans from *Brassica fruticulosa*: effects on seed germination and plant growth. J Agric Food Chem 51:6165–6172
28. Nishiwaki H, Kumamoto M, Shuto Y, Yamauchi S (2011) Stereoselective syntheses of allstereoisomers of lariciresinol and their plant growth inhibitory activities. J Agric Food Chem 59:13089–13095
29. Carillo P, Cozzolino C, D'Abrosca B, Nacca F, DellaGreca M, Fiorentio A, Fuggi A (2011) Effects of the allelochemicals dihydrodiconiferylalcohol and lariciresinol on metabolism of *Lactuca sativa*. Open Bioact Compd J 3:18–24
30. Heinonen S, Nurmi T, Liukkonen K, Poutanen K, Wähälä K, Deyama T, Nishibe S, Adlercreutz H (2001) In vitro metabolism of plant lignans: new precursors of mammalian lignans enterolactone and enterodiol. J Agric Food Chem 49:3178–3186
31. Lampe JW, Atkinson C, Hullar MA (2006) Assessing exposure to lignans and their metabolites in humans. J AOAC Int 89:1174–1181
32. Liu Z, Saarinen NM, Thompson LU (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
33. Mueller SO, Simon S, Chae K, Metzler M, Korach KS (2004) Phytoestrogens and their human metabolites show distinct agonistic and antagonistic properties on estrogenreceptor alpha (ERα) and ERβ in human cells. Toxicol Sci 80:14–25
34. Penttinen P, Jaehrling J, Damdimopoulos AE, Inzunza J, Lemmen JG, van der Saag P, Pettersson K, Gauglitz G, Mäkelä S, Pongratz I (2007) Diet-derived polyphenol metabolite enterolactone is a tissue-specific estrogen receptor activator. Endocrinology 148:4875–4886
35. During A, Debouche C, Raas T, Larondelle Y (2012) Among plant lignans, pinoresinolhas the strongest antiinflammatory properties in human intestinal Caco-2 cells. J Nutr 142:1798–1805
36. Adlercreutz H (2007) Lignans and human health. CRC Crit Rev Clin Lab Sci 44:483–525
37. Bergman Jungeström M, Thompson LU, Dabrosin C (2007) Flaxseed and its lignansinhibit estradiol-induced growth, angiogenesis, and secretion of vascular endothelial growth factor in human breast cancer xenografts in vivo. Clin Cancer Res 13:1061–1067
38. Power KA, Saarinen NM, Chen JM, Thompson LU (2006) Mammalian lignans enterolactone and enterodiol, alone and in combination with the isoflavone genistein, do not promote the growth of MCF-7 xenografts in ovariectomized athymic nude mice. Int J Cancer 118:1316–1320
39. Mense SM, Hei TK, Ganju RK, Bhat HK (2008) Phytoestrogens and breast cancer prevention: possible mechanisms of action. Environ Health Perspect 116:426–433
40. Saarinen NM, Wärri A, Dings RPM, Airio M, Smeds AI, Mäkelä S (2008) Dietary lariciresinol attenuates mammary tumor growth and reduces blood vessel density inhuman MCF-7 breast cancer xenografts and carcinogen-induced mammary tumors in rats. Int J Cancer 123:1196–1204
41. Adolphe JL, Whiting SJ, Juurlink BHJ, Thorpe LU, Alcorn J (2010) Health effects with consumption of the flax lignan secoisolariciresinol diglucoside. Br J Nutr 103:929–938
42. Barre DE, Mizier-Barre KA, Stelmach E, Hobson J, Griscti O, Rudiuk A, Muthuthevar D (2012) Flaxseed lignan complex administration in older human type 2diabetics manages central obesity and prothrombosis-an invitation to further investigation into poly pharmacy reduction. J Nutr Metab 585170

43. Hano C, Renouard S, Molinié R, Corbin C, Barakzoy E, Doussot J, Lamblin F, Lainé E (2013) Flaxseed (*Linum usitatissimum* L.) extract as well as(+)-secoisolariciresinol diglucoside and its mammalian derivatives are potentinhibitors of α-amylase activity. Bioorg Med Chem Lett 23:3007–3012
44. Sirato-Yasumoto S, Katsuta M, Okuyama Y, Takahashi Y, Ide T (2001) Effect of sesame seeds rich in sesamin and sesamolin on fatty acid oxidation in rat liver. J Agric Food Chem 49:2647–2651
45. Nakano D, Itoh C, Takaoka M, Kiso Y, Tanaka T, Matsumura Y (2002) Antihypertensive effect of sesamin. IV. Inhibition of vascular superoxide production by sesamin. Biol Pharm Bull 25:1247–1249
46. Nakai M, Harada M, Nakahara K, Akimoto K, Shibata H, Miki W, Kiso Y (2003) Novel antioxidative metabolites in rat liver with ingested sesamin. J Agric Food Chem 51:1666–1670
47. Akimoto K, Kitagawa Y, Akamatsu T, Hirose N, Sugano M, Shimizu S, Yamada H (1993) Protective effects of sesamin against liver damage caused by alcohol or carbon tetrachloride in rodents. Ann Nutr Metab 37:218–224
48. Tada M, Ono Y, Nakai M, Harada M, Shibata H, Kiso Y, Ogata T (2013) Evaluation of antioxidative effects of sesamin on the in vivo hepatic reducing abilities by a radiofrequency ESR method. Anal Sci 29:89–94
49. Liu C-M, Zheng G-H, Ming Q-L, Cheng C, Sun J-M (2013) Sesamin protects mouse liver against nickel-induced oxidative DNA damage and apoptosis by the PI3K/Akt pathway. J Agric Food Chem 61:1146–1154
50. Saarinen NM, Wärri A, Airio M, Smeds A, Mäkelä S (2007) Role of dietary lignans inthe reduction of breast cancer risk. Mol Nutr Food Res 51:857–866
51. Velentzis LS, Cantwell MM, Cardwell C, Keshtgar MR, Leathem AJ, Woodside JV (2009) Lignans and breast cancer risk in pre- and post-menopausal women: meta-analysesof observational studies. Br J Cancer 100:1492–1498
52. Velentzis LS, Keshtgar MR, Woodside JV, Leathem AJ, Titcomb A, Perkins KA, Mazurowska M, Anderson V, Wardell K, Cantwell MM (2011) Significant changes in dietary intake and supplement use after breast cancer diagnosis in a UK multicentre study. Breast Cancer Res Treat 128:473–482
53. Buck K, Zaineddin AK, Vrieling A, Linseisen J, Chang-Claude J (2010) Meta-analysesof lignans and enterolignans in relation to breast cancer risk. Am J Clin Nutr 92:141–153
54. Buck K, Zaineddin AK, Vrieling A, Heinz J, Linseisen J, Flesch-Janys D, Chang-Claude J (2011) Estimated enterolignans, lignan-rich foods, and fibre in relationto survival after postmenopausal breast cancer. Br J Cancer 105:1151–1157
55. Zaineddin AK, Buck K, Vrieling A, Heinz J, Flesch-Janys D, Linseisen J, Chang-Claude J (2012) The association between dietary lignans, phytoestrogen-rich foods, and fiber intake and postmenopausal breast cancer risk: a German case–control study. Nutr Cancer 64:652–665
56. Buck K, Vrieling A, Zaineddin AK, Becker S, Hüsing A, Kaaks R, Linseisen J, Flesch-Janys D, Chang-Claude J (2011) Serum enterolactone and prognosis of postmenopausal breast cancer. J Clin Oncol 29:3730–3738
57. Chen JM, Saggar JK, Corey P, Thompson LU (2009) Flaxseed and pure secoisolariciresinol diglucoside, but not flaxseed hull, reduce human breast tumor growth (MCF-7) in athymic mice. J Nutr 139:2061–2066
58. Truan JS, Chen JM, Thompson LU (2012) Comparative effects of sesame seed lignan and flaxseed lignan in reducing the growth of human breast tumors (MCF-7) at high levels of circulating estrogen in athymic mice. Nutr Cancer 64:65–71
59. Yousefzadi M, Sharifi M, Behmanesh M, Moyano E, Bonfill M, Cusido RM, Palazon J (2010) Podophyllotoxin: current approaches to its biotechnological production and future challenges. Eng Life Sci 10:281–292
60. Davin LB, Lewis NG (2003) A historical perspective on lignan biosynthesis: monolignol, allylphenol and hydroxycinnamic acid coupling and downstream metabolism. Phytochem Rev 2:257–288

61. Ono E, Nakai M, Fukui Y, Tomimori N, Fukuchi-Mizutani M, Saito M, Satake H, Tanaka T, Katsuta M, Umezawa T, Tanaka Y (2006) Formation of two methylenedioxy bridges by a *Sesamum* CYP81Q protein yielding a furofuran lignan, (+)-sesamin. Proc Natl Acad Sci U S A 103:10116–10121
62. Noguchi A, Fukui Y, Iuchi-Okada A, Kakutani S, Satake H, Iwashita T, Nakao M, Umezawa T, Ono E (2008) Sequential glucosylation of a furofuran lignan, (+)-sesaminol, by *Sesamum indicum* UGT71A9 and UGT94D1. Plant J 54:415–427
63. Marques JV, Kim K-W, Lee C, Costa MA, May GD, Crow JA, Davin LB, Lewis NG (2013) Next generation sequencing in predicting gene function in podophyllotoxin biosynthesis. J Biol Chem 288:466–479
64. Marques JV, Dalisay DS, Yang H, Lee C, Davin LB, Lewis NG (2014) A multi-omics strategy resolves the elusive nature of alkaloids in *Podophyllum* species. Mol BioSyst 10:2838–2849
65. Wang L, Yu S, Tong C, Zhao Y, Liu Y, Song C, Zhang Y, Zhang X, Wang Y, Hua W, Li D, Li D, Li F, Yu J, Xu C, Han X, Huang S, Tai S, Wang J, Xu X, Li Y, Liu S, Varshney RK, Wang J, Zhang X (2014) Genome sequencing of the high oil crop sesame provides insight into oil biosynthesis. Genome Biol 15:R39
66. Wang L, Han X, Zhang Y, Li D, Wei X, Ding X, Zhang X (2014) Deep resequencing reveals allelic variation in Sesamum indicum. BMC Plant Biol 14:225
67. Wu K, Yang M, Liu H, Tao Y, Mei J, Zhao Y (2014) Genetic analysis and molecular characterization of Chinese sesame (*Sesamum indicum* L.) cultivars using insertion-deletion (InDel) and simple sequence repeat (SSR) markers. BMC Genet 15:35
68. Babu PR, Rao KV, Reddy VD (2013) Structural organization and classification of cytochrome P450 genes in flax (*Linum usitatissimum* L.). Gene 513:156–162
69. Bhattacharyya D, Sinha R, Hazra S, Datta R, Chattopadhyay S (2013) De novotranscriptome analysis using 454 pyrosequencing of the Himalayan Mayapple, *Podophyllum hexandrum*. BMC Genomics 14:748
70. Dinkova-Kostova AT, Gang DR, Davin LB, Bedgar DL, Chu A, Lewis NG (1996) (+)-pinoresinol/(+)-lariciresinol reductase from *Forsythia intermedia*. J Biol Chem 271:29473–29482
71. Gang DR, Kasahara H, Xia Z-Q, Vander-Mijnsbrugge K, Bauw G, Boerjan W, Van Montagu M, Davin LB, Lewis NG (1999) Evolution of plant defense mechanisms. Relationships of phenylcoumaran benzylic ether reductases to pinoresinol-lariciresinol and isoflavone reductases. J Biol Chem 274:7516–7527
72. Hemmati S, Schmidt TJ, Fuss E (2007) (+)-pinoresinol/(−)-lariciresinol reductase from *Linum perenne Himmelszelt* involved in the biosynthesis of justicidin B. FEBS Lett 581:603–610
73. Bayindir Ü, Alfermann AW, Fuss E (2008) Hinokinin Biosynthesis in *Linum corymbulosum* Reichenb. Plant J 55:810–820
74. Nakatsubo T, Mizutani M, Suzuki S, Hattori T, Umezawa T (2008) Characterization of *Arabidopsis thaliana* pinoresinol reductase, a new type of enzyme involved in lignan biosynthesis. J Biol Chem 283:15550–15557
75. Wankhede DP, Biswas DK, Rajkumar S, Sinha AK (2013) Expressed sequence tags and molecular cloning and characterization of gene encoding pinoresinol/lariciresinolreductase from *Podophyllum hexandrum*. Protoplasma 250:1239–1249
76. Ono E, Kim H-J, Murata J, Morimoto K, Okazawa A, Kobayashi A, Umezawa T, Satake H (2010) Molecular and functional characterization of novel furofuran-class lignan glucosyltransferases from *Forsythia*. Plant Biotechnol 27:317–324
77. Kim H-J, Ono E, Morimoto K, Yamagaki T, Okazawa A, Kobayashi A, Satake H (2009) Metabolic engineering of lignan biosynthesis in *Forsythia* cell culture. Plant Cell Physiol 50:2200–2209
78. Morimoto K, Satake H (2013) Seasonal alteration in amounts of lignans and their glucosides and gene expression of the relevant biosynthetic enzymes in the *Forsythia suspense* leaf. Biol Pharm Bull 36:1519–1523

79. Okazawa A, Kusunose T, Ono E, Kim H-J, Satake H, Shimizu B, Mizutani M, Seki H, Muranaka T (2014) Glucosyltransferase activity of *Arabidopsis* UGT71C1 towards pinoresinol and lariciresinol. Plant Biotechnol 31:561–566. doi:10.5511/plantbiotechnology.14.0910a
80. Xia Z-Q, Costa MA, Pélissier HC, Davin LB, Lewis NG (2001) Secoisolariciresinol dehydrogenase purification, cloning, and functional expression. J Biol Chem 276:12614–12623
81. Ghose K, Selvaraj K, McCallum J, Kirby CW, Sweeney-Nixon M, Cloutier SJ, Deyholos M, Datla R, Fofana B (2014) Identification and functional characterization of a flax UDP-glycosyltransferase glucosylating secoisolariciresinol (SECO) into secoisolariciresinol monoglucoside (SMG) and diglucoside (SDG). BMC Plant Biol 14:82
82. Umezawa T, Ragamustari SK, Nakatsubo T, Wada S, Li L, Yamamura M, Sakakibara N, Hattori T, Suzuki S, Chiang VL (2013) A lignan *O*-methyltransferase catalyzing the regioselective methylation of matairesinol in *Carthamus tinctorius*. Plant Biotechnol 30:97–109
83. Ragamustari SK, Yamamura M, Ono E, Hattori T, Suzuki S, Suzuki H, Shibata D, Umezawa T (2014) Substrate-enantiomer selectivity of matairesinol *O*-methyltransferases. Plant Biotechnol 31:257–267
84. Ragamustari SK, Nakatsubo T, Hattori T, Ono E, Kitamura Y, Suzuki S, Yamamura M, Umezawa T (2013) A novel *O*-methyltransferase involved in the first methylation step of yatein biosynthesis from matairesinol in *Anthriscus sylvestris*. Plant Biotechnol 30:375–384
85. Wang Z, Hobson N, Galindo L, Zhu S, Shi D, McDill J, Yang L, Hawkins S, Neutelings G, Datla R, Lambert G, Galbraith DW, Grassa CJ, Geraldes A, Cronk QC, Cullis C, Dash PK, Kumar PA, Cloutier S, Sharpe AG, Wong GKS, Wang J, Deyholos MK (2012) The genome of flax (*Linum usitatissimum*) assembled de novo from short shotgun sequence reads. Plant J 72:461–473
86. Barvkar VT, Pardeshi VC, Kale SM, Kadoo NY, Gupta VS (2012) Phylogenomic analysis of UDP glycosyltransferase1 multigene family in *Linum usitatissimum* identified genes with varied expression patterns. BMC Genomics 2012:13,175
87. Eyberger AL, Dondapati R, Porter JR (2006) Endophyte fungal isolates from *Podophyllum peltatum* produce podophyllotoxin. J Nat Prod 69:1121–1124
88. Schmitt J, Petersen M (2002) Pinoresinol and matairesinol accumulation in a *Forsythia* x *intermedia* cell suspension culture. Plant Cell Tissue Organ Cult 68:91–98
89. Morimoto K, Ono E, Kim H-J, Okazawa A, Kobayashi A, Satake H (2011) The construction of transgenic *Forsythia* plants: comparative study of three *Forsythia* species. Plant Biotechnol 28:273–280
90. Renouard S, Tribalatc M-A, Lamblin F, Mongelard G, Fliniaux O, Corbin C, Marosevic D, Pilard S, Demailly H, Gutierrez L, Hano C, Mesnard F, Lainé E (2014) RNAi-mediated pinoresinol lariciresinol reductase gene silencing in flax (*Linum usitatissimum* L.) seed coat: consequences on lignans and neolignans accumulation. J Plant Physiol 171:1372–1377
91. Rosati C, Cadic A, Renou J-P, Duron M (1996) Regeneration and agrobacterium-mediated transformation of *Forsythia* x *intermedia* "Spring Glowly". Plant Cell Rep 16:114–117
92. Rosati C, Simoneau P, Treutter D, Poupard P, Cadot Y, Cadic A, Duron M (2003) Engineering of flower color in forsythia by expression of two independently-transformed dihydroflavonol 4-reductase and anthocyanidin synthase genes of flavonoid pathway. Mol Breed 12:197–208
93. Murase K, Sugai Y, Hayashi S, Suzuki Y, Tsuji K, Takayama S (2015) Generation of transgenic *Linum perenne* by *Agrobacterium* mediated transformation. Plant Biotechnol 32:349–352
94. Morimoto K, Kim H-J, Ono E, Kobayashi A, Okazawa A, Satake H (2011) Effects of light on production of endogenous and exogenous lignans by *Forsythia koreana* wildtype and transgenic cells. Plant Biotechnol 28:331–337
95. Yousefzadi M, Sharifi M, Behmanesh M, Ghasempour A, Moyano E, Palazon J (2012) The effect of light on gene expression and podophyllotoxin biosynthesis in *Linum album* cell culture. Plant Physiol Biochem 56:41–56

96. Hata N, Hayashi Y, Okazawa A, Ono E, Satake H, Kobayashi A (2012) Effect of photoperiod on growth of the plants, and sesamin content and CYP81Q1 gene expression in the leaves of sesame (*Sesamum indicum* L.). Environ Exp Bot 75:212–219
97. Kobayashi T, Niino T, Kobayashi M (2005) Simple cryopreservation protocol with an encapsulation technique for tobacco BY-2 suspension cell cultures. Plant Biotechnol 22:105–112. doi:10.5511/plantbiotechnology.22.105
98. Ogawa Y, Sakurai N, Oikawa A, Kai K, Morishita Y, Mori K et al (2011) High-throughput cryopreservation of plant cell cultures for functional genomics. Plant Cell Physiol 53:943–952. doi:10.1093/pcp/pcs038, PMID: 22437846
99. Sarasan V, Cripps R, Ramsay MM, Atherton C, Mcmichen M, Prendergast G (2006) Conservation *in vitro* of threatened plants – progress in the past decade. In Vitro Cell Dev Biol Plant 42:206–214. doi:10.1079/IVP2006769
100. Chen Q, Lai H, Hurtado J, Stahnke J, Leuzinger K, Dent M (2013) Agroinfiltration as an effective and scalable strategy of gene delivery for production of pharmaceutical proteins. Adv Tech Biol Med 1:103
101. Zhao J, Davis LC, Verpoorte R (2005) Elicitor signal transduction leading to production of plant secondary metabolites. Biotechnol Adv 23:283–333
102. Berim A, Spring O, Conrad J, Maitrejean M, Boland W, Petersen M (2005) Enhancement of lignan biosynthesis in suspension cultures of *Linum nodiflorum* by coronalon, indanoyl-isoleucine and methyl jasmonate. Planta 222:769–776
103. Van Fürden B, Humburg A, Fuss E (2005) Influence of methyl jasmonate on podophyllotoxin and 6-methoxypodophyllotoxin accumulation in *Linum album* cell suspension cultures. Plant Cell Rep 24:312–317
104. Yousefzadi M, Sharifi M, Behmanesh M, Ghasempour A, Moyano E, Palazon J (2010) Salicylic acid improves podophyllotoxin production in cell cultures of *Linum album* by increasing the expression of genes related with its biosynthesis. Biotechnol Lett 32:1739–1743
105. Bhattacharyya D, Sinha R, Ghanta S, Chakraborty A, Hazra S, Chattopadhyay S (2012) Proteins differentially expressed in elicited cell suspension culture of *Podophyllum hexandrum* with enhanced podophyllotoxin content. Proteome Sci 10:34
106. Vardapetyan HR, Kirakosyan AB, Oganesyan AA, Penesyan AR, Alfermann WA (2003) Effect of various elicitors onlignan biosynthesis in callus cultures of *Linum austriacum*. Russ J Plant Physl 50:297–300
107. Ionkova I (2009) Effect of methyl jasmonate on production of ariltetralin lignans in hairy root cultures of *Linum tauricum*. Pharmacogn Res 1:102–105
108. Schmitt J, Petersen M (2002) Influence of methyl jasmonate and coniferyl alcohol on pinoresinol and matairesinol accumulation in a *Forsythia* × *intermedia* suspension culture. Plant Cell Rep 20:885–890
109. Muranaka T, Miyata M, Ito K, Tachibana S (1998) Production of podophyllotoxin in *Juniperus chinensis* callus cultures treated with oligosaccharides and a biogenetic precursor. Phytochemistry 49:491–496
110. Bahabadi SE, Sharifi M, Safaie N, Murata J, Yamagaki T, Satake H (2011) Increased lignan biosynthesis in the suspension cultures of *Linum album* by fungal extracts. Plant Biotechnol Rep 5:367–373
111. Bahabadi SE, Sharifi M, Chashmi NA, Murata J, Satake H (2014) Significant enhancement of lignans accumulation in hairy root cultures of *Linum album* using biotic elicitors. Acta Physiol Plant 36:3325–3331
112. Bahabadi SE, Sharifi M, Murata J, Satake H (2014) The effect of chitosan and chitin oligomers on gene expression and lignan production in *Linum album* cell cultures. J Med Plant 13:46–53
113. Hano C, Addi M, Bensaddek L, Crônier D, Baltora-Rosset S, Doussot J, Maury S, Mesnard B, Chabbert B, Hawkins S, Lainé E, Lamblin F (2006) Differential accumulation of monolignol-drived compounds in elicited flax (*Linum usitatissimum*) cell suspension cultures. Planta 223:975–989

114. Bahabadi SE, Sharifi M, Behmanesh M, Safaie N, Murata J, Araki R, Yamagaki T, Satake H (2012) 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
115. Tahsili J, Sharifi M, Safaie N, Bahabadi SE, Behmanesh M (2014) Induction of lignans and phenolic compounds in cell culture of Linum album by culture filtrate of *Fusarium graminearum*. J Plant Interact 9:412–417
116. Chun C, Kozai T (2001) A closed transplant production system, a hybrid of scaled-up micropropagation system and plant factory. J Plant Biotechnol 3:59–66
117. Hirai T, Fukukawa G, Kakuta H, Fukuda N, Ezura H (2010) Production of recombinant miraculin using transgenic tomatoes in a closed cultivation system. J Agric Food Chem 58:6096–6101
118. Kato K, Yoshida R, Kikuzaki A, Hirai T, Kuroda H, Hiwasa-Tanase K, Takane K, Ezura H, Mizoguchi T (2010) Molecular breeding of tomato lines for mass production of miraculin in a plant factory. J Agric Food Chem 58:9505–9510