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# Effect of light on gene expression and shikonin formation in cultured *Onosma paniculatum* cells

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## Abstract

The cell culture in a production medium in the dark is capable of producing a large quantity of shikonin and its derivatives, which were completely inhibited when the cell cultures were irradiated with continuous white light and blue light and partially repressed under continuous red light. The expressions of *PAL1*, *4CL1*, and *CYP98A6* were induced under continuous red, blue and white light. Transcript levels of *HMGR* and *LDI2* gradually were decreased in the dark and under continuous red light, while no transcriptional product of *LDI2* was detected under blue and white light. *C4H2* and *LePGT1* genes were constantly expressed irrespective of different light conditions. Among the genes studied, the expression of *LDI2* was critical for light-regulated shikonin formation. The inducible expression of *PAL1*, *4CL1*, and *CYP98A6* as well as the inhibitory transcription of *LDI2* mediated by continuous red light irradiation were likely accounted for the reduced accumulation of shikonin.

## Introduction

The root of *Lithospermum erythrorhizon* Sieb. et Zucc. (Boraginaceae), a medicinal plant, produces red naphthoquinone compounds, shikonin and its derivatives, which have been used medicinally as antibacterial, anti-inflammatory or anti-tumor drugs and for inhibition of HIV virus, and also as colorants for cosmetics, fabrics, and foods (Tabata et al., 1974; Yazaki et al., 1999; Yoon et al., 1999; Chen et al., 2003). Cell cultures of *L. erythrorhizon* in the production medium are capable of producing a large quantity of shikonin and its derivatives (Tabata et al., 1974; Fujita et al., 1981). Various chemical, physical and biological factors, such as light, plant growth regulators, medium nutrients, fungal elicitors, play a regulatory role in shikonin biosynthesis in *L. erythrorhizon* cell cultures (Tabata et al., 1974; Kim and Chang, 1990; Gaisser and Heide, 1996; Ning et al., 1998; Brigham et al., 1999). At present, the metabolic pathway of shikonin biosynthesis is well-characterized (Heide et al., 1989; Yamaga et al., 1993; Yazaki et al., 1999). Moreover, some genes, *PAL* (Yazaki et al., 1997b), *C4H* (Yamamura et al., 2001), *4CL* (Yazaki et al., 1995), *LePGT* (Yazaki et al., 2002), *HMGR* (Lange et al., 1998), *CYP98A6* (Matsuno et al., 2002), and *LEPS-2* (Yamamura et al., 2003), encoding metabolic enzymes closely related in shikonin formation and secretion and five genes, *LDI1–LDI5* (Yazaki et al., 1997a, 1999, 2001), preferentially expressed in the dark were cloned and identified from the cultured cells. Therefore, the cell cultures of shikonin formation may become one of the promising model systems suitable for biochemical and molecular study of the regulation of plant secondary metabolism.

Plants use light not only as an energy source for photosynthesis but also as an important environmental signal. Plants can detect almost all facets of light such as direction, duration, quantity, and wavelength by using three classes of photoreceptors: the red/far-red light (600–750 nm) absorbing phytochromes, the blue/UV-A (320–500 nm) absorbing cryptochromes and phototropins, and the UV-B receptors (282–320 nm). These photoreceptors perceive, interpret, and transduce light signals, via distinct intracellular signaling pathways, to mediate a broad range of physiological responses to light in addition to plant growth and development (Briggs and Olney, 2001; Lin, 2002; Wang and Deng, 2002). It is well known that carotenoids are widespread pigments in organisms and carotenogenesis is photo-regulated in plants (Molina Grima et al., 1999; Sandmann, 2001). In most plant cell cultures, secondary metabolism, including the production of phenolic, terpenoid, and alkaloid compounds, is stimulated by light (Weisshaar and Jenkins, 1998; Hahlbrock and Scheel, 1989). For example, light promoted vindoline and serpentine biosynthesis in *Catharanthus roseus* callus cultures (Zhao et al., 2001). However, the formation of secondary metabolites – shikonin and its derivatives is strongly inhibited by white light in *L. erythrorhizon* cell cultures (Tabata et al., 1974; Heide et al., 1989; Gaisser and Heide, 1996). Furthermore, tests conducted under continuous irradiation with monochromatic light revealed that the marked inhibition of shikonin biosynthesis is caused by blue light but not by red or green light (Tabata et al., 1974; Yamamoto et al., 2002). Many plant genes, which encode proteins involved in photosynthesis or biosynthetic enzymes in secondary metabolism, have been found to change their expression patterns in response to light (Terzaghi and Cashmore, 1995; Procissi et al., 1997). A variety of genes, such as *CHS*, *CAB*, and *RBCS*, are up-regulated by light, while *PHYA*, *AS1*, and *pra2* are down-regulated by light (Yoshida et al., 1993; Terzaghi and Cashmore, 1995; Neuhaus et al., 1997). The mechanism of light-regulated gene expression has been widely investigated, several *cis*-regulatory DNA elements and their correspondingly binding transcription factors of light-regulated genes have been isolated and characterized (Terzaghi and Cashmore, 1995; Inaba et al., 2000), but the accumulated evidence has shown that the mechanism is very complex.

Although several biochemistry and molecular studies of the light regulation of shikonin biosynthesis have been reported in cultured *L. erythrorhizon* cell (Gaisser and Heide, 1996; Yazaki et al., 1997a, 1999, 2001), the molecular mechanism of the inhibition of the secondary metabolism in the light is an open question, especially in cultured *Onosma paniculatum* cells (another species for shikonin and its derivatives) (Ning and Cao, 1995). In the present study, the seven cDNA clones (*PAL*, *C4H*, *4CL*, *HMGR*, *LePGT*, and *LDI2*) isolated from cultured *L. erythrorhizon* cell, closely related to shikonin biosynthesis were selected to study the expression patterns of genes and inhibitory regulation of shikonin formation in response to white, red, and blue light in *Onosma paniculatum* cell cultures.

## Materials and methods

### *Plant materials and cell culture conditions*

The callus used is a somatic line YN12, derived from young shoots of *Onosma paniculatum* (Yang et al., 2003). The two-stage culture system was used, including a growth stage for cell proliferation, in which the callus was maintained in a $B_5$ medium at 25 °C in light (80 μmol $m^{-2}$ $s^{-2}$ 8 h/day) and the subculture was carried out every 16–18 days, and a production stage for the formation of shikonin and its derivatives in $M_9$ liquid medium (Fujita et al., 1981; Ning and Cao, 1995). IAA at 0.05 mg $l^{-1}$ and BA at 1 mg $l^{-1}$ were added to B5 medium as a basic growth regulator combination, while IAA at 0.1 mg $l^{-1}$ and BA at 1 mg $l^{-1}$ were added to $M_9$ production medium (Ning and Cao, 1995; Yang et al., 1999, 2003). To produce shikonin, about 2 g of callus produced during the growth stage in $B_5$ medium was inoculated in a 250 ml Erlenmayer flask containing 50 ml of $M_9$ medium and cultured on a rotary shaker at 120 rpm at 25 ± 1 °C in the dark. The calluses were harvested after 18–20 days to measure shikonin production (Yang et al., 1999).

### *Light treatments*

The *Onosma paniculatum* cell cultures were treated with complete darkness, continuous white light, continuous red light, and continuous blue light, respectively, after inoculating in $M_9$ production

medium. For continuous white light irradiation, white light fluorescent tubes (TLD36W/54, Philips) were used at an intensity of 25 W $m^{-2}$. The Philips TLD36W/15 (600–700 nm in wavelength, $\lambda_{max}$ = 660 nm) and TLD36W/18 (380–560 nm in wavelength, $\lambda_{max}$ = 470 nm) were used for red and blue light, respectively, at a light intensity of 80 μmol $m^{-2}$ $s^{-1}$. The calluses in production medium treated with different light and darkness were harvested after 18–20 days to measure shikonin production. All treatments were performed twice with five replicates each. In addition, *Onosma paniculatum* cells cultured in $M_9$ treated with different light conditions and darkness mentioned above for 2, 4, 8, and 12 days, respectively, after inoculation, were harvested by filtration, frozen immediately in liquid nitrogen, and stored at −80 °C for RNA extraction.

*Measurement of cell growth, shikonin and its derivatives content*

The cells were weighed at the beginning and end of the culture. The cell increase ratio based on fresh weight determination (CIRFW) was defined as the difference between the end and beginning weights divided by the beginning weight.

The shikonin and its derivative in the cells were determined as described by Heide and Tabata (1987) with a slight modification. The shikonin was extracted from fresh cells with petrol ether, and its absorption was measured at 520 nm. The amount of shikonin and its derivatives was finally reported as mg $g^{-1}$ FW (fresh weight) cells, according to the standard curve.

*Total RNA extraction and analyses of mRNA levels of gene expression by RT-PCR*

Total RNA was extracted by an improved cold phenol method (Liu et al., 1999). The concentration of each RNA preparation was measured by UV spectrophotometer (HITACHI U-3000).

About 1 μg of total RNA was used for the first-strand cDNA synthesis according to the protocol of Reverse Transcription System (Promega Co.). PCR mixture (total volume 20 μl) contained 0.5 μl reverse transcription reaction mixture (20 μl in total), 1× PCR buffer, 60 pmol each of primers, 0.2 mM dNTP, 1.5 mM $MgCl_2$, and 1.5 U Taq DNA polymerase (Promega Co.). The amplification program consisted of one cycle of a 3-min denaturation at 94 °C, 30 cycles of a 30-s denaturation at 94 °C, a 30 min primer annealing at 55 °C and a 30-s elongation at 72 °C, one final elongation cycle 3 min at 72 °C. The amplified products were separated by 1.2% agarose gel electrophoresis and visualized by ethidium bromide staining.

The cDNA clones selected for analyses of mRNA levels of gene expression and their specific primers are as follows: *C4H2*, forward primer 5′CATGATGCCAAGCTTGGTGAA3′and reverse primer 5′TGACCATAAACCACCCTTCC TA3′; *LDI2*, forward primer 5′CTACTTGG AAATCTTGTTG CAC3′and reverse primer 5′AATCCCATCAACACATACAC3′; *LePGT1*, forward primer 5′TTCTAGCTTGGCCAGCGT TC3′and reverse primer 5′CCCCAATCACTAG AGCAGCA3′; *PAL1* forward primer 5′GAG GTGATGAATCGAAAGCCTG3′and reverse primer 5′ACTCTGGACATGGTTGGTGACT3′; *CYP98A6*, forward primer 5′AGTTTGTTGGT GAGGGAGTA3′and reverse primer 5′TCTTGA GCCTTTCGTTGC3′; *4CL1*, forward primer 5′ATCTTCAG AT CAAAACTCCCTG3′and reverse primer 5′CTCCATCAACTTGTTGTGCA AC3′; *HMGR*, forward primer 5′ATGGCTACA ACTGAAGG3′and reverse primer 5′GGCCC CTCGATCCAA TT3′; *GAPDH*, forward primer 5′GGGTGGTGCTAAGAAGGTTGTC3′and reverse primer 5′CACGAGAGCTGTACCCATT CG3′. The amplified fragments of these genes are 530 bp, 310 bp, 612 bp, 570 bp, 470 bp, 622 bp, 443 bp, and 630 bp in size, respectively. *GAPDH* cDNA clone was included as a internal control for a constitutively expressed isolated from *L. erythrorhizon* cell cultures (Yamamura et al., 2001).

## Results

*Effect of light on shikonin formation in* Onosma paniculatum *cell cultures*

As shown in Figure 1, a large quantity of shikonin and its derivatives (mean content, 45.3 mg $g^{-1}$ FW) was produced, nearly without the cells growth, in cultured *Onosma paniculatum* cells grown in $M_9$ production medium at the dark, while shikonin formation was completely inhibited when the cell cultures illuminated under continuous white light and blue light. Under continuous

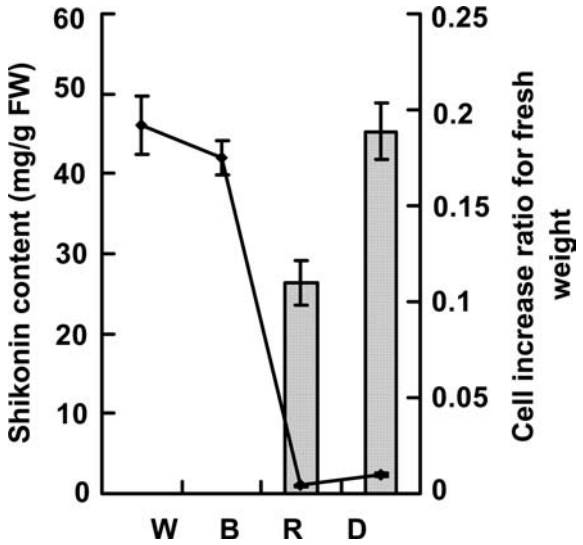


*Figure 1.* Effect of different light treatments on the cell growth and shikonin formation in cultured *Onosma paniculatum* cells. The cells cultured in M9 production medium in the complete darkness (D), or under continuous white light (W), blue light (B), red light (R) at 25 ± 1°C, and harvested 18–20 d after inoculation. The line denotes the growth (cell increase ratio for fresh weight), and column denotes shikonin content. The bars indicate standard deviation of the means with five replications.

red light, shikonin production was only partially repressed and its content was about 58% of the dark-grown control, but also without the cell growth. During the culture process, a color change in cell cultures and $M_9$ medium was observed from 4 days after inoculation at the dark and red light conditions and the color became deeply red when the cells were harvested. There was nearly no change in color but a little increase in cell growth (less than one fifth in the cell increase ratio for fresh weight at the dark or under red light in $M_9$ medium) occurred in cell cultures treated with continuous white light and blue light. The shikonin and its derivatives excreted into culture medium was also measured, the ratio of shikonin content in the cell cultures to that in the culture liquid under red light treatment was about 30%, which was the same as that in the dark.

*Gene expression pattern in response to different light conditions in* Onosma paniculatum *cell cultures*

The transcripts levels of several genes, which include metabolic enzyme genes *C4H*, *PAL*, *4CL*, *HMGR*, *LePGT*, and *CYP98A6* and a dark-inducible gene *LDI2*, in cultured *Onosma paniculatum* cells in the complete darkness or under continuous white, blue, and red light, respectively, were analyzed by RT-PCR (Figure 2). The *PAL1* mRNA kept at steady state level in the dark, while the transcript was a little elevated under continuous red light, blue light, and white light. The *C4H2* gene was constantly expressed irrespective of different light conditions. In dark-grown cultures, mRNA steady state level of *4CL1* declined with days after inoculation. However, illumination with continuous white, blue, and red light resulted in an up-regulation of *4CL1* mRNA level. Transcript levels of *HMGR* gradually decreased and dropped below detectable limit under complete darkness and continuous red light for 12 d after inoculation, the expression of *HMGR* kept steady state level and was slightly elevated under blue and white light, respectively. The expression of *LePGT1* was nearly constant under continuous white, blue, and red light except for a slight up-regulation in the dark. Expression of *LDI2* was dramatically induced in the dark, but no amplified products were detected under continuous white and blue light. The gene *LDI2* expressed weakly under continuous red light and

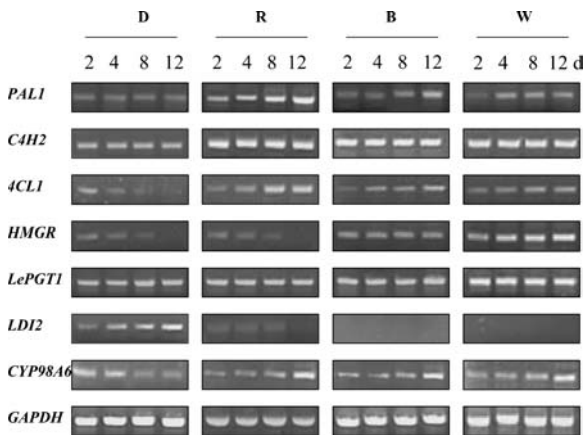


*Figure 2.* Gene expression pattern at mRNA level in cultured *Onosma paniculatum* cells in M9 medium under different light conditions by RT-PCR. The cells cultured in M9 production medium in the complete darkness (D), or under continuous white light (W), blue light (B), red light (R) at 25 ± 1°C, and harvested 2, 4, 8, and 12 d after inoculation, respectively, for RNA extraction. 1 μg of total RNA was used for reverse transcription in total volume of 20 μl reaction mixture, 0.5 μl of the mixture as template was amplified as described in Materials and Methods. The total 20 μl PCR products were separated by 1.2% agarose gel electrophoresis and visualized by ethidium bromide staining.

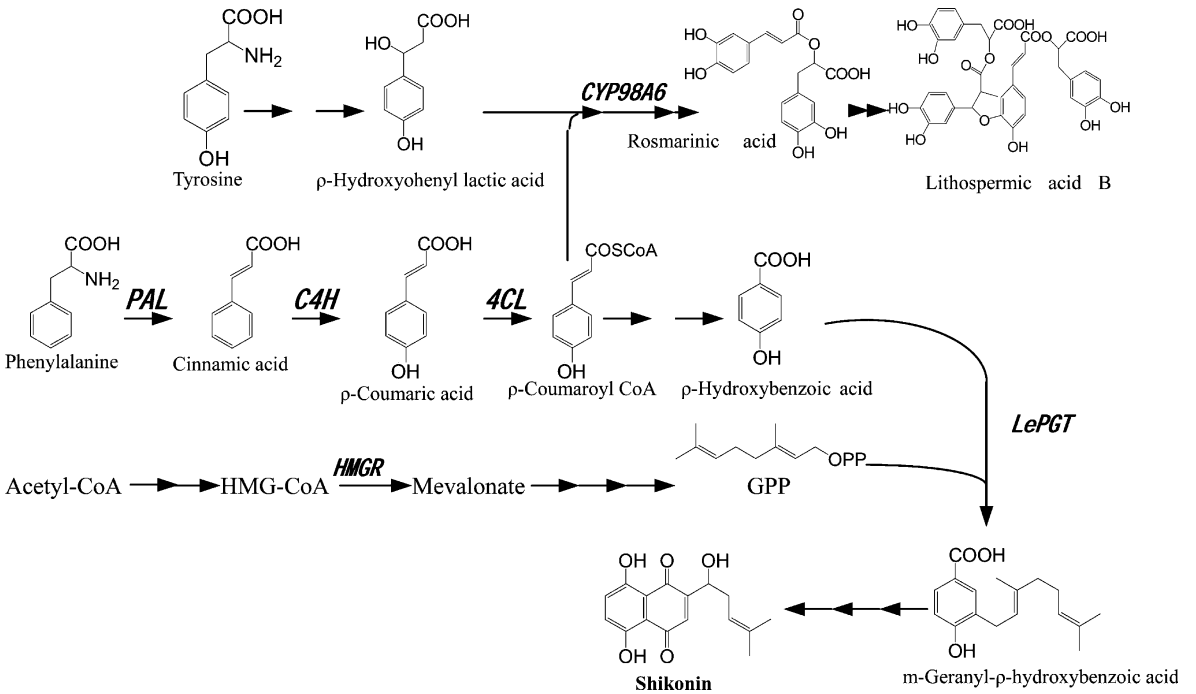


*Figure 3*. A brief pathway for shikonin biosynthesis. The genes of metabolic enzymes selected for expressional analysis were marked in bold and italic.

decreased to be undetectable during culturing process. *CYP98A6* was down-regulated by darkness and up-regulated in response to white, blue, and red light, whose expression pattern was the same as that of *4CL1* and *PAL1*.

Among the genes tested, the expression pattern of *LDI2* played a critical role in light-regulated shikonin formation in *Onosma paniculatum* cell cultures. The transcript of *LDI2* was undetectable under continuous white and blue light, where the shikonin formation was completely inhibited (Figures 1 and 2). The inducible expression of *PAL1*, *4CL1*, and *CYP98A6* as well as the repressible transcription of *LDI2* mediated by continuous red light irradiation were likely accounted for the reduced accumulation of shikonin and its derivatives in *Onosma paniculatum* cell cultures.

## Discussion

The shikonin and its derivatives are biosynthetically derived from two key precursors *p*-hydroxybenzoic acid (PHB), formed from phenylpropanoid metabolites, and geranyl pyrophosphate (GPP), derived from the isoprenoid pathway in cultured *L. erythrorhizon* cells (Figure 3) (Heide et al., 1989; Yamaga et al., 1993; Gaisser and Heide, 1996). PAL, C4H, and 4CL are sequential and important enzymes on the common phenylpropanoid pathway in plant phenolic metabolism (Hahlbrock and Scheel, 1989). The reaction catalyzed by enzyme HMGR is an early committed step of isoprenoid pathway in mammals and plants (Goldstein and Brown, 1990; McGarvey and Croteau, 1995). The geranyldiphosphate: *p*-hydroxybenzoate 3-geranyltransferase (LePGT) catalyses PHB with GPP to form *m*-geranyl-*p*-hydroxybenzoic acid (GBA), finally leading to shikonin biosynthesis in *L. erythrorhizon* cells (Gaisser and Heide, 1996; Mühlenweg et al., 1998; Yazaki et al., 2002). The expression patterns of the *PAL*, *C4H*, and *4CL* genes in *Arabidopsis* were found to be similar, which were induced by light and other influential factors (Bell-Lelong et al., 1997; Logemann et al., 1995). In the present study, expression of *PAL1* and *4CL1* was shown to be induced not only by white light but also by red and blue light in *Onosma paniculatum* cell cultures, while *C4H2* was constitutively expressed in the culture system

irrespective of different light conditions, these results were consistent with the characteristics of expression of the *PAL1*, *4CL1*, and *C4H2* in cultured *L. erythrorhizon* cells in previous reports (Yazaki et al., 1995, 1997b; Yamamura et al., 2001). The expression of *C4H2* seemed to be not involved in the light negative regulation of shikonin biosynthesis. The HMGR and LePGT enzymes have been reported to play a significant role in the regulation of shikonin biosynthesis, and white light was shown to strongly suppress *HMGR* and *LePGT1* gene expression in *L. erythrorhizon* cell cultures (Lange et al., 1998; Yazaki et al., 2002). In contrast to the previous results, the transcripts of *HMGR* was slightly stimulated under continuous blue and white light. The expression of *LePGT* was nearly constant irrespective of different light conditions. The *Onosma paniculatum* cell cultures were irradiated by the light conditions at high intensity in present study, which likely led to different expression patterns of the two genes. Bohne and Linden (2002) reported that the carotenoid biosynthesis genes of phytoene synthase and phytoene desaturase in cultured *Chlamydomonas reinhardtii* cells showed somewhat different expression patterns when the cells cultivated under low light were transferred into high light. The *LDI2* was strictly inducible expressed in the dark and its function may be related to the structural stability and secretion of shikonin and its derivatives (Yazaki et al., 1999, 2001). In *Onosma paniculatum* cell cultures, expression of the gene was dramatically induced in the dark and was completely repressed under continuous white and blue light. When cultivated under continuous red light, *LDI2* was weakly expressed and decreased to be undetectable during culturing process. The expression of the gene was shown to be pivotal for light-regulated shikonin production among the genes investigated in *Onosma paniculatum* cell cultures. The encoding product of *CYP98A6* participates in lithospermic acid B (LAB) biosynthesis, which was produced in large amount when cultured in shikonin production medium $M_9$, and the colorless compound and red pigments, shikonin and its derivatives, are formed through a common phenylpropanoid pathway in cultured *L. erythrorhizon* cells (Matsuno et al., 2002; Yamamoto et al., 2002). The LAB production was stimulated under red, blue, and white light, where shikonin formation was partially or completely inhibited (Tabata et al., 1974; Heide et al., 1989; Gaisser and Heide, 1996; Yamamoto et al., 2002). The expression of *CYP98A6* as well as *PAL1*, and *4CL1* was gradually increased under continuous white, red, and blue light possibly enhanced LAB biosynthesis, which led to decreasing or terminating production of shikonin and its derivatives in *Onosma paniculatum* cell cultures. However, the gene resources involved in shikonin biosynthetic metabolism seem to be limited and the detailed mechanism of regulation of shikonin production at molecular level is still unknown, which requests large-scale cloning of genes and function study in the shikonin formation.

Light signal perceived by three plant photoreceptor systems, the red/far-red ligh phytochromes, the blue/UV-A absorbing cryptochromes and phototropins, and the UV-B receptors, triggers alternations in gene expression (Terzaghi and Cashmore, 1995; Wang and Deng, 2002; Lin, 2002). Many studies have revealed that these photoreceptors mediated expression of a number of genes in positive and negative manners to date (Gilmartin et al., 1990; Tsai and Coruzzi, 1991; Christie and Jenkins, 1996; Im et al., 1996; Yoshida et al., 1993, 1999; Bohne and Linden, 2002). In present study, the expression of *PAL1*, *4CL1*, *LDI2*, *HMGR*, and *CYP98A6* was regulated by white, blue, and red light at transcriptional level in *Onosma paniculatum* cell cultures, which indicates that expression of these genes may be controlled by the phytochromes and blue light receptors systems. The light-regulatory mechanisms of the genes and identification of phytochromes and blue light receptors in *Onosma paniculatum* cell cultures need to be addressed further.

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