**Lithospermum erythrorhizon** cell cultures: Present and future aspects

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**Abstract**  *Lithospermum erythrorhizon* cell cultures have been used to produce plant secondary metabolites, as well as in biosynthetic studies. Shikonin, a representative secondary metabolite of *L. erythrorhizon*, was first produced industrially by dedifferentiated cell cultures in the 1980s. This culture system has since been used in research on various plant secondary metabolites. Other boraginaceaeous plant species, including *Arnebia*, *Echium*, *Onosma* and *Alkanna*, have been shown to produce shikonin, and studies have assessed shikonin regulation, including transgene expression, in these plants. This review summarizes current knowledge of shikonin production by *L. erythrorhizon* cell and hairy root cultures, including the historical aspect of large-scale production, and discusses future biochemical and biological research using this species.

**Key words:** lithospermic acid B, *Lithospermum erythrorhizon*, plant cell cultures, shikonin derivatives.

**Introduction**

Higher plants produce a large number of secondary metabolites, many of which are utilized in medicines, pesticides, spices, dyes, and fragrances. These compounds generally have complicated structures exhibiting chirality, preventing the cost-effective chemical synthesis of many of these natural compounds. Market demands may be fulfilled with field-grown or cultivated plants (Balandrin et al. 1985; Dicosmo and Misawa 1995). However, many of these plant species cannot be cultivated, and growth for many years, especially of woody plants, may be required to obtain sufficient material (Kieran et al. 1997).

Plant cell culture systems provide an effective method for the stable production of valuable natural compounds, including secondary metabolites. Efforts in the 1970s and 1980s were triggered by the industrial production, by Mitsui Chemicals (formerly Mitsui Petrochemical Industries), of shikonin by cell cultures of *Lithospermum erythrorhizon*. This was the first industrial scale production of a secondary metabolite by dedifferentiated plant cells, and was followed by the large-scale production of other plant products, e.g., the production of the cancer chemotherapeutic agent paclitaxel (Taxol). The key to the successful production of shikonin derivatives is dependent on the development of a production medium tailored for shikonin. In fact, the production medium M9 was optimized solely for the production of shikonin derivatives.

The chemotaxonomy of shikonin derivatives is fairly narrow. These compounds have been detected to occur in some boraginaceaeous plants belonging to the genera *Lithospermum*, *Arnebia*, *Anchusa*, *Echium*, *Onosma* and *Alkanna*. Culture of cells from these other, non-*Lithospermum* plant species in M9 medium, has yielded findings similar to those observed in *Lithospermum* (Malik et al. 2016). This review summarizes previous research on shikonin production by *L. erythrorhizon*, including the mechanisms that regulate shikonin biosynthesis. In addition, this review discusses future aspects of shikonin research, especially research related to its biochemistry and cellular biology.

**Characteristics of Lithospermum erythrorhizon and shikonin derivatives**

*Lithospermum erythrorhizon* Sieb. et Zucc. (Boraginaceae) is a perennial herbaceous plant native to Japan, Korea, and China. Because the root bark (cork layers) of this plant contains large amounts of

**Abbreviations:** 2,4-D, 2,4-dichlorophenoxyacetic acid; 4CL, 4-coumarate:CoA ligase; ARF, adenosine diphosphate (ADP)-ribosylation factor; C4H, cinnamate 4-hydroxylase; DMAPP, 3,3-dimethylallyl diphosphate; ER, endoplasmic reticulum; GBA, m-geranyl-p-hydroxybenzoic acid; GEF, guanine nucleotide exchange factor; GHQ, geranylhydroquinone; GPP, geranyl diphosphate; GUS, β-glucuronidase; HPT, hygromycin phosphotransferase; IPP, isopentenyl diphosphate; LEDI, *L. erythrorhizon* dark-inducible; LEPS, *L. erythrorhizon* pigment callus-specific gene; LS, Linsmaier-Skoog; MJ, methyl jasmonate; PAL, phenylalanine ammonia-lyase; PGT, PHB:geranyltransferase; PHB, p-hydroxybenzoic acid.

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red naphthoquinone pigments (shikonin derivatives; Figure 1), the roots of *L. erythrorhizon* are red-purple in color and have been used as a dye since ancient times. Historically, the purple dye extracted from *L. erythrorhizon* was used to dye clothes of high-class bureaucrats in Japan. In addition, the dried roots have been used as a crude drug (Fujita and Yoshida 1937), including as an ingredient of the ointment ‘Shi-Un-Koh,’ which is used to treat skin disorders, such as wounds, burns, frostbite, swelling, and hemorrhoids (Ootsuka et al. 1972). The effectiveness of this ointment has been described in detail (Hayashi 1977a). Shikonin and its fatty acid ester derivatives, like acetylshikonin, occurring naturally in *L. erythrorhizon* roots, have shown various pharmacological activities, including antibacterial (Kyogoku et al. 1973; Tabata et al. 1982; Tanaka and Odani 1972), wound-healing (Hayashi 1977b), anti-inflammatory (Hayashi 1977c), tumor-inhibiting (Papageorgiou 1980; Sankawa et al. 1977), anti-angiogenic (Hisa et al. 1998), and anti-topoisomerase (Ahn et al. 1995) properties. *L. erythrorhizon* roots have also been used in the traditional, orally administered anti-tumor medicine ‘Shikon-Borei-Toh.’

The chemical structure of shikonin, first proposed in 1918 (Kuroda 1918), was finally determined to be an optical isomer of alkannin (Brockmann 1935), with shikonin being the R-form and alkannin the S-form (Arakawa and Nakazaki 1961). However, naturally occurring shikonin isolated from the roots and callus cultures of *Lithospermum* was later found to be a mixture of 84–93% R-form and 7–16% S-form (Papageorgiou et al. 1999). Other sources of natural naphthoquinone pigments include the roots of *Macrotomia euchroma* Pauls and *Alkanna tinctoria*, although these components are ester derivatives of alkannin. The absolute configurations of these compounds have little effect on
their wound healing and anti-inflammatory activities (Seto et al. 1992).

The properties of shikonin derivatives are dependent on their handling. Red pigments in native form are extracted from dried materials, including roots and cultured cells. If shikonin is extracted from fresh tissues, it gradually darkens over several days, finally becoming black precipitates, which are thought to be polymers. Contact of shikonin solution with metals tends to result in the formation of insoluble complexes. For example, the ash of Camellia trees, which contains high amounts of Al, is used as a dye mordant; when mixed with shikonin solution, it forms of a beautiful purple color, which is utilized to dye clothes.

L. erythrorhizon, the representative source of shikonin, has faced the risk of extinction in recent years, and its cultivation is very difficult. Its germination rate is low, its seedlings are very susceptible to infection by viruses, and the plants are sensitive to disinfectants. According to the Amato Pharmaceutical Company, which expended considerable effort to maintain this plant species, desirable conditions for its cultivation in the field include moderate sunlight and relatively low nutrition, as well as special care conditions for its cultivation in the field include moderate sunlight and relatively low nutrition, as well as special care to prevent its lower leaves from contact with soil upon watering (personal communication). Commercial use of this plant requires growth for three years to obtain a sufficient quantity of roots, from which crude drugs can be prepared, but growth for three years is usually very difficult. However, the yield of shikonin by chemical biosynthesis was only 0.7%, an amount insufficient for the industrial production of this compound (Terada et al. 1983).

Establishment of a high shikonin-producing line and production medium M9

Callus tissues of L. erythrorhizon were originally derived from seedlings and were grown on Linsmaier–Skoo (LS) basal (Linsmaier and Skoog 1965) agar medium containing 10^{-6} M 2,4-dichlorophenoxyacetic acid (2,4-D) and 10^{-5} M kinetin. The tissues were subsequently subcultured in the same medium containing 10^{-6} M indole-3-acetic acid (IAA) in place of 2,4-D and grown in the dark at 25°C, to produce the red pigmented shikonin derivatives (Mizukami et al. 1977; Tabata et al. 1974). The chemical composition of shikonin derivatives in callus cultures were the same as in the intact roots (Mizukami et al. 1978). Repeated cell selection of L. erythrorhizon callus cultures resulted in a high-yielding cell line, M18, with a shikonin content of 1.2 mg/g fresh weight of cells, a 20-fold increase over the original strain (Mizukami et al. 1978). Selection of 478 single-cell clones from a single callus yielded eight relatively stable cell lines, with an average shikonin content of 2.3 mg/g fresh weight (4.8% of dry weight), higher than the shikonin content of intact roots (ca. 1.3%) (Tabata et al. 1978). This strain was subsequently used for large-scale cell cultures.

The development of an industrial scale shikonin production system (Tabata and Fujita 1985) was dependent on the development of the M9 pigment production medium (Fujita et al. 1981a, 1982). This medium was designed by thoroughly optimizing the constituents present in White's medium (White 1954). Essentially, M9 medium does not contain ammonium ions, which are abundant in LS medium, while having a >10-fold greater concentration of Cu^{2+} ion than LS medium. The process of developing M9 medium led to the identification of many chemical and physical regulatory factors (Fujita et al. 1981b; Fukui et al. 1983; Kim and Chang 1990) (Table 1). Positive regulators include methyl jasmonate (MJ), acidic oligosaccharide, Cu^{2+} and cold temperature (<21°C), whereas negative regulators include light, NH_{4}, ethanol, and high temperatures (Tabata et al. 1974).
temperature (>28°C). The regulatory mechanisms are complex. For example, even in the presence of a high concentration of NH₄⁺ in LS medium, the presence of acidic oligosaccharide may induce shikonin production, explaining the feasibility of callus selection on LS agar medium (Tani et al. 1992, 1993). MJ is also a strong inducer of shikonin production in LS medium (Gaisser and Heide 1996; Yazaki et al. 1997). Light is the greatest inhibitor of shikonin production, almost completely inhibiting its production in M9 medium. Blue light had the strongest inhibitory effect (Yamamoto et al. 2002), suggesting the involvement of a flavin-linked enzyme in shikonin biosynthesis or of a flavoprotein in signal transduction (Tabata et al. 1993). Details of the effects of these factors and other regulatory elements are described elsewhere (Touno et al. 2000, 2005; Yazaki et al. 1999; Yoshikawa et al. 1986).

**Large-scale production of shikonin**

Because M9 medium was optimized solely for shikonin production, it is inferior to LS medium in promoting cell growth due to lack or low concentrations of essential nutrients, such as phosphate, in M9 medium. Industrial scale production of shikonin utilizes a “two-stage culture system” of *L. erythrorhizon* cells. In the first stage, cells of the high-producing strain M18 are allowed to proliferate in “growth medium”, without producing shikonin derivatives; in the second stage, these cells were transferred to M9 medium for shikonin production (Fujita et al. 1982) (Figure 2). To improve cell growth during the first stage, each component of LS medium was optimized, with the resulting “growth medium” MG-5 containing one-third of the original concentrations of Ca²⁺, NH₄⁺/(NH₄⁺+NO₃⁻), SO₄²⁻, and BO₃⁻. The cell yield was 15% higher with MG-5 medium than with LS medium, while the use of both MG5 and M9 media in the dual-culture system resulted in an 11.5-fold increase in shikonin yield compared with growth in the LS/White dual culture system.

To produce shikonin on an industrial scale, cultured cells were grown in 200 l fermentation tank containing MG5 medium for 9 days. The cultures were subsequently filtered to remove MG5 medium, and the cells were transferred to a second 750 l tank containing M9 and cultured for 14 days. The red-colored cells were harvested by filtration and dried, and the red pigment was extracted from the harvested cells with *n*-hexane. The chemical composition of the extracted shikonin derivatives was very similar to that of the intact roots. The differences in the relative amounts of each shikonin derivative between the extracts and the intact roots were much lower than the differences between batches of intact roots purchased in the market (Tabata and Fujita 1985). To isolate free shikonin, the extracted red pigments were hydrolyzed with 2% KOH, which turned the shikonin blue (Papageorgiou et al. 1999). The aqueous solution was acidified, and free shikonin was recovered by extraction into the organic phase. Pure shikonin was subsequently obtained by recrystallization.

Comparing the yields of shikonin by plant cultivation (48 months, shikonin content 1.4%) and cell culture (0.8 month, 20%) indicated that the latter method is about 800 times more productive than the former. Another calculation showed that a 750 l culture tank containing 600 l medium can produce 2 g/l shikonin in 2 weeks.
This was equivalent to the yield over 4 years from a field of 17.6 hectares, with *L. erythrorhizon* plants cultivated at a density of 3.5 plants/m², resulting in 25 g of intact roots per plant with a shikonin content of 1.0%. Shikonin production by plant cell culture was far more economical than by chemical synthesis (Terada et al. 1983, 1987).

Beginning in 1984, shikonin produced by this method was used commercially in Japan for cosmetics, such as lipsticks, lotions, and soaps; at present, however, these products are not available.

**Shikonin biosynthetic pathway and key regulatory enzymes**

**Removal of shikonin from living cells with liquid paraffin**

Shikonin derivatives are highly accumulated on cell surfaces. These naphthoquinone compounds strongly inhibit many enzymes, as well as interfere with the isolation of pure RNA and DNA. Thus, biochemical studies of shikonin biosynthesis require removal of shikonin from living cultured cells prior to their homogenization. To overcome this problem, liquid paraffin was overlaid on M9 medium throughout the entire culture period to remove shikonin pigments from the cell surface (Heide and Tabata 1987a). Cell growth was not affected by the presence of 3–10 ml paraffin in 30 ml M9 medium in a conical flask, and production of shikonin derivatives was only slightly reduced. Most of the shikonin derivatives move to the paraffin layer, whereas pigments remaining on the cells can be largely reduced during early growth phase. This process was necessary to obtain active enzymes from cells producing shikonin and was also advantageous for the preparation of high quality RNA samples.

**Biosynthetic pathway and its regulation**

Early studies of shikonin biosynthesis involved tracer experiments in *Lithospermum* callus cultures, showing that shikonin was biosynthesized through the prenylation of *p*-hydroxybenzoic acid (PHB), which was derived from *L*-phenylalanine and two molecules of mevalonic acid (Inouye et al. 1979). This pathway was analogous to that of alkannin in *Plagiotroty arizonicus* (Schmid and Zenk 1971). Many subsequent studies have elucidated the shikonin biosynthetic pathway (Figure 3). Two key precursors of shikonin, geranyl diprophosphate (GPP) derived via the mevalonate pathway (Li et al. 1998b) and PHB derived via the shikimate pathway (Inouye et al. 1979), are coupled by a geranyltransferase to yield the intermediate, *m*-geranyl-4-hydroxybenzolic acid (GBA) (Heide and Tabata 1987b). GBA is subsequently converted to geranylhydroquinone, followed by the hydroxylation of the geranyl chain (Yamamoto et al. 2003). The formation of a naphthalene ring by this intermediate yields shikonin, whereas cyclization of the geranyl moiety to form a furan ring yields dihydroechinofuran (Yazaki et al. 1986a). Thus, the formation of the naphthalene ring is regarded as critical for shikonin synthesis, although details of the latter half of the biosynthetic route have not yet been clarified. One exception is the finding that deoxyshikonin, a shikonin derivative lacking the hydroxyl residue on the side chain, is a biosynthetic precursor of acetylshikonin, as shown by tracer experiments with radio-labeled phenylalanine and deoxyshikonin (Okamoto et al. 1995), suggesting that the oxygen atom on the side chain of shikonin is introduced during the last step of shikonin formation. It is to be noted that the biosynthesis takes place in symplast, while the final products, shikonin derivatives, are accumulated in apoplast.

Several genes encoding shikonin biosynthetic enzymes have been identified in *L. erythrorhizon*. With regard to the biosynthesis of PHB, cDNAs encoding genes involved in the general phenylpropanoid pathway, including phenylalanine ammonia-lyase (PAL) (Yazaki et al. 1997), cinnamate 4-hydroxylase (C4H) (Yamamura et al. 2003), and 4-coumarate:CoA ligase (4CL) (Yazaki et al. 1995), have been isolated. These three enzymes are almost constantly expressed in cultured *L. erythrorhizon* cells. The enzyme involved in coupling these aromatic and prenyl substrates, PHB:geranyltransferase (PGT) (Heide and Tabata 1987b; Inouye et al. 1979), was found to require a divalent cation, with Mg²⁺ being optimal, while its optimal pH ranged widely from 7.1 to 9.3. This enzyme was highly specific for both substrates, PHB and GPP, and its activity was 35 times higher in shikonin-producing than in non-producing cells. This enzyme was detectable only in the microsomal fraction with a density (ρ) of 1.09–1.10 g·cm⁻³, similar to that of the endoplasmic reticulum (ER) (Heide and Tabata 1987b). Because conventional purification of this membrane protein was difficult (Mühlenweg et al. 1998), we used homology-based PCR to isolate cDNAs encoding PHB-geranyltransferase. These cDNAs, LePGT-1 and -2, were isolated by using primers for the conserved amino acid sequences of the PHB-polyprenyltransferase (gene: *coq2*) family involved in ubiquinone biosynthesis (Yazaki et al. 2002). The transcriptional regulation of PGT was found to contribute to the regulation of shikonin biosynthesis, including enhancement by MJ and suppression by light and ammonium ions.

Regulation of the quantity of prenyl chain was found to parallel the induction of shikonin production. HMG-CoA reductase, which regulates the mevalonate pathway in many organisms, was found to catalyze a rate-limiting reaction for GPP biosynthesis in *L. erythrorhizon* (Köhle et al. 2002; Lange et al. 1998). The regulatory pattern of gene expression was similar to that of PGT. In contrast, GPP synthase, which catalyzes the synthesis of GPP from
isopentenyl pyrophosphate (IPP) and 3,3-dimethylallyl diphosphate (DMAPP) in the presence of Mg\textsuperscript{2+} or Mn\textsuperscript{2+}, was constitutively active in *L. erythrorhizon* cell suspension cultures (Heide 1988; Heide and Berger 1989). This enzyme differed from other plant GPP synthases, in that it localized to the cytosol and utilized as substrates IPP and DMAPP from the mevalonate pathway (Li et al. 1998). In contrast, all other GPP synthases localized to plastids and were involved in non-mevalonate pathways.

Geranylhydroquinone 3‴-hydroxylase, which provides a common intermediate for shikonin and dihydroechinofuran from geranylhydroquinone (GHQ), was identified in suspension cultures of non-pigmented cells (Yamamoto et al. 2000a). This enzyme, which introduces a hydroxyl residue to the isoprenoid side chain of GHQ at position 3‴, localizes to the microsomal fraction. Further analyses suggested that this enzyme was a cytochrome P450 dependent monooxygenase with high affinity for GHQ (*Km*= 1.5 µM).

Although these enzyme activities have been reported involved in shikonin biosynthesis, the genes encoding these proteins have not yet been identified. Proteins involved in this pathway include GPP synthase (Sommer et al. 1995), PHB O-glucosyl transferase (Bechthold et al. 1991; Li et al. 1997; Yazaki et al. 1995) and geranylhydroquinone 3‴-hydroxylase (Yamamoto et al. 2000a). Molecular biological approaches are required to isolate these genes, including their regulation by light in cultured *L. erythrorhizon* cells.

**Gene determination by subtractive hybridization**

The finding, that shikonin production is reversibly regulated by irradiation with light, suggested cDNAs potentially related to shikonin production in cultured *L. erythrorhizon* cells could be isolated by conventional subtractive hybridization. The genes preferentially expressed in the dark were designated *L. erythrorhizon* dark-inducible (LEDI) genes. These genes included the oxidoreductase-like genes LEDI-3 through -5 (Yazaki...
et al. 1999), as well as LEDI-2, which encodes a small polypeptide of 114 amino acids and shows the strictest dark-selectivity (Yazaki et al. 2001). These dark-selective genes also included a fragment of LePGT. However, biosynthetic enzymes encoded by the genes described in the above paragraph were not found.

We therefore utilized a more powerful technique, PCR-selected subtraction, to search for dark-inducible genes. Of about 800 candidate clones, 240 showed dark-induction. These included several biosynthetic genes previously identified by other methods, including genes encoding LePGTs, HMG-CoA reductase and LEDIs (our unpublished data). In addition, more systematic transcriptome information has been obtained by RNA-seq analysis. We intend to expand this analysis to identify genes involved in the later regulation of shikonin biosynthesis.

Other metabolites highly accumulated in L. erythrorhizon cells

Intact L. erythrorhizon plants produce caffeic acid derivatives, such as rosmarinic acid (Figure 3), and cultured L. erythrorhizon cells were shown to produce this caffeic acid dimer (Mizukami et al. 1992, 1993). Moreover, transfer of L. erythrorhizon cells from LS medium to M9 medium resulted in an increase in production of lithospermic acid B, a caffeic acid tetramer, comparable to the level of shikonin derivatives (Yamamoto et al. 2000b). In addition, lower amounts of other caffeic acid derivatives like rabdosin were detected in M9 medium (Fukui et al. 1984; Yamamoto et al. 2000c). A striking difference, however, was seen upon illumination, which strongly inhibited the production of shikonin but not of lithospermic acid B (Yamamoto et al. 2002). Another difference between lithospermic acid B and shikonin derivatives were their sites of accumulation, with lithospermic acid B accumulating inside cells, presumably in vacuoles, in contrast to shikonin derivatives, which accumulate on cell surfaces. Lithospermic acid B is biosynthesized by branching from 4-coumaric acid or its CoA ester, however, thus sharing a common phenylpropanoid pathway with shikonin (Figure 4).

Secretion of Shikonin

Intracellular biosynthesis and transport of shikonin

The aromatic shikonin precursor PHB is derived from shikimate, which localizes to plastids, whereas another key intermediate, GPP, is produced in the cytosol via the mevalonate pathway. The enzyme coupling both substrates, LePGT, localizes to the ER. In LS medium or under illumination in M9 medium, however, the expression of PGT is strongly suppressed, with excess amounts of PHB being glucosylated and accumulating in vacuoles (Yazaki et al. 1986b, 1995). UDPG:PHB glucosyltransferase was purified from shikonin-free L. erythrorhizon cell cultures (Bechthold et al. 1991) and shown to localize in the cytosol (Yazaki et al. 1995). If shikonin production ceases, the biosynthesis of GPP, another key intermediate, is downregulated at the HMG-CoA reductase step, thereby avoiding the over-accumulation of GPP. The geranylated intermediate is hydroxylated by cytochrome P450, but the steps and cellular location involved in ring formation, resulting in the production of shikonin or dihydroechinofuran, have not yet been determined (Figure 3).

Little information is available about the transport mechanisms of shikonin derivatives. An early electron microscopic analysis of shikonin-producing L. erythrorhizon cells suggested that shikonin pigments or their intermediates accumulated in “secretion vesicles”, which originate from electron dense, spherical swellings formed in highly elongated rough ER (Tabata et al. 1982; Tsukada and Tabata 1984). These secretion vesicles are thought to be present in the microsomal fraction (ρ = 1.09–1.10 g·cm⁻³) containing PHB-geranyltransferase activity (Yamaga et al. 1993), because in vitro incubation of ¹⁴C-labeled deoxyshikonin with these vesicles resulted in the generation of ¹⁴C-labeled...
shikonin and 14C-labeled shikonin esters, including acetyl- and β-hydroxyisovaleryl-shikonin, indicating that these vesicles contain enzymes responsible for both the hydroxylation and acylation of deoxyshikonin (Okamoto et al. 1995). Moreover, granules attached to the cell surface were thought to contain shikonin derivatives secreted from these cells (Tsukada and Tabata 1984). Because these electron micrographs were taken after chemical fixation, the granules appeared to be empty, suggesting that lipophilic substances are removed by the dehydration process but that these structures are surrounded by membranes. Thus, intracellular shikonin vesicles were thought to fuse to plasma membranes and form extracellular granules containing high amounts of shikonin derivatives (Tabata 1996) (Figure 4).

There are, however, several drawbacks to this model of shikonin derivative secretion. First, as shikonin derivatives are very hydrophobic, these shikonin vesicles are surrounded by a phospholipid monolayer rather than a lipid bilayer like oil bodies. Those oleophilic particles may not simply fuse to plasma membranes, because fusion would result in a strong phospholipid imbalance between the inner and outer leaflets. Second, this model cannot explain the origin of the large quantities of membrane lipids observed in large extracellular granules or the high shikonin production rate, amounting to about 10% of cell weight. Third, this model cannot explain how these shikonin-containing structures on plasma membranes cross the cell wall, as such images were not observed.

Our recent report may, however, provide a key to the mechanism underlying the secretion of shikonin derivatives. Using hairy roots of *L. erythrorhizon* (Figure 5), a model system closer to intact roots than dedifferentiated cells, we observed similar extracellular granules on epidermal cells, along with many small vesicle-like structures inside cell walls (Tatsumi et al. 2016). This study also showed that shikonin secretion was stopped by depolymerization of actin filaments or inhibition of the adenosine diphosphate (ADP)-ribosylation factor/guanine nucleotide exchange factor (ARF/GEF) system, suggesting that shikonin secretion utilizes, at least partly, the same pathway as the sec system (Tatsumi et al. 2016) (Figure 4).

To date, two genes have been reported to be associated with the shikonin secretion process. *LeDI-2*, which was isolated by subtractive hybridization, encodes a small, membrane-bound polypeptide. The expression pattern of *LeDI-2* paralleled that of shikonin production. Suppression of *LeDI-2* by antisense RNA also suppressed the shikonin accumulation, but did not affect membrane-bound LePGT enzyme activity (Yazaki et al. 2001). Another small protein, LEPS-2, containing 184 amino acids, was cloned by differential display between shikonin-producing and non-producing strains (Yamamura et al. 2003). LEPS-2 expression was also closely associated with shikonin production. Moreover, LEPS-2 was found to be a cell wall protein, suggesting

Figure 5. Hairy roots of *L. erythrorhizon*. (A) Hairy root cultures in M9 medium under illumination (left) and in the dark (right). (B, C) Longitudinal sections of a hairy root grown under illumination (B) and in the dark (C). Shikonin derivatives accumulated in epidermal cells. (D) Whole mount picture of a GUS transformant of an *L. erythrorhizon* hairy root. Scale bars are 20 μm.
its involvement in the accumulation of shikonin pigment in cell walls. To date, however, details of the biochemical functions of LEDI-2 and LEPS-2 remain unknown.

**Stable transformation of L. erythrorhizon**

Establishment of a stable transformation method is in general a hurdle for non-model plants, such as *L. erythrorhizon*, but it can improve the productivity of secondary metabolites by genetic engineering and enable reverse genetic methods for the functional analyses of genes. Attempts to stably transform cultured cells have been unsuccessful. In 1998, we utilized intact shoots and *Agrobacterium rhizogenes* (ATCC C15834) to introduce desired genes into hairy roots of *L. erythrorhizon* (Yazaki et al. 1998) (Figure 5). Briefly, a foreign gene was introduced into *A. rhizogenes* prior to infecting the plants; following infection, this gene would integrate into the plant genome, in parallel with the *rol* genes of the Ri plasmid. Root tissue is the specific site for the production and accumulation of shikonin derivatives in intact plants, and the synthesis of shikonin derivatives has been detected in hairy roots (Shimomura et al. 1991). Moreover, no special apparatus was needed for transformation via direct infection with *A. rhizogenes*. A binary vector containing a gene encoding β-glucuronidase (GUS), driven by a CaMV35S promoter, as well as a gene encoding hygromycin phosphotransferase (HPT) as a selection marker was constructed to show that a desired foreign gene could be expressed in *L. erythrorhizon* hairy roots. The survival rate on hygromycin plates depended on the gene subcloned into the binary vector, being 20%, 25%, and 50% for GUS (Yazaki et al. 1998), *ubiC* (Sommer et al. 1999), and LEDI-2-antisense (Yazaki et al. 2001), respectively. Histochemical analyses of the transgenic hairy roots using the GUS reporter gene showed that the CaMV35S promoter was active in most of the root tissues (Figure 5).

Shikonin production patterns and responses to various regulatory factors of hairy roots in M9 medium are generally similar to those observed in dedifferentiated cells (Yazaki et al. 1998), suggesting that hairy roots provided an alternative plant material to study shikonin biosynthesis and transport in *L. erythrorhizon*. For example, this transformation system was utilized in a reverse-genetic study to show that the LeDI-2 gene was involved in shikonin production (Yazaki et al. 2001). Practical differences between cultured cells and hairy root cultures are summarized in Table 2.

**Future aspects**

Current ‘omics’ technologies provide very powerful tools to analyze expressed genes and proteins as well as synthesized metabolites. We have used these techniques to thoroughly analyze biological events that occur in cultured cells and the hairy roots of *L. erythrorhizon*. Transcriptomics data in several shikonin-producing boraginaceous plants were recently published (Wu et al. 2017). Active competition in this field will accelerate the progress in understanding the pathways and regulatory mechanisms of shikonin biosynthesis and transport.

Natural plant products may include large numbers of valuable, potentially useful resources. Along with conventional plant cell culture systems involved in the production of compounds like shikonin, natural compounds may be produced by metabolic engineering. For example, foreign genes can be introduced into various host plants, or expressed in microorganisms (Yazaki 2004). Although the production of these secondary metabolites requires basic knowledge of their biosynthetic pathways, the pathways involved in the biosynthetic reactions of certain secondary metabolites have not been completely elucidated. Further basic studies are required for successful metabolic engineering and synthetic biology. Moreover, efforts are needed to determine the mechanisms involved in the accumulation of these natural products in plant cells, because secondary metabolites often accumulate in specific organelles like vacuoles, or in specific cell layers such as the epidermis, and can be transported from source cells to sink organs (Yazaki 2005, 2006). Integrated studies of the biosynthesis and transport of these secondary metabolites may improve future metabolic engineering.

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