Technical Note

Effects of light on production of endogenous and exogenous lignans by *Forsythia koreana* wildtype and transgenic cells

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Abstract   In a previous study, we reported the production of the exogenous lignan, sesamin, using the *Forsythia koreana* transgenic cells (CPi-Fk cells) in which an exogenous sesamin-synthase CYP81Q1 is stably expressed while an endogenous pinoresinol-lariciresinol reductase is suppressed by RNA interference. Here, we present the effects of light on the production of sesamin and an endogenous lignan pinoresinol which is a precursor of sesamin in CPi-Fk cells. CPi-Fk cells showed a 2.3-fold, 2.7-fold, or 1.6-fold increase in sesamin production after two-week irradiation with white fluorescent, blue LED, or red LED light, respectively, compared with the level obtained under the dark condition. Likewise, CPi-Fk cells showed an approximately 1.5 to 3.0-fold increase in pinoresinol (aglycone and glucosides) production. Furthermore, expression of the pinoresinol-glucosylating enzyme UGT71A18 was suppressed in CPi-Fk cells under blue or red light. Considering that white fluorescent light contains the blue wavelength and that CYP81Q1 fails to convert pinoresinol glucosides to sesamin, it is concluded that blue light plays a major role in the up-regulation of the production of sesamin by CPi-Fk via an enhancement of the production of pinoresinol aglycone and a reduction of UGT71A18. This is the first report on the elevation of lignan biosynthesis by light.

Key words: *Forsythia koreana*, light, lignan, transgenic cell line.

Lignans are naturally occurring phenylpropanoid dimers (C6-C3 units; e.g., coniferyl alcohol), in which the phenylpropane units are linked by the central carbons of the side chains (Ayres and Loike 1990; Hearon and MacGregor 1995). These secondary plant metabolites are commonly included in the human diet (Milder et al. 2005; Peñalvo et al. 2008; Umezawa 2003). Plant lignans are thought to play various important physiological and/or ecological roles in the interaction of plants with insects (Harmatha and Dinan 2003; Schroeder et al. 2006). In the human digestive tract, plant lignans are metabolized by the intestinal microflora into the phytoestrogen enterodiol and enterolactone (Heinonen et al. 2001; Saarinen et al. 2007). Phytoestrogens are believed to reduce the risk of several types of cancer and cardiovascular disease (Heinonen et al. 2001; Saarinen et al. 2007).

Sesamin, classified as a furofuran lignan, is the most abundant water-insoluble lignan in sesame seeds, and is biosynthesized through the formation of two methyl-enedioxy bridges (MDBs) of a precursor lignan, pinoresinol (Figure 1), by the sesame cytochrome P450, CYP81Q1 (Liu et al. 2006; Lee and Choe 2006; Ono et al. 2006; Umezawa 2003). Sesamin exerts an anti-oxidative activity through the metabolism of the MDB moieties, and an anti-hypertensive effect by inhibition of vascular superoxide production (Nakai et al. 2003; Nakano et al. 2002). Moreover, sesamin has been shown to lessen the liver damage caused by ethanol and lipid oxidation (Akimoto et al. 1993; Sirato-Yasumoto et al. 2001). These beneficial effects on human health suggest that there will be a significant increase in demand for sesamin in the near future. However, the acquisition of sesamin depends on its extraction from sesame seed oil, and sesamin occupies at most 0.4–0.6% (w/w) of sesame seed oil, which is known to produce the most abundant sesamin of all plants (Umezawa 2003).

The engineering of various plant secondary metabolites, such as alkaloids and flavonoids, resulted in increased amounts of target compounds or the generation
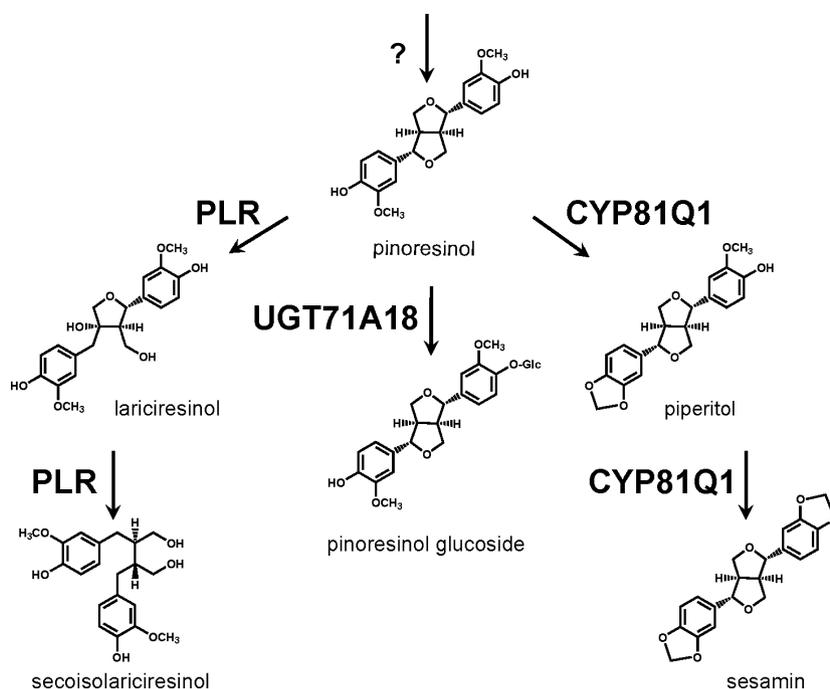

Figure 1.   Major lignan biosynthetic pathways in *Forsythia koreana* (Fk). PLR and UGT71A18 are endogenous enzymes of Fk. Pinoresinol is converted into pinoresinol glucoside by UGT71A18 or into secoisolariciresinol by PLR. Secoisolariciresinol is further converted into matairesinol. CYP81Q1 is an exogenous enzyme responsible for the conversion of pinoresinol into sesamin in CPi-Fk cells but not in the Fk wildtype. PLR is suppressed by RNA interference in CPi-Fk cells. Consequently, CPi-Fk cells have the ability to produce pinoresinol and sesamin and abolish the biosynthetic pathway for larisiresinol, secoisolarisiresinol and matairesinol. In addition, a pinoresinol synthase has not so far been identified in plants.

of exogenous compounds with improved pharmacological properties both in plants and in cell cultures (Allen et al. 2004; Ayella et al. 2007; Badejo et al. 2009; Butelli et al. 2008; Leonard et al. 2009; Ogita et al. 2003). In the previous study, we stably introduced CYP81Q1 and an RNA interference (RNAi) sequence against an Fk endogenous lignan-biosynthetic enzyme, pinoresinol/lariciresinol reductase (PLR), into *Forsythia koreana* (Fk) cultured cells (Kim et al. 2009). The Fk transgenic cells, designated CPi-Fk cells, were shown to accumulate approximately 1.5-fold pinoresinol in its glucosides in comparison with the non-transformant, and to produce the exogenous lignan, sesamin, whereas the Fk wildtype cells lacked the capacity to biosynthesize sesamin (Figure 1) (Kim et al. 2009; Ono et al. 2006; Umezawa 2003). This is the first establishment of the transgenic production of an exogenous lignan in a plant species, indicating that CPi-Fk cells are promising platforms for efficient production of sesamin.

There have been several reports on the functional correlation between types of light and production of secondary metabolites. White light stimulation of an *Arabidopsis icx1* mutant line (increased chalcone synthase expression) led to a marked accumulation of anthocyanin compared with that in the wildtype (Bharti and Khurana, 1997; Jackson et al. 1995). Moreover, a large increase in anthocyanin accumulation was observed when the mutant plants grown at a low fluorescence rate of white light ($25\,mmol\,m^{-2}\,s^{-1}$) were cultivated at a higher fluorescence rate ($175\,mmol\,m^{-2}\,s^{-1}$) (Jackson et al. 1995). The increase in chlorophyll content by continuous red, blue or white light resulted in elevated carotenoid levels in *Arabidopsis* (von Lintig et al. 1997). On the other hand, blue light completely inhibited shikonin production in *Lithospermum* (Yazaki et al. 1999). These findings suggest that the production of sesamin by CPi-Fk cells is up- or down-regulated by certain types of light, given that CPi-Fk cells produce sesamin under the dark condition (Kim et al. 2009). In this study, we show the effects of light on production of an endogenous lignan (pinoresinol) and exogenous lignan (sesamin) by CPi-Fk cells under white fluorescent, blue LED, and red LED light.

Wildtype Fk cells and CPi-Fk cells were maintained and proliferated as previously reported (Kim et al. 2009). For light treatment, cell cultures were grown in the same medium for two weeks under continuous irradiation of no, red LED (450–550 nm, 470 nm peak), blue LED (600–700 nm, 630 peak), or white light (white fluorescent tubes) at $100\,\mu mol\,m^{-2}\,s^{-1}$ PPFD (photosynthetic photon flux density). Both Fk wild type and CPi-Fk cells were grown under the dark condition for two weeks, and then were transferred to the new B5 liquid medium followed by 2-week culturing under no,
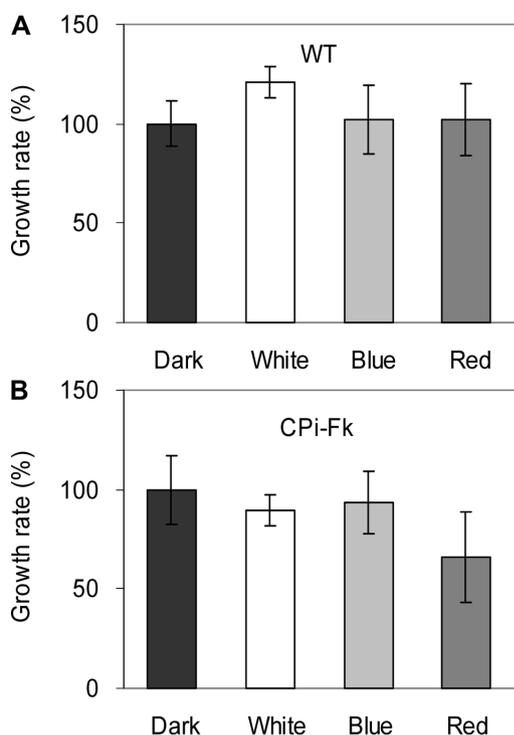
Figure 2 Cell growth of the wildtype (A) and CPi-Fk cells (B) after two weeks under the dark, white, blue and red continuous light conditions. Each point represents the mean±S.E.M. of three preparations.

Figure 3 Gene expression of PLR, UGT71A18, ribosome RNA, and CYP81Q1 in the Fk wildtype (A) and CPi-Fk cells (B) in the presence or absence of light.

white, blue or red light. Both of the suspended cell cultures had a grayish white appearance under the dark condition. No light types exhibited a significant effect on the growth of the wildtype (Figure 2A). CPi-Fk cells under red light showed relatively low growth, compared with the cell growth under dark conditions (Figure 2B) and had a brownish appearance (supplemental data). In contrast, CPi-Fk cells under white light or blue light grew to a similar extent, compared with those under the dark condition (Figure 2B).

To examine the effects of light on gene expression of *PLR* and a pinoresinol-glucosylating enzyme gene, *UGT71A18* (Ono et al. 2010), reverse transcription polymerase chain reaction (RT-PCR) was performed as previously reported (Kim et al. 2009; Ono et al. 2010). In Fk wildtype cells, no gene expression was affected by any light types, with the exception that *PLR* expression was slightly lower under blue light (Figure 3A). RT-PCR analysis confirmed a high expression of the exogenous *CYP81Q1* and RNAi-directed suppression of the endogenous *PLR* in CPi-Fk cells under all of the light conditions (Figure 3B). Notably, the gene expression of *UGT71A18* was down-regulated in CPi-Fk cells under blue or red light (Figure 3B), whereas such an effect was not detected in the wildtype cells (Figure 3A).

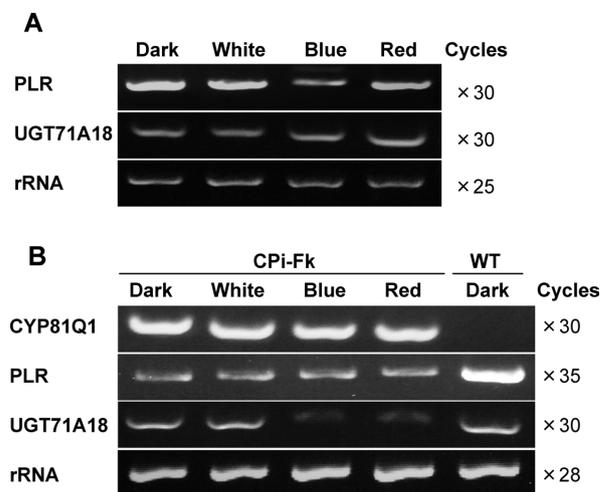In the previous study, we verified that Fk wildtype cells produced pinoresinol and matairesinol as major endogenous lignans, while CPi-Fk cells under the dark condition had enhanced the production of pinoresinol and sesamin but almost completely lost the ability to produce matairesinol due to the over expression of *CYP81Q1* and *PLR*-RNAi (Kim et al. 2009). In the present study, we also evaluated the effects of light on production of pinoresinol and sesamin in the wildtype and CPi-Fk cells using reverse-phase high-performance liquid chromatography (HPLC) as previously reported (Kim et al. 2009; Ono et al. 2010). The total amounts of pinoresinol (pinoresinol aglycone plus glucosides) were calculated using the data from the HPLC analysis of lignan extracts treated with β-glucosidase, and pinoresinol aglycone and sesamin level was assessed using the data from the HPLC analysis of the untreated extract (Kim et al. 2009; Schmitt and Petersen 2002). Abundant pinoresinol was detected in the lignan extract of the cells treated with β-glucosidase compared with the untreated extracts (Table 1, Figures 4, 5), demonstrating that a large portion of pinoresinol in its glucosylated form accumulated in both the wildtype and CPi-Fk cells. In the wildtype cells under the dark condition, the pinoresinol aglycone and total pinoresinol were $0.2 \, \mathrm{mg \, g^{-1}}$ and $0.7 \, \mathrm{mg \, g^{-1}}$ of the dry weight (DW) of the cells (Table 1, Figure 4). These data are consistent with our previous study (Kim et al. 2009). The white fluorescent light was found to cause a 2.2-fold increase in pinoresinol aglycone production and 5.2-fold increase in total pinoresinol production, compared with the levels of production under the dark condition (Table 1, Figure 4). Blue or red light irradiation exhibited no effect on the production of the pinoresinol aglycone, but induced an approximately 2-fold increase in total pinoresinol production (Table 1, Figure 4). It is noteworthy that such non-proportional increase in pinoresinol aglycone and total pinoresinol led to a reduction of the relative ratio of pinoresinol aglycone to total pinoresinol (Table 1).

0.3 mg g^{-1} DW pinoresinol aglycone and 1.2 mg g^{-1} DW total pinoresinol were detected in CPi-Fk cells under the dark condition (Figure 5A, Table 1). Intriguingly, all of the light types were found to up-regulate the production of pinoresinol aglycone and its glucosylated form. As depicted in Figure 5A, B, and Table 1, CPi-Fk cells under the white light showed an approximately 3-fold (1.0 mg g^{-1} DW) increase in production of pinoresinol aglycone and 1.8-fold (3.4 mg g^{-1} DW) increase in production of total pinoresinol, compared with CPi-Fk cells under the dark condition. Likewise, an approximately 2.3-fold (0.7 mg g^{-1} DW) increase in pinoresinol aglycone production and 1.5-fold (1.8
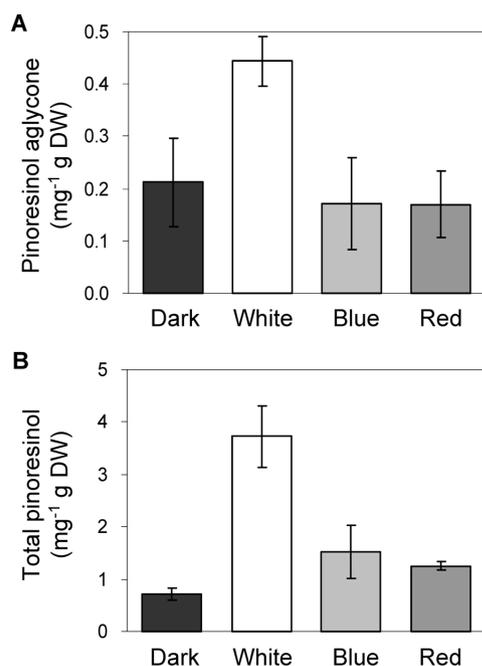

Figure 4 Comparison of the lignan content in Fk wildtype cells under the dark, white, blue, and red continuous light conditions. (A) pinoresinol aglycone. (B) total pinoresinol (aglycone and glucosides). Lignans were determined separately for each sample and then presented as the average values of three independent experiments after a culture period of two weeks in the same medium. Each point represents the mean±S.E.M. of three preparations.

mg g^{-1} DW) increase in total pinoresinol production were obtained in CPi-Fk cells under the blue light, compared with the same cells under the dark condition (Table 1, Figure 5A, B). The red light also exhibited a near-equivalent increase in accumulation of pinoresinol aglycone (2.3-fold) and total pinoresinol (1.9-fold). These results revealed that the white, blue, and red light exerted positive effects on the production of pinoresinol aglycone and its glucosides by CPi-Fk cells.

The Fk wildtype reportedly produces no sesamin (Ono et al. 2006; Umezawa 2003), supporting the result that no sesamin was detected in the lignan extract from the wildtype cells (Table 1, Figure 5C). In contrast, sesamin (1.02 mg g^{-1} DW) was detected in the CPi-Fk cell extracts under the dark condition. A striking feature is that sesamin production was also up-regulated by light. CPi-Fk cells under the white, blue, or red light showed an approximately 2.3-fold (2.32 mg g^{-1} DW), 2.6-fold (2.65 mg g^{-1} DW), or 1.6-fold (1.6 mg g^{-1} DW) increase in sesamin production, respectively, compared with CPi-Fk cells under the dark condition (Table 1, Figure 5C). Taken together, these quantitative analyses provide evidence that light up-regulates the production of the exogenous lignan sesamin as well as of that of the endogenous lignan pinoresinol by CPi-Fk cells. To our knowledge, this is the first report on the upregulation of both endogenous and exogenous lignans by light in a plant.

Increase of both pinoresinol and sesamin (Table 1, Figure 5) indicates that the elevation of the sesamin production resulted mainly from the up-regulation of the pinoresinol aglycone by light. It is most likely that pinoresinol synthase is up-regulated by light. Unfortunately, however, pinoresinol synthase has yet to be identified in plants. Elucidation of the molecular mechanism underlying the enhancement of pinoresinol and sesamin production awaits further investigation. Furthermore, different increase in total pinoresinol production in CPi-Fk cells is induced between white, blue, or red light (Table 1, Figure 5B). These results demonstrate that white light exhibits a more potent effect

Table 1 Lignan content in Fk wildtype and CPi-Fk cells

| Cell strain | Light | Pinoresinol (mg g^{-1} DW) | | | Sesamin (mg g^{-1} DW) |
		Aglycone	Total	Percent (%) Aglycone/Total	
Wild type	Dark	0.2 ± 0.08	0.7 ± 0.11	29 ± 6.6	ND
	White	0.4 ± 0.27	3.7 ± 0.60	10 ± 5.8	ND
	Blue	0.2 ± 0.09	1.5 ± 0.51	11 ± 5.2	ND
	Red	0.2 ± 0.06	1.3 ± 0.08	11 ± 3.1	ND
CPi-Fk	Dark	0.3 ± 0.09	1.2 ± 0.06	23 ± 5.1	1.0 ± 0.2
	White	1.0 ± 0.16	3.4 ± 0.45	30 ± 3.3	2.3 ± 0.2
	Blue	0.7 ± 0.05	1.8 ± 0.42	40 ± 7.7	2.7 ± 0.5
	Red	0.7 ± 0.15	2.2 ± 0.49	33 ± 1.4	1.6 ± 0.3

'Total' indicates the total amount of the aglycone and glucosylated forms of pinoresinol.
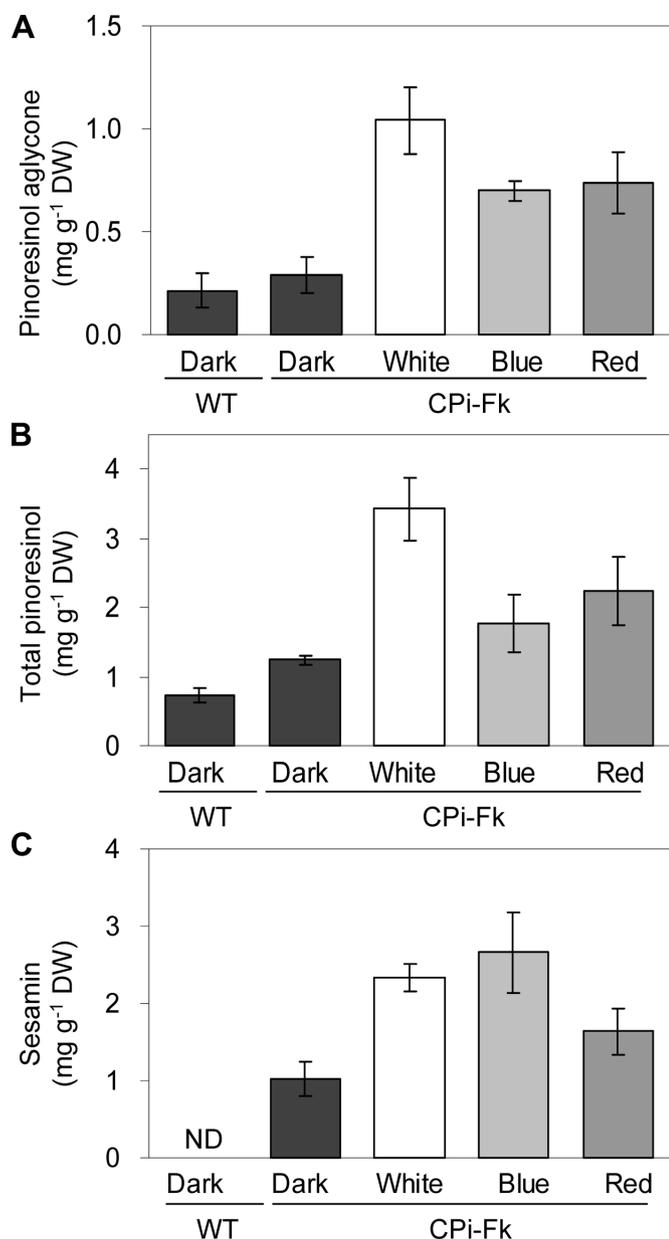
Figure 5 Comparison of the lignan content in CPi-Fk cells under the dark, white, blue, and red continuous light conditions. (A) pinoresinol aglycone. (B) total pinoresinol (aglycone and glucosides). (C) sesamin. Lignans were determined separately for each sample and then presented as the average values of three independent experiments after a culture period of two weeks in the same medium. Each point represents the mean±S.E.M. of three preparations.

pinoresinol as a substrate (Ono et al. 2006; Ono et al. 2010), and CYP81Q1 cannot convert pinoresinol glucosides into sesamin (Ono et al. 2006). These findings are compatible with the view that higher ratio of pinoresinol aglycone/total pinoresinol leads to more efficient production of sesamin. Moreover, the ratio of pinoresinol aglycone/total pinoresinol in CPi-Fk cells under blue light was 40% (Table 1), which is higher than that in CPi-Fk cells under the dark condition (23%) or white light (30%) (Table 1). Altogether, the reduction of the gene expression of UGT71A18 by blue light (Figure 3B) is also expected to potentiate sesamin production by CPi-Fk cells. Intriguingly, red light also reduced *UGT71A18* expression (Figure 3B), whereas the ratio of pinoresinol aglycone/total pinoresinol (30%) was lower than that under blue light (40%), and less sesamin was produced than that in CPi-Fk cells under white or blue light (Table 1, Figure 5C). These results demonstrate that the reduction of the *UGT71A18* expression under the red light failed to lead to the level of elevation of the pinoresinol aglycone/total pinoresinol and enhancement of the sesamin production in CPi-Fk cells that was observed under the blue light, and thus suggest light types-specific activation pathways for lignan synthesis in Fk cells. For instance, other lignan biosynthetic pathways involving pinoresinol may be triggered exclusively by red light. Alternatively, red light may induce degradation of pinoresinol aglycone, which is suppressed by other types of light. Several enzymes for biosynthesis of secondary metabolites are found to be affected by light, leading to an increase or decrease of the production of the relevant compounds. For example, the gene expression of multiple carotenoid-biosynthesis enzymes is activated by white light or red light irradiation in *Sinapis alba* and *Arabidopsis thaliana* (von Lintig et al. 1997). In *Glycine max*, various flavonoid-biosynthetic enzymes for precursors of anthocyanin are up-regulated by white fluorescent light (Suita et al. 2007). These findings suggest that the expression of biosynthetic enzymes for pinoresinol and/or its precursors is also enhanced by blue light. In addition, white fluorescent light includes the wavelengths of blue and red light. Collectively, we conclude that white fluorescent, blue, and red light up-regulate the production of sesamin via an increase in the substrate of CYP81Q1, pinoresinol, and the reduction of *UGT71A18* expression also contributes to the enhanced production of sesamin under blue light.

Also of interest are the different effects of light on pinoresinol biosynthesis and glucosylation between the wildtype and CPi-Fk cells. The expression of *UGT71A18* was reduced in CPi-Fk cells (Figure 3B) under blue or red light, but not in the wildtype under the same condition (Figure 3A). Moreover, the production of the pinoresinol aglycone was markedly enhanced in CPi-Fk

on the total pinoresinol production than blue or red light, whereas sesamin is more efficiently produced under blue light than under white or red light. In other words, the additional pinoresinol is not proportionately supplied to the production of sesamin in CPi-Fk cells under light irradiation. Such a difference in the utility of the additional pinoresinol is highly likely to be attributable to light-sensitive alteration in the metabolic pathways of pinoresinol aglycone and/or its glucosides. It is noteworthy that CYP81Q1 and UGT71A18 share

cells under blue or red light (Figure 5A), but not in the wildtype (Figure 4A). These data suggest that light-sensitive biosynthetic pathways are altered in CPi-Fk cells compared with the wildtype. In particular, the production of Fk endogenous lignans, lariciresinol, secoisolariciresinol, and matairesinol, which are biosynthesized downstream of pinoresinol in Fk (Figure 1), is completely blocked via *PLR*-RNAi in CPi-Fk cells (Kim et al. 2009), implying that the absence of these lignans affects light-sensitive lignan biosynthesis. Investigation of the molecular mechanisms underlying light-sensitive lignan biosynthesis is expected to lead to the elucidation of the functional correlation between light and lignan biosynthesis and the biological significance of lignans in plants. Such studies are currently in progress.

The metabolic engineering of *in vitro* cultured cells possesses a variety of advantages for efficient production of secondary metabolites of interest, given that the growth of cultured cells is regulated under optimal conditions including temperature, medium components, and supplements. CPi-Fk cells have been conferred with the capacity to produce an exogenous lignan sesamin by a simple cultivation procedure under dark conditions without the need of any supplements (Kim et al. 2009). Moreover, the present study provides evidence that light, in particular, blue light, enhances the production of sesamin as well as pinoresinol in CPi-Fk cells. Hence, this study has paved the way for the establishment of efficient lignan production platforms by metabolic engineering of plant transgenic cells at an industrial level.

Acknowledgements

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