Co-overexpression of geraniol-10-hydroxylase and strictosidine synthase improves anti-cancer drug camptothecin accumulation in Ophiorrhiza pumila

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Camptothecin (CPT) belongs to a group of monoterpenoidindole alkaloids (TIAs) and its derivatives such as irinotecan and topotecan have been widely used worldwide for the treatment of cancer, giving rise to rapidly increasing market demands. Genes from Catharanthus roseus encoding strictosidine synthase (STR) and geraniol 10-hydroxylase (G10H), were separately and simultaneously introduced into Ophiorrhiza pumila hairy roots. Overexpression of individual G10H (G lines) significantly improved CPT production with respect to non-transgenic hairy root cultures (NC line) and single STR overexpressing lines (S lines), indicating that G10H plays a more important role in stimulating CPT accumulation than STR in O. pumila. Furthermore, co-overexpression of G10H and STR genes (SG Lines) caused a 56% increase on the yields of CPT compared to NC line and single gene transgenic lines, showed that simultaneous introduction of G10H and STR can produce a synergistic effect on CPT biosynthesis in O. pumila. The MTT assay results indicated that CPT extracted from different lines showed similar anti-tumor activity, suggesting that transgenic O. pumila hairy root lines could be an alternative approach to obtain CPT. To our knowledge, this is the first report on the enhancement of CPT production in O. pumila employing a metabolic engineering strategy.

Camptothecin (CPT), originally isolated from the bark of the Chinese happy tree Camptotheca acuminata1, is a modified terpenoidindole alkaloid (TIA) and exhibits excellent anti-tumor activity, which is due to its ability to inhibit DNA topoisomerase I2. Its two derivatives, irinotecan and topotecan, have been approved by the US Food and Drug Administration (FDA) in 1994 and used extensively for the treatment of metastatic colorectal cancer, ovarian cancer, cervical cancer and small cell lung cancer throughout the world3. Besides their anti-tumor activity, CPT derivatives have also been found to show good activity against viruses such as the human immunodeficiency virus (HIV)4. More derivatives of CPT are now in clinical trials, such as 9-nitrocamptothecin, 9-aminocamptothecin and rubitecan5, which will potentially result in growing demand for these drugs in the future. Due to the excellent pharmacological activity of CPT derivatives, the world market for these CPT analogues has increased rapidly. The combined sales of two CPT analogs (irinotecan and topotecan) in only 2008 had reached 2.2 billion US dollars and is expected to increase further6,7. At present, the annual production of CPT throughout the world is only 600 kg, while approximately 3000 kg of CPT is needed in the international market8. However, all the CPT derivatives which are consumed are synthesized from natural CPT, which is mainly obtained by extraction from the trees C. acuminata and Nothapodytes foetida9,10. Since the limited supply of CPT is from the above two woody plants with slow growth rates and low yields, it is an important and urgent task to develop sustainable and alternative production sources of CPT in order to resolve the worldwide scarcity of natural sources of CPT.
Due to several advantages such as rapid growth rate, unlimited branching, and genetic stability, in vitro hairy root induced by *Agrobacterium rhizogenes* has been considered as an alternative means to produce high-value secondary metabolites including CPT\(^{10,11}\). To overcome the low yield of active components, the development and application of metabolic engineering strategies provides a promising approach to increase CPT production by introducing multiple CPT biosynthetic genes into CPT-producing plant cells or tissue\(^{22,23}\), followed by culturing transgenic cell lines, hairy roots or regenerated plants on a large scale. Therefore, detailed understanding of the CPT biosynthesis pathway, especially for the committed steps, will be helpful to improve CPT production by genetic manipulation.

Camptothecin belongs to the family of monoterpenoid indole alkaloids (TIA), which are found in some plant species such as *Apocynaceae, Loganiaceae, Rubiaceae* and *Nyssaceae*. Although CPT is one of the most promising natural plant-derived anti-tumor drugs, its biosynthetic pathway and regulatory mechanism of production remain unclear\(^{14}\). The biosynthesis of CPT is a complicated process and involves several catalytic steps (Fig. 1). Tryptamine is synthesized via the shikimate pathway, and the secologanin part is synthesized via the MEP pathway\(^{21}\). Stricosidine is then converted to strictosamide, but the remaining details and precise intermediates between strictosamide and CPT are not completely defined\(^{6}\). All TIA-s including CPT are derived from the common precursor strictosamide, which is formed via the condensation of the indoletryptamine and the monoterpenoid secologanin\(^{7}\). This important step is catalyzed by strictosidine synthase (STR), which is considered to be a key enzyme in TIA biosynthesis. The STR gene has been successfully isolated from *Rauvolfia serpentina*\(^{16}\), *C. roseus*\(^{17}\), *O. pumila*\(^{18}\) and *O. japonica*\(^{7}\). Over-expression of STR gene in transgenic *C. roseus* showed ten-fold higher STR activity than wild-type cultures, which exhibited a great enhancement effect on TIA biosynthesis\(^{19}\). Geraniol 10-hydroxylase (G10H), being a cytochrome P450 monooxygenase, can hydroxylate geraniol at the C-10 position to generate 10-hydroxy-geraniol, which is considered to be a committed step in the biosynthesis of secoliganin and even TIA-s\(^{19}\). The G10H gene was firstly cloned from *C. roseus*\(^{17}\) and recently from *C. acuminata*\(^{20}\). G10H has been reported to be a rate-limiting enzyme in the biosynthesis of terpenoid indole alkaloids in transgenic *C. roseus*\(^{21-23}\).

Although much is known about biosynthesis of TIA in *C. roseus*, little is known about regulation of CPT biosynthesis in CPT-producing plants\(^{24}\). The difficulties in establishing a stable transformation system for the CPT-producing woody plant *C. acuminata* led to few successful reports on introducing a CPT biosynthetic gene into *C. acuminata* by metabolic engineering in the past two decades\(^{25}\), although much effort was put into optimization of transformation procedures and conditions for *C. acuminata*. The findings that CPT exists in herbs such as *O. mungos*\(^{26}\) and the established hairy root culture system of *O. pumila* provided an alternative experimental model system for CPT biosynthesis and production\(^{23}\). Until now, there has been no report on the introduction of CPT and/or G10H genes into any CPT-producing plants including *O. pumila*. In this present work, we investigated the effects of overexpressing *CgG10H* and *CgSTR* individually and simultaneously in hairy root cultures of *O. pumila*.

**Results**

**Optimization of *O. pumila* hairy root induction procedure.** An efficient sterile plant culture system was established and to induce hairy root formation in *O. pumila* (Fig. 2). In this study, different explants derived from lamina, petioles and stems from *O. pumila* sterile plants were co-cultivated with *Agrobacterium* A4, 15834, and C58C1 on hormone free B5 medium for hairy root induction. Hairy root formation occurred most rapidly with strain C58C1 (induced about 10–15 days after infection, Fig. 2) among the three strains tested, suggesting that the modified strain C58C1 was more competent than the other strains as reported for *Anisodus acutangularis*\(^{20}\), and was therefore chosen for further experiments. The influence of bacterial strains on transformation frequency has been previously documented in different plant species\(^{28}\). The types of explants also affected hairy root induction frequencies. Hairy roots could be induced from wounded sites on the different explants including lamina, petioles and stems after infection with C58C1. The hairy root induction efficiency of lamina and petioles only reached to about 2% and 8%, respectively. And the explants often went brown,
the hairy root derived from lamina and petioles grew slowly. The highest hairy root induction efficiency of 80.4% was achieved using modified strain C58C1 with stem explants, suggesting that stems were more susceptible than lamina and petioles. Out of three tested media (MS, GB5 and White), the hairy root in B5 medium grew much better with normal branching than the other two media (White and MS). After about 14 days, the hairy roots in B5 medium began to branch largely, whereas hairy roots in the other two media didn’t branch and grew slowly, became abnormal and aged. The results suggested the liquid B5 medium was the most suitable for O. pumila hairy root growth. Apical tips of hairy roots (1 mg segments) were inoculated in the liquid medium, and the fresh weight of the tissue typically reached about 3 g after 45 days of culture. The above efficient hairy root induction system for the medicinal plant O. pumila was successfully developed and optimized for further genetic transformation.

Acquisition of transgenic O. pumila hairy roots with CrSTR and CrG10H. Three plasmids containing the cDNAs encoding CrSTR and/or CrG10H under the control of the CaMV 35S promoter were separately introduced into O. pumila hairy roots by using disarmed A. tumefaciens C58C1 strain after infection of young stems of O. pumila. Hairy root lines generated from transformations with an empty vector that did not contain CrSTR or CrG10H genes was used as a control (NC line). After two weeks, transgenic O. pumila hairy roots were generated with phenotypic characteristics such as being long, thin and golden yellow (Fig. 2). The abbreviations S, G, and SG refer to the transgenic hairy root lines generated from CrSTR single gene, CrG10H single gene, and CrSTR/CrG10H double gene transformations, respectively. In total, 53 STR single gene transformed lines (S line), 34 G10H single gene transformed lines (G line) and 95 STR-G10H double gene transformed lines (SG line) were generated, and 48 S, 31 G, 88 SG hygromycin-resistant (2 mg/L) hairy root lines with normal phenotype were maintained for PCR analysis (Table 1).

Genomic DNA of all the above independent hairy roots were isolated and used for PCR analysis using primers specially designed to overlap part of the CrG10H, CrSTR and the CaMV 35S promoter sequences. The C58C1 strains harboring plasmids pCAMBIA1304-CrSTR, pCAMBIA1304-CrG10H and pCAMBIA1304-CrSTR-CrG10H were also amplified as positive controls (PC). Control hairy roots generated from empty vector transformations were used as negative controls (NC), and water was used as a blank control (BC). The rolC gene as a marker gene of C58C1 was also detected in all the PCR-positive clones (Fig. 3). No amplicons of CrG10H and CrSTR were detected in the NC and BC lines. In total, the PCR-positive clones amounted to 25.7% (43/167), with 35% (17/48) for S lines, 16% (5/31) for G lines, and 24% (21/88) for SG lines (SG). These results preliminarily suggest that the CrSTR and CrG10H genes were introduced into the genome of S and G lines, while both CrSTR and CrG10H genes were introduced into SG lines. Here we randomly selected 4 S, 3 G and 5 SG of PCR-positive lines with normal growth phenotypes for further establishment of hairy root culture lines for qRT-PCR and HPLC experiments (Table 1).

Transcript analysis of CrG10H and CrSTR gene in hairy roots. qRT-PCR was determined to further analyze the expression level of the exogenous target gene (CrG10H and CrSTR) and reference gene alpha-tubulin (Tub) was used for the internal control gene. qRT-PCR results indicated that CrG10H and CrSTR effectively expressed with varying levels in the S, G and SG lines, respectively (Fig. 4), while no expression can be detected in the NC line as expected. Among all the transgenic lines, the G1 line had the highest expression level of CrG10H, while SG26 had the highest expression levels of CrSTR.

### Table 1 | Gene constructs and derived root cultures

| Gene constructs | Number of root lines | PCR-positive Established root lines |
|-----------------|----------------------|-----------------------------------|
| STR             | 53                   | 17 S8, S14, S16, S26              |
| G10H            | 34                   | 5 G1, G3, G10                     |
| G10H + STR      | 95                   | 21 SG1, SG20, SG26, SG28, SG43    |
expression level of CrSTR (Fig. 4). These results indicated that CrG10H and CrSTR genes were expressed in corresponding transgenic lines, but with varied expression levels.

**Accumulation patterns of camptothecin.** The hairy roots of *O. pumila* were inoculated into 50 mL liquid B5 medium and cultured for 6 weeks followed by collection for further study. The camptothecin that accumulated in the hairy roots was detected and quantified by HPLC analysis (Fig. 5). Higher levels of CPT ranging from 1.25 to 1.28 mg/g existed in G10H-transformed lines than in NC hairy root (1.05 mg/g) and wild-type plant root (0.68 mg/g dw) (Fig. 5). The average CPT content (1.27 mg/g) of all the four G lines was obviously higher than NC and wild-type root, implying that overexpression of G10H can efficiently promote the accumulation of CPT in *O. pumila*. The levels of CPT in S lines were variable with a range of 0.83-1.20 mg/g dw, which was higher than wild-type roots.
but not as good as in G lines, implying that the effect of single STR is much weaker than G10H for CPT biosynthesis in *O. pumila*. Interestingly, hairy root lines co-overexpressing G10H and STR produced higher levels of CPT with variation from 1.54 to 1.77 mg/g when compared to NC hairy roots and wild-type roots, and even higher than single overexpression of G10H or STR (Fig. 5). These results indicated that co-expression of G10H and STR can produce a synergistic effect for CPT accumulation in *O. pumila*.

One-way analysis of variance (ANOVA) was used to detect the difference of the average total content of CPT among S, G, SG, NC and WT lines to analyze the functional role of STR or/and G10H in CPT biosynthesis. It showed that the average content of CPT was higher in S lines (1.04 mg/g) and NC lines (1.05 mg/g) than WT (0.68 mg/g), while much higher in G (1.27 mg/g) and SG (1.64 mg/g) lines compared with NC and WT lines (P < 0.05) (Fig. 5). Overexpression of G10H in G and SG lines both effectively promote CPT biosynthesis, suggesting that G10H is a key regulation target gene for metabolic engineering of CPT biosynthetic pathway. CPT production is higher in SG lines than G lines, implying that STR also plays an important role in CPT synthesis under some conditions (when G10H was overexpressed). Therefore, compared with a single gene, co-introduction of G10H and STR can produce a coordination effect for camptothecin biosynthesis in *O. pumila* hairy root.

**Inhibitory activity on myelogenous leukemia cells.** The anti-tumor activity of CPT extracted from different hairy root lines was estimated by the MTT test in this study. The crude CPT extracts from some selected transgenic *O. pumila* hairy root lines and wild type *O. pumila* plant root were used for the MTT test, to compare their anti-tumor activities on the human chronic myelogenous leukemia K562 cell line. The MTT test showed that crude CPT extracts from line S14, G1, SG26 and WT at the same concentration with 6 levels showed similar anti-tumor activity (Fig. 6). When CPT from various sources was normalized at concentrations of 100 μg/ml, the inhibiting rate varied from 64.59% to 68.57%. The MTT assay results showed that there are no significant differences of anti-tumor activity among these transgenic lines and WT lines, and it can be deduced that genetic engineering did not change CPT activity.
C. acuminata

Discussion

Transgenic hairy root cultures have been shown to have the potential to enhance production of secondary metabolites in different plants by metabolic engineering, but an effective transformation system is necessary for successful metabolic engineering of hairy root. The supply of camptothecin mainly relies on extraction from the tree C. acuminata at present, but it is very hard to obtain transgenic lines of C. acuminata by genetic engineering. Weedy plants such as O. pumila were also found to produce CPT, which provided an alternative experimental model system for CPT biosynthesis and production. Since CPT mainly existed in the roots of perennial medicinal herb O. pumila, which rarely distributed in limited regions in China and other Asia countries, collection of its roots as drug source may result in its extinction. In vitro hairy root culture is regarded as a promising strategy to obtain CPT without threatening the survival of related resource plants. A hairy root culture system of O. pumila with good CPT production ability has been reported for the first time by scientists in Japan, but with too long time span for induction of hairy roots (emerging 80 days after infection). In this study, we successfully optimized the procedures of hairy root induction from O. pumila and shortened the time taken for the emergence of hairy roots to only 10–15 days with high efficiency, which greatly simplifies the operation, and obviously improves research efficiency. Meanwhile, CPT content was obviously enhanced in transgenic O. pumila hairy roots using the above optimized system by means of metabolic engineering. Further MTT assay results showed that there are no obvious differences on anti-tumor activity of CPT extracted from transgenic O. pumila hairy root lines and WT plant roots. The above results suggested transgenic O. pumila hairy root lines could be an alternatively promising approach to produce more CPT in place of natural plant resources including O. pumila, which will reduce environmental concerns.

G10H, a cytochrome P450 monoxygenase, was considered to be a key enzyme involved in the biosynthesis of TIAs in different plant species. Previous studies showed that G10H is a possible bottleneck for TIAs production and a good candidate target for genetic manipulation. However, there is no report on the effect of overexpression of G10H in CPT-producing plants. In our study, the CPT content of G10H-transformed lines ranged from 1.25 to 1.28 mg/g, with an average CPT content (1.27 mg/g) of all the five G lines being obviously higher than NC and wild-type root (Fig 5). The above results indicate that expression of CrG10H obviously enhanced production of CPT in O. pumila, which was in good agreement with previous results that overexpression of CrG10H increased accumulation of TIAs in C. roseus plants and hairy roots. Our study, for the first time, provides direct evidence that overexpression of heterologous G10H is sufficient to promote CPT synthesis in O. pumila, suggesting that G10H is an effective regulation target for metabolic engineering of CPT synthesis at least in O. pumila. Furthermore, overexpression of single CrG10H showed much more powerful driving effect than single CrSTR in increasing the production of CPT, which suggested that G10H may play a more important role in the regulation of CPT synthesis.

STR condenses tryptamine and the iridoid secologonin to yield strictosidine which is the universal precursor of TIAs including CPT. Overexpression of STR in transgenic C. roseus showed tenfold higher STR activity than wild-type cultures, which indicated that STR plays a critical role in biosynthesis of TIAs. However, the real effect of the overexpression of the STR gene in CPT-producing plants remains unknown. In the present study, the camptothecin content in S lines varied from 0.83 mg/g to 1.20 mg/g, which are all higher than that of wild type root (0.68 mg/g DW). However, the CPT content increased in two S lines (S8, S14), but decreased in the other two S lines (S16, S26) when compared to the non-transgenic control line (NC, 1.05 mg/g). The above puzzling result makes it hard to generalize the overall effect of a single STR gene on CPT biosynthesis, reflecting some kind of uncertainty in regulating metabolite flux through the biosynthetic pathway by manipulation of only a single enzyme. The introduction of STR in O. pumila hairy roots resulted in mixed results, and the average effect of STR overexpression is not as good as G10H (Fig 5). One possible explanation for the result is that the effect of STR overexpression may be blocked by another key enzymes such as G10H.

The “push-pull” strategy has been successfully applied to increase pharmaceuticals and flavors yields in many plants such as C. roseus, Solanum lycopersicum, Artemisia annua, Salvia miltiorrhiza and Anisodus acutangulus, respectively. But sometimes overexpression of a single gene encoding a rate-limiting enzyme to increase contents of secondary metabolites leads to unexpected consequences, and simultaneous overexpression of several enzymes within the same pathway is more effective to increases metabolites production. In our study, SG lines possessed higher average CPT content (1.64 mg/g DW) than S lines (1.04 mg/g DW) and G lines (1.27 mg/g DW) (Fig 5B), and all the tested SG lines have a higher level of campto-
cin than other lines, which varied from 1.54 mg/g to 1.77 mg/g. The CPT content of 5G lines is relatively higher than the reported CPT content in the 5-week cultured O. pumila hairy roots (0.1% per dry weigh) and the intact plant (approx. 0.03–0.04% dry weight in the leaves, 0.1% dry weight in the young roots)^35. Expression of single CrSTR has less effect than CrG10H in increasing the camptothecin content, but co-expression of CrG10H and CrSTR displayed a steady and more powerful pulling effect compared with S lines and G lines. The results suggested that multiple key enzymes can effectively promote the accumulation of camptothecin. Alternatively, introduction of a global transcription factor that positively regulates the pathway is more effective than modifying multiple biosynthetic pathway genes at one time^12,31. Recently, we found a considerable increase of a global transcription factor that positively regulates the pathway to generate a high level of camptothecin. Alternatively, introduction of a global transcription factor that positively regulates the pathway may be another promising strategy to increase CPT yield in the near future.

Methods

Plant materials. Wild-type O. pumila plants were collected from Fujian Province, China. Shoot tips of wild type plants were rinsed overnight with running tap water, soaked in 70% (v/v) ethanol for 30 sec and then in 0.1% (v/v) mercuric chloride for 10–15 min, and finally thoroughly rinsed four times with sterilized distilled water. The treated shoot tips were cultured on solid Murashige and Skoog (MS) medium to obtain sterile O. pumila plants using a previously reported method^8. Sterile O. pumila plants were cultured and maintained at 25°C under a 14 h light/10 h dark photoperiod with light provided by cool white fluorescent lamps at an intensity of 250 μmol m⁻² s⁻¹.

Construction of plant expression vectors. The complete G10H and STR cDNAs were cloned from the sterile seedlings of C. roseus according to the sequences reported in NCBI (X61992.1 for STR and AJ251269.1 for G10H). The full-length ORF of CrG10H cDNA was inserted into pCAMBIA1304^-CRSTR, the full-length CrG10H cDNA was used to replace the mGFp5 and gusA gene in pCAMBIA1304^-CRSTR under the digestion of BglII and BsrEI (Takara Biotech Co., Ltd) to generate the expression vector pCAMBIA1304^-CRSTR-CrG10H containing both CRSTR and CrG10H genes (Fig. 7C). The genes CrSTR and CrG10H were under the control of the strong cauliflower mosaic virus (CaMV) 35S promoter. The blank vector pCAMBIA1304^- without exogenous genes was used as the control. The disarmed Agrobacterium tumefaciens strain C58C1 harboring both the Agrobacterium rhizogenes Ri plasmid pHAl1^11,12,30 and each of the four plasmids constructed above was used for plant genetic transformation.

Plant transformation and hairy root culture. Different explants including leaf blades, petals or stems were isolated from 4-week-old in vitro grown young aseptic O. pumila plants cultured on B5 medium supplemented with 100 mg/L LH, 0.1 mg/L NAA and 0.1 mg/L KT. The isolated explants were cut into small pieces (about 1 cm) followed by pre-incubation on B5 in the dark for 2 days, and then submersed in three A. rhizogenes strains (A4, 15834, and C58C1) for 10 min, blot-dried on sterile filter paper and then placed on B5 medium in the dark for 2 days. The explants were subsequently rinsed with sterile water, and transferred onto B5 medium supplemented with 300 μg/L carbenicillin. The apical tips of hairy root induced from transformed explants were excised and sub-cultured on B5 solid medium with 200 mg/L carbenicillin at 2-week intervals, the concentration of carbenicillin was gradually lowered and finally omitted after 2 month. When cultures had been cleaned of agrobacterium, hairy roots were transferred onto B5 solid medium and the apical tips of rapidly growing hairy roots were inoculated into 50 mL liquid B5 media in 250 mL-shake flasks to establish hairy root lines, on a shaker at a gyration rate of 120 rpm at 25°C for further study.

DNA isolation and PCR analysis. Genomic DNA was isolated from harvested hairy root samples using the cetyltrimethyl ammonium bromide (CTAB) method^37. The DNA was then used as the template in PCR analysis for detecting the presence of CrSTR and CrG10H genes in transgenic hairy roots. The sequences of 35SF23 (forward primer) located at the 35S promoter of the pBI121 vector and CrG10HR214 (reverse primer) in the interior of the CRSTR gene for the G10H detection were as follows: 5’-GAGGACCTAACAGAACTCGCC-3’ and 5’-TCCTCCACACACTGTCCTTTT-‘3’, respectively. PCR was carried out in a total volume of 25 μL reaction mixture, containing 1 μL of each primer, 0.5 μL of 10 mmol/L dNTPs, 2.5 μL of 10× PCR buffer (Mg²⁺ plus), 1 μL genomic DNA as

Figure 7 | The scheme of recombinant vector. (A): pCAMBIA1304^-CRSTR, (B): pCAMBIA1304^-CrG10H, (C): pCAMBIA1304^-CRSTR+CrG10H. 35S P: promoter of CaMV35S, 35ST:terminator of CaMV35S, NOST: terminator of noster, LB: left border of T-DNA, RB: right border of T-DNA, hyg+: hygromycin.
DNA synthesis and qPCR amplification. Total RNA was extracted from the hairy root samples using the RNA prep pure plant kit (Tiangen Biotech.) according to the manufacturer’s protocol. The quality and quantity of DNA were checked and stored as described before. Aliquots of total RNA (1 μg) were used as templates to generate cDNA with Oligo dT primer using avian myeloblastosis virus (AMV) reverse transcriptase (TaKaRa) in Bio-Rad T100 Thermal Cycler (Bio-Rad, USA), and then used for further quantitative real-time fluorescence analysis. Gene-specific primers of the alpha-tubulin gene (TUB-QF: CCAGATAACTTTTGGC, TUB-QR: GTGAACTCCATTTCATCCAT) was used as reference gene. The qPCR amplification was performed in an Applied Biosystem Step One (Applied Biosystem). The delta Ct (threshold cycle value) method was used to estimate the initial amount of template present in the reactions by Applied Biosystems SDS 2.0.

Determination of camptothecin by HPLC. The 6-week-old hairy roots were dried to constant weight in an oven at 50 °C, powdered into powder and weighed. 0.5 mL of methanol (analytic grade) was then added to the powder at a ratio of 1:50 (W/V) and the mixture was concentrated to 2 mL, 95% ethanol was added to redissolve it at a ratio of 1:10 (V/V) overnight after a 60 min’s ultrasonication. The extracts were clarified twice by centrifugation at 12,000 rpm for 10 min at 4 °C, and the supernatant was collected and used as a reversed-phase HPLC detector. Then the sample was dissolved in 2 mL methanol and passed through a 0.22 mm nylon filter (Pall Corporation). HPLC analysis was performed to determine the contents of CPT on a seapax C18 reversed-phase symmetry column (4.6 × 250 mm, 5 μm). The mobile phase consisted of 35% acetonitrile (HPLC grade) and 75% double distilled water. The flow rate was 1.0 mL/min, and the injection volume was 20 μL. The chromatogram was monitored at 220 nm on a Hitachi Diode Array Detector L2455. CPT standard substance was dissolved in methanol at 100 μg/mL, the retention time of the CPT was 9.58 min. The accuracy and reproducibility of HPLC analysis were confirmed by analyzing different quantities of the sample including the control group. Samples were quantified using standard curve fitting with linear regression.

Anti-tumor activity of transgenic hairy roots by MTT analysis. The 6-week-old hairy roots from line S14, G1, SG26 and WT were dried and dissolved in methanol using the same extraction method for HPLC determination, and used to measure in vitro anti-tumor activity by MTT assay as described previously, and methanol as negative control. The crude CPT extract was diluted to the same concentration with a serial of grades (100 μg/mL, 50 μg/mL, 25 μg/mL, 12.5 μg/mL, 6.25 μg/mL, 3.13 μg/mL) of CPT using methanol and the final concentration of samples were adjusted using RPMI 1640 medium in a ratio of 1:100. K562 cells were cultured and the cell density was adjusted to 2 × 10^5/mL. Then the K562 cells were seeded in the 96-well plate and incubated at 37 °C for 44 h in a humidified atmosphere consisting of 5% CO2, and 6–8 replicates were set for each sample. Then, 10 μL MTT [3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide] (Sigma-Aldrich) was added to the medium at 5 μg/mL, and the medium was incubated for an additional 4 h. Then 100 μL of 0.5% hydrochloric acid was added to dissolve the formazan crystals and the absorbance of the solution was measured with a spectrophotometer (Biomek 1000) at test and reference wavelengths of 570 nm.

Data analysis. All the experiments including PCR identification, qR-PCR, HPLC analysis and anti-tumor activity analysis were repeated three times. Results of CPT concentration are presented as the mean ± standard error. The error bars are due to biological variation. The statistical significance of CPT content difference was analyzed by one sample t test and the errors of different hairy root clones were used in the one-way analysis of variance (ANOVA) using SPSS 11.5 software (SPSS, Inc.).
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G.K. and L.C. designed research, L.C., X.N., Q.J., X.T., Y.Y. and C.W. performed research, L.C., G.K. and D.S.Z. analyzed data, G.K., L.C., X.N. and D.Z. wrote the paper.

Additional information
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