Camptothecin: Therapeutic Potential and Biotechnology

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Abstract: Camptothecin (CPT) and its derivatives have been received considerable attention recently. Two semi-synthetic derivatives, topotecan and irinotecan, are currently prescribed as anticancer drugs. Several more are now in clinical trial. CPT is produced in many plants belonging to unrelated orders of angiosperms. At present, CPT supplied for pharmaceutical use is extracted from the plants, *Camptotheca acuminata* and *Nothapodytes foetida*. Several efforts have been made to sustain a stable production of CPT by *in vitro* cell cultures of *C. acuminata, N. foetida* and *Ophiorrhiza pumila*. Recent report showed that plants are not the only sources that produce CPT. CPT was reported to be produced from the endophytic fungus isolated from the inner bark of *N. foetida*. The hairy root cultures of *C. acuminata* and *O. pumila* produce and secrete CPT into the medium in large quantities. These reports suggest the possibility to develop large-scale production of CPT. In addition, recent advance in the cloning and characterization of biosynthetic enzymes involved in CPT biosynthetic pathway provides valuable information for developing genetically engineered CPT-producing plants.

Key Words: Camptothecin, *Camptotheca acuminata*, *Nothapodytes foetida*, *Ophiorrhiza pumila*, terpenoid indole alkaloid, anticancer agent, biosynthesis and biotechnology.

1. INTRODUCTION

Camptothecin (CPT) (**1**), a terpenoid indole alkaloid (TIA), was firstly isolated from the Chinese tree *Camptotheca acuminata* (Nyssaceae) in 1966 [1]. It has been shown that camptothecin exhibits anticancer property by inhibiting DNA topoisomerse I [2]. Its semi-synthetic derivatives including topotecan and irinotecan, are presently used as anticancer agents. These two drugs are the most advanced CPT derivatives and have nearly US$1 billion in annual reported worldwide sales [3].

Besides *C. acuminata*, many plants in unrelated orders and families of angiosperms are reported to produce CPT (for review, see [4]). Some of those plants, including *C. acuminata*, *Nothapodytes foetida* [5] and *Ophiorrhiza pumila* [6], have been much studied for their potential to produce CPT (Fig. (**1**)). At present, CPT supplied for pharmaceutical market is mainly extracted from the intact plants *C. acuminata* and *N. foetida*. Regarding the shortage of natural resources and environmental problem, biotechnological production of CPT has become an important issue. In this paper, we review the CPT derivatives in clinical use, the study on CPT biosynthetic pathway and the *in vitro* cultures of CPT producing plants. The possibility for construction of genetically engineered plants or plant cultures is also discussed.

2. CAMPTOTHECIN AND ITS DERIVATIVES IN CLINICAL USE AS AN ANTICANCER AGENT

CPT represents one of the most promising classes of anticancer drug. The structure of CPT that is essential for activity include alpha-hydroxy lactone ring, the pyridine moiety of the D-ring, the lactone moiety of the E-ring, conformation at C-20, and the planarity of the five-membered ring system [7, 8]. Since CPT itself is highly toxic and insoluble, its derivatives, such as topotecan and irinotecan, have been developed. These two derivatives have already been approved by the FDA and currently prescribed. Topotecan (Hycamptin®) (**2**), 9-[(dimethylamino)methyl]-10- hydroxycamptothecin, is indicated for treatment of ovarian cancer and small-cell lung cancer [9, 10], and manufactured by GlaxoSmithKline. Irinotecan (Camptosar®) (**3**), 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin, is indicated for treatment of colorectal cancer [11, 12], and manufactured by Pharmacia. Besides these two derivatives, there are still several derivatives that enter clinical trials as anticancer drug candidates. Rubitecan (Oratecin®) (**4**), 9-nitrocamptothecin, has not yet been approved by the FDA and is now being developed in a phase II trial for the treatment of pancreatic cancer and other solid tumors by SuperGen [13]. Lurtotecan (**5**), 10,11-(methylethylenedioxy)-7-((N-methylpiperazino)methyl) camptothecin, is currently being investigated in a phase II trial for breast, colorectal and small cell lung cancers [14]. OSI-221, the liposome encapsulated form of lurtotecan, has been developed to enhance tumor growth inhibition [15]. Gimatecan (**6**), 7-t-butoxyiminomethylcamptotecin, is a novel oral lipophilic CPT that showed an impressive antitumor efficacy in a large panel of human tumor xenografts [16]. Exatecan (DX-8951f) (**7**), developed by Daiichi Pharmaceutical, demonstrated broad antitumor activity in preclinical studies compared with available CPT derivatives [17, 18].

Recently, crystal structure of human topoisomerase I in ternary complexes with DNA and topotecan has been reported [19]. This would provide valuable information for

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Fig. (1). Camptothecin producing plants.

drug design that might improve efficacy of the original derivatives.

3. CAMPTOTHECIN BIOSYNTHESIS

The first study on camptothecin biosynthesis in *C. acuminata* has been reported in the early 1970s [20]. However, until now the camptothecin biosynthetic pathway has not been completely elucidated yet (Fig. **3**). Hutchinson *et al*. (1979) reported the key intermediate, uniquely to the pathway, called strictosamide which is yielded from the intermolecular cyclization of strictosidine, a common precursor of all TIAs [21]. The latter step until CPT is formed has remained largely unexplored. Pumiloside, which is proposed to be the intermediate precursors between strictosamide and camptothecin, have been isolated from *Ophiorrhiza* species (Fig. **3**) [6, 22]. In addition, pumiloside has also been isolated from *C. acuminata* [23].

It has been known that isopentenyl diphosphate (IPP), the precursor of terpenoid biosynthesis, can be synthesized from one of these two different pathways, the mevalonate (MVA) pathway (for review, see [24]) and 2C-methyl-D-erythritol 4-phosphate (MEP) pathway (for review, see [25]). Recently, Yamazaki *et al*. (2004) conducted a tracer experiment in the hairy root of *O. pumila* by the incorporation of [1-^{13}C] glucose into camptothecin together with *in silico* approach using the Atomic Reconstruction of Metabolism software [26]. The results show that the monoterpene-secologanin moiety of camptothecin was synthesized *via* the MEP pathway, not *via* the MVA pathway, and quinoline moiety from tryptophan was synthesized *via* the shikimate pathway. In addition, the inhibition of the MEP pathway by fosmidomycin in the hairy root that caused lower CPT production supports MEP pathway-derived secologanin moiety.

The biosynthetic pathway prior to the formation of strictosidine has been extensively studied including cDNA cloning of several biosynthetic enzymes listed in Table **1**. Strictosidine is produced from the condensation of seloganin and tryptamine by the enzyme strictosidine synthase (STR) (Fig. **3**). The cDNA encoding STR has been isolated from *O. pumila* hairy roots (Fig. **4**) [27]. The recombinant *Op*STR protein expressed in *E. coli* exhibits STR activity. It shows 55% and 51% identity to STR from *Rauvolfia serpentina* [28] and *Catharanthus roseus*, [29] respectively. Both *Op*STR mRNA and protein were detected in hairy roots, roots and stems and their abundance paralleled that of CPT except for young leaves which show no STR activity but contain CPT [27]. These results suggest that roots and stems are possibly the main organs for camptothecin biosynthesis and CPT is transported to other parts. The mechanism of transport and accumulation of CPT remains unexplored. Recent data from the crystal structure of STR from *R. serpentina* suggest that STR from different plant species seems to conserve their overall active site structures and use a similar catalytic mechanism [30].

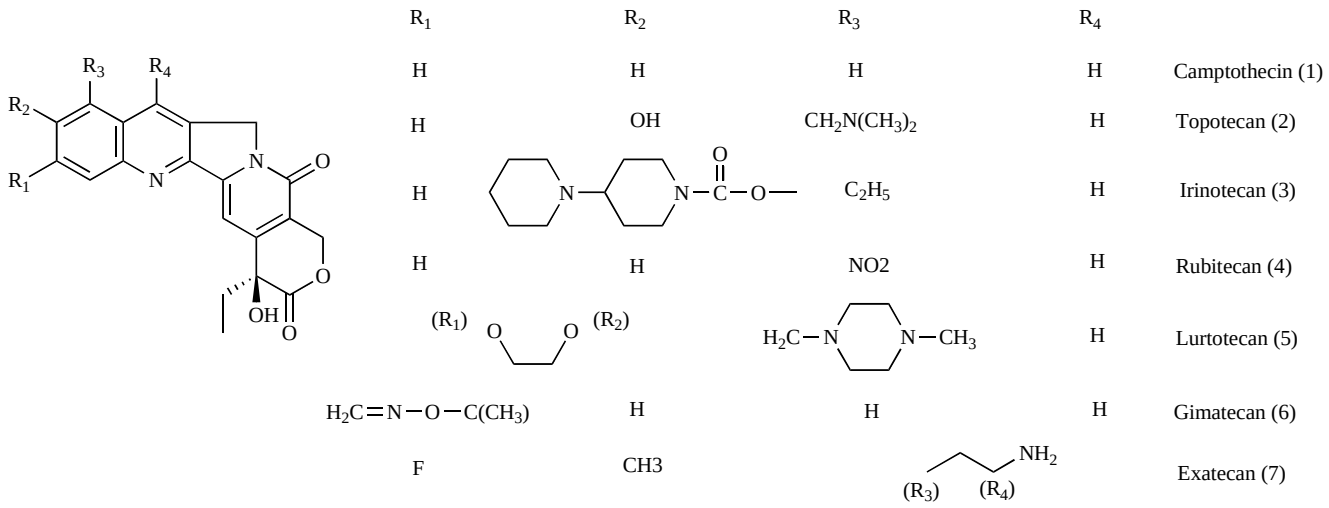

Fig. (2). Camptothecin and its derivatives.

Table 1. Cloned and Characterized Biosynthetic Enzymes in CPT Biosynthetic Pathway

Enzyme	Abbreviation	Source	Reference
Strictosidine synthase	STR	*O. pumila*	[27]
NADPH:cytochrome P450 reductase	CPR	*O. pumila*	[27]
Anthranilate synthase	ASA	*C. acuminata*	[35]
β -subunit of tryptophan synthase	TSB	*C. acuminata*	[36]
Tryptophan decarboxylase	TDC	*O. pumila* *C. acuminata*	[27] [37]

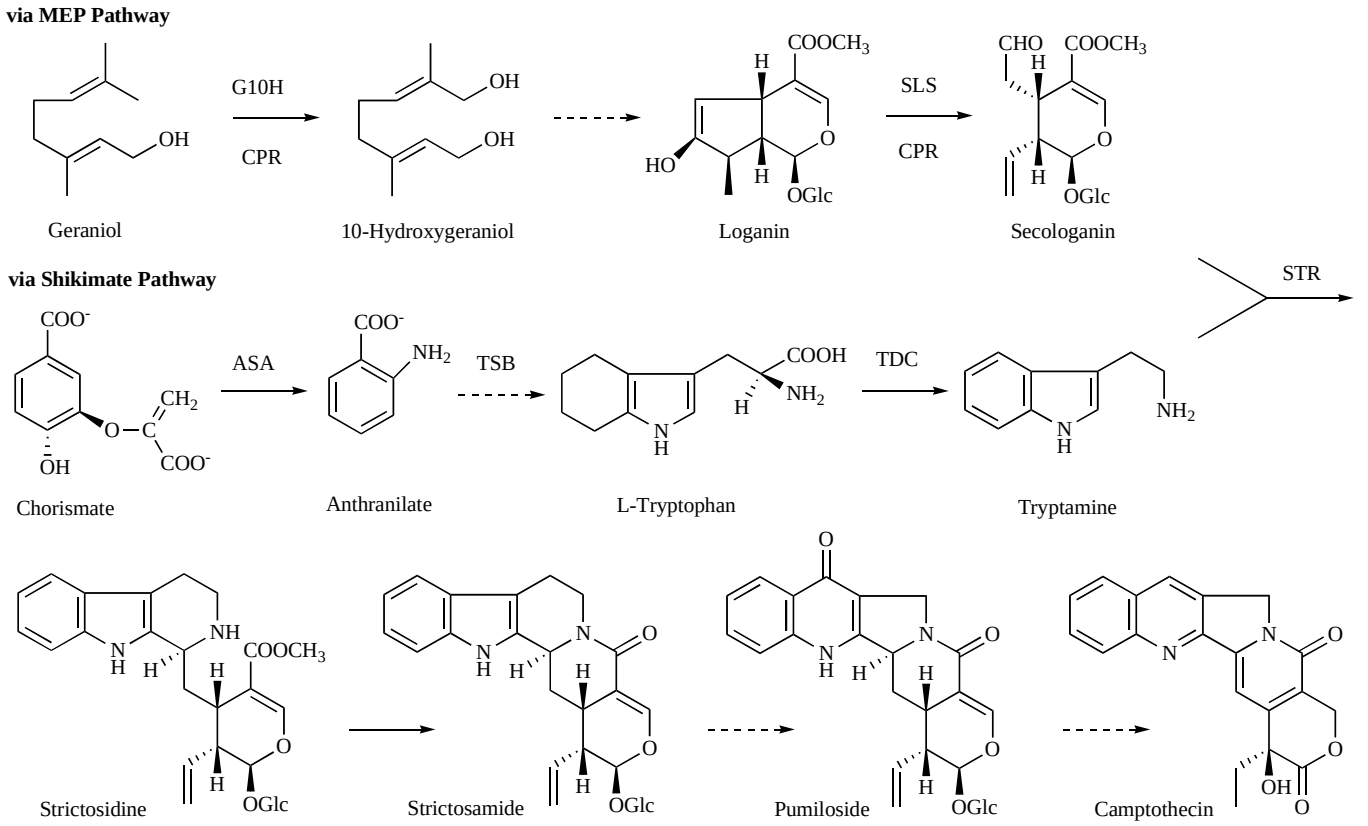

Fig. (3). The biosynthetic pathway of camptothecin. The enzymes are: G10H, geraniol 10-hydroxylase; CPR, NADPH:cytochrome P450 reductase; SLS, secologanin synthase; ASA, anthranilate symthase; TSB, β-subunit of tryptophan synthase; TDC, tryptophan decarboxylase; STR, strictosidine synthase. Dashed arrows indicate involvement of multiple enzymatic steps.

It has been reported that a NADPH:cytochrome P450 reductase (CPR) is essential for the activity of geraniol 10-hydroxylase (G10H) and secologanin synthase (SLS) , which both enzymes are involved in the formation of secologanin (Fig. **3**) [31]. G10H converts monoterpenoid geraniol to 10-hydroxygeraniol. This step is recognized as the first committed step in the formation of secologanin [32]. SLS catalyzed the final step for the biosynthesis of secologanin [33, 34]. The cDNA encoding CPR from the hairy root of *O. pumila* has been isolated. The deduced amino acid of *Op*CPR showed 72%, 66%, 65%, and 67% sequence identity to *Arabidopsis thaliana*, *Petroselium crispum*, *Pisum sativum*, and *Triticum aestivum* CPR, respectively [27]. *OpCPR* is a single-copy gene that expresses in all tissues [27].

The cloning and characterization of the enzymes anthranilate synthase (ASA) and β-subunit of tryptophan synthase (TSB) from *C. acuminata* have been performed [35, 36]. Both enzymes are responsible for the biosynthesis of tryptophan, utilized for protein biosynthesis and indole alkaloid production (Fig. **3**). ASA involves the first step of tryptophan biosynthesis where chorismate is converted to anthranilate. Two differentially regulated non-identical copies of ASA genes (ASA1 and ASA2) have been identified in *C. acuminata*. *Ca*ASA1 mRNA is induced prior to the peak accumulation of CPT in seedling of *C. acuminata*, in contrast to the constitutively low expression level of ASA2. These results suggest that *Ca*ASA1 is involved in the early indole pathway of CPT biosynthesis in *C. acuminata* and

CPT biosynthesis are coordinately regulated [35]. TSB is responsible for the final step of tryptophan biosynthesis. The expression levels of TSB mRNA and protein are similar to the level of CPT accumulation. Similar to *Ca*ASA1, *Ca*TSB is also highly expressed during early seedling development at a stage corresponding to peak accumulation of camptothecin [36].

Tryptophan is later converted to tryptamine by the enzyme tryptophan decarboxylase (TDC) (Fig. **3**). It has been reported that TDC is encoded by multi-copy genes in *C. acuminata* and *O. pumila* [37, 38], in contrast to single-copy TDC gene from *C. roseus* which TDC has been extensively studied [39]. The deduced amino acid of TDC from *C. acuminata* and *O. pumila* show high identity (~67%) to that from *C. roseus*. Recombinantly expressed TDC of *C. acuminata* and *O. pumila* in *E. coli* exhibit the enzyme activity [27, 37].

The yield of plant secondary metabolites can be improved by treating the cultures with various elicitors, signal compounds, and abiotic stresses (for review, see [40]). In *C. roseu*s cell suspension culture, many studies have demonstrated that the genes encoding STR, TDC and CPR are induced by fungal elicitors [31, 41, 42] and methyl jasmonate (MeJA) [32, 43]. Interestingly, in *O. pumila*, it has been shown that the expressions of STR, TDC and CPR were not induced when the hairy roots were treated with various stress compounds such as MeJA, salicylic acid and yeast extract. These results suggest the different regulation mechanism of TIA biosynthesis between *C. roseus* and *O. pumila* [27, 44].

Little is known about the biosynthetic and accumulation sites of CPT. In general, different plant tissues including epidermis, endodermis, laticifers, idioblasts, pericycle and cortex, involve the biosynthesis and/or accumulation of alkaloid [45]. Recently, it has been demonstrated that CPT accumulates in the glandular trichomes of the leaf and stem, parenchymatic and/or epidermic cells of the root, stem, and leaf, but not in the laticifer cells of *C. acuminata* [46, 47]. At the subcellular level, CPT accumulates in crystalline form in the vacuole of segregator idioblasts [47]. Since the pathway of CPT biosynthesis has not been completed yet, obstacle continues to hinder the mechanism of transport and accumulation of CPT.

4. BIOTECHNOLOGICAL PRODUCTION OF CAMPTOTHECIN

Despite the great demand of CPT in pharmaceutical market, CPT is still supplied exclusively from the intact plants, mainly *C. acuminata* and *N. foetida*. Chemical synthesis of CPT has been extensively studied (for review, see [48, 49]). However, large-scale synthesis has not yet been reported yet. With regard to a shortage of the plants and environmental issues, developing sustainable and alternative sources become the main issue.

The first established cell suspension culture of *C. acuminata* was reported in 1974. However, the culture produced CPT in amount as low as 2.54×10^{-4} % of dry weight while the young leaves contained 0.4% of dry weight [50]. There have been several efforts to improve the CPT production in culture of *C. acuminata*. Van Hengel *et al.* (1992) established the cell suspension cultures with a CPT production of 0.004 % of dry weight [51]. The production of CPT in callus cultures of *C. acuminata* was about 0.2% of dry weight [52]. In addition to CPT, 10-hydroxycamptothecin (HCPT), a more potent and less toxic CPT derivative, was reported to produce in the callus with a maximum amount of 0.08% of dry weight [52]. It has been known that the nitrogen source might significantly affect the cell growth and formation of many alkaloid. Recently, Pan *et al.* (2004) reported that altering nitrogen source to NH_4^+/NO_3^- molar ratio of 5:1 (a total of 40 mM) in cell suspension cultures of *C. acuminata* increased the CPT content up to 280% when compared with a control culture [53].

Callus and suspension cultures of *N. foetida* were reported to produce very small amounts of CPT [54-57]. Unlike the callus cultures of *C. acuminata* and *N. foetida*, the callus cultures of *O. pumila* do not accumulate camptothecin and any camptothecin-related alkaloids [58].

Agrobacterium rhizogenes-transformed hairy root has been known as an excellent culture having high stability of the production of secondary metabolites. The hairy root culture of *O. pumila* has been established (Fig. **4**) [44]. This culture produces CPT up to 0.1% of dry weight. Interestingly, the hairy root cultures not only accumulate CPT in the cells but also excrete CPT into the culture medium (Fig. **5**). CPT content in the medium was increased by the presence of a polystyrene resin (Diaion HP-20) that absorbed CPT. CPT was later easily recovered from the resin by elution with methanol [44]. A large-scale culture of hairy root of *O. pumila* using a modified 3 l bioreactor was established [59]. The concentration of CPT from the 8-week culture was found to be 0.0085 % of fresh weight tissue and the total CPT production was 22 mg which 16.5% (3.6 mg) was excreted into the medium. This report can be applied for a commercial production of CPT.

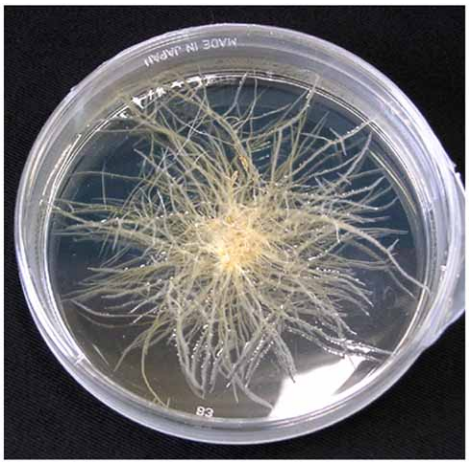

Fig. (4). *Agrobacterium rhizogenes*-transformed hairy root culture of *O. pumila*.

Asano *et al.* (2004) have established the aseptic plants and hairy root cultures of other *Ophiorrhiza* species, *O. liukiuensis* and *O. kuroiwai*, in addition to *O. pumila* [60]. Metabolite profiles of these aseptic plants and cultures were examined by HPLC/DAD/ESI/MS. 10-Methoxycamptothe-

cin, which might be a better synthetic precursor of topotecan and irinotecan, was accumulated in the hairy root of *O. liukiuensis* and *O. kuroiwai* but not in *O. pumila* [60]. However, the CPT production and the growth rate of hairy root of *O. pumila* seem to be the best of *in vitro* culture to be reported so far.

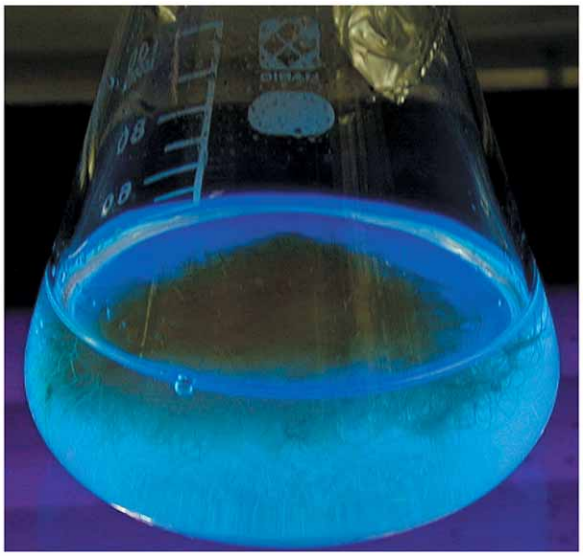

Fig. (5). Excretion of CPT from four-week-old hairy root culture of *O. pumila* into the medium. CPT excreted into the medium shows autofluorescence when exposed to ultraviolet light.

The hairy root of *C. acuminata* was also established that the yield of CPT from this culture was 0.1% of dry weight. This hairy root culture also secreted CPT into the medium as well as 10-hydroxycamptothecin, the more potent and less toxic natural derivative [61]. Recently, hairy root culture technology has developed into large-scale industrial production of pharmaceuticals. The company ROOTec aims to produce bioactive plant-derived compounds including CPT from broad-scale hairy root cultures (http://www.rootec.com).

Regeneration of transformed plant can contribute to the establishment of genetically modified plants feasibly producing CPT in the future. A method for regeneration of *O. pumila* from hairy roots has been reported [62]. The regeneration frequency was over 83% and the regenerated plants accumulated CPT in amounts of 66-111% compared with that in the wild-type plants.

Interestingly, plants are not the only source that produces CPT. It has been known that some endophytes can produce phytochemicals (for review, see [63]). Taxol, an anticancer diterpene found in *Taxus* species, was also produced from the endophytic fungal, *Taxomyces andreanae*, isolated from the plant, *Taxus brevifolia* [64]. Recently, an endophytic fungal producing CPT has been isolated, for the first time, from the inner bark of *N. foetida* from the Western coast of India [65]. The yield of CPT produced from a culture grown in a semi-synthetic liquid medium was 0.575 ± 0.031 % of dry cell mass in 96 h in shake flasks while as much as 4.96 ± 0.73 % of dry cell mass was produced in 48 h in a bioreactor [66]. This report provides another alternative non-plant source for commercial CPT production.

5. FUTURE PROSPECTS

How can biotechnology be used to improve the CPT production? This is an interesting question that many research groups are trying to find a way to achieve the ultimate yield and sustainable production of CPT. Metabolic engineering has been a keen issue to be performed. Since the biosynthetic pathway of CPT remained largely unclear, metabolic engineering in CPT-producing plant becomes more difficult. As mentioned previously, only several genes of the upper part of CPT biosynthetic pathway have been isolated including *ASA*, *TSB* and *TDC* from *C. acuminata* [35-37], and *CPR*, *TDC* and *STR* from *O. pumila* (Table **1**) [27]. Overexpression of these genes might affect CPT production as well as other alkaloids. The successes of hairy root induction by agrobacterium-mediated transformation and regeneration of transformed plants clearly suggest that genetically modified CPT-producing plant can be performed easily [44, 61, 62]. However, in *C. roseus*, it has been reported that direct overexpression of encoding genes seems to increase only intermediate products. The accumulation of the end product is difficult to obtain. Overexpression of TDC or recombinant ASA in *C. roseus* calluses or hairy roots, respectively, showed the increase in tryptophan and tryptamine but not monoterpene indole alkaloids in the latter part of the pathway [67, 68]. However, overexpression of STR which is restricted to terpene route seems to enhance several alkaloids accumulation [69]. Hughes *et al.* (2004) reported the successful engineering of the indole pathway of *C. roseus* hairy roots using an inducible promoter [70]. Results and discussion on those reports should be seriously considered before performing an experiment with CPT-producing plants. In addition, discovery of unknown genes in CPT biosynthetic pathway is still a main objective to be solved. Recently, Hirai *et al.* (2004) reported the investigation for gene-to-metabolite networks regulating sulfur and nitrogen nutrition and secondary metabolism in *Arabidopsis*, with integration of metabolomics and transcriptomics [71]. The study on integration of metabolomics and transcriptomics in CPT-producing plants could be useful for identification of gene function and improvement of CPT production.

Transcription factors play an important role in the regulation of TIA production. This issue has also been extensively studied in *C. roseus*. The gene encoding transcription factor ORCA3 (Octadecanoid-derivative Responsive Catharanthus AP2-domain protein 3) has been isolated from *C. roseus* [72]. Induction of this gene by fungal elicitor and jasmonate leads to enhance expression of several biosynthetic genes such as *TDC*, *STR* and *CPR*, resulting in increased TIAs accumulation [72]. In addition to ORCA3, several other transcription factors including G-box binding factor (GBF1 and GBF2) [72] and MYC-type bHLH transcription factor (MYC1) [73] from *C. roseus* can bind to *STR* promoter region suggesting their involvement in the regulation of TIA biosynthesis. In contrast to *C. roseus*, it has been shown that the expression of *STR*, *TDC* and *CPR* from *O. pumila* was not induced by fungal elicitor and jasmonate, suggesting the difference in regulation mechanism [27]. Isolation of transcription factors that recognize promoter region of CPT biosynthetic genes would be an interesting task. Manipulation of transcription factor expression might lead to improved CPT production in the plants.

Goossens *et al*. (2003) showed that a transporter can be used to stimulate the secretion of secondary metabolites and might enhance secondary metabolite production in plant cell cultures [74]. Several studies demonstrated the role of ATP-binding cassette (ABC) transporter in the mechanism of resistance to CPT derivatives by reducing drug accumulation in human cancer cells [75-78]. Moreover, yeast SNQ2 and PDR5 transporters were reported to play a role in CPT resistance [79]. These results suggest that a transporter might play a role in CPT transport in plant. The ABC transporters involve in berberine alkaloid and anthocyanin transport have already been described [80, 81]. If such a CPT transporter exists in plants, it would be interesting to study. Increased transporter expression might affect the CPT production.

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ABBREVIATIONS

CPT	=	Camptothecin
TIA	=	Terpenoid indole alkaloid
IPP	=	Isopentenyl diphosphate
MVA	=	Mevalonate
MEP	=	2C-Methyl-D-erythritol 4-phosphate
STR	=	Strictosidine synthase
CPR	=	NADPH:cytochrome P450 reductase
G10H	=	Geraniol 10-hydroxylase
ASA	=	Anthranilate synthase
TSB	=	β-Subunit of tryptophan synthase
TDC	=	Tryptophan decarboxylase
MeJA	=	Methyl jasmonate
ORCA3	=	Octacecanoid-derivative Responsive Cathar-anthus AP2-domain protein 3
ABC	=	ATP-binding cassette

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