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Biosynthesis and biotechnological production of anti-cancer drug Camptothecin

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Abstract Camptothecin (CPT) is a kind of modified monoterpene indole alkaloid firstly identified from woody plant *Camptotheca acuminata,* and its semisynthetic CPT analogs irinothecan and topothecan are clinically used for the treatment of various cancers throughout the world. However, the extraction of CPT from limited natural CPT-producing plant resources couldn't meet the rapidly increasing market need. The development of plant metabolic engineering provides one alternative way to increase CPT yield by genetic manipulation, which relies on in-depth understanding of the CPT biosynthesis pathway. Several attempts have been also made to obtain CPT by biotechnological approaches such as cell suspensions, endophytic fungi, hairy roots, elicitation as well as metabolic engineering in the past decade. Here, recent advances in knowledge of biosynthesis of CPT, gene isolation, molecular regulation, production improvement and biotechnological methods are summarized and future perspectives are also discussed in this review.

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Introduction

Camptothecin (CPT) is a kind of modified monoterpene indole alkaloid (Fig. 1), which was firstly isolated from endemic Chinese happy tree *Camptotheca acuminata* (Nyssaceae) (Wall et al. 1966). CPT was discovered to own one specific anti-cancer mechanism by inhibition of DNA topoisomerase I to kill cancer cells, which is different from other famous anticancer agents such as Taxol®, and it attracted considerable attention for clinical trials (Hsiang et al. 1985). Due to its quite low water solubility and severe side effects, CPT itself was not suitable for clinical application (Lorence et al. 2004). However, some semisynthetic water-soluble CPT derivatives such as topotecan and irinotecan (Fig. 1) were approved by the US Food and Drug Administration (FDA) in 1994 and were successfully used for the treatment of various cancers such as ovarian, lung, colorectal cancers and so on throughout the world (Kai et al. 2008; Lu et al. 2009).

Because of their excellent anti-cancer activity and extensive clinical use, the combined sales of irinotecan and topotecan had reached 1.5 billion US dollars in 2002 and rose to 2.2 billion US dollars in 2008 with

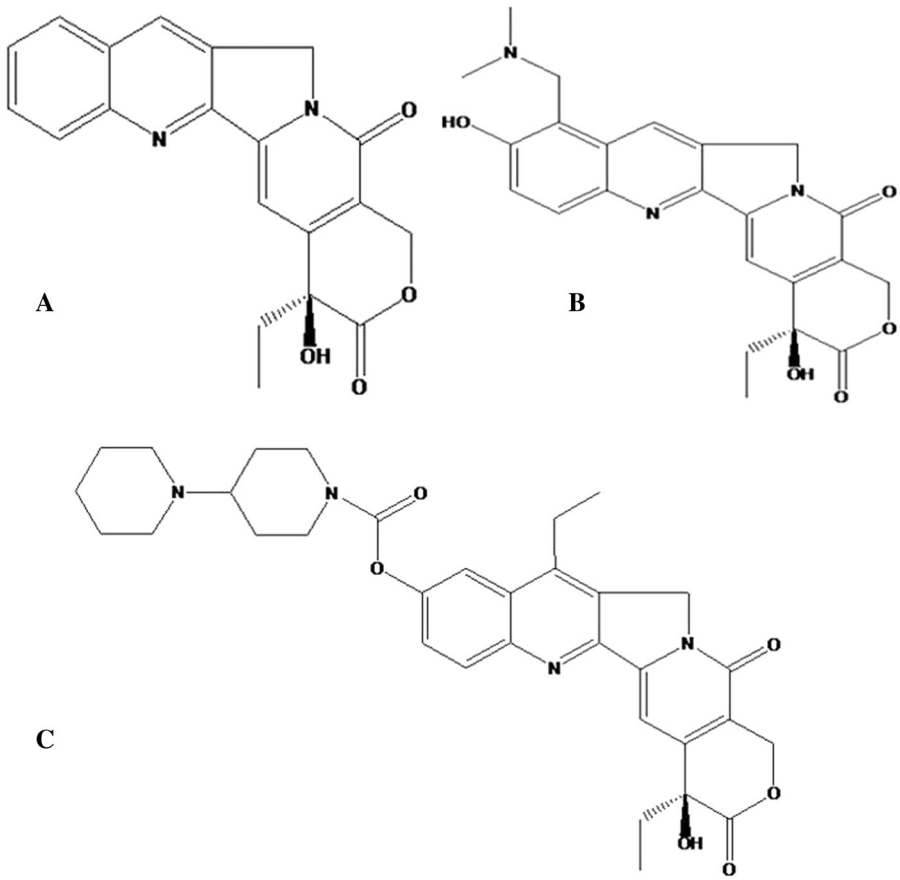


Fig. 1 Chemical structures of Camptothecin and its two derivatives as anticancer drugs. **a** Camptothecin, **b** topotecan, **c** irinotecan

the tendency of increase (Lorence and Nessler 2004; Kai et al. 2014). Currently, these CPT analogs mentioned above are all synthesized from natural camptothecin which is mainly extracted from plants such as *C. acuminata* (Beegum et al. 2007). Till now, Camptothecin has been also found the existence in other distantly related plants such as *Ervatamia heyneana* (Apocynaceae) (Gunasekera et al. 1979), *Merrilliodendron megacarpum* (Icacinaceae) (Arisawa et al. 1981), *Nothapodytes foetida* (Icacinaceae) (Govindachari and Viswanathan 1972) and some *Ophiorrhiza* species (Rubiaceae) (Tafur et al. 1976; Aimi et al. 1989; Beegum et al. 2007). However, the extraction of CPT from limited natural CPT-producing plant resources, which may result in environmental concerns, wouldn't meet the expanding need of the market (Lorence and Nessler 2004; Sirikantaramas et al. 2007; Yamazaki et al. 2010; Kai et al. 2008, 2013, 2014). Due to low content of CPT in plants (about 1 mg/g dry weight (DW), Lopez-Meyer et al. 1994), it is very important to increase CPT production and develop sustainable methods to obtain CPT for clinical applications (Ni et al. 2011; Cui et al. 2015).

The rapid development of plant biotechnology alternatively provides one promising approach to enhance CPT production by transferring key CPT biosynthetic genes (and/or transcript factor) into CPT-producing plant cell, and then large scale culture of transgenic cell lines, hairy roots or regenerated plants to obtain CPT (Lu et al. 2009; Cui et al. 2015). This, however, significantly relies upon the in-depth understanding of CPT biosynthetic pathway and molecular regulation mechanism (Ni et al. 2011; Kai et al. 2013). In the past decade, much progress has been made in CPT biosynthesis and biotechnological production of CPT. This review is to summarize the recent advances in the understanding of CPT biosynthesis pathway, molecular regulations and various biotechnological ways for increasing CPT production and the future prospects.

Isolation, biosynthesis and regulation of CPT in plants

Isolation of CPT from natural resource plants

Early in 1966, extraction of Camptothecin was firstly reported from dried *C. acuminata* stems using so-called standard fractionation method, which consists of continuous and hot hexane-heptane extraction followed by similar extraction with 95 % ethanol, and Camptothecin was then isolated by silica column chromatography followed by recrystallization (Wall et al. 1966). Until 1992, methanol was not attempted as alternative solvent to extract Camptothecin (van Hengel et al. 1992), and later methanol with concentrations of 70 % was found to display optimal effect for the CPT extraction (Zhang et al. 2007).

Normally, there are three drying methods including oven-drying, air-drying, and freeze-drying was used to dry natural CPT-producing plant resources such as *C. acuminata*. Freeze-drying method showed a 27 % higher CPT concentration than oven- or air-dried ways, implied that oven- and air-drying caused some degree of degradation of CPT (Liu et al. 1998). Among different extraction methods such as stirring extraction, Soxhlet extraction, ultrasonic extraction and microwave-assisted extraction (MAE), MAE was testified more efficient in short time (only need 3 min) than the other extraction techniques at least in *N. foetida* (Fulzele and Satdive 2005).

Biosynthetic pathway of CPT

CPT belonging to one kind of monoterpenoid indole alkaloids (TIA), its biosynthesis pathway is a very complicated process consisted of many distinct enzymatic steps (Fig. 2), which is not completely defined especially in later specific stage to form CPT (Lorence and Nessler 2004). CPT as well as other TIAs, are derived from the common precursor strictosidine, which is condensed product by the monoterpenoid secologanin and the amino acid derivative indole tryptamine catalyzed by the enzyme strictosidine synthase (Kutchan 1995; Lu et al. 2009). Tryptamine is synthesized via the shikimate pathway and secologanin comes from the terpene biosyntheis pathway (Yamazaki et al. 2004; Kai et al. 2014).

In higher plants, there are two different isoprenoids biosynthesis pathways (MVA occurring in the cytosol and MEP pathway in the plastids) responsible for the synthesis of universal five-carbon precursor isopentenyl pyrophosphate (IPP) to form various isoprenoids including tanshinone, Taxol and CPT (Liao et al. 2009; Kai et al. 2011a, 2013; Shi et al. 2014). More and more studies revealed that some degree of crosstalk between the above two pathways (Aule et al. 2003; Kai et al. 2011a, 2014), which means that both MVA and MEP pathway provide common terpene precursor for CPT biosynthesis and the latter is main resource (Kai et al. 2011a, 2014). Then strictosidine is transformed into strictosamide, and the remaining steps and precise intermediates between strictosamide and CPT still remains to be identified now (Lorence and Nessler 2004). Recently several genes involved in CPT biosynthesis have been isolated and characterized from CPT-producing plants such as *C. acuminata* and *Ophiorrhiza pumila* by various research groups as describe below (Table 1).

Cloning and characterization of CPT biosynthetic genes

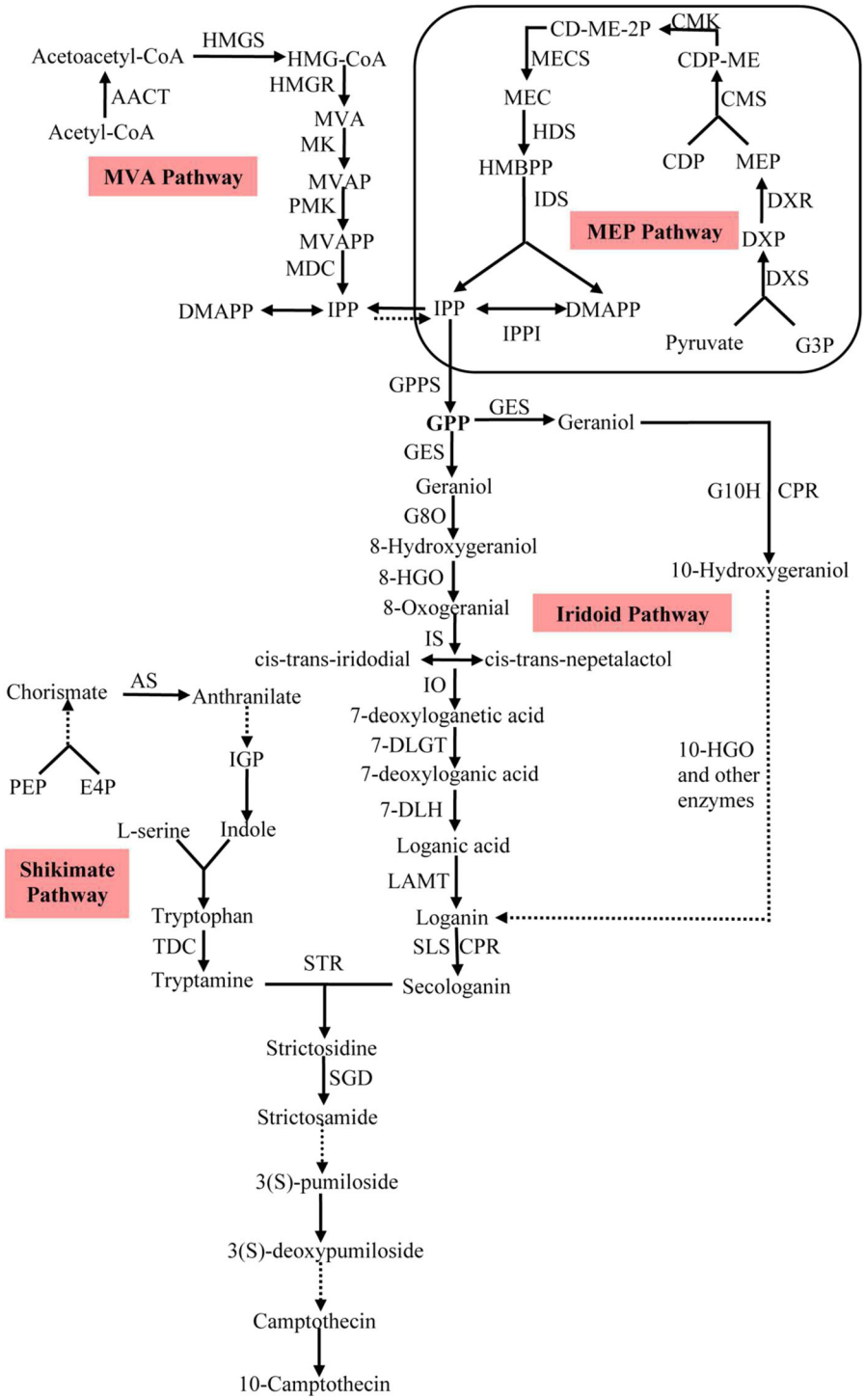
Genes in early stage

3-hydroxy-3-methylglutaryl-CoAreductase (HMGR)

The 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), which catalyzes the conversion of 3-hydroxy-methylglutaryl-CoA (HMG-CoA) to MVA, has been considered as the first key step in the MVA pathway in plants (Liao et al. 2009). A small gene family of HMGR is found and three alleles, *HMGR*1, *HMGR* 2, *HMGR* 3 have been isolated from *C. acuminata* with different expression profiles (Maldonado-Mendoza et al. 1997). *HMGR* 1 is only be detected in seedling but not nutritive organs of veteran, whose expression could be induced by the development and damage and suppressed by MeJA in transgenic tobacco (Burnett et al. 1993). Interestingly, *HMGR* 2 and *HMGR* 3 can express normally without being affected by damage or MeJA addition (Maldonado-Mendoza et al. 1997), implying the diverse role of each HMGR gene member in *C. acuminata*.

3-hydroxy-3-methylglutaryl-CoA synthase (HMGS)

3-hydroxy-3-methylglutaryl-CoA synthase (HMGS) catalyzes the condensation of acetyl CoA and acetoacetyl CoA to form 3-hydroxy-3-methylglutaryl-CoA


Acetoacetyl-CoA
HMGS
HMG-CoA
HMGR
AACT
Acetyl-CoA
MVA
MK
MVAP
PMK
MVAPP
MDC
MVA Pathway
DMAPP
IPP
IPP
DMAPP
IPPI
CD-ME-2P
CMK
MECS
CDP-ME
MEC
CMS
HDS
CDP
MEP
HMBPP
DXR
IDS
MEP Pathway
DXP
DXS
Pyruvate
G3P
GPPS
GPP
GES
Geraniol
GES
Geraniol
G10H
CPR
G8O
8-Hydroxygeraniol
10-Hydroxygeraniol
8-HGO
8-Oxogeranial
Iridoid Pathway
IS
cis-trans-iridodial
cis-trans-nepetalactol
IO
Chorismate
AS
Anthranilate
7-deoxyloganetic acid
7-DLGT
IGP
7-deoxyloganic acid
10-HGO
and other
enzymes
PEP
E4P
7-DLH
L-serine
Indole
Loganic acid
Shikimate
Pathway
LAMT
Tryptophan
Loganin
TDC
SLS
CPR
Tryptamine
STR
Secologanin
Strictosidine
SGD
Strictosamide
3(S)-pumiloside
3(S)-deoxypumiloside
Camptothecin
10-Camptothecin

◀**Fig. 2** Proposed Camptothecin biosynthetic pathway in plants. *Dotted line arrows* indicate multiple steps between intermediates. *CMS,* 4-(cytidine 5-diphospho)-2-C-methylerythritol synthase; *CMK*, 4-(cytidine 5-diphospho)-2-C-methylerythritolkinase; *MECS*, 2-C-methylerythritol-2,4-cyclodiphosphate synthase; *HDS*, hydroxymethylbutenyl 4-diphosphate synthase; *IDS*, IPP/DMAPP synthase; *IPI*, IPP isomerase; *PTS*, isopreny-ltransferase; *GPPS*, geranyl pyrophosphate synthase; *TPS*, terpene synthase; *G10H*, Geraniol-10-hydroxylase; *CPR*, NADPH-Cytochrome P450 reductase; *10-HGO*, 10-hydroxy-geraniol oxidoreductase; *8-HGO*, 8-hydroxy-geraniol oxidoreductase; *IS*, iridoid synthase(IS); *IO*, iridoid oxidase (CYP76A26); *DLGT*, 7-deoxyloganetic acid UDP-glucosyltransferase; *DLH*, 7-deoxyloganic acid hydroxylase (CYP72A224); *LAMT*, loganic acid O-methyltransferase; *SLS*, Secologanin synthetase; *AACT*, acetyl-CoA: acetyl-CoA C-acetyltransferase; *HMGS*, 3-hydroxy-3-methylglutaryl-CoA synthase; *HMGR*, 3-hydroxy-3-methylglutaryl-CoA reductase; *MK*, mevalonate kinase; *PMK*, phosphomevalonate kinase; *MDC*, mevalonate 5-diphosphate decarboxylase; *AS*, anthranilic acid synthetase; *PAT*, 5-phosphoribosylanthranilate transferase; *PAI*, 5-phosphoribosylanthranilate isomerase; *IGS*, indole glycerol phosphate synthase; *TSB*, tryptophan synthase beta; *TDC*, tryptophan decarboxylase; *STR*, strictosidine synthase; *SGD*, Strictosidine beta-glucosidase (Kai et al. 2014; Cui et al. 2015)

as an early step in the MVA pathway (Kai et al. 2006; Zhang et al. 2011). Based on homology-cloning strategy by rapid amplification of cDNA ends (RACE) (Kai et al. 2012a), a full-length cDNA of HMGS (EU677841) was successfully isolated from young leaves of *C. acuminata*which consists of 1801 bp with a 1413 bp open reading frame (ORF) encoding a 471 amino acid protein (Kai et al. 2013). As revealed by southern blotting, at least two *HMGS* gene members existed in the *C. acuminata* genome, *HMGS* expressed strong in hypocotyls and cotyledons but undetectable in roots, in accordance with CPT distribution in these tissues (Kai et al. 2013).

DXP reductoisomerase (DXR) DXP reductoisomerase (DXR, EC: 1.1.1.267) which converts DXP to MEP, is the second enzyme of MEP pathway and has been regarded as a committed step (Lois et al. 2000). For example, overexpression of *DXR* in Peppermint can lead to increased production of monoterpenes essential oil (Mahmoud and Croteau 2001) and MEP-derived plastid diterpenoids such as tanshinone in *Slavia miltiorrhiza* (Shi et al. 2014). The full-length *DXR* cDNA sequence was isolated from *C. acuminata*, and *CaDXR* expressed strongly in stem, weak in leaf and root while it is significantly induced by exogenous elicitor methyl jasmonate (Yao et al. 2008).

1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase (HDR) 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase (HDR) is the last catalytic enzyme of the MEP pathway (Hsieh and Goodman 2005). A full-length cDNA of HDR was successfully isolated from *C. acuminata* and functionally identified in *Escherichia coli*, which consists of 1686 bp with a 1377 bp open reading frame (ORF) encoding a 459 amino acid protein (Wang et al. 2008a). As revealed by southern blotting, *HDR* of *C. acuminata* belonged to a low-copy gene family, *which* expressed constitutively in all tested plant organs with the highest level in flowers and it is induced by methyl-jasmonate (MeJA) but not salicylic acid (SA) (Wang et al. 2008a).

Isopentenyl diphosphate isomerase (IPI) Isopentenyl diphosphate isomerase (IPI) catalyzes the revisable conversion of isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP), which are the essential common precursors for biosynthesis of isoprenoids including CPT (Pan et al. 2008). A full-length cDNA of IPI (DQ839416) was successfully isolated from *C. acuminata* and functionally identified in *E. coli*, which consists of 930 bp open reading frame (ORF) encoding a 309 amino acid protein (Pan et al. 2008). Tissue expression analysis results showed that *IPI* expressed high in stems, moderate in roots and tender in leaves but not in mature leaves and fruits (Pan et al. 2008).

Anthranilate synthase (ASA) Anthranilate synthase catalyzes the conversion of chorismate into anthranilate, is the first committed step in the indole pathway (Herrmann and Weaver 1999). In plants, anthranilate synthases are commonly composed of two non-identical subunits, namely alpha (ASA) and beta subunit (ASB) (Crawford 1989). The beta subunit of tryptophan synthase (TSB) whose expression parallels CPT production in seedlings, was isolated from *Camptotheca* early (Lu and McKnight 1999). The two ASA genes were isolated from *C. acuminata* (*asa* 1 and *asa* 2) (Lu et al. 2005). Expression of *ASA*2 is constitutively low in *C. acuminata* while asa1 expressed in all the tested organs with varying levels, whose spatial and developmental regulation of *ASA* 1 is consistent with *TSB* as well as CPT accumulation, suggesting that *ASA* 1 other than *ASA* 2 is involved in CPT biosynthesis.

Table 1 Isolated genes involved in CPT biosynthetic pathway in plants

Genes	Encoding enzymes	Accession no.	Organism	Length of AA	Expression profiling	References
CaTDC1	Tryptophan decarboxylase	U73656	*C. acuminata*	502	Regulated by developmental stage and associated with CPT biosynthesis	López-Meyer and Nessler (1997)
CaTDC2	Tryptophan decarboxylase	U73657	*C. acuminata*	498	Responsive to elicitors such as fungal elicitor or methyl jasmonic acid (MJ)	López-Meyer and Nessler (1997)
OpTDC	Tryptophan decarboxylase	AB086168	*O. pumila*	506	Expressed in hairy roots, root and stem, but not in leaves; repressed by salicylic acid (SA) and YE but induced by DMSO	Yamazaki et al. (2003)
CaG10H	Geraniol-10-hydroxylase	JF508378	*C. acuminata*	501	Constitutively expressed and responsive to MJ	Sun et al. (2011)
OpG10H	Geraniol-10-hydroxylase	LC010422	*O. pumila*	512	Not examined	Asano et al. (2013)
CaSTR	Strictosidine synthase	JF508375	*C. acuminata*	330	Constitutively expressed and responsive to MJ and SA	Sun et al. (2011)
OpSTR	Strictosidine synthase	AB060341	*O. pumila*	351	Expressed in hairy roots, root and stem, but not in leaves; repressed by SA and YE but induced by DMSO	Yamazaki et al. (2003)
OjSTR	Strictosidine synthase	EU670747	*O. japonica*	353	Constitutively expressed and responsive to elicitors including MJ and SA	Lu et al. (2009)
OpCPR	NADPH-cytochrome P-450 reductase	AB086169	*O. pumila*	690	Constitutively expressed in hairy roots, roots, stems and leaves; NOT responsive to elicitors including SA and YE	Yamazaki et al. (2003)
CaSLS	secologanin synthase	HQ605982	*C. acuminata*	524	MJ-responsive and higher expression in young leaves and petioles	Sun et al. (2011)
OpSLS	secologanin synthase	LC010421	*O. pumila*	520	Not examined	Asano et al. (2013)
CaTSB	Tryptophan synthase beta subunit	AF042320	*C. acuminata*	466	Constitutively expressed with higher levels in apex, bark, young leaf and young stem	Lu and McKnight (1999)
CaIPI	isopentenyl diphosphate isomerase	DQ839416	*C. acuminata*	309	Expressed higher in stems than in roots and tender leaves, but not in mature leaves and fruits	Pan et al. (2008)
CaHMGS	3-hydroxy-3-methylglutaryl-CoA synthase	EU677841	*C. acuminata*	471	Expressed strong in hypocotyls and cotyledons and MJ-responsive	Kai et al. (2013)
CaHMGR1	3-hydroxy-3-methylglutaryl CoA reductase	L10390	*C. acuminata*	593	Wounding and MJ responsive	Burnett et al. (1993)
CaHMGR2	3-hydroxy-3-methylglutaryl CoA reductase	U72146	*C. acuminata*	589	Not responsive to wounding and MJ	Maldonado-Mendoza et al. (1997)
CaHMGR3	3-hydroxy-3-methylglutaryl CoA reductase	U72145	*C. acuminata*	575	Not responsive to wounding and MJ	Maldonado-Mendoza et al. (1997)
CaHDR	hydroxymethylbutenyl diphosphate reductase	DQ864495	*C. acuminata*	459	Constitutively expressed with highest level in flowers, responsive to MJ but not by SA	Wang et al. (2008a, b)

Table 1 continued

Genes	Encoding enzymes	Accession no.	Organism	Length of AA	Expression profiling	References
CaDXR	1-deoxy-D-xylulose 5-phosphate reductoisomerase	DQ355159	*C. acuminata*	472	Constitutively expressed with strong level in stem and weak in root and leaf	Yao et al. (2008)
CaASA1	anthranilate synthase alpha	AY753655	*C. acuminata*	581	Constitutively expressed and regulated by seedling development; associated with CPT biosynthesis	Lu et al. (2005)
CaASA2	anthranilate synthase alpha	AY753656	*C. acuminata*	579	Constitutively expressed with quite low levels and not regulated by seedling development	Lu et al. (2005)
CaSGD	strictosidine beta-D-glucosidase	JF508379	*C. acuminata*	532	Not examined	Sun et al. (2011)

Genes in late stage

Tryptophan decarboxylase (TDC) During the process of CPT biosynthesis, tryptophan decarboxylase (TDC) catalyzes tryptophan into tryptamine which is necessary for indole ring formation of CPT and its derivate (De Luca et al. 1989). Since this reaction represents a branching point from primary into a secondary metabolism, TDC is looked as one key enzyme in biosynthesis of monoterpene indole alkaloids including CPT. The cDNA clone encoding TDC has been firstly isolated from *Catharanthus roseus* (De Luca et al. 1989), then from *C. acuminata* (López-Meyer and Nessler 1997). The *TDC 1* gene is regulated by developmental stage and expressed highest in the stem tip, caulicle and bark, consistent with the distribution of CPT, which is associated with CPT accumulation (López-Meyer and Nessler 1997). Whereas *TDC 2* expression cannot be detected in undisposed organs or buds of development period and it could be induced by fungal elicitor or methyl jasmonic acid but not for tdc1, demonstrating *TDC* 2 is part of the defense system (López-Meyer and Nessler 1997). CPT accumulation was detected in epidermal idioblasts, some glandular trichomes, and groups of idioblast cells localized in parenchyma tissues and CPT accumulation could be increased by drought-stress (Valletta et al. 2010). Gene *TDC* was also isolated from *O. pumila*, the highest *TDC* expression occurred in hairy roots, followed by roots and stems, and undetected in leaves of plant (Yamazaki et al. 2003).

Geraniol-10-hydroxylase (G10H) Geraniol-10-hydroxylase (G10H), being a cytochrome P450 monooxygenase, can hydroxylate geraniol at the C-10 position to generate 10-hydroxy-geraniol, which is also considered to be a committed step in the biosynthesis of secologanin and even TIAs (Collu et al. 2002). G10H has been reported to be a rate-limiting enzyme in the biosynthesis of terpenoid indole alkaloids in transgenic *C. roseus* (van der Fits and Memelink 2000; Wang et al. 2010; Pan et al. 2012). The *G10H* gene was firstly cloned from *C. roseus* (Collu et al. 2002) and then from *C. acuminata* recently (Sun et al. 2011). G10H were regulated in a waveform manner by MJ treatment, including two expression peaks during 24 h and constitutively expressed in young leaves, old leaves, petioles,

stems, root bark and root but with highest level in petioles (Sun et al. 2011).

Strictosidine synthase (STR) Strictosidine synthase (STR) catalyses tryptamine and the secologanin into the important TIA intermediate Strictosidine (Lu et al. 2009), has been identified as a key enzyme for biosynthesis of TIA including CPT. The *STR* gene was firstly isolated from *Rauvolfia serpentina* (Kutchan 1989) and then from *C. roseus* (McKnight et al. 1990). In transgenic *C. roseus*, over-expression of *STR* showed tenfold higher STR activity than wild-type, which exhibited great enhancement effect on TIA biosynthesis (Canel et al. 1998). The first *STR* gene isolated from CPT-producing plant comes from *O. pumila* (Yamazaki et al. 2003). Using RACE-PCR, we successfully isolate a full-length *str* cDNA from young leaves of *O. japonica* and *STR* constitutively expressed in all the tested tissues including flower, leaf, root and stem (from high to low expression level) and responsive to methyl jasmonate and salicylic acid (Lu et al. 2009). Due to low activity of STR in *C. acuminata* plants and suspension culture cells, it is not easy to obtain this gene using forward genetics method. Recently, by employing transcriptomic sequencing a *STR* gene was successfully identified in C. *acuminata* (Sun et al. 2011).

Secologanin synthase (SLS) Secologanin synthase (SLS), the second CYP450, is the last enzyme in the biosynthesis of secologanin (Sun et al. 2011). The precursor, secologanin, is biosynthesized from loganin by secologanin synthase (SLS) (Yamamoto et al. 2000). The gene CYP72A1 from *C. roseus* was firstly identified to encode SLS (Irmler et al. 2000). One putative *SLS* gene in *C. acuminata* was cloned (GenBank ID: HQ605982) by transcriptomic sequencing and was likely targeted to the endoplasmic reticulum (ER) membrane by subcellular localization prediction (Sun et al. 2011). The *SLS* gene were also MJ-responsive but with much weaker level than *G10H* and expression of *SLS* is highest in young leaves and then petioles, but very weak in old leaves, stems, root bark and root n (Sun et al. 2011).

CPT is one of the most promising plant anti-tumor drugs and much progress has made in gene isolation, but yet several gaps existed in the whole CPT biosynthetic pathway and very little is known about molecular regulatory mechanism of production, for example no any related functional transcription factor was identified from CPT-producing plants until now.

Metabolic engineering of CPT biosynthesis

Due to the shortage of natural resources for CPT extraction and related environmental concerns, it has become a keen issue to produce CPT by genetically engineered plant cell cultures (Yamazaki et al. 2003; Sirikantaramas et al. 2007; Asano et al. 2013). Isolation of the above CPT biosynthetic genes provide possibility to genetic modification of pathway in CPT-producing plants but with very slow advances (Ni et al. 2011; Asano et al. 2013; Cui et al. 2015).

Since most of CPT-producing plants are woody, it is difficult to establish a stable transformation system for woody plant *C. acuminata* and led to few successful reports on introducing a CPT biosynthetic gene into *C. acuminata* by metabolic engineering in the past two decades (Ni et al. 2011), although much effort was put into optimization of transformation procedures and conditions for *C. acuminata* (Wang et al. 2008a, b).

As mentioned above, successful establishment of hairy root culture system for *O. pumila*, which provided an alternative experimental model system for CPT biosynthesis and production (Saito et al. 2001). In most *TDC*- and *SLS*-suppressed *O. pumila* hairy root lines by RNA interference (RNAi), accumulation of CPT and related alkaloids, strictosidine, strictosamide, pumiloside, and deoxypumiloside was reduced, suggesting they was possibly involved in CPT biosynthesis (Asano et al. 2013). Based on our optimized *O. pumila* hairy root culture system *STR* and *G10H* genes from *C. roseus* were separately and simultaneously introduced into *O. pumila* hairy roots and co-overexpression of *G10H* and *STR* genes caused a 56 % increase on the yields of CPT (1.77 mg/g) with respect to the control hairy root culture (Cui et al. 2015). Furthermore, ORCA3 is a jasmonate responsive APETALA2-domain transcript factor isolated from *C. roseus*, with strong ability to up-regulate expression of several key genes involved in TIA biosynthetic pathway (van der Fits and Memelink 2000). Overexpression of *ORCA3* in transgenic *C. acuminata* hairy root lines can effectively enhance the production of CPT with 1.5-fold compared with the control (1.12 mg/g dw) (Ni et al. 2011). The above results revealed that metabolic engineering is an

effective strategy for improving CPT biosynthesis in the future.

Biotechnological production of CPT

In vitro culture system

Plant cell suspension culture technology is an effective way for production of valuable secondary metabolites such as taxol (Kai et al. 2006, 2014). The first callus induction and culture study of *C. acuminata* was performed 40 years before, which only produced 2.5 ug/g CPT DW (Sakato et al. 1974). Later reports showed that from 4 to 41 μg/g CPT DW could be produced in cell suspensions of *C. acuminata* (van Hengel et al. 1992; Zhang et al. 2002; Ma 2007). The above results implied that too lower content of CPT (2.5–41 μg/g) was produced in vitro callus or cell cultures of CPT-producing plants than soil-grown plant such as *C. acuminata* (about 0.2–1 mg/g). Even no CPT was produced in cell cultures of *O. pumila* (Kitajima et al. 1998), which reflected complicated situations between different plants and limited cell suspension culture to obtain CPT further.

Hairy root, which is caused by *Agrobacterium rhizogenes*, owns some advantages such as rapid growth rate, unlimited branching, and biochemical and genetic stability and is considered as an effective means to produce high-value secondary metabolites (Li et al. 2008; Georgiev et al. 2012; Kai et al. 2011a, b, 2012b, c, d; Hao et al. 2015; Shi et al. 2014). Hairy root was induced from *C. acuminata* by *A. rhizogenes* strains ATCC 15834 and R-1000, and were able to synthesize about 1.0 and 0.15 mg/g dry weight for CPT and the HCPT similar to roots of plants (Lorence et al. 2004). We also optimized hairy root induction conditions and established hairy root system of *C. acuminata*, but they generally grew slowly with very limited branching (Wang et al. 2008a, b).

Excitingly, a hairy root culture of *O. pumila* transformed by *A. rhizogenes* strain 15,834 was established by Japan scientist and the hairy root culture grew well with rapid biomass increase and produced high level of CPT (reached up to 0.1 % per dry weight), which provides an alternative experimental model system for CPT biosynthesis and production (Saito et al. 2001). Recently hairy root induction conditions of *O. pumila* were also optimized with much more induction efficiency in our group (Cui et al. 2015). Indeed, *O. pumila*, *O. liukiuensis*, and *O. kuroiwai* hairy root cultures have shown good results for CPT production (Saito et al. 2001; Sudo et al. 2002; Asano et al. 2004). Hairy roots was also induced from *O. alata* infected by *A. rhizogenes* TISTR 1450 and accumulated CPT at level of 785 mg/g dry weight which was twice that of roots of soil-grown plants and similar to *O. pumila* hairy roots (Ya-ut et al. 2011). All the above resulted suggested that hairy root systems of some CPT-producing *Ophiorrhiza* weedy plant are a promising way to obtain CPT in the future.

Elicitors treatment

Elicitation by treatment of plant cell or tissue with elicitors, is one kind of effective strategy for enhancing plant secondary metabolite accumulation (Luo et al. 2012; Wang and Wu 2013; Kai et al. 2014; Hao et al. 2015). Abiotic elicitors such as metal ions and inorganic compounds and biotic elicitors as fungi, bacteria and viruses have been widely used with good effects (Zhao et al. 2005; Zabala et al. 2010; Luo et al. 2012; Kai et al. 2012b, c). The most common elicitors used in plant tissue cultures include yeast extract (Kai et al. 2012b, c), and some important plant hormones molecules such as jasmonic acid (JA) and salicylic acid (SA) (Liao et al. 2009; Fujimoto et al. 2011; Hao et al. 2015; Kai et al. 2014).

The addition of yeast extract, jasmonic acid and methyl jasmonic acid could significantly enhance the content of CPT (Song and Byun 1998). However, in the hairy roots of *O. pumila*, elicitors and signal compounds did not significantly increase CPT production (Saito et al. 2001). Addition of $CuCl_2$ with optimum concentration of 0.008 mg/mL, increased CPT production for 30 times (1.17 mg/g fresh weight, FW) than control (0.04 mg/g FW) with no inhibitive effects on cell growth (Gu et al. 2006). Among various tested treatments, UV-B showed the most notable effects for CPT (11-fold increase, 0.00115 mg/g dw) while salicylic acid showed a 25-fold increase (0.00056 mg/g dw) for 10-hydroxycamptothecin (Pi et al. 2010). Abscisic acid (ABA, 100 μM), methyl jasmonate (MJ, 100 μM) and salicylic acid (SA, 1 mM) were used to treat *C. acuminata* seedlings and the results showed that all three elicitors enhanced both CPT and 10-hydroxycamptothecin accumulation, among which abscisic acid (ABA) exhibited the most

effective elicitation with the increment of 1.44-fold for CPT (1.81 mg/g DW) and 1.21-fold for HCPT (2.6 mg/g DW), respectively (Kai et al. 2014). Expression profiles results indicated that there is a positive correlation between gene expression and alkaloid accumulation, suggesting that CPT is accumulated by synchronous up-regulation of several CPT biosynthetic genes such as HMGR, DXR and TDC (Kai et al. 2014).

Endophytic fungi

Endophytes are the microorganisms that reside in the tissues of living host plants without causing apparent disease symptoms and are considered as a potential resource to produce natural bioactive compounds (Chandra 2012). Being a kind of readily renewable, reproducible, and inexhaustible source (Chandra 2012), plant endophytic fungi is much easier for mass-production by fermentation in bioreactors which is different from plant cell or tissues.

The first taxol-producing endophytic fungus *Taxomyces andreanae* was isolated in 1993 (Stierle et al. 1993), reflecting that endophytic fungi own the ability to produce some important native product as dose in the plant host during long coexistence process. After more than 10 years, the first CPT-producing endophytic fungus *Entrophospora infrequens,* belonging to the family Phycomycetes, was obtained from the inner bark of *Nothapodytes foetida* from India (Puri et al. 2005). When it was grown in a synthetic liquid medium (Sabouraud broth) under shake flask and bench scale fermentation conditions, CPT could be produced as identification by means of chromatographic and spectroscopic methods as well as anticancer activity, providing an easily accessible source for the production of CPT (Puri et al. 2005). Amna et al. (2006) investigated the growth and CPT production of *E. infrequens* isolated from *N. foetida* in bioreactor, which was testified to have the potential to produce CPT. CPT and two of its analogues, 9-methoxycamptothecin and 10-hydroxycamptothecin could be produced by a novel endophytic fungus isolated from the inner bark of *C. acuminata*, in rich mycological medium under shake-flask fermentation conditions (Kusari et al. 2009). CPT and 10-hydroxycamptothecin was discovered in endophytic fungi *Fusarium solani* from *Apodytes dimidiata* (Icacinaceae) in India (Shweta et al. 2010). Shweta et al. (2010) reported the production of CPT, 9-methoxycamptothecin and 10-hydroxycamptothecin by endophytic fungi strains isolated from *Apodytes dimidiata* (Icacinaceae). Interestingly, the production of CPT and 9-methoxy CPT (9-MeO-CPT) in culture was firstly reported by endophytic bacteria isolated from *Miquelia dentate* Bedd. (Icacinaceae), independent of the host tissue (Shweta et al. 2013).

The above studies indicated that CPT and its derivatives could be produced by endophytes isolated from CPT-producing host plants (Table 2), provide an alternative method to obtain CPT and other new drugs. However, the CPT yield of all the above endophytic fungi is very low than that in host plants and is genetically unstable (the ability of CPT production would frequently be attenuated or even lost over subculture generations), which is one of the major challenges that needs to be conquered for in vitro production by CPT-producing endophytes (Pu et al. 2013).

Conclusion and future prospects

Despite of excellent anti-cancer active of CPT derivatives such as irinothecan and topothecan and rapid increasing market need, CPT is still obtained by the extraction from natural plant resources such as *C. acuminata*, which cannot meet the heavy demand from the global market. Therefore, it is of significance to increase CPT production and develop sustainable methods to obtain CPT to meet the rapidly increasing market need by biotechnological approaches in the future. The rapid development of mordern biotechnology provides a new promising way to improve CPT production by genetic manipulation of CPT producing plants, however which depended on in-depth understanding of the CPT biosynthesis pathway and its molecular regulation mechanism (Lu et al. 2009). Much progress has been made to understanding the CPT biosynthesis pathway in the past decade, however, the precise steps between strictosidine and CPT are not very clear in CPT-producing plants, which involves a series of oxidation and hydroxylation reactions. Much more attention should be paid to dissect the CPT biosynthetic pathway to identify those rate-limiting steps by metabolic flux analysis and isolate the genes encoding key enzymes, as well as regulatory gene such as upstream transcription factors.

Table 2 A list of CPT-producing endophytic fungi

Host	Endophytic fungi	Fungal strain	Accession no.	CPT yield	CPT analogues yield	References
N. foetida	*Entrophospora infrequens*	–	–	+	–	Puri et al. (2005)
N. foetida	*Entrophospora infrequens*	MTCC 5121	–	49.6 μg/g	–	Amna et al. (2006)
N. foetida	*Neurospora* sp.	ZP5SE	–	5.5 μg/g	–	Rehman et al. (2008)
N. foetida	*Nodulisporium* sp.	–	–	5.5 μg/g	–	Rehman et al. (2009)
C. acuminata	*Fusarium solani*	INFU/CA/KF/3	FM179605	6.0 μg/g	+ (9-MeOCPT and 10-OHCPT)	Kusari et al. (2009)
C. acuminata	Unidentified	XK001	–	+	–	Min and Wang (2009)
C. acuminata	*Xylaria* sp.	M20	GQ414524	–	5.4 mg/L(10-OHCPT)	Liu et al. (2010)
A. dimidiata	*Fusarium solani*	MTCC 9667	GQ465774	0.37 μg/g	+ (9-MeOCPT)	Shweta et al. (2010)
A. dimidiata	*Fusarium solani*	MTCC 9668	GQ465775	0.53 μg/g	+ (9-MeOCPT and 10-OHCPT)	
N. nimmoniana	Unidentified	UAS001	FJ158119	+	–	Gurudatt et al. (2010)
N. nimmoniana	*Fusarium sacchari*	UAS013	FJ158129	+	–	
N. nimmoniana	*Phomopsis* sp.	UAS014	FJ158130	21.7 μg/g(2nd*)	–	
N. nimmoniana	*Botryosphaeria Parva*	UAS015	FJ158131	+	–	
N. nimmoniana	*Fusarium subglutinans*	UAS017	FJ158133	+	–	
M. dentata	*Fomitopsis* sp.	MTCC 10177	–	+	–	Shweta et al. (2013)
M. dentata	*Alternaria alternata*	MTCC 5477	–	+	–	
M. dentata	*Phomposis* sp.	–	–	+	–	
C. acuminata	*Botryosphaeria dothidea*	X4	HQ416954	–	+	Ding et al. (2013)
C. acuminata	*Aspergillus* sp.	LY341	–	7.93 μg/l	–	Pu et al. (2013)
C. acuminata	*Aspergillus* sp.	LY355	–	42.92 μg/l	–	
C. acuminata	*Trichoderma atroviride*	LY357	KC469612	197.82 μg/l	–	

N. foetida, *Nothapodytes foetida*; *A. dimidiata*, *Apodytes dimidiata*; *N. Nimmoniana*, *Nothapodytes nimmoniana*; *M. dentata*, *Miquelia dentata*; *C. acuminata*, *Camptotheca acuminata.* *2nd, generation 2

Quick development and application of combined new omics-based approaches such as transcriptomics, proteomics and metabolomics would greatly speed up the dissection of CPT biosynthesis pathway and unveiled those unknown steps.

Biotechnological approaches such as plant cell suspension, hairy root and endophytic fungi as alternative platforms to produce CPT, appeared to exhibit the potential of fulfill the increasing market demand, independent of natural CPT-producing plant. Recently, hairy root culture system of CPT-producing plants such as *O. pumila* has showed good application potential to study CPT biosynthesis and produce CPT by coupling with associated bioreactor mass production technology. The special morphology of hairy roots normally hampers the scale-up processes, so modifications of the existing bioreactors and even re-design of suitable bioreactors to overcome shear stress problems, are still needed for further mass-production of CPT. The combination of metabolic engineering

with those in vitro CPT-production and enhancement approaches, may be the most promising way to produce CPT and its derivatives via biological approaches in the future.

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