Original Paper Plant Biotechnology, **17** (2), 127-130 (2000)

Histological Observation of Red Pigment Formed on Shoot Stem of *Lithospermum erythrorhizon*

Kaori TOUNO*, Kyuya HARADA*, Kayo YOSHIMATSU**, Kazufumi YAZAKI*** and Koichiro SHIMOMURA**[1)]

**Graduate School of Sciences and Technology, Chiba University, 648 Matsudo, Matsudo, Chiba, 271-0092, Japan*
***Tsukuba Medicinal Plant Research Station, National Institute of Health Sciences, 1 Hachimandai, Tsukuba, Ibaraki, 305-0843, Japan*
****Graduate School of Biostudies, Kyoto University, Kitashirakawa, Oiwake-cho, Sakyo-ku, Kyoto, 606-8502, Japan*
[1)] *Corresponding author* E-mail address: shimomur@nihs. go. jp

Received 20 December 1999; accepted 28 December 1999

Abstract

Shikonin production on the stem of shoot cultures of *Lithospermum erythrorhizon* was controlled by use of various culture media and light irradiation. Microscopic analysis of the shoot cultures revealed that the red pigment formation was only observed on the stem surface and the stem hairs of shoots cultured in the dark. Cross section of the shoot stem which formed red pigment in the dark was morphologically similar to that of shoots cultured under illumination. Red pigment accumulation was strictly localized in the outer surface of epidermal cells. The localization of these pigments was similarly observed in root tissues generated from the cultured shoots as well as field-grown roots. Northern blot analysis indicated that LEDI-2 gene, which is one of the candidates for the regulatory element of shikonin biosynthesis and specifically expressed in the root system of the intact plants, was also expressed in the stem of shoot cultures when producing shikonin.

1. Introduction

Lithospermum erythrorhizon Sieb. et Zucc. (Boraginaceae) is a perennial herb which grows spontaneously in Japan, Korea and China. *L. erythrorhizon* accumulates red pigment (shikonin derivatives) in its root, which are used as a natural dye and its roots are prescribed in the ointment (Shi-Un-Koh) for treating burns. Production of shikonin derivatives by callus, cell suspension and hairy root cultures, has been reported (Deno *et al.*, 1987, Fujita *et al.*, 1983, Shimomura *et al.*, 1991). In cell cultures, molecular genetic study has been performed to elucidate the regulatory mechanism of shikonin biosynthesis (Yazaki, 1997). One of those cDNAs isolated from a library of this plant, LEDI-2, specifically expressed in the cells of *L. erythrorhizon* in the dark, was cloned by a subtractive hybridization technique (Yazaki *et al.*, 1997).

Recently we established a shoot culture, which is capable of producing shikonin derivatives on the stem (Shimomura *et al.*, 1998, Touno *et al.*, in press). The shoot culture, when subcultured on solid or in liquid Murashige and Skoog's (MS, Murashige and Skoog, 1962) media without phytohormones, was stable in producing shikonin derivatives. The shoots cultured in Gamborg B5 (B5, Gamborg *et al.*, 1968) liquid medium at 25 ℃ in the dark showed the highest shikonin derivative content (2.3 % as dry weight, *ca.* 1.5 mg / flask) (Touno *et al.*, 1998, Touno *et al.*, in press). In cell suspension cultures of *L. erythrorhizon*, it is generally known that shikonin derivative production was inhibited by the addition of NH_4^+ (Fujita *et al.*, 1981a). However, the shoots cultured on solid and in liquid MS medium, which contained high concentration of NH_4^+, produced shikonin derivatives (Touno *et al.*, 1998, Touno *et al.*, in press). These results indicate that there might be a different regulation mechanism involved in shikonin biosynthesis of the cultured shoot stem.

In this paper we describe histological observation of red pigment formed on shoot stem by microscopy. In addition, the expression of LEDI-2, whose function shows a close correlation with shikonin accumulation (Yazaki *et al.*, 1997, 1999), was investigated in the shoot stem.

2. Material and Methods

2.1 Plant material and culture methods

The seeds of *L. erythrorhizon* were surface-sterilized with 75 % ethanol for 30 sec followed by 2 % sodium hypochlorite with Tween 20 (1 drop / 40 m*l*) for 10 min and rinsed with sterilized water 3 times. The axenic shoots germinated from seeds cultured on agar medium (0.5 % agar and 0.5 % sucrose) were maintained on HF Woody Plant (WP, Lloyd and McCown, 1980) solid medium (3 % sucrose, 0.2 % Gelrite, 30 m*l* medium / 4 cm i.d. X 13 cm tube) at 25 ℃ under 14 h / day light (7 μEm^{-2}s^{-1}) for 10 years at Tsukuba Medicinal Plant Research Station, NIHS, Japan.

The shoots (*ca.* 2 cm in length) were horizontally placed on solid medium of either Root Culture (RC, Thomas and Davey, 1982) or MS (containing 0.05 mg / *l* IBA, 3 % sucrose, 25 m*l* medium / 9 cm i.d. petri dish) and cultured at 25 ℃ in the dark. Newly developed shoots which produced shikonin derivatives on stem were subcultured on HF MS solid medium at 25 ℃ in the dark. To cultivate the shoots in liquid medium, shoot tips (*ca.* 2 cm in length) were inoculated in B5 liquid medium (3 % sucrose, 10 m*l* medium / 100 m*l* flask, 60 rpm) for 5 weeks at 25 ℃ in the dark. All media were adjusted to pH 5.7 and autoclaved at 121 ℃ for 15 min.

2.2 Microscopic observation

Surface of the shoot stem was directly observed under a binocular stereo-microscope (SZH, Olympus) and an inverted-microscope (IMT-2, Olympus). For light microscopy, samples were embedded in O.C.T. compound (MILES), freezed at -20 ℃, and cross-sectioned (40-50 μm thickness) by cryostat microtome (MICROM). Sections were immediately observed under a light microscope (VANOX AH-3, Olympus).

2.3 RNA preparation and Northern blot hybridization

Total RNA was isolated using an RNeasy Plant mini kit (QIAGEN) from the shoots (*ca.* 2 cm in length) vertically placed on HF MS solid medium (3 cm i.d. X 13 cm tube) and cultured at 25 ℃ in the dark. The red pigments were not formed on the stem of developed shoots under this condition. RNA sample was also prepared from shikonin-producing stems cultured horizontally on HF MS solid medium (petri dish) and hairy roots cultured in HF MS liquid medium with the same kit. The total RNA (6 μg each) was loaded on a formaldehyde-containing agarose gel (1 %), then blotted onto a nylon membrane Hybond-N (Amersham). The blotted RNA was hybridized in a formamide-containing solution following the standard method (Sambrock *et al.*, 1989). The membrane was probed with the full-length LEDI-2 cDNA.

3. Results and discussion

3.1 Observation by stereo- and inverted-microscopes

We reported the establishment of shoot cultures producing shikonin derivatives on stem. To confirm where shikonin derivatives were produced, stem of shoot cultures (*ca.* 2 cm), which had been cultured in B5 liquid medium (10 m*l* medium / 100 m*l* flask, rotated at 60 rpm) for 5 weeks at 25 ℃ in the dark, was observed under the binocular stereo-microscope. Red pigment was formed on the surface of the stems and stem hairs of the shoot cultures **(Fig. 1 a)**. Similarly in the cases of hairy root cultures of *L. erythrorhizon* transformed with *Agrobacterium rhizogenes* 15834 (Shimomura *et al.*, 1991), roots derived from shoot cultures and intact roots grown in the field, shikonin derivatives were formed only on the surface of the roots and lateral roots.

The red pigments, *i. e.* shikonin derivatives, were released into the medium when the cultured shoots, roots derived from cultured shoots and hairy roots were grown in liquid medium in the dark. A microscopic observation of liquid medium cultured with hairy roots or shoots was then carried out. Red granules containing shikonin derivatives and some cell aggregates holding the red pigments were found in all the liquid media (data not shown). These results indicated that the outer surface of epidermal cells of shoot stem or roots producing red pigments had peeled off due to hydrodynamic stress caused by shaking.

Observation of the shoot stem cultured horizontally on HF MS solid medium by the inverted-microscope showed that the red pigments were formed on the epidermal cells of the stem and stem hairs of shoot cultures **(Fig. 1 b)**. The red pigments were located as oil droplets in the outer surface of epidermal cells **(Fig. 1 b)**. In addition, pigments in stem hairs were observed as granule-like structures. In the case of hairy root cultures, the red pigments were also located on the epidermis as reported earlier (Shimomura *et al.*, 1991). These results indicate that the red pigments biosynthesized on shoot cultures were located in the outer surface of epidermal layer similar to pigments found in intact roots and hairy roots.

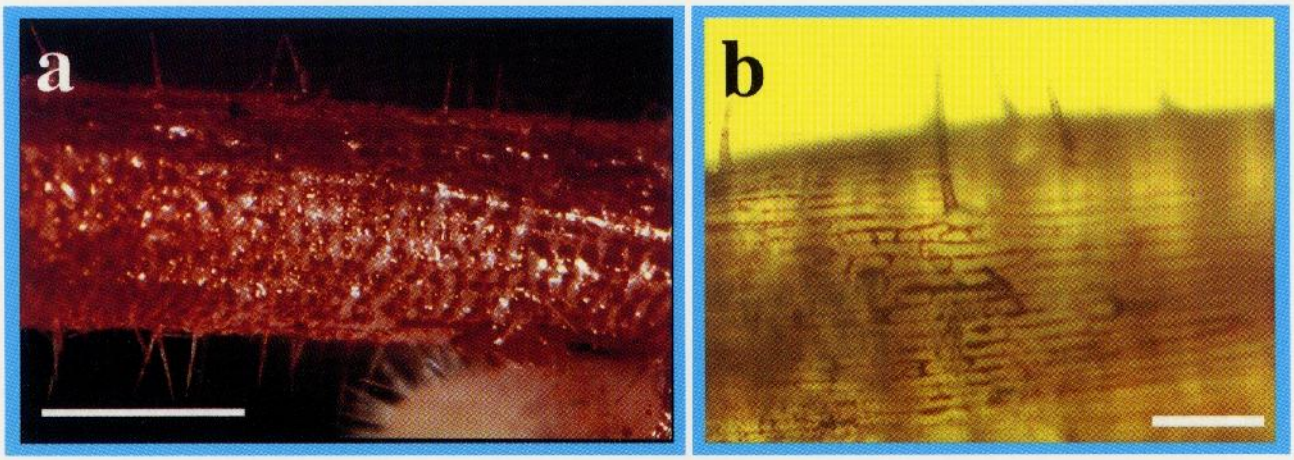

Fig. 1 Stereo-and inverted microscopic observation of red pigment on stem of cultured shoot in *L. erythrorhizon*

a; Stem surface of shoot cultured in B5 liquid medium at 25°C for 5 weeks in the dark. Bar=1 mm b; Stem surface of shoots cultured on MS solid medium at 25°C in the dark. Bar=100 μm

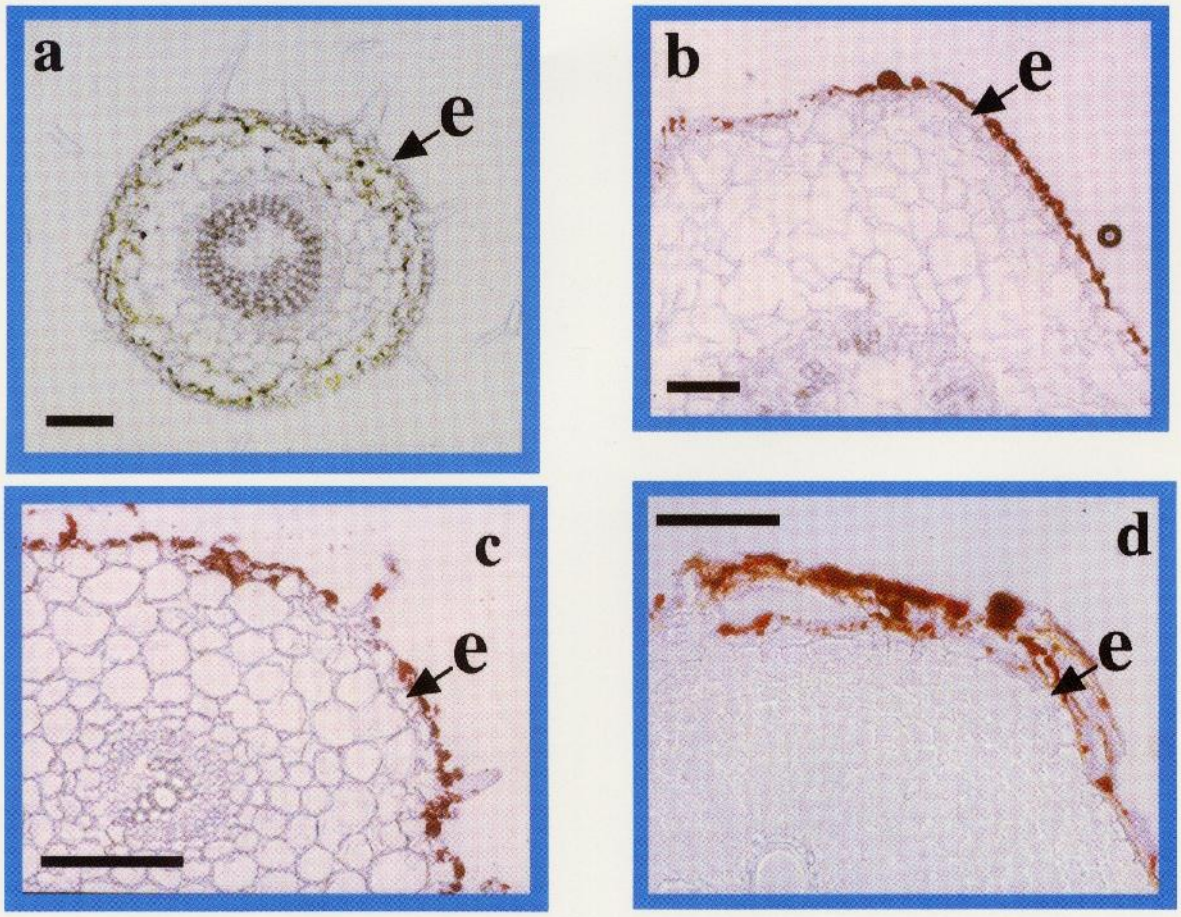

Fig. 2 Cross sections of shoot cultures and field-grown roots

a; Section of plantlet stem cultured on WP solid medium in 14 h/day light. b; Section of shoot stem cultured in B5 liquid medium in the dark. c; Section of plantlet root cultured in B5 liquid medium in the dark. d; Section of 3-month-old plant root cultivated in the field. e; epidermal cell Bars=100 μm

3.2 Microscopic analysis

The cross section of shoot stem were observed under the bright-field microscope. The shoot stem cultured on WP solid medium (4 cm i.d. X 13 cm tube) in the light did not accumulate red pigments but chloroplast development was observed in the cortex (**Fig. 2 a**). On the other hand, stems and roots developed from the shoots cultured in B5 liquid medium in the dark accumulated red pigment and no chloroplast formation was found (**Fig. 2 b** and **2 c**). The stem of cultured shoots accumulating red pigment was morphologically similar to that of shoots cultured in the light (**Fig. 2 a** and **2 b**). In cell suspension cultures, the intracellular localization and secretion process of red pigment were investigated by electron microscope (Tsukada and Tabata, 1984).

By microscopic observation, red pigments on the shoot stem were accumulated as oil droplets in the outer surface of epidermal cells (**Fig. 2 b**). In the case of the roots derived from the shoot cultures or the intact roots cultivated in the field, red pigments were formed in the outer surface of the epidermis (**Fig. 2 c** and **2 d**) as was in shoot stems. These results indicated that the shoot stem had the same potential to produce and to accumulate shikonin derivatives as the root when grown in the dark.

3.3 LEDI-2 expression in shoot stem

Cell suspension cultures of *L. erythrorhizon*, when cultured in M9 medium in the dark, produced shikonin derivatives (Fujita *et al.*, 1981b), whereas the formation of shikonin derivatives was shown to be strongly inhibited in the light. LEDI-2 cDNA, whose expression was specifically observed in the dark, was cloned from cell cultures by a subtractive hybridization technique (Yazaki *et al.*, 1997). The deduced polypeptide sequence shares a significant homology to the known plant proteins specifically expressed in the root, such as ZRP3, RCc2, PVR5 (John *et al.*, 1992, Xu *et al.*, 1995, Choi *et al.*, 1996), but the biological roles of those polypeptides have so far been unknown. Northern blot hybridization and recent functional analyses of LEDI-2 indicated that the gene product of LEDI-2 has a close correlation with the production of shikonin in the cell cultures (Yazaki *et al.*, 1997). To investigate the expression of LEDI-2 in the shikonin-producing stem tissues, we extracted total RNAs from the shoot stems cultured horizontally on MS solid medium (petri dish) in the dark (producing red pigment), the shoot stems cultured vertically on MS solid medium (3 cm i.d. X 13 cm tube) in the dark (no red pigment), and hairy roots cultured in the dark as the positive control (Yazaki *et al.*, 1999).

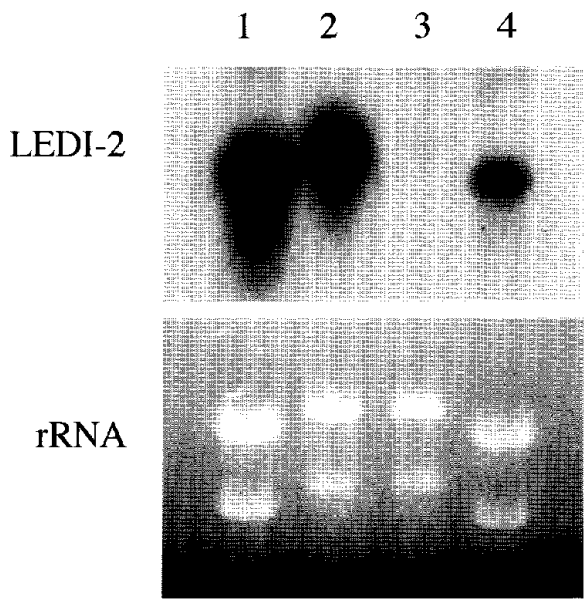

Fig. 3. Northern blot hybridization of LEDI-2 in shoot cultures and hairy roots
Total RNAs (6 μg each) from hairy roots cultured for 7 days (lane 1), stem of shoot cultured on MS solid medium (petri dish) which formed red pigment (lane 2), stem of shoot cultured on MS solid medium (3 cm i. d. X 13 cm tube) producing no red pigment (lane 3) and hairy roots cultured for 14 days (lane 4) were separated in a formaldehyde-containing agarose gel and transblotted to a nylon membrane, and hybridized to a LEDI-2 full-length cDNA as a prode. Ribosomal RNA are shown as the load control.

Northern blot analysis using LEDI-2 full-length cDNA as a probe revealed that LEDI-2 mRNA was highly expressed in shoot stem when red pigments were produced similar to hairy roots (**Fig. 3**). On the other hand, LEDI-2 mRNA was not detectable in shoot stem which did not form the red pigment (**Fig. 3**). This result suggests that LEDI-2 gene which is specifically expressed in the root of an intact plant (unpublished data), can be induced to form in the stem tissue when cultured in the dark, and these shoot cultures will be utilized as an alternative plant material for biochemical and molecular genetic studies on shikonin production.

Acknowledgements

The authors thank Ms. W. Shu (Singapore Polytechnic) for critically reviewing the manuscript.

References

Choi, DW., Song, JY., Kwon, YM., Kim, SG., 1996. Characterization of a cDNA encoding a proline-rich 14 kDa protein in developing cortical cells of the roots of bean (*Phaseolus vulgaris*) seedlings. Plant Molecular

Biology., **30** (5):973-982.

Deno, H., Suga, C., Morimoto, T., Fujita, Y., 1987. Production of shikonin derivatives by cell suspension cultures of *Lithospermum erythrorhizon*. VI Production of shikonin derivatives by a two-layer culture containing an organic solvent. Plant Cell Reports., **6:** 197-199.

Fujita, Y., Hara, Y., Ogino, T., Suga, C., 1981a. Production of shikonin derivatives by cell suspension cultures of *Lithospermum erythrorhizon*. I. Effects of nitrogen sources on the production of shikonin derivatives. Plant Cell Reports., **1:** 59-60.

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

Fujita, Y., Maeda, Y., Suga, C., Morimoto, T., 1983. Production of shikonin derivatives by cell suspension cultures of *Lithospermum erythrorhizon*. III. Comparison of shikonin derivatives of cultured cells and Koshikon. Plant Cell Reports., **2:** 192-193.

Gamborg, OL., Miller, RA., Ojima, K., 1968. Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res., **50:** 151-158.

John, I., Wang, H., Held, BM., Wurtele, ES., Colbert, JT., 1992. An mRNA that specifically accumulates in maize roots delineates a novel subset of developing cortical cells. Plant Molecular Biology., **20** (5):821-831.

Lloyd, GB., McCown, BH., 1980. Commercially feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot-tip culture. Intnl. Plant Propag. Soc. Combd. Proc., **30:** 421-427.

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

Sambrook, J., Fritsch, EF., Maniatis, T., 1989. Molecular cloning. A laboratory manual, second edn. Cold Spring Harbor Laboratory Press. New York.

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

Shimomura, K., Touno, K., Harada, K., Yoshimatsu, K., Yazaki, K., 1998. Red pigment formation on stem of cultured shoots in *Lithospermum erythrorhizon* (Abstract no. 2B-07). XVI Symposium of The Japanese Society for Plant Cell and Molecular Biology.

Thomas, E., Davey, MR., 1982. Plant tissue culture media (5) Root Culture Medium. In EMBO Course on Ti Plasmids, Lab, Genetics Riaks Universiteit, Gent, Belgium, p 109.

Touno, K., Harada, K., Yoshimatsu, K., Yazaki, K., Shimomura, K., 1998. Red pigment formation on stem of cultured shoots in *Lithospermum erythrorhizon*. Effect of phytohormones (Abstract no. 2B-08). XVI Symposium of The Japanese Society for Plant Cell and Molecular Biology.

Touno, K., Harada, K., Yoshimatsu, K., Yazaki, K., Shimomura, K.: Shikonin derivative formation on the stem of cultured shoots in *Lithospermum erythrorhizon*. (Plant Cell Reports, in press).

Tsukada, M., Tabata, M., 1984. Intracellular localization and secretion of naphthoquinone pigments in cell cultures of *Lithospermum erythrorhizon*. Planta Medica., **50:** 338-340.

Xu, Y., Buchholz, WG., DeRose, RT., Hall, TC., 1995. Characterization of a rice gene family encoding root-specific proteins. Plant Molecular Biology., **27**(2):237-248.

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

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

Yazaki, K., Matsuoka, H., Ujihara, T., Sato, F., 1999. Shikonin biosynthesis in *Lithospermum erythrorhizon*: Light-induced negative regulation of secondary metabolism. Plant Biotechnology., **16:** 335-342.