Review

Plant Biotechnology, **16** (5), 335-342 (1999)

Shikonin Biosynthesis in *Lithospermum erythrorhizon*: Light-induced Negative Regulation of Secondary Metabolism

Kazufumi YAZAKI*, Hideaki MATSUOKA, Tomomi UJIHARA, and Fumihiko SATO

Molecular & Cellular Biology of Totipotency, Division of Integrated Life Sciences, Graduate School of Biostudies, Kyoto University, Kitashirakawa, Kyoto 606-8502, Japan
**Corresponding author* E-mail: yazaki@kais.kyoto-u.ac.jp

Received 22 June 1999; accepted 8 August 1999

Abstract

Cell suspension cultures of *Lithospermum erythrorhizon*, a medicinal plant, produce red naphthoquinone pigments, which are derivatives of shikonin, when cultured in the production medium M9 in darkness, whereas light as well as ammonium ion which is a major component of Linsmaier and Skoog's medium, completely inhibit shikonin production. In the non-pigmented cells, a large amount of shikonin precursor *p*-hydroxybenzoic acid is accumulated as its *O*-glucoside instead of shikonin. An overview of the regulation of shikonin biosynthesis and the localization of important intermediates is presented in this review. We also discuss the effect of light in more detail, and characterize the dark-inducible genes (LEDI) which have been isolated from shikonin-producing cells, as well as the establishment of stable transformation of this plant. Furthermore, the latest results of functional analyses of LEDI genes in hairy root cultures are also reported.

1. Introduction

The root of *Lithospermum erythrorhizon* Sieb. et Zucc. is an important crude drug used for the treatment of wounds, burns and skin disorders in Asian countries. The active principles are red naphthoquinone compounds, shikonin derivatives which show antibacterial (Tanaka and Odani, 1972), anti-inflammatory (Hayashi *et al.*, 1969), anti-tumor (Sankawa *et al.*, 1977; Papageorgiou, 1980), anti-angiogenesis (Hisa *et al.*, 1998), and anti-topoisomerase-I (Ahn *et al.*, 1995) activities. Callus cultures of this plant producing shikonin derivatives were established by Tabata *et al.* (1974), and the industrial production of shikonin with the cell suspension cultures was accomplished by Mitsui Petrochemical Industries (Tabata and Fujita, 1985) by developing the pigment production medium M9 (Fujita *et al.* 1981). Formation of shikonin derivatives in hairy root cultures of this plant has also been reported in recent years (Shimomura *et al.*, 1991; Brigham *et al.*, 1999). In both dedifferentiated cultured cells and hairy roots, shikonin derivatives are compartmented in the red granules localized on the surface or apoplastic space of the cells. Especially, accumulation of shikonin granules is observed in epidermal cells in the hairy roots (**Fig. 1**).

2. Inducible and inhibitory elements of shikonin biosynthesis

Shikonin biosynthesis is regulated by various chemical and physical factors. Shikonin is not synthesized when the cells are cultured in Linsmaier and Skoog's (LS) liquid medium (1965) used for subculture, because of the high concentration of ammonium ion, a strong inhibitor of shikonin biosynthesis, and of the shortage of copper ion, an important enhancer of the biosynthesis (Fujita *et al.*, 1981). If ammonium ion is removed and copper ion (1 μM) is supplemented, shikonin production is inducible in LS medium although the administration of copper ion alone shows no effect (**Fig. 2A**). Under a high concentration of ammonium ion in LS medium, however, shikonin biosynthesis can be induced by addition of agar (Yoshikawa, 1987). Tani *et al.* (1992) demonstrated that the active principle of agar was acidic oligosaccharides, in particular oligogalacturonides with a polymerization degree of 12 to 20. Interestingly, the signal compound in elicitor treatments, i. e., methyl jasmonate also showed a similar inducing effect on shikonin biosynthesis as the oligogalacturonides (Yazaki *et al.*, 1997d).

A low concentration of ammonium ion, as well as

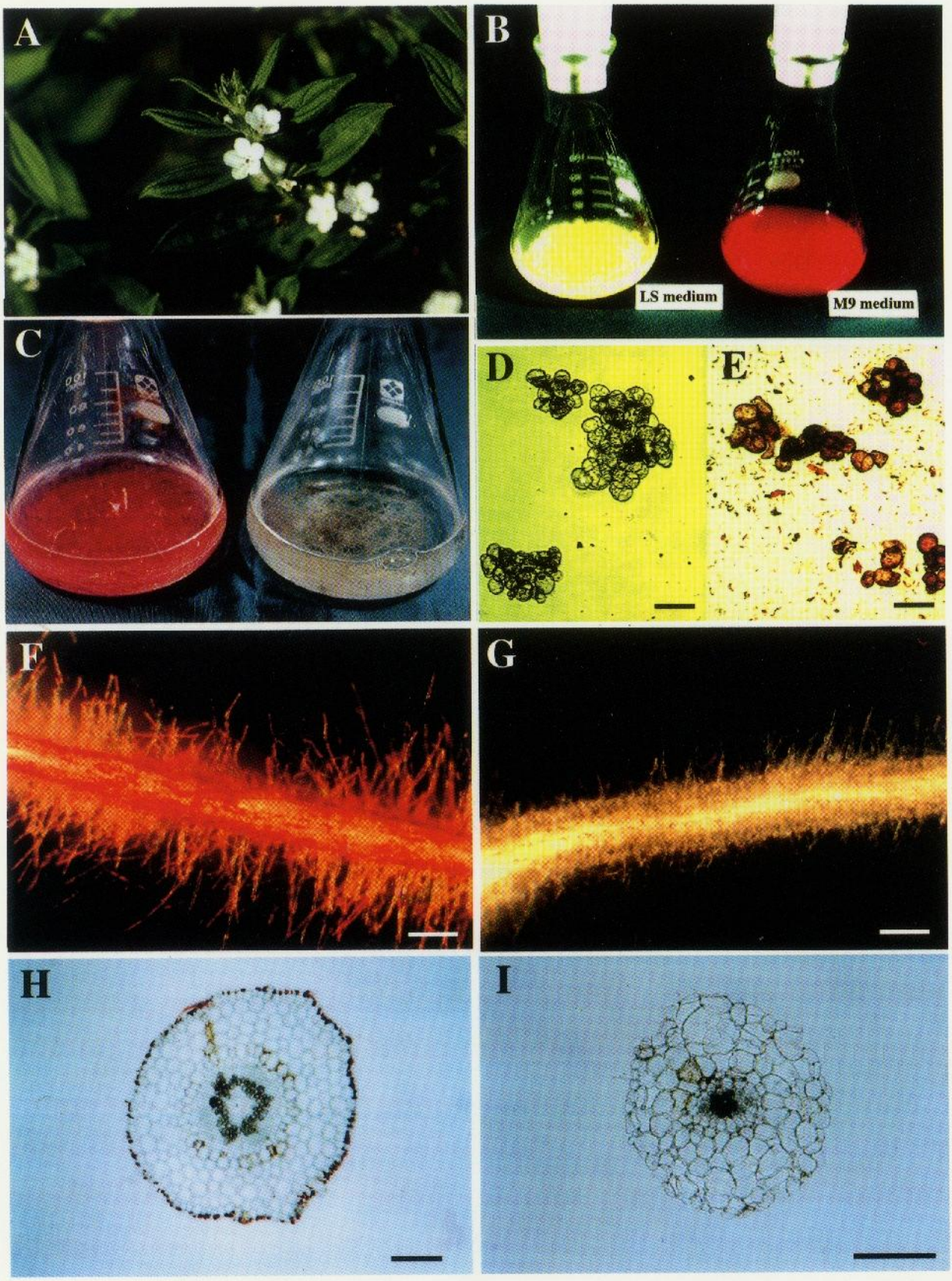


Fig. 1 **A** : Intact plant of *Lithospermum erythrorhizon*. **B** : Cell suspension cultures of *L. erythrorhizon* in LS medium (left) and M9 medium (right). **C** : Hairy root cultures of *L. erythrorhizon* in M9 medium in the dark (left) and under illumination (right). **D** : Cell clusters of *L. erythrorhizon* in LS medium, and **E** : M9 medium. **F** and **G** : Hairy root producing shikonin in M9 medium in the dark, and hairy root grown under illumination, respectively. **H** and **I** : Cross sections of hairy root cultured in M9 medium in the dark and under illumination, respectively. Bars shown in panel **D** and **E** indicate 50 μm, those in **F** and **G** 500 μm, and those in **H** and **I** 100 μm. The brown color observed in endodermis is not attributable to shikonin.

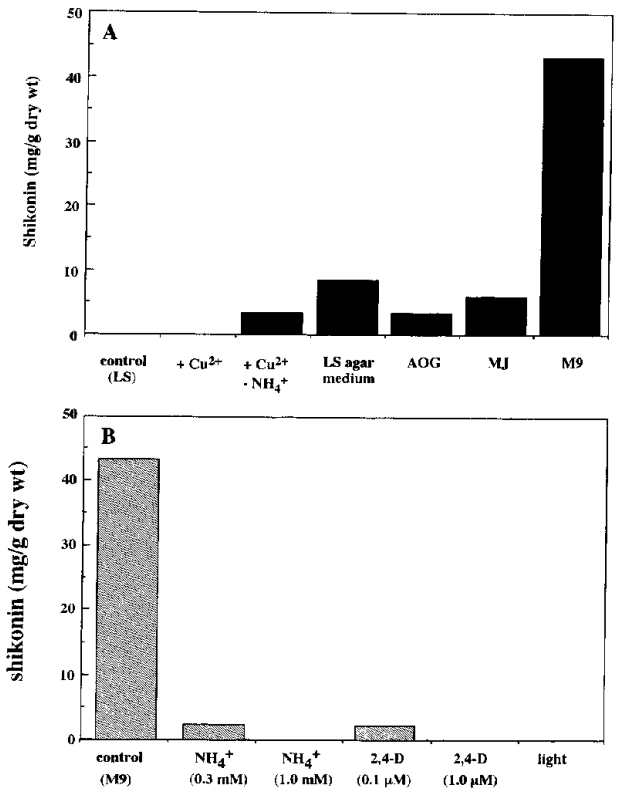


Fig. 2. Inducers and inhibitors of shikonin production in *L. erythrorhizon* cell cultures. **A:** Inducers of shikonin production. These elements were added to LS liquid medium. Cu^{2+}: 1 μM $CuSO_4$, $-NH_4^+$: ammonium-free LS medium, agar medium: solid medium with 1% agar, AOG: 100 μg/ml acidic oligogalacturonide, MJ: 10 μM methyl jasmonate. M9: shikonin production in M9 medium as the positive control. **B:** Inhibitors of shikonin production. These inhibitors were present in M9 medium in the dark. 2,4-D: 2,4-dichlorophenoxyacetic acid. Light: M9 culture under illumination by fluorescent lamps.

2,4-D is sufficient to completely inhibit shikonin production in M9 medium (**Fig. 2B**). The inhibitory effect of the former is reversible but that of the latter is not. Light, which is a strong inhibitor of shikonin biosynthesis, also shows reversible inhibition. As the quality of light, blue is known to be most effective for the inhibitory effect (Gaisser and Heide, 1996), indicating that a flavoprotein is involved in the biosynthetic pathway (Tabata *et al.*, 1993). Another possibility is that a blue light photoreceptor, cryptochrome identified in *Arabidopsis* may play an important role in light signal sensing (Cashmore *et al.* 1999) which shuts down the gene expression necessary for shikonin production.

3. Biosynthetic pathway of shikonin and other secondary metabolites

Fig. 3 shows the biosynthetic pathway of shikonin and other secondary metabolites produced by *L. erythrorhizon* cell cultures. *p*-Hydroxybenzoic acid (PHB), an important precursor of shikonin, accumulates as the *O*-glucoside form (PHBOG) in the cells cultured in LS medium (Yazaki *et al.*, 1986a), or in M9 medium in the light (Heide *et al.*, 1989), whereas after hydrolysis of the glucoside PHB is immediately prenylated by geranyltransferase (Heide and Tabata, 1987; Muehlenweg *et al.*, 1998) to give *m*-geranyl-*p*-hydroxybenzoic acid, which is converted to shikonin derivatives as the end products in M9 medium in the dark. Dihydroechinofuran (DHEF) is a benzofuran derivative formed from geranylhydroquinone, the same intermediate as shikonin (Fukui *et al.*, 1992), and it is easily oxidized to yield an orange compound echinofuran B (Fukui *et al.* 1984). Although these compounds were first thought to be unusual metabolites only produced by the cultured cells, they have recently been found to be common components in intact plants (Yamamoto *et al.*, in preparation).

Shikonin-producing cells in M9 medium also contains a caffeic acid tetramer, lithospermic acid B, in a considerable quantity, which is nearly the same level as that of shikonin derivatives in M9 medium (Yamamoto *et al.*, in preparation). This phenolic compound as well as a caffeic acid dimer, rosmarinic acid (Mizukami *et al.*, 1992), is constitutively formed in a detectable amount in LS-cultured cells, but the production of the former is strongly induced only in M9 medium.

4. Dark-inducible genes in *L. erythrorhizon*

The cell cultures of *L. erythrorhizon* seem to be a suitable model system to investigate the light regulation of secondary metabolism, because the product is visible, the inhibition of shikonin synthesis by light is very rapid and reversible (Heide *et al.*, 1989). An inhibitor experiment has shown that light inhibits PHB-geranyltransferase presumably at the transcriptional level, while it promotes the activity of glucosyltransferase to form PHBOG (Gaisser and Heide, 1996). However, the regulatory mechanism by light is still open to question.

In order to study the light regulation of shikonin biosynthesis, we isolated cDNAs preferentially expressed in the dark by subtractive hybridization (Yazaki *et al.*, 1995a). These clones were desig-

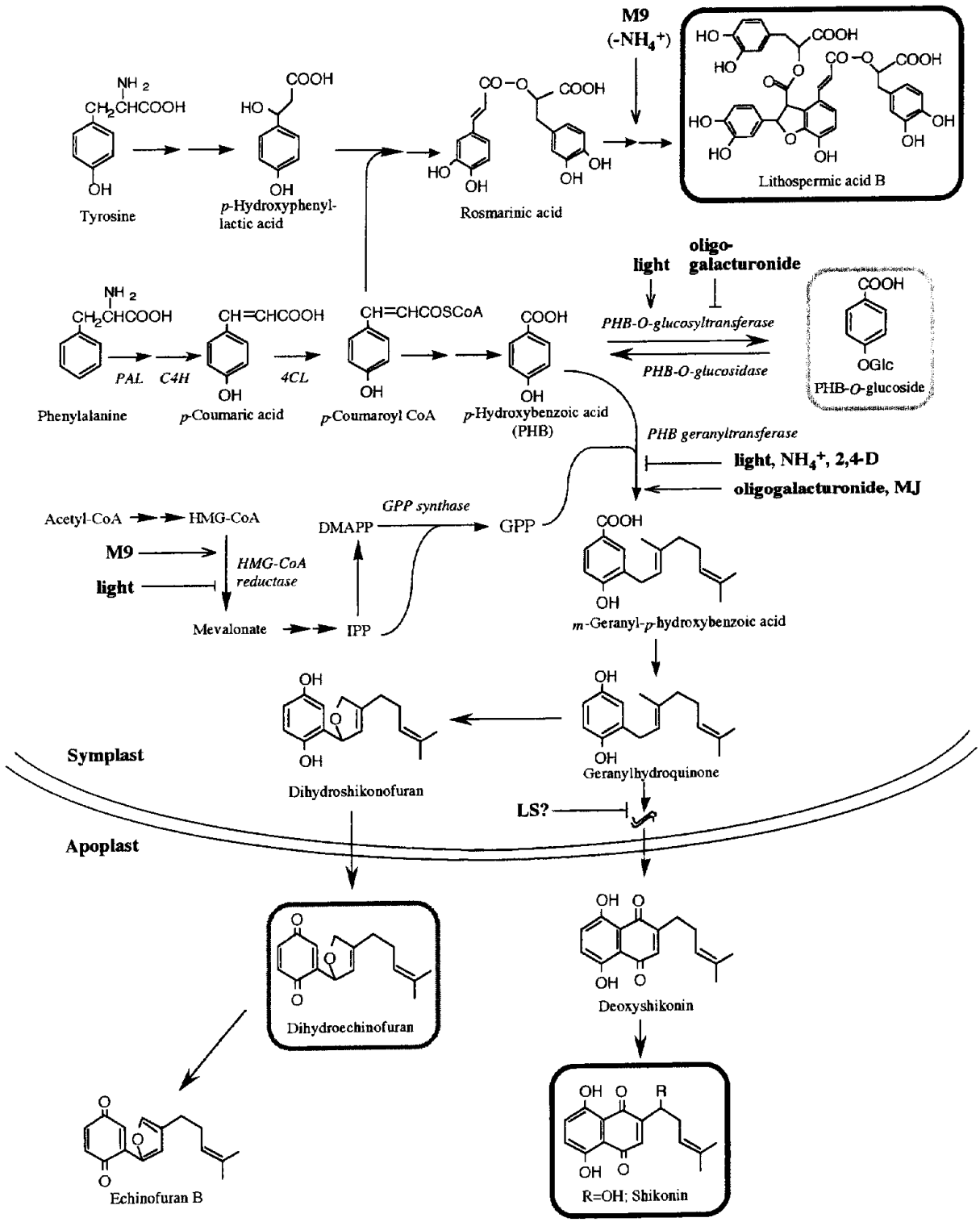


Fig. 3. Secondary metabolic pathway in *L. erythrorhizon* cell cultures. Compounds high-lighted by circles are the end products which accumulated in high quantities, i. e., shikonin (only in M9 medium in the dark), dihydroechinofuran (DHEF, in LS culture in the dark), *p*-*O*-*β*-glucosylbenzoic acid (PHBOG, in LS culture and M9 culture under light), and lithospermic acid B (in M9 medium both in the dark and light). The former three compounds are synthesized *via* *p*-hydroxybenzoic acid, and the latter compound is the end product conserving a phenylpropanoid skeleton.

nated LEDI (*L. erythrorhizon* dark-inducible) genes. As shown in **Table 1**, some of them shared apparent similarity to known proteins (Yazaki *et al.*, 1995a; Xu *et al.*, 1995; Yazaki *et al.*, 1997a). Among them, LEDI-2, which showed significant similarity to the root specific genes of several plant species, showed the most strict dark-selectivity in the RNA blot analysis (Yazaki *et al.*, 1997c). The deduced amino acid sequence of LEDI-2 was hydrophobic, indicating that the gene product is

Table 1. cDNA clones isolated by subtractive hybridization from *Lithospermum erythrorhizon*.

designation	No. of clone (%)	insert size (kb)	dark selectivity	similarity	ref.
LEDI-1	82(50.6%)	0.8	±	PR-1 protein	(1)
LEDI-2	2(1.2%)	0.7	+++	root sp. protein	(2)
LEDI-3	12(7.4%)	1.0	±	WrbA	(3)
LEDI-4	8(4.9%)	1.2	++	oxidoreductase*	–
LEDI-5	39(24.1%)	1.6	+	oxidoreductase	–

(1) Yazaki *et al.*, 1995a, (2) Xu *et al.*, 1995, (3) Yazaki *et al.*, 1997a.
*The deduced amino acid sequence shares a weak similarity to that of NADPH quinone oxidoreductase

membrane-bound. The time-course of mRNA accumulation of LEDI-2 during the culture period paralleled the accumulation of shikonin in the cell cultures (data not shown). These data strongly suggest that the LEDI-2 polypeptide has a close correlation to shikonin production. Because shikonin or its precursor are probably localized in vesicles like the oil bodies (Tsukada and Tabata, 1984), it is considered that a hydrophobic protein like LEDI-2, would be needed to stabilize the vesicle structure and to accumulate these lipophilic compounds. To examine this hypothesis, we established a stable transformation method in *Lithospermum*.

5. Stable transformation of *L. erythrorhizon*

We used *Agrobacterium rhizogenes* (ATCC 15834) to establish hairy root cultures of *L. erythrorhizon*. The root is the site of the production and accumulation of shikonin in the intact plants and the formation of shikonin derivatives has been reported in the hairy root (Shimomura *et al.*, 1991). The binary vector used in a model experiment contained β-glucuronidase (GUS) gene which was driven by CaMV 35S promoter and hygromycin phosphotransferase (HPT) gene as a selection marker (Yazaki *et al.*, 1998). Histochemical analysis of the transgenic hairy root with the GUS gene showed that the CaMV 35S promoter was active in most of the root tissues (Yazaki *et al.*, 1998). The survival rate on hygromycin plate depended on the gene subcloned in the binary vector, for instance 20 %, 25 %, and 50 % for GUS (Yazaki *et al.*, 1998), *ubiC* (Sommer *et al.*, 1999), and LEDI-2-antisense, respectively.

6. Comparison of secondary metabolism in the cell cultures and hairy root cultures of *L. erythrorhizon*

Because a stable transformation method for *L. erythrorhizon* has been established, functional analyses of LEDI genes became feasible *via* reverse genetics using the hairy root cultures as an alternative plant material to cell cultures. Thus, the phenotypes of the hairy root cultures, e. g., light responsiveness, have been compared with those of conventional cell suspension cultures. Shikonin production in hairy root cultures was strongly induced in M9 medium when cultured in the dark, and it was specifically and completely inhibited by white light irradiation in a very similar manner as cell suspension cultures (Yazaki *et al.*, 1998). A slight difference between them was that a small amount of shikonin was formed even in Murashige-Skoog's medium (1962) used for subculture of the hairy roots, that would be because, different from the dedifferentiated cells, the hairy roots possess developed epidermal tissues which may be advantageous for accumulation of red granules containing shikonin derivatives (see **Fig. 1**).

The accumulation pattern of mRNAs involved in several important reaction steps, i. e., phenylalanine ammonia-lyase (PAL: Yazaki *et al.*, 1997b), cinnamate 4-hyroxylase (C4H: Mizutani *et al.*, 1997), 4-coumarate CoA-ligase (4CL: Löscher and Heide, 1994; Yazaki *et al.*, 1995c), and HMG-CoA-reductase (Lange *et al.*, 1998), involved in the shikonin pathway in hairy root cultures are indicated in **Fig. 4**. The expression patterns of these genes in hairy root cultures were similar to those in cell suspension cultures, except for 4CL which had a much higher expression level in hairy roots than in the cell cultures (Yazaki *et al.*, 1995c). This might be

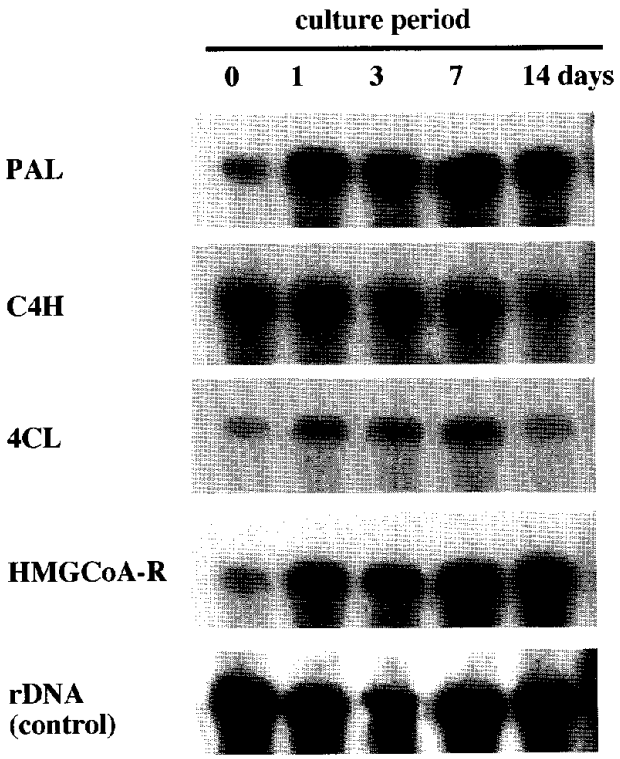
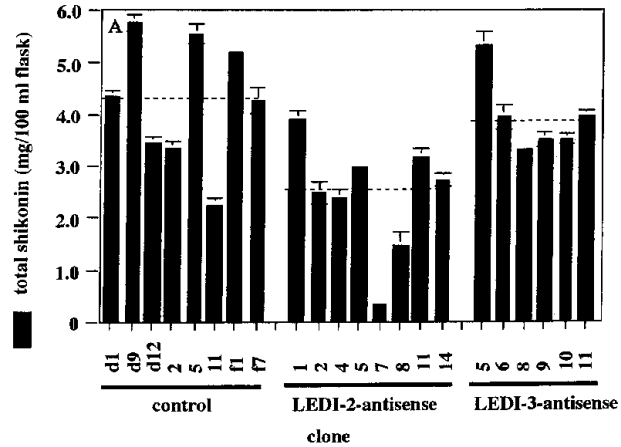


Fig. 4. Gene expression of phenylalanine ammonia-lyase (PAL; Yazaki *et al.*, 1997b), cinnamate 4-hyroxylase (C4H; Mizutani *et al.*, 1997), 4-coumarate CoA-ligase (4CL; Yazaki *et al.*, 1995c), and HMG-CoA-reductase (HMGCoA-R; Lange *et al.*, 1998), which are involved in shikonin biosynthesis, in *L. erythrorhizon* hairy root cultures in the dark. Total RNA was separated on a formamide-containing agarose gel, blotted and hybridized with each cDNA as probe.



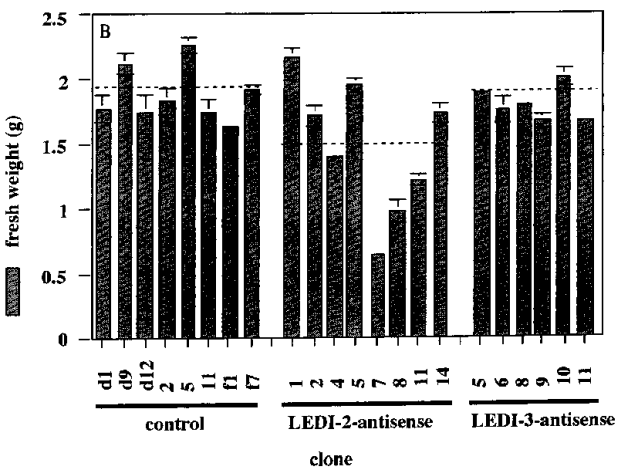


Fig. 5. Shikonin production in hairy root cultures transformed with antisense DNAs of LEDI-2 and LEDI-3, as well as the control hairy root cultures. For LEDI-2 and -3, each coding region was subcloned into binary vector in antisense orientation which is driven by CaMV 35S promoter. The control hairy root clones were obtained by the infection with *A. rhizogenes* containing no binary vector. Dotted lines show the average value of each subgroup.

because such high 4CL expression is necessary for lignin formation in xylem and other root tissues. In general, however, we conclude that hairy roots can be utilized as an alternative culture system to investigate the secondary metabolism of this plant.

7. Functional analyses of LEDI genes in *L. erythrorhizon* hairy root cultures

Using the above transformation method, both LEDI-2 and LEDI-3 genes were induced in the hairy root of *L. erythrorhizon* in the antisense orientation, to observe the alteration of the phenotype. **Fig. 5** depicts the shikonin production of LEDI-2 antisense transformants in which the LEDI-2 mRNA level was suppressed, compared with that of the GUS transformant as the control. As clearly seen in the figure, LEDI-2 antisense showed much lower shikonin productivity than the control. Although the suppression of mRNA accumulation of LEDI-2 by its antisense construct was not very strong, such transformants having obviously lower shikonin production were observed only among the LEDI-2 antisense transformants. This suggests that the gene product of LEDI-2 plays an important role in the biosynthesis or the accumulation of shikonin in *L. erythrorhizon*. By contrast, the antisense of LEDI-3 (Yazaki *et al.*, 1997a), which shared a significant homology with Trp repressor binding protein in *Escherichia coli*, showed almost no effects on either shikonin production or cell growth.

8. Localization of secondary metabolites biosynthesized by *L. erythrorhizon* cell cultures

Fig. 6 summarizes the accumulation and the biosynthetic flow of the intermediates leading to shikonin. In non-pigmented cells, all of PHBOG is accumulated in the vacuoles of the cells, whereas two enzymes involved in PHBOG metabolism, glucosyltransferase and β-glucosidase, seem to be localized in the cytosolic space (Yazaki *et al.*, 1995b; Yazaki, 1997). Once shikonin production is induced in M9 medium in the dark, PHBOG is hydrolyzed to give PHB, which is immediately prenylated by geranyltransferase (Heide and Tabata, 1987; Muehlenweg *et al.*, 1998) localized on ER membrane in the presence of geranyldiphosphate to form *m*-geranyl-*p*-hydroxybenzoic acid (Inouye *et al.*, 1979; Yazaki *et al.*, 1986b). If the next intermediate, geranylhydroquinone, forms a naphthalene ring, the biosynthesis leads to shikonin derivatives (Inouye *et al.*, 1979) which are localized in vesicles to be secreted out of the cells (Tsukada and Tabata, 1984; Tabata, 1996). However, if a furan ring is formed in the geranyl moiety, it gives DHEF through a hydroquinone intermediate, dihydroshikonofuran (Yazaki *et al.*, 1987). A large amount of DHEF is often produced by "inoculation shock" on subculture, and it is secreted into the medium in a soluble form, while lithospermic acid B is solely localized inside of the cells.

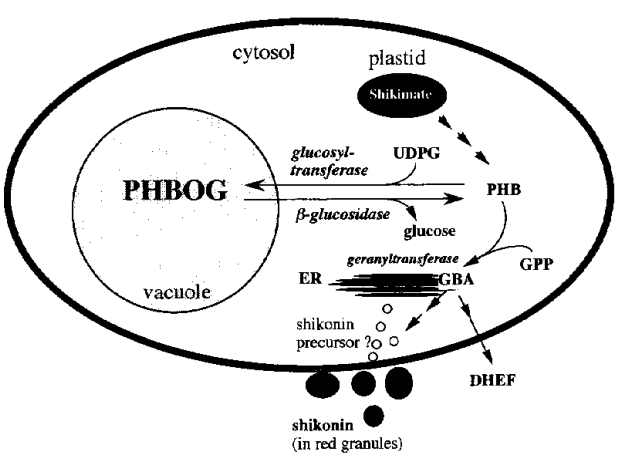


Fig. 6. Subcellular localization of shikonin and PHBOG, as well as the biosynthetic enzymes in *L. erythrorhizon* cell cultures. PHB: *p*-hydroxybenzoic acid, GPP: geranylpyrophosphate, GBA: *m*-geranyl-*p*-hydroxybenzoic acid, DHEF: dihydroechinofuran, UDPG: UDP-glucose, ER: endoplasmic reticulum.

9. Concluding remarks

Plants are always exposed to microorganisms in the environment. In particular, root tissues are always in close contact with soil-borne bacteria and fungi, some of which are pathogenic. Secondary metabolites are thought to play an important role to protect the plant from the microorganisms (Brigham *et al.*, 1999). In *L. erythrorhizon*, shikonin derivatives are specifically accumulating in epidermal cells of the root (**Fig. 1**). Since the establishment of a cell culture system having high productivity of shikonin, several regulatory factors on shikonin biosynthesis have been identified, and the biosynthetic pathway of shikonin has been intensively studied, as summarized in this review. The most critical regulator of shikonin biosynthesis is light. There are numerous reports about light-inducible secondary metabolism in higher plants (Hahlbrock and Scheel, 1989), but very little is known about the light-suppressive secondary metabolism, in other words, dark-inducible secondary metabolism. Shikonin biosynthesis of *L. erythrorhizon* is a representative of this, and a suitable experimental system for studies on the regulatory mechanism, because the inhibition is very clear, specific, and reversible. In order to investigate the negative regulatory mechanism, we have isolated cDNAs which shows preferably high expression in darkness. One of them, LEDI-2 has a very short open reading frame for 114 amino acids, and is unlikely a biosynthetic enzyme. For the reverse genetic study, we have also established a stable transformation method by aid of *A. rhizogenes*, in which hairy roots are obtained as transformants. Cultured hairy roots can be utilized for analyzing the gene function in terms of shikonin production, because they have very similar shikonin productivity and light responsiveness as well. The antisense of LEDI-2 clearly decreases the amount of shikonin accumulated in the hairy roots compared to the control, suggesting that the gene product of LEDI-2 has a close correlation to the shikonin production. Another clone, LEDI-3 did not seem to affect the shikonin production. The detailed functional analysis of LEDI-2 *in vivo* is on going.

The transformation method established here should also be useful for genetic engineering of the secondary metabolism of *L. erythrorhizon*. Recently, we succeeded in producing a shikonin molecule through a bacterial gene *ubiC* in the plant (Sommer *et al.*, 1999). We are expecting that this transformation system will be further applied to attractive model experiments for alteration of sec-

ondary metabolism in *L. erythrorhizon* by genetic manipulation. Moreover, plant regeneration from callus cultures of this plant is also possible (Yu *et al.*, 1997), which will enable more diverse application of reverse genetics and alteration of secondary metabolic engineering.

Acknowledgments

We thank Prof. M. Tabata for providing *L. erythrorhizon* cell cultures and advice during this research. We are grateful to Dr. M. Mizutani, Institute for Chemical Research of our University, for giving us mung bean C4H cDNA used as a hybridization probe. A part of this work was supported by a Grant-in-aid for Scientific Research fro Ministry of Education, Science and Culture of Japan.

References

Ahn, B. Z., Baik, K. U., Kweon, G. R., Lim, K., Hwang, B. D., J. Med. Chem., 1995. Acylshikonin analogues: synthesis and inhibition of DNA topoisomerase-I. **38**: 1044-1047.

Brigham, L. A., Michaels, P. J., Flores, H. E., 1999. Cell-specific production and antimicrobial activity of naphthoquinones in roots of *Lithospermum erythrorhizon*. Plant Physiol., **119**: 417-428.

Cashmore, A. R., Jarillo, J. A., Wu, Y. J., Liu, D., 1999. Cryptochromes: blue light receptors for plants and animals. Science, **284**: 760-765.

Fujita, Y., Hara, Y., Suga, C., and Morimoto, T. 1981. Production of shikonin derivatives by cell suspension cultures of *Lithospermum erythrorhizon*. A new medium for the production of shikonin derivatives. Plant Cell Rep., **1**: 61-63.

Fukui, H., Yoshikawa, N., Tabata, M., 1984. Induction of benzoquinone formation by activated carbon in *Lithospermum erythrorhizon* cell suspension cultures. Phytochem., **23**: 301-305.

Fukui, H., Tani, M., Tabata, M., 1992. An unusual metabolite, dihydroechinofuran, released from cultured cells of *Lithospermum erythrorhizon*. Phytochem., **31**: 519-521.

Gaisser, S., Heide, L., 1996. Inhibition and regulation of shikonin biosynthesis in suspension cultures of *Lithospermum*. Phytochem. **41**: 1065-1072.

Hahlbrock, K., Scheel, D., 1989. Physiology and molecular biology of phenylpropanoid metabolism. Annu. Rev. Plant Physiol. Plant Mol. Biol. **40**: 347- 369.

Hayashi, M., Tsurumi, S., and Fujimura, H. 1969. Pharmacological activities of *Lithospermum* root. Japan J. Pharmacol., **65**: 195-196.

Heide, L., Nishioka, N., Fukui, H., and Tabata, M. 1989. Enzymatic regulation of shikonin biosynthesis in *Lithospermum erythrorhizon* cell cultures. Phytochem., **28**: 1873-1877.

Heide, L., Tabata, M., 1987. Geranylpyrophosphate: *p*-hydroxybenzoate geranyltransferase activity in extracts of *Lithospermum erythrorhizon* cell cultures. Phytochem., **26**: 1651-1655.

Hisa, T., Kimura, Y., Takada, K., Suzuki, F., Takigawa, M., 1998. Shikonin, an ingredient of *Lithospermum erythrorhizon*, inhibits angiogenesis *in vivo* and *in vitro*. Anticancer Res., **18(2A)**: 783-90.

Inouye, H., Ueda, S., Inoue, K., Matsumura, H., 1979. Biosynthesis of shikonin in callus cultures of *Lithospermum erythrorhizon*. Phytochem., **18**: 1301-1308.

Lange, B. M. Severin, K., Bechthold, A., Hiede L., 1998. Regulatory role of microsomal 3-hydroxy-3-methylglutaryl-coenzyme A reductase for shikonin biosynthesis in *Lithospermum erythrorhizon* cell suspension cultures. Planta, **204**: 234-241.

Linsmaier, E.M., Skoog, F. 1965. Organic growth factor requirements of tobacco tissue cultures. Physiol. Plant., **18**: 100-127.

Löscher R., Heide, L., 1994. Biosynthesis of *p*-hydoxybenzoate from *p*-coumarate and *p*-coumaroyl-coenzyme A in cell-free extracts of *Lithospermum erythrorhizon* cell cultures. Plant Physiol., **106**: 271-279.

Mizukami, H., Ogawa, T., Ohashi, H., Ellis, B. E., 1992. Induction of rosmarinic acid biosynthesis in *Lithospermum erythrorhizon* cell suspension cultures by yeast extract. Plant Cell Rep., **11**: 480-483.

Mizutani, M., Ohta, D., Sato, R., 1997. Isolation of a cDNA and a genomic clone encoding cinnamate 4-hydroxylase from Arabidopsis and its expression manner in Planta. Plant Physiol., **113**: 755-763.

Muehlenweg, A., Melzer M., Li, S-M, Heide, L., 1998. 4-Hydorxybenzoate 3-geranyltransferase from *Lithospermum erythrorhizon*: purification of a plant membrane-bound prenyltransferase. Planta, **205**: 407-413.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassays with tissue cultures. Physiol. Plant. **15**: 473-497.

Papageorgiou, V.P., 1980. Naturally occurring isohexenylnaphthazarin pigments: a new class of drugs. Planta Med., **38**: 193.

Sankawa, U., Ebizuka, Y., Miyazaki, T., Isomura, Y., Otsuka, H., Shibata, S., Inomata, M., and Fukuoka, F. 1977. Anti-tumor activity of shikonin and its derivatives. Chem. Pharm. Bull., **25**: 2392-2395.

Shimomura, K., Sudo, H., Saga, H., Komada, H., 1991. Shikonin production and secretion by hairy root cultures of *Lithospermum erythrorhizon*. Plant Cell Rep. **10**: 282-285.

Sommer, S., Koehle, A., Yazaki, K., Shimomura, K., Bechthold, A., Heide, L., 1999. Genetic engineering of shikonin biosynthesis. Hairy root cultures of *Lithospermum erythrorhizon* transformed with the bacterial *ubiC* gene. Plant Mol. Biol., **39**: 683- 693.

Tabata, M., 1996. The mechanism of shikonin biosynthesis in *Lithospermum* cell cultures. Plant Tissue Culture Lett., **13**: 117-125.

Tabata, M., Fujita, Y., 1985. Biotechnology in Plant Science, M. Zaitlin, P. Day, A. Hollaender (Eds.), Academic Press, Orlando, p. 207-218.

Tabata, M., Mizukami, H., Hiraoka, N., Konoshima, M.,

1974. Pigment formation in callus cultures of *Lithospermum erythrorhizon*. Phytochemistry, **13**: 927-932.

Tabata, M., Yazaki, K., Nishikawa, Y., Yoneda, F., 1993. Inhibition of shikonin biosynthesis by photodegradation products of FMN. Phytochem., **32**: 1439-1442.

Tanaka, Y., Odani, T. 1972. Pharmacodynamic study on "shiunko". I. Antibacterial effect of "shiunko". Yakugaku Zasshi, **92**: 525-530.

Tani, M., Fukui, H., Shimomura, M., Tabata, M., 1992. Structure of endogenous oligogalacturonides inducing shikonin biosynthesis in *Lithospermum erythrorhizon*. Phytochem., **31**: 2719-2723

Tsukada, M., Tabata, M., 1984. Intracellular localization and secretion of naphthoquinone pigments in cell cultures of *Lithospermum erythrorhizon*. Planta Med., **50**: 338-341.

Xu, Y., Buchholz, W. G., DeRose, R. T. Hall, T. C., 1995. Characterization of a rice gene family encoding root-specific proteins. Plant Mol. Biol., **27**: 237-248.

Yazaki, K., 1997. Recent advances in quinone biosynthesis and related gene expression in *Lithospermum erythrorhizon*. Research Tre., **1**: 125-135.

Yazaki, K., Bechthold, A., and Tabata, M. 1995a. Nucleotide sequence of a cDNA from *Lithospermum erythrorhizon* homologous to PR-1 of parsley. Plant Physiol., **108**: 1331-1332.

Yazaki, K., Bechthold, A., Tabata, M., 1997a. Molecular cloning and expression of a cDNA encoding a WrbA homologue from *Lithospermum erythrorhizon* (PGR97-159). Plant Physiol., **115**: 1287-1288.

Yazaki, K., Fukui, H., Tabata, M., 1986a. Accumulation of *p*-*O*-β-D-glucosylbenzoic acid and its relation to shikonin biosynthesis in *Lithospermum* cell cultures. Phytochem., **25**: 1620-1632.

Yazaki, K., Fukui, H., Tabata, M., 1986b. Isolation of the intermediates and related metabolites of shikonin biosynthesis from *Lithospermum erythrorhizon* cell cultures. Chem. Pharm. Bull., **34**: 2290-2293.

Yazaki, K., Fukui, H., Tabata, M., 1987. Dihydroshikonofuran, an unusual metabolite of quinone biosynthesis in *Lithospermum* cell cultures. Chem. Pharm. Bull., **35**: 898-901.

Yazaki, K., Inushima, K., Kataoka, M., Tabata, M., 1995b. Intracellular localization of UDPG: *p*-hydroxybenzoate glucosyltransferase and its reaction product in *Lithospermum* cell cultures. Phytochem., **38**: 1127-1130.

Yazaki, K., Kataoka, M., Honda, G., Severin, K., Heide, L., 1997b. cDNA cloning and gene expression of phenylalanine ammonia-lyase in *Lithospermum erythrorhizon*. Biosci. Biotech. Biochem., **61**: 1995-2003.

Yazaki, K., Matsuoka, H., Sato, F. 1997c. cDNA cloning and functional analysis of LEDI-2, a gene preferentially expressed in the dark in *Lithospermum erythrorhizon* cell suspension cultures (abstract no. 1348). Plant Physiol., **114**: S-261.

Yazaki, K., Ogawa, A., Tabata, M. 1995c. Isolation and characterization of two cDNAs encoding 4-coumarate:CoA ligase in *Lithospermum* cell cultures. Plant Cell Physiol., **36**: 1319-1329.

Yazaki, K., Takeda, K., and Tabata, M., 1997d. Effects of methyl jasmonate on shikonin and dihydroechinofuran production in *Lithospermum* cell cultures. Plant Cell Physiol., **38**, 776-782.

Yazaki, K., Tanaka, S., Matsuoka, H., Sato, F., 1998. Stable transformation of *Lithospermum erythrorhizon* with *Agrobacterium rhizogenes* and shikonin production of the transformants. Plant Cell Rep., **18**: 214-219.

Yoshikawa, N., 1987. Studies on quinone pigment induction in *Lithospermum erythrorhizon*. Ph. D. Thesis, Kyoto University, 66 pp.

Yu, H. J., Oh, S. K., Oh, M. H., Choi, D. W., Kwon, Y. M., Kim, S. G., 1997. Plant regeneration from callus cultures of *Lithospermum erythrorhizon*. Plant Cell Rep. **16**: 261-266.