Chapter 3
Camptothecin Production and Biosynthesis in Plant Cell Cultures

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Abstract Camptothecin, a well-known monoterpenoid indole alkaloid originally identified in the extracts of the Chinese tree *Camptotheca acuminata* (Nyssaceae), exhibits antitumor activity due to its ability to kill cancer cells via topoisomerase I poisoning. Other plant species have since been shown to produce camptothecin and related compounds. In particular, *Ophiorrhiza* species (Rubiaceae) are important resources for the production of various alkaloids, including camptothecin. This chapter describes the production of camptothecin-related alkaloids and the elucidation of the mechanisms of camptothecin biosynthesis using plant cell and tissue cultures. In particular, aseptically grown plants, callus cultures, and hairy root cultures were established for several species, *O. liukiuensis, O. kuroiwai*, and *O. pumila*, which were then evaluated for production of camptothecin and related alkaloids. The metabolite profiles differed between the species, and between tissues of the same species; for example, profiles from hairy roots were not identical to those of aseptic plants. The complementary DNAs (cDNAs) for strictosidine synthase, tryptophan decarboxylase, and cytochrome P450 reductase were cloned from *O. pumila* and evaluated for involvement in production of camptothecin in this species. RNA interference (RNAi)-mediated knockdown of gene expression indicated that

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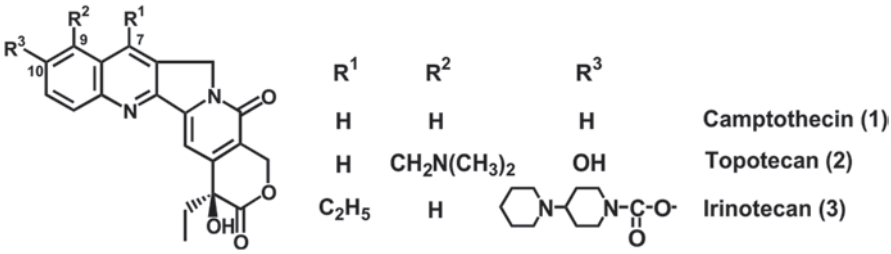

Fig. 3.1 Camptothecin (*1*) and its clinically used derivatives, topotecan (*2*) and irinotecan (*3*). (With permission from Ref. [38])

the production of camptothecin, strictosidine, and camptothecin-related alkaloids was suppressed in a *TDC* expression-dependent manner in RNAi hairy roots.

3.1 Introduction

Alkaloids are nitrogen-containing basic compounds known from about 20% of all plant species. Many alkaloids are pharmacologically active and have been used traditionally in the form of medicinal plant extracts as treatments for various diseases [1]. A few dozen pharmacologically active alkaloids, including camptothecin, are widely used in modern medicine, and worldwide sales of alkaloid-containing drugs were projected to exceed US$ 4 billion in 2002 [2].

Camptothecin (1) is a well-known monoterpenoid indole alkaloidand was originally identified in the extracts of the Chinese tree *Camptotheca acuminata* (Nyssaceae) [3]. Camptothecin exhibits antitumor activity, which is due to its ability to kill cancer cells via topoisomerase I poisoning [4]. At present, the semi-synthetic water-soluble camptothecin derivatives, topotecan (2) and irinotecan (3), are used worldwide as clinical antitumor agents against cancers of the lung, cervix, ovaries, colon [5], and other organs [6] (Fig. 3.1). In addition, a number of reports are available announcing the therapeutic values of camptothecin derivatives against acquired immunodeficiency syndrome (AIDS) [7] and falciparum malaria [8]. Consequently, the demand for camptothecin will continue to increase in the future.

Despite the rapid growth of the pharmaceutical market for this compound, camptothecin is still supplied exclusively from intact plants, mainly *C. acuminata* and *Nothapodytes foetida* [9]. However, the extraction of this compound from intact plants is problematic because of the shortage of natural resources and the resultant environmental concerns. Thus, the production of secondary metabolites by genetically engineered plant cell cultures, particularly for compounds such as camptothecin, has become a keen issue [10].

Camptothecin-related alkaloids have been reported to be produced in a relatively wide array of plant species, besides *C. acuminata* and *N. foetida* [11]. For instance,

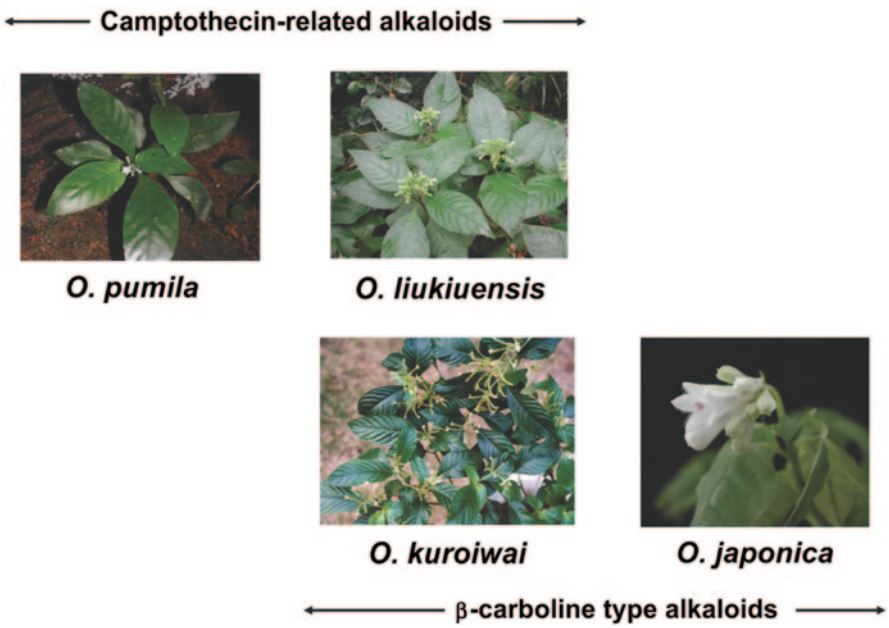

Fig. 3.2 The genus *Ophiorrhiza* species distributed in Japan

Merrilliodendron megacarpum [12], *Pyrenacantha klaineana* (Icacinaceae) [13], *Ervatamia heyneana* (Apocynaceae) [14], *Mostuea brunonis* (Loganiaceae) [15], *Ophiorrhiza mungos* [16], and *O. filistipula* (Rubiaceae) [17] have been reported to produce camptothecin-related compounds. Moreover, the results of phytochemical studies of the genus *Ophiorrhiza* have shown that camptothecin also accumulates in some *Ophiorrhiza* species (e.g., *O. pumila*) distributed in Japan [18, 19].

The genus *Ophiorrhiza* is widely distributed around tropical and subtropical Asia and comprises about 150 species [20]. Moreover, some of these species produce indole alkaloids [21]. With regard to the chemical constituents of *Ophiorrhiza* species distributed in Japan, *O. pumila* accumulated camptothecin and related alkaloids [18, 22] and *O. japonica* accumulated β-carboline-type alkaloids, such as lyalosidic acid and harman [23, 24]. Meanwhile, *O. liukiuensis* [25] and *O. kuroiwai* [26], which was shown to be an interspecies hybrid of *O. pumila* and *O. liukiuensis*, accumulated both camptothecin-related alkaloids and β-carboline-type alkaloids (Fig. 3.2). Therefore, these *Ophiorrhiza* species are important as resources for the production of various alkaloids, including camptothecin.

In this chapter, we describe the production of camptothecin-related alkaloids and the elucidation of the mechanisms of camptothecin biosynthesis by use of plant cell and tissue cultures.

3.2 In Vitro Cultures of Camptothecin-Producing Plants

3.2.1 Establishment of In Vitro Cultures

Cell and tissue cultures of several camptothecin-producing plants have been investigated as alternative sources for camptothecin production [27]. Sakato et al. [28] reported the first establishment of a rapidly growing cell suspension culture of *C. acuminata*, although the camptothecin productivity was insufficient (0.002 mg g^{-1} dry weight) for practical use. Callus cultures of *C. acuminata* established by Wiedenfeld et al. [29] produced comparatively adequate amounts of camptothecin (2 mg g^{-1} dry weight). These callus cultures were also reported to contain 10-hydroxycamptothecin, from trace amounts up to 0.08–0.1 mg g^{-1} dry weight [29]. Callus cultures of *N. foetida* were found to accumulate small amounts of camptothecin and 9-methoxycamptothecin [30–32], but the level of alkaloid production was 100- to 1000-fold lower than that from soil-grown plants. Callus cultures of *O. pumila* produced no camptothecin-related alkaloids but accumulated only anthraquinones [33, 34].

Since alkaloid biosynthesis and accumulation are under the strict control of cell developmental and environmental factors [35], establishing cultures of cell types suitable for the production of the camptothecin is important. Accordingly, aseptic plants and hairy roots of *Ophiorrhiza* species have been established as an effective means of producing camptothecin (Fig. 3.3) [36–38].

3.2.2 Camptothecin Production and Metabolite Profiles in Tissue Cultures of Ophiorrhiza Species

In shoots and roots of established aseptic plants of *Ophiorrhiza* species, camptothecin production per tissue weight was the highest in the roots of *O. pumila*. On the other hand, the production per tube was the highest in *O. kuroiwai* because it showed the higher growth rate of the two species. The concentration and total amount of camptothecin in *O. liukiuensis* were lower than those of *O. kuroiwai* and *O. pumila*.

Camptothecin accumulated to higher levels in hairy root lines of *O. pumila* than in those of *O. liukiuensis* and *O. kuroiwai* [38]. Camptothecin accumulation and increased growth rate of *O. pumila* hairy roots have the best results in the reports of camptothecin production by *in vitro* tissue cultures [37, 39].

The patterns of secondary metabolite production in the aseptic plants and hairy roots of *Ophiorrhiza* species were profiled by high-performance liquid chromatography–diode array detection–electrospray ion trap tandem mass spectrometry (Fig. 3.4 and Table 3.1) [38]. The metabolite profiles of *O. liukiuensis* and *O. kuroiwai* were highly similar in the shoot and root. 10-Methoxycamptothecin (5) and lyalosidic acid (6) were detected in the roots and shoots, respectively, of both *O. liukiuensis* and *O. kuroiwai* but not in those of *O. pumila*. Moreover, 3(*S*)- and

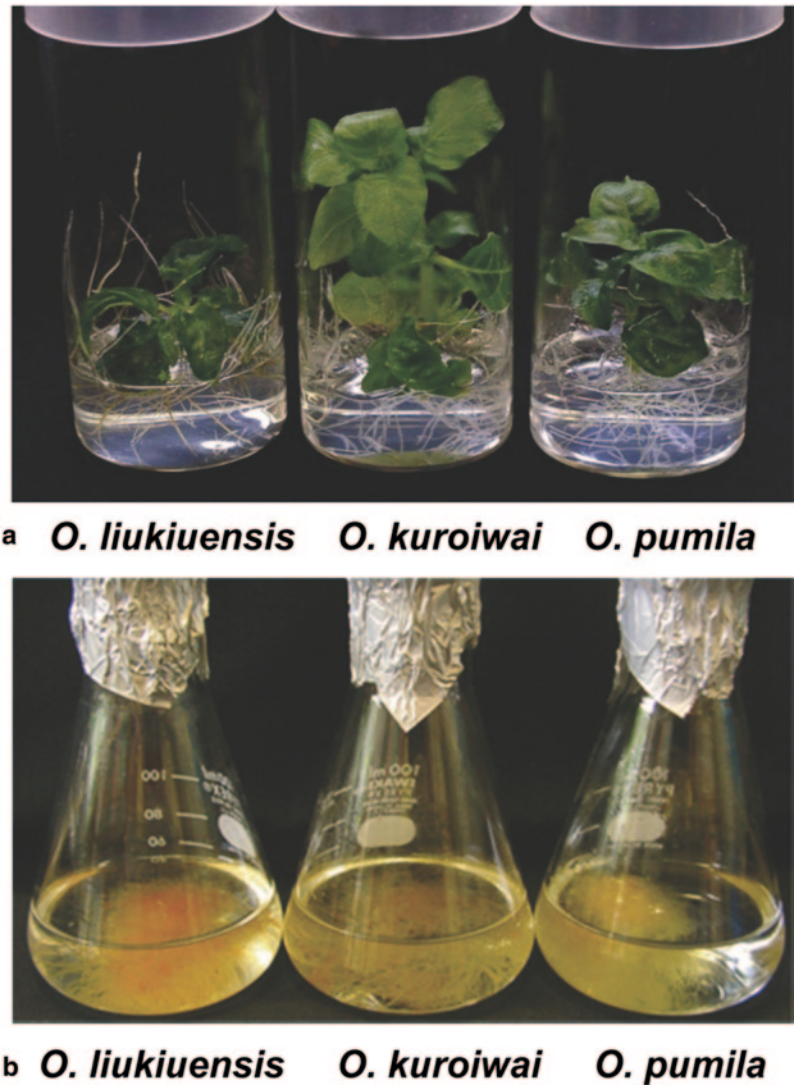

Fig. 3.3 Established tissue cultures of *Ophiorrhiza liukiuensis, O. kuroiwai*, and *O. pumila*. **a** Aseptic plants cultured for 5 weeks on 1/2 MS medium containing 1 % sucrose and 0.2 % gellan gum in test tubes. **b** Hairy roots cultured for 4 weeks in B5 liquid medium containing 2 % sucrose. (With permission from Ref. [38])

3(*R*)-deoxypumilosides (9, 10) were detected only in *O. pumila*. Camptothecin (1), 9-methoxycamptothecin (4), strictosamide (7), pumiloside (8), strictosidinic acid (11), and 3-*O*-caffeoylquinic acid (13) were detected in all three species. The metabolite profiles of the hairy roots were not identical to those of aseptic plants.

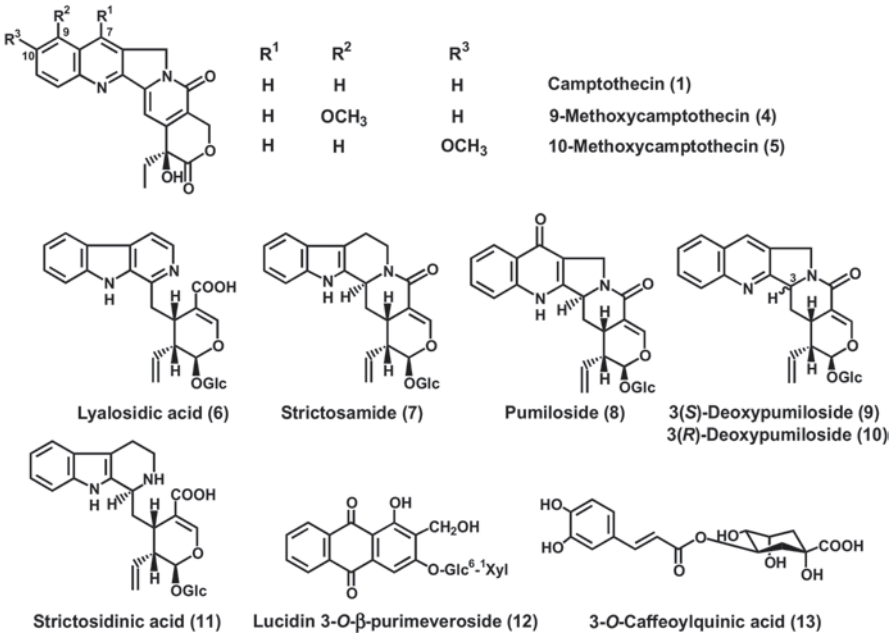

Fig. 3.4 Chemical structures of secondary metabolites detected in tissue cultures of *Ophiorrhiza* species. (With permission from Ref. [38])

Table 3.1 Alkaloids and anthraquinones detected in tissue cultures of *Ophiorrhiza* species

Compound	*O. liukiuensis*			*O. kuroiwai*			*O. pumila*		
	Shoot	Root	Hairy root	Shoot	Root	Hairy root	Shoot	Root	Hairy root
1 Camptothecin	+	+	+	+	+	+	+	+	+
4 9-Methoxycamptothecin	+	+		+	+		+	+	
5 10-Methoxycamptothecin		+			+				
6 Lyalosidic acid	+			+					
7 Strictosamide		+	+		+	+	+	+	
8 Pumiloside	+	+	+	+	+	+	+	+	+
9 3(S)-Deoxypumiloside[a]							+	+	
10 3(R)-Deoxypumiloside[a]									
11 Strictosidinic acid	+			+			+		
12 Lucidin 3-O-β-purimeveroside									+
13 3-O-Caffeoylquinic acid	+			+	+		+	+	+

[a] 3(*S*)-Deoxypumiloside (9) and 3(*R*)-deoxypumiloside (10) cannot be separated in this condition

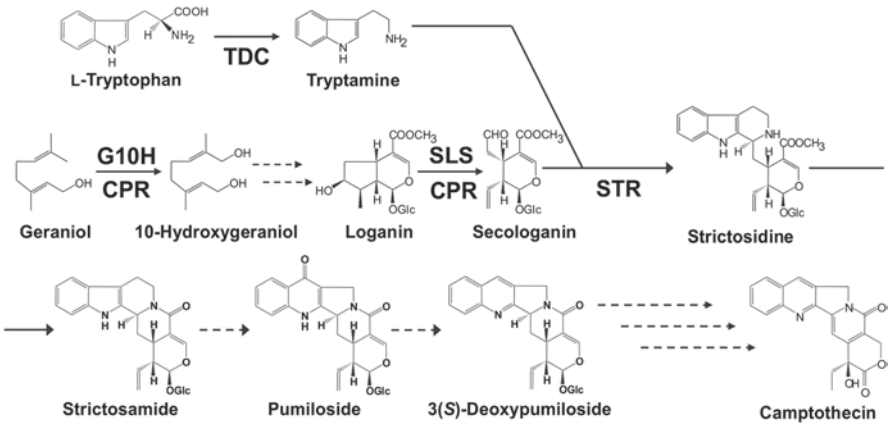

Fig. 3.5 Predicted camptothecin biosynthetic pathway in *O. pumila*. The enzymes are as follows: *TDC*, tryptophan decarboxylase; *G10H*, geraniol 10-hydroxylase; *CPR*, NADPH:cytochrome *P450* reductase; *SLS*, secologanin synthase; *STR*, strictosidine synthase. Plausible intermediates of camptothecin biosynthesis are provided in *parentheses*

3.3 Biosynthesis of Camptothecin

3.3.1 Camptothecin Biosynthetic Genes

Monoterpenoid indole alkaloids, including camptothecin, are derived from strictosidine, which is a common intermediate formed by condensation of the indole tryptamine with the iridoid glucoside secologanin by the enzyme strictosidine synthase (STR) [40–42] (Fig. 3.5). The intramolecular cyclization of strictosidine results in strictosamide, which is an intermediate peculiar to camptothecin biosynthesis, as proven by the incorporation of radiolabeled precursor [43]. The remaining details between strictosamide and camptothecin are not completely defined. However, camptothecin has been postulated to be formed potentially from strictosamide by three transformations: (1) oxidation–recyclization of the B- and C-rings, (2) oxidation of the D-ring and removal of the C-21 glucose moiety, and (3) oxidation of ring E [43]. Plausible camptothecin precursors, such as pumiloside and 3(*S*)-deoxypumiloside, were isolated from *Ophiorrhiza* species [18, 19]. Pumiloside has been found also in *C. acuminata* [44].

The cloning of complementary DNAs (cDNAs) from *O. pumila* hairy roots has been successfully performed to isolate the genes encoding the biosynthetic enzymes involved in the upper part of camptothecin biosynthesis, including STR, tryptophan decarboxylase (TDC) [45], and nicotinamide adenine dinucleotide phosphate, reduced form (NADPH):cytochrome P450 reductase (CPR), in this species [46] (Fig. 3.5). The full-length STR cDNA sequence isolated from *O. pumila* (*OpSTR*) contained a 1,056-bp open reading frame (ORF) encoding a protein of 351 amino acids with a molecular mass of 38.9 kDa. The deduced amino acid sequence of

OpSTR exhibited 55% and 51%identities with STRs from *Rauwolfia serpentina* [41] and *Catharanthus roseus* [47], respectively. OpSTR most likely localizes to the vacuole, as predicted by the PSORT program. Southern blot analysis suggested that a single STR-encoding gene is present in the genome of *O. pumila*. The highest *OpSTR* expression occurred in hairy roots, followed by the root, and the stem, whereas *OpSTR* was apparently not expressed in leaves. STR enzymatic activity was detected in the protein extracts of stems, roots, and hairy roots; however, no activity was detected in leaf and callus extracts. The distribution of STR activity correlated with the messenger RNA (mRNA) accumulation pattern and the camptothecin concentrations in *O. pumila* tissues, with the exception of the young leaves, suggesting that roots and stems are the main tissues for camptothecin biosynthesis [34].

Tryptamine, a precursor of strictosidine, is formed by the decarboxylation of tryptophan by the enzyme TDC. The cDNA clone encoding TDC was first isolated from *C. roseus* [48]. The full-length TDC cDNA sequence isolated from *O. pumila* (*OpTDC*) contained a 1,521-bp ORF encoding a protein of 506 amino acids with a molecular mass of 56.6 kDa. The deduced amino acid sequence of OpTDC showed high identity to TDCs from *C. acuminata* [49] and *C. roseus* [48] (71 and 67%, respectively). Southern blot analysis suggested that at least TDC-encoding genes are present in the genome. The expression patterns of *OpSTR* and *OpTDC* were nearly the same.

The enzyme CPR is essential for the activity of cytochrome P450 monooxygenases, such as geraniol 10-hydroxylase (G10H) and secologanin synthase (SLS), which are involved in camptothecin biosynthesis [50] (Fig. 3.5). The full-length CPR cDNA sequence isolated from *O. pumila* (*OpCPR*) contained a 2,073-bp ORF encoding a protein of 690 amino acids with a molecular mass of 76.6 kDa. The deduced amino acid sequence of OpCPR showed high identity with *Arabidopsis thaliana, Petroselinum crispum*, *Pisum sativum*, and *Triticum aestivum* CPRs (72, 66, 65, and 67%, respectively). Southern blot analysis suggested that only a single CPR-encoding gene was present in the genome of *O. pumila*. Mirroring the general importance of the enzyme, *OpCPR* was expressed in all tissues.

Studies have been performed to investigate the effects of stress compounds, such as methyl jasmonate (MeJA), salicylic acid (SA), and yeast extract (YE), on the expression of *OpSTR*, *OpTDC*, and *OpCPR* in *O. pumila* hairy roots [46]. The changes in the expression patterns of *OpSTR* and *OpTDC* in response to these various compounds were highly similar. In particular, *OpSTR* and *OpTDC* expression was repressed by SA and YE treatments but unaffected by MeJA. Meanwhile, no treatment resulted in the induction or repression of *OpCPR* transcripts. In addition, no change in STR activity was observed after treatment with either stress compounds or phytohormones.

3.3.2 In Silico and In Vitro Tracer Studies with [1-13C] glucose

Both the mevalonate (MVA) pathway [51] and the 2*C*-methyl-D-erythritol 4-phosphate (MEP) pathway [52–54] have been recognized for their role in the formation

of isopentenyl diphosphate, the precursor of terpenoid biosynthesis. Yamazaki et al. [55] investigated the incorporation of [1-^{13}C]glucose into camptothecin in the hairy roots of *O. pumila* by *in silico* computation using the Atomic Reconstruction of Metabolism (ARM) [56] program and by *in vivo* tracer experiments. The ^{13}C-nuclear magnetic resonance (^{13}C-NMR) analysis clearly showed that the secologanin moiety of camptothecin was synthesized via the MEP pathway. Furthermore, in *O. pumila* hairy root cultures, treatment with fosmidomycin, a specific inhibitor of the MEP pathway, resulted in a significant decrease in camptothecin production. These results support the conclusion that the secologanin moiety of camptothecin is derived from the MEP pathway.

3.4 Metabolic Modification in Hairy Roots of *O. pumila* by RNA Interference

A detailed understanding of camptothecin production, including the enzymatic pathway for its biosynthesis, will be essential to the ultimate goal of the metabolic engineering of this compound. In *Papaver somniferum* (opium poppy), genetic approaches using antisense RNA [57, 58] or RNA interference (RNAi)-mediated silencing [59] of biosynthetic enzymes have been performed, leading to rapid progress in the metabolic engineering of benzylisoquinoline alkaloids. Therefore, it is considered that RNAi technology is an effective strategy for investigating camptothecin biosynthesis. In our study, the production of camptothecin, strictosidine, and camptothecin-related alkaloids was suppressed in a *TDC* expression-dependent manner in RNAi hairy roots. Among the hairy root-specific peaks correlated with *TDC* expression in the liquid chromatography/Fourier transform ion cyclotron resonance mass spectrometry (LC-FTICR-MS) analysis, two unknown peaks with a positive correlation were annotated as alkaloids and six unknown peaks with a negative correlation, as flavonoids. The exact mass of several non-annotated peaks was similar to those of predicted intermediates in camptothecin biosynthesis, suggesting that most peaks that positively correlated with *TDC* expression could be intermediates in camptothecin biosynthesis [60].

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