Characterization of shikonin vesicle transport mechanisms in *L. erythrorhizon* root cultures

Mariko Yano
Labolatory of Plant Gene expression, RISH, Kyoto University

Lithospermum erythrorhizon Sieb. et Zucc. (Boraginaceae) is a medicinal herb that accumulates red naphthoquinone pigments shikonin derivatives in roots. These compounds show antibacterial, wound-healing and anti-tumor activities and the dried roots are used as natural medicine. Two key precursors, *p*-hydroxybenzoic acid (PHB) derived from the phenylpropanoid pathway, and geranyl pyrophosphate synthesized through the mevalonate pathway, couple to form *m*-geranyl *p*-hydroxyl benzoic acid, and further reaction such as ring-forming and oxidation lead the formation of shikonin. It was reported that shikonin biosynthesis in *L. erythrorhizon* suspension cultures were regulated by various elements, such as Cu^{2+} ion, methyl jasmonate and oligogalacturonide which induced shikonin production, whereas ammonium ion, glutamine and light irradiation inhibited shikonin formation. In particular, light is the strongest inhibitor of shikonin biosynthesis.

Shikonin is secreted out of the cell presumably via vesicle transport pathway from ER to plasma membrane. In *L. erythrorhizon* pigment-producing cells, a large number of spherical bodies (0.1-0.2 um) or swellings which were filled with substance of high electron density were specifically found in ER by electron microscopic observation. To investigate shikonin transport by a molecular biological approach, genetic viewpoint, we applied PCR-select subtraction to *L. erythrorhizon* cells cultured in dark (shikonin producing) or light irradiation (non-producing) to isolate genes involved in shikonin production systematically.

In the PCR-select subtraction, we picked up 242 clones that were induced in the dark, whereas only 7 clones were obtained as UP-regulated genes under illumination, which were more than 5 times down or up regulated out of 1152 clones investigated. The high preference for dark-inducible genes was a characteristic for *L. erythrorhizon* cultured cells that synthesize the lipophilic secondary metabolite specifically in root. The BLAST search revealed that 70% of dark-inducible 242 genes were coding for metabolic enzymes, in which known shikonin biosynthetic enzymes like Le-PGT (*L. erythrorhizon* PHB Geranyltransferase) and HMG-CoA reductase. Beside these biosynthetic genes, 3% were transporters, 4% were receptors, transport signals, and transcription factors, as well as 23% of genes of unknown. However no genes related to vesicle transport were found in the list, suggesting that capable of transporting shikonin were not regulated by light at reast at the transcriptional level.

To thoroughly analyze genes involved in shikonin production, we carried out EST analysis of *L.erythrorhizon* cells in shikonin-producing condition. BLAST analysis with 10,000 clones revealed that adding to some known shikonin biosynthetic enzymes like Le-PGT or HMGCoA reductase, many genes relevant to vesicle transport were obtained. They are classified into several groups, i.e., COP II components which carry transport vesicles from ER to Golgi anterograde pathway, COP I components which transport vesicles from Golgi to ER retrograde pathway, clathrin heavy and light chains which play important role in various route of vesicle transport, and clathrin adopter proteins.

As shikonin derivatives are very hydrophobic compounds, they are presumed to be compartmented in lipid monolayer vesicles. All known vesicle transport mechanisms so far are elucidated for lipid bilayer vesicles, and lipid monolayer vesicle transports is yet unidentified. However, these maybe some common protein members which COP I, COP II, and clathrin-mediated vesicles taking part in the intercellular transport of lipid monolayer vesicles in *L. erythrorhizon*. I expect that EST information obtained in this study will contribute to clarify the shikonin vesicle transport mechanism at molecular revel.