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Biosynthetic system of camptothecin: An anticancer plant product*

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Abstract: Camptothecin is one of the clinically used anticancer compounds derived from plants. We have established a hairy root culture *Ophiorrhiza pumila*, which efficiently produces camptothecin. The strictosidine synthase cDNA was obtained from *O. pumila*, and its properties were characterized using recombinant protein expressed in *Escherichia coli*. The mechanisms of camptothecin transport and self-resistance of producing plant cells have also been investigated. These studies offer a basis for pathway engineering of camptothecin in the future.

Keywords: anticancer alkaloids; camptothecin; hairy roots; *Ophiorrhiza pumila*; strictosidine synthase.

INTRODUCTION

When we look at how many new drug entries are derived from natural products, still nearly half of them from natural origin [1], we are always astonished by the how much man enjoys the benefits of the huge chemical diversity of plants for medicines, flavors, and other specialized compounds. As anticancer drugs, four plant products, i.e., vincristine/vinblastine, paclitaxel (taxol), podophyllotoxin, and camptothecin, are used currently in the clinical field. Camptothecin, a pentacyclic quinoline alkaloid, has been discovered through an extensive screening by the U.S. National Cancer Institute [2]. This compound exhibits an antitumor activity due to its inhibitory action to DNA topoisomerase I [3]. At present, semisynthetic water-soluble camptothecin analogs, topotecan and irinotecan, are prescribed as clinical antitumor drugs throughout the world. The worldwide market size of irinotecan/topotecan in 2003 was estimated at about US$1 billion [4]. Despite the rapid growth of the market, camptothecin is still obtained by the extraction from natural resources such as the seeds of *Camptotheca acuminata* and the bark of *Nothapodytes foetida*. For the stable supply of camptothecin and for the development of more potent anticancer compounds, the biotechnological investigation of the biosynthesis of camptothecin is urgently anticipated [5]. As illustrated in Fig. 1, camptothecin is synthesized from tryptophan and geranyldiphosphate (GPP) as the precursors via strictosidine as a key intermediate. Thus, though camptothecin is structurally classified as a quinoline alkaloid, it belongs to a family of monoterpenoid indole alkaloids from the viewpoint of biosynthesis. A number of studies on biotechnological production of camptothecin have been reported (for reviews, see refs. [4–6]). These are

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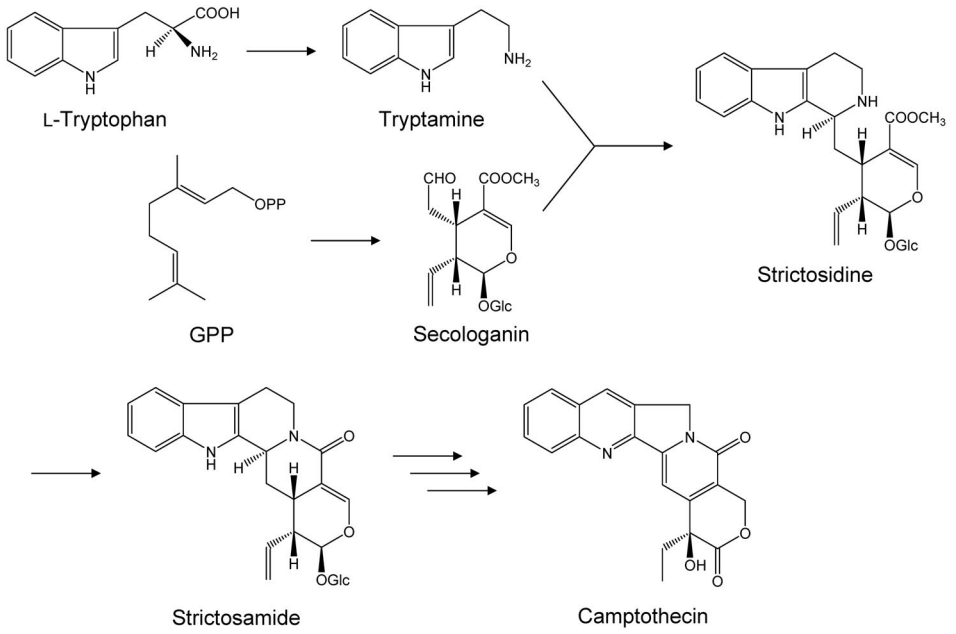


Fig. 1 Biosynthetic pathway of camptothecin.

mostly focused on cell/tissue cultures including transformed hairy roots (as described below) or adventitious roots [7] of the plants which accumulate camptothecin. The investigation of organogenesis of the explants derived from the camptothecin-producing plants has been conducted (for reviews, see refs. [4–6,8]). These studies may potentially be useful for propagation of elite plants for a more feasible supply of camptothecin from cultivated plants.

RESULTS AND DISCUSSION

Biosynthetic system by hairy roots: Feasible production and pathway elucidation

Although camptothecin was originally isolated from a Chinese tree, *C. acuminata* (Nyssaceae), this compound has been known to be distributed in a relatively wide range of plant species, 12 species in 5 families, such as *N. foetida* (Icacinaceae), *Ophiorrhiza pumila*, *O. liukiuensis*, *O. mungos* (Rubiaceae), *Merrilliodendron megacarpum* (Icacinaceae), and *Ervatamia heyneana* (Apocynaceae) [9–14].

We have established a hairy root culture of *O. pumila* incited by infection of *Agrobacterium rhizogenes* [15]. This hairy root culture grew rapidly by 16-fold increase of the weight during 5 weeks in liquid culture, and it produced camptothecin as a main alkaloid up to 0.1 % per dry weight of the cells. The culture also produces a high level of anthraquinones besides camptothecin. Although camptothecin was accumulated in all differentiated plant tissues and hairy roots but not in calli of *O. pumila*, anthraquinones regarded as phytoalexins were present in the hairy roots and calli but not in the differentiated plants tissues [16]. Interestingly, camptothecin was present not only in hairy root cells, but excreted out to medium at a substantial amount. Camptothecin content in the medium was increased by the presence of a polystyrene resin (Diaion HP-20) that absorbed camptothecin. Then, camptothecin of substantially high purity could be easily recovered from the resin simply by eluting with methanol [15]. Two new camptothecin-related alkaloids have been isolated from *O. pumila* hairy roots [17]. By using the hairy root cultures, the tracer study using ^{13}C-glucose has been conducted to show the operation of

combination of the 2C-methyl-D-erythritol 4-phosphate pathway and the shikimate pathway for the biosynthesis of camptothecin [18].

Besides *O. pumila*, adventitious roots of *O. prostrata* were shown to produce a relatively high level of camptothecin [19]. The hairy roots of *O. liukiuensis* and *O. kuroiwai* have also been established and exhibited to accumulate camptothecin [20]. Interestingly 10-methoxycamptothecin was only produced in tissue cultures of *O. liukiuensis* and *O. kuroiwai* [20]. Since 10-methoxycamptothecin is presumed to be an efficient synthetic precursor of topotecan and irinotecan, tissue cultures of *O. liukiuensis* and *O. kuroiwai* would be useful sources for production of 10-methoxycamptothecin for further development of camptothecin analogs.

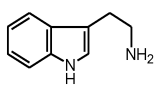
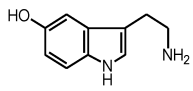
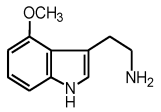
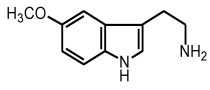
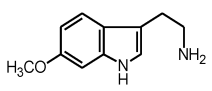
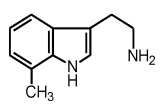
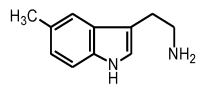
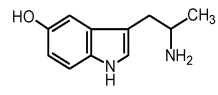
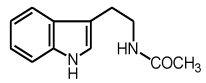
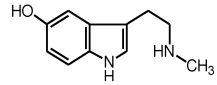
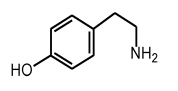
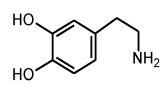
Semi-large-scale production of camptothecin was achieved by the culture of hairy roots in a 3-l jar bioreactor [21]. The final concentration of camptothecin reached ~0.01 % fresh wt of tissue, and the total camptothecin production was 22 mg in 8 weeks culture, suggesting the potential application of hairy root culture for industrial production of camptothecin. Approximately 17 % of the total camptothecin produced was excreted into the culture medium of bioreactor.

Characterization of strictosidine synthase from *O. pumila*

From the hairy roots, the cDNAs of two key enzymes in the biosynthesis of monoterpenoid indole alkaloids, strictosidine synthase (*Op*STR; EC 4.3.3.2) and tryptophan decarboxylase (*Op*TDC; EC 4.1.1.28) were isolated [22]. The high expression of *Op*STR and *OpTDC* observed in hairy roots, roots, and stems was closely correlated with STR protein accumulation. Plant stress compounds such as salicylic acid down-regulated the expression of these two genes.

The recombinant protein of *Op*STR expressed in *Escherichia coli* exhibited the STR activity, showing the similar kinetic properties of the native proteins from hairy root cells [16,22]. A number of tryptamine analogs were examined whether they act as substrates of *Op*STR. The analogs, 4-methyltryptamine, 5-methoxytryptamine, 6-methoxytryptamine, 5-hydroxytryptamine, and 7-methyltryptamine, were accepted as substrates with a very low activity (Table 1).

Table 1 Substrate acceptance of STR isolated from *O. pumila*.

Accepted as substrate	Not accepted as substrate
     	     

We have generated several point mutants of *Op*STR and assayed the STR activity of these mutants (Fig. 2). According to the recent structural determination of STR protein from *Rauvolfia serpentine* [23], the Cys-97 is the site of the conserved disulfide bond which connected two helices of α1 and α2. Mutation of this Cys to Ser resulted in the loss of activity, suggesting an important role of the disulfide bridge. The mutation of His-317 to Leu adjacent to a Phe-316, one of the hydrophobic active sites [24],

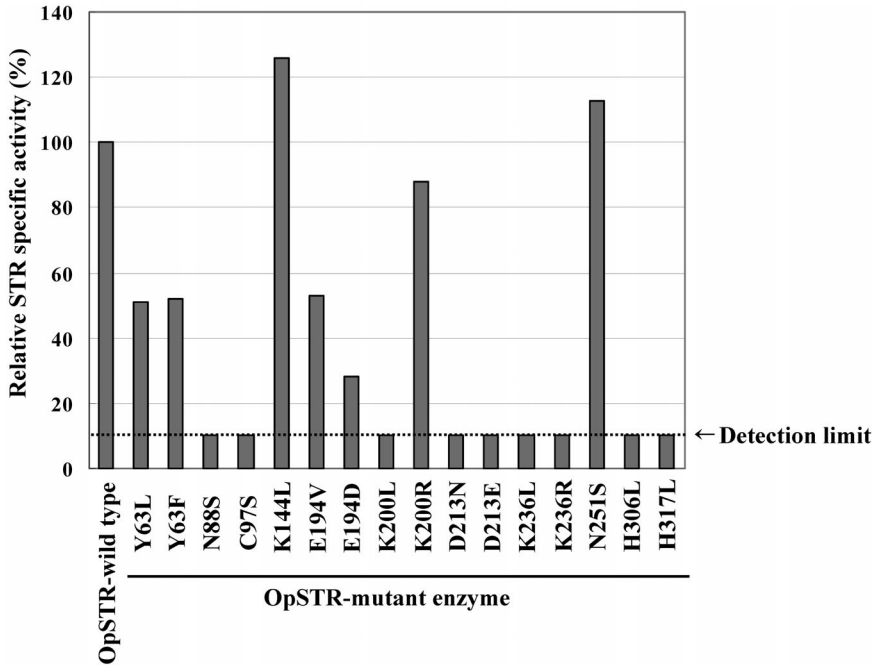


Fig. 2 STR activities of site-directed mutant enzymes. The relative STR specific activity of the mutant *Op*STR is given with that of the wild-type enzyme as 100 %.

diminished the activity. In contrast, the change of Lys-144 to Leu also adjacent to Trp-145, one of the hydrophobic active sites, slightly increased the activity. In summary, most of the mutations of conserved amino acid residues resulted in decrease of the activity, indicating the importance of these residues.

Transport of camptothecin and self-resistance mechanism, co-evolved with camptothecin production

Camptothecin exhibits eukaryotic topoisomerase I poisoning activity, resulting in cell death. Because of such toxicity to a house-keeping enzyme of cells, we addressed the question of how the camptothecin-producing plant cells survive in the presence of camptothecin. Despite its potent toxicity to most organisms, including plants, camptothecin-producing plants can resist its toxicity, indicating the presence of a specific self-resistance mechanism in those plants. Sequestration of toxic metabolites is generally believed as a self-resistance mechanism. Indeed, camptothecin seems to be localized in vacuoles [25]. However, the experiments using transport inhibitors suggested that camptothecin excretion is a passive transport driven by the concentration gradient of the compound and independent from active transporters. The higher concentration of camptothecin in the cytoplasm resulted in the increased rate of excretion to outside of cells. Thus, camptothecin seems to be biosynthesized at the endoplasmic reticulum and transported to vacuoles, at least partly, by vacuolar protein-sorting mechanism [25].

Camptothecin is passively excreted, and no particular sequestration mechanisms operate as mentioned above, suggesting the functioning of a distinct mechanism apart from expected sequestration. Therefore, we hypothesized that these plants might possess camptothecin-resistant-type topoisomerase I [25,26]. In fact, the recombinant topoisomerase I from *O. pumila* (*Op*TOP1) expressed in yeast was resistant to camptothecin. Amino acid sequence analysis of *Op*TOP1 revealed that the highly conserved residue next to the catalytic site has been mutated [26]. One of the mutations is identical to that found in camptothecin-resistant human cancer cells, suggesting the presence of a similar

mechanism of resistance to camptothecin with keeping the topoisomerase activity. All the topoisomerase I from the plants producing camptothecin examined so far possess the conserved mutations, which confer resistance to camptothecin. The results demonstrate the novel molecular mechanism of self-resistance to endogenously produced toxic compounds and also suggest the possibility of adaptive co-evolution of topoisomerase I with camptothecin biosynthetic pathway in camptothecin-producing plants [26].

Possibility of camptothecin biosynthetic pathway engineering

From the hairy root culture, we have established the non-differentiated cell-suspension culture that does not produce secondary products, camptothecin-related alkaloids and anthraquinones. Thus, the comparison of these hairy roots and cell suspension cultures is a desirable experimental system for research of molecular biology and biochemistry of camptothecin biosynthesis. We have conducted polymerase chain reaction (PCR)-select cDNA subtraction for those two cultures to isolate cDNA fragments which are specifically expressed in camptothecin-producing hairy roots. Functional identification of those cDNAs that are presumed to be involved in biosynthesis of camptothecin is now undertaken by RNAi strategy in transformed roots and analysis of recombinant proteins. The methods of foreign gene transfer by *A. rhizogenes* and the regeneration of plants from the transformed hairy roots were established [27]. Thus, we have now all the necessary platforms for gene identification and subsequent engineering of camptothecin production in our hands. We hope that the engineering of biosynthetic pathway of camptothecin will be achieved by using these research platforms in the future as nicely exemplified for engineering of the structural complexity city of periwinkle alkaloids by a modified enzyme [28].

METHODS

Hairy root culture of *O. pumila*

For induction of hairy roots, the aseptic plants of *O. pumila*, *O. liukiuensis*, and *O. kuroiwai* was infected with *A. rhizogenes* 15834 by scratching the stems as described [15,20]. The established hairy root was subcultured every three weeks in B5 liquid medium containing 2 % sucrose at 25 °C on a rotary shaker (80 rpm) under dark conditions.

Recombinant STR and site-directed mutagenesis

The *Op*STR ORF was cloned into pETBlue-2 blunt vector (Novagen, Madison, WI, USA) to give construct for the expression of *Op*STR, and the *Op*STR was expressed in *E. coli* Tuner(DE3)pLacI (Novagen) as described previously [22]. Site-directed mutagenesis of *Op*STR was performed by QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) using *PfuTurbo* DNA polymerase and the oligonucleotide primers containing the desired mutation. The constructed vector DNA containing the desired mutations was then transformed into *E. coli* XL1-Blue (Stratagene).

Assay of STR activity and topoisomerase I against camptothecin

The STR activity in the recombinant protein extracts from *E. coli* was measured as the quantity of strictosidine formed by the condensation of tryptamine with secologanin as described [22]. The camptothecin sensitivity assay was conducted by using *Saccharomyces cerevisiae* RS190 (MATa, *top1*Δ) transformed with various topoisomerase I constructs as described [26]. Individual transformed cells were spotted onto selective plates supplemented with 2 % glucose or galactose and 0 or 5 μg/ml camptothecin at a final Me_2SO concentration of 0.25 %.

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