**Suppression of camptothecin biosynthetic genes results in metabolic
modification of secondary products in hairy roots of *Ophiorrhiza
pumila***

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Abstract

Camptothecin is a monoterpenoid indole alkaloid that exhibits anti-tumor activity. In *Ophiorrhiza pumila*, production of camptothecin and its related alkaloids was high in the hairy roots, but not in the cell suspension culture derived from hairy roots. To identify the intermediates in camptothecin biosynthesis, expression of genes encoding tryptophan decarboxylase (TDC) and secologanin synthase (SLS), the 2 enzymes catalyzing the early steps in camptothecin biosynthesis, were suppressed in the hairy roots of *O. pumila* by RNA interference (RNAi), and metabolite changes were investigated. In most *TDC*- and *SLS*-suppressed lines, accumulation of camptothecin and related alkaloids, strictosidine, strictosamide, pumiloside, and deoxypumiloside was reduced. The accumulation levels of secologanin exhibited a strong negative correlation with the expression level of *TDC*, and that of loganin exhibited a negative correlation with the expression level of *SLS*. Some hairy root-specific chromatographic peaks detected by liquid chromatography Fourier transform ion cyclotron resonance mass spectrometry (LC-FTICR-MS) exhibited positive or negative correlation with *TDC* expression, suggesting their possible involvement in camptothecin biosynthesis.

Keywords

Ophiorrhiza pumila; Rubiaceae; Hairy root; RNAi; FTICR-MS; LC-FTICR-MS;
Monoterpenoid indole alkaloids; Camptothecin; Tryptophan decarboxylase (TDC);
Secologanin synthase (SLS)

1. Introduction

A success story of alkaloids began with the discovery of nitrogen-containing plant products 200 years ago (Zenk and Juenger, 2007). Alkaloids are basic nitrogen-containing compounds found in about 20% of all plant species (Facchini and De Luca, 2008). Many alkaloids are pharmacologically active and have traditionally been used as treatments for various diseases (Kutchan, 1995). Camptothecin is a well-known alkaloid biosynthetically belonging to the monoterpenoid indole family, and was originally identified in the extracts of a Chinese tree *Camptotheca acuminata* (Wall et al., 1966). It exhibits anti-tumor activity by inhibiting topoisomerase-I, thereby killing cancer cells (Hsiang et al., 1985). At present, semi-synthetic water-soluble camptothecin derivatives such as topotecan and irinotecan are used as clinical anti-tumor agents against cancers of the lung, cervix, ovaries, colon, and other organs throughout the world (Pizzolato and Saltz, 2003). Consequently, the demand for camptothecin will increase in the future. In spite of the rapid growth of the pharmaceutical market, camptothecin is exclusively produced from intact plants, mainly *C. acuminata* and *Nothapodytes foetida* (Govindachari and Viswanathan, 1972). Because of the shortage of natural resources and the associated environmental concerns, production of secondary metabolites by genetically engineered plant cell cultures has

become a keen issue (Oksman-Caldentey and Saito, 2005; Sirikantaramas *et al.*, 2007).

Camptothecin and related alkaloids are produced in relatively wide array of plant species belonging to different families, such as Nyssaceae, Icacinaceae, Rubiaceae, and Apocynaceae. In spite of many trials, it is still difficult to obtain tissue cultures or transgenic plants in these species. We have performed genetic transformation of *Ophiorrhiza* species, including *O. pumila*, *O. liukiensis*, and *O. kuroiwai*, and succeeded in establishing hairy root cultures (Asano *et al.*, 2004; Saito *et al.*, 2001; Watase *et al.*, 2004), which produce a good yield of camptothecin and the related alkaloids, namely, strictosamide, pumiloside, and deoxypumiloside (Asano *et al.*, 2004; Kitajima *et al.*, 2002; Yamazaki *et al.*, 2003a).

Monoterpenoid indole alkaloids (TIAs), including camptothecin, are derived from strictosidine, which is a common intermediate formed by the condensation of tryptamine, an indole compound, with secologanin, an iridoid glucoside, catalyzed by strictosidine synthase (STR) (Kutchan *et al.*, 1988; Stöckigt *et al.*, 1977; Yamazaki *et al.*, 2004) (Fig. 1). Tryptamine is formed by decarboxylation of tryptophan catalyzed by tryptophan decarboxylase (TDC) (De Luca *et al.*, 1989; Noé *et al.*, 1984). The other precursor, secologanin, is biosynthesized from loganin by secologanin synthase (SLS) (Yamamoto *et al.*, 2000). The gene *CYP72A1* from *Catharanthus roseus* was identified

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3 to encode SLS (Irmeler et al., 2000). Using this sequence information, the cDNAs
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6 encoding STR and TDC were cloned from hairy roots of *O. pumila* (Yamazaki et al.,
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9 2003b).

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12 Intramolecular cyclization of strictosidine produces strictosamide, an
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15 intermediate in camptothecin biosynthesis, as proven by incorporation of radiolabeled
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18 precursor (Hutchinson et al., 1979). Possible precursors of camptothecin, such as
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21 pumiloside and 3(*S*)-deoxypumiloside, were isolated from *Ophiorrhiza* species (Aimi et
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24 al., 1989; Kitajima et al., 2005), and pumiloside has also been found in *C. acuminata*
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27 (Carte et al., 1990). However, intermediates in the later steps after strictosamide
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30 generation in camptothecin biosynthesis have not been identified.
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35 We have used RNA interference (RNAi) technology as an effective way to
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38 investigate camptothecin biosynthesis. In this study, hairy roots of *O. pumila* were
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41 suppressed for the expression of *TDC* and *SLS* using RNAi, and the accumulation
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44 patterns of camptothecin and related alkaloids were correlated with the expression
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47 levels of the targeted genes in RNAi hairy roots. Cell suspension cultures induced from
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50 hairy roots did not accumulate alkaloids. Specific metabolites in *O. pumila* hairy roots
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53 were selected by comparing the metabolite patterns of hairy roots and cell suspension
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56 cultures using Fourier transform ion cyclotron resonance mass spectrometry
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(FTICR-MS). From these specific metabolites, candidates for intermediates in camptothecin biosynthesis were selected based on their metabolic changes in RNAi hairy roots.

2. Results and Discussion

2.1. Establishment of cell suspension culture induced from hairy roots

O. pumila hairy roots produce a large amount of camptothecin and a variety of related alkaloids (Saito *et al.*, 2001; Yamazaki *et al.*, 2003a). Light yellow calli induced from leaves and shoots of *O. pumila* plants do not produce alkaloids, but accumulate a variety of anthraquinones (Kitajima *et al.*, 1998). In this study, new callus was induced from *O. pumila* hairy roots to produce camptothecin, and was subcultured in liquid medium in the dark to establish a cell suspension culture. The accumulation patterns of secondary metabolites in cell suspension culture were profiled by HPLC-photodiode array detection-electrospray ionization mass spectrometer (HPLC/DAD/ESI/MS) and compared with those of hairy roots. Hairy roots produced many alkaloids and anthraquinones; however, the cell suspension culture accumulated no alkaloids and little anthraquinones (Fig. 2). The accumulation of camptothecin in cell suspension culture was also not confirmed by fluorescence-HPLC analysis (data not shown).

2.2. Establishment of RNAi hairy roots

To investigate the modulation of camptothecin biosynthesis using RNAi

technology, *TDC* and *SLS* were chosen as target genes, because these 2 genes are known to be involved in camptothecin biosynthesis. For induction of RNAi hairy roots, the stem segments of young aseptic plants of *O. pumila* were infected with *Agrobacterium rhizogenes* 15834 harboring the RNAi plasmid pGWB80-TDCi or pGWB80-SLSi. Fifteen TDCi hairy root (TDCi-HR) lines and 16 SLSi hairy root (SLSi-HR) lines were established. As a negative control, hairy roots harboring pGWB80-GUSi were established (GUSi-HR). To evaluate the suppression of *TDC* and *SLS* genes in TDCi-HR and SLSi-HR, respectively, gene expression was analyzed in RNAi hairy roots by semi-quantitative RT-PCR. *TDC* expression was downregulated in TDCi-HR, and 5 TDCi-HR lines expressed less than 40% compared to the average expression of *TDC* in the GUSi-HR control (Fig. 3A). Similarly, *SLS* expression was downregulated in SLS-HR, and 7 SLSi-HR lines expressed less than 80% compared to the average expression in GUSi-HR (Fig. 3B).

2.3. Changes in accumulation patterns of camptothecin-related alkaloids in RNAi

hairy roots

The camptothecin content in RNAi hairy roots used for gene expression analysis was quantified by fluorescence-HPLC. As seen in Fig. 3, camptothecin

production was suppressed in TDCi-HR and SLSi-HR, and the suppression positively correlated with the targeted gene expression levels. In most *TDC*- and *SLS*-suppressed lines, the production of camptothecin was about one-half of that of the average in GUSi-HR. Furthermore, metabolite profiling of TDCi-HR and SLSi-HR by HPLC/DAD/ESI/MS indicated that the production of strictosamide, pumiloside, and other unknown compounds with similar eluting times was suppressed (Fig. 4). These unknown compounds may be the candidates for intermediates in camptothecin biosynthesis.

2.4. Non-targeted metabolite analysis by infusion FTICR-MS

We identified potential candidates for post-strictosamide intermediates in camptothecin biosynthesis by non-targeted metabolite analysis of hairy roots and camptothecin non-producing cell suspension culture using infusion FTICR-MS. In this analysis, 832 and 659 ion peaks were detected in hairy roots and cell suspension culture, respectively. The *m/z* values for the detected ion peaks ranged from 116 to 1800. To identify the principal candidates, differentially expressed peaks between hairy root and cell suspension culture were narrowed down. By this process, 241 of the 832 peaks in hairy roots were categorized as hairy root-specific peaks (Fig. 5), which included peaks

corresponding to camptothecin, strictosidine and the camptothecin-related alkaloids such as strictosamide, pumiloside, and deoxypumiloside.

2.5. *Semi-quantitative mass analysis of camptothecin-related alkaloids in RNAi*

hairy roots

As previously mentioned, the production of camptothecin and its related alkaloids strictosamide and pumiloside were suppressed in TDCi-HR and SLSi-HR. Camptothecin content was quantified by fluorescence-HPLC; however, other camptothecin-related alkaloids were impossible to quantify using the same method, because these alkaloids lacked specific fluorescence and were co-eluted with several compounds in an HPLC separation detected by photodiode array. As a comprehensive method of targeted metabolite quantification, the peak area of a mass signal acquired by LC-FTICR-MS analysis was used. As the FTICR-MS analysis provides mass values with very high accuracy and resolution, the greater part of metabolites are acquired as a single mass peak. We used this method for the identification and semi-quantification of the targeted metabolites. As seen in Fig. 6, the production of camptothecin, strictosidine, and the camptothecin-related alkaloids, such as strictosamide, pumiloside, and deoxypumiloside, was suppressed, and the suppression was positively correlated with

the expression levels of *TDC* and *SLS*. Strictosidine, strictosamide, and pumiloside, in particular, exhibited a strong correlation.

2.6. Semi-quantitative analysis of secologanin biosynthesis intermediates in RNAi hairy roots

To understand the effect of targeted gene suppression on pre-strictosidine events, we examined the changes in accumulation of secologanin and its biosynthetic intermediates in RNAi hairy roots. Secologanin accumulated in TDCi-HR, and exhibited a strong negative correlation with *TDC* expression. Loganin accumulated in SLSi-HR and exhibited a negative correlation with *SLS* expression (Fig. 7). Furthermore, we examined the accumulation changes in RNAi hairy roots for peaks of accurate mass corresponding to the predicted intermediates in secologanin biosynthesis (Fig. 8). Other intermediates, such as 7-deoxyloganic acid and loganic acid, did not exhibit a correlation with *TDC* and *SLS* expression (Fig. 7).

2.7. Narrowing down of new candidates for intermediates in camptothecin biosynthesis

The 241 hairy root-specific peaks obtained by non-targeted metabolite analysis

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3 using infusion FTICR-MS should include many possible intermediates in camptothecin
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6 biosynthesis. Because the production of camptothecin-related alkaloids, such as
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9 strictosidine, strictosamide and pumiloside, were significantly suppressed in TDCi-HR,
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12 hairy root-specific peaks that exhibit a good correlation with *TDC* expression represent
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15 potential candidates for intermediates in camptothecin biosynthesis. To effectively
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18 narrow down the candidates, we selected the potential peaks related to camptothecin
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21 biosynthesis from the 241 hairy root-specific peaks according to the schematic flow
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24 shown in Fig. 5. Twelve chromatographic peaks exhibited positive correlation, and 11
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29 chromatographic peaks exhibited negative correlation with *TDC* expression.
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32 Among the hairy root-specific peaks that exhibited positive or negative
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35 correlations with *TDC* expression, probable metabolites were annotated based on
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38 elemental composition calculated from accurate *m/z* values using the public database
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41 KNApSAcK (<http://kanaya.naist.jp/KNApSAcK>) (Afendi et al., in press). Among the
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44 peaks with positive correlation, 2 peaks had probable annotations of secondary
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47 metabolites (Table 1). Gelsemicine is an indole alkaloid isolated from *Gelsemium*
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50 species (Kitajima et al., 2006; Przybylska and Marion, 1961). Lyaloside is an indole
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53 alkaloid isolated from *Pauridiantha lyalli* (Aimi et al., 1992). Although a direct
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56 comparison with standard compounds revealed that these 2 peaks were not due to these
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3 annotated compounds, these compounds may be potential biosynthetic intermediates
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6 with identical chemical composition. Among the peaks with negative correlation, 6
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9 peaks were annotated as flavonoids (Table 2). Many peaks were not annotated by the
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12 KNApSAcK search; however, two non-annotated peaks had exact masses almost the
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15 same as those of the possible intermediates in camptothecin biosynthesis (Fig. 9).
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3. Conclusions

The production of camptothecin, strictosidine, and camptothecin-related alkaloids, such as strictosamide, pumiloside, and deoxypumiloside, was suppressed in a *TDC* and *SLS* expression-dependent manner in RNAi hairy roots. Among the hairy root-specific peaks that correlated with *TDC* expression in LC-FTICR-MS analysis, 2 unknown peaks with positive correlation were annotated as alkaloids and 6 unknown peaks with negative correlation were annotated as flavonoids. The exact mass of some non-annotated peaks were similar to those of the predicted intermediates in camptothecin biosynthesis. These results suggest that most of the peaks that positively correlated with *TDC* expression could be intermediates in camptothecin biosynthesis. We are also aware that if the biosynthetic reactions take place in a protein complex or ‘metabolon’ with unstable intermediates, it would be difficult to detect and see the correlations the levels of those metabolites with gene expression.

4. Experimental

4.1. Plant materials

Sterile *O. pumila* plants were cultured and maintained at 25°C with a photoperiod of 18-h light (2000 lux.)/6-h darkness, as described previously (Kitajima *et al.*, 1997).

4.2. Induction and culture of callus

One-centimeter fragments of 3-week-old hairy roots of *O. pumila* (Saito *et al.*, 2001) were cultured on B5 medium (Gamborg *et al.*, 1968) containing 2% sucrose, 0.2% gellan gum, 0.5 µM 1-naphthaleneacetic acid (NAA), and 5 µM N⁶-benzyladenine (BA) at 25°C under dark conditions. After 3 weeks, a yellowish-white callus was induced from the hairy root fragments. The induced callus was maintained by subculturing once a month under the same conditions. Cell suspension cultures were established by culturing the callus in B5 liquid medium containing 2% sucrose, 0.5 µM NAA, and 5 µM BA at 25°C on a rotary shaker (125 rpm) under dark conditions. The established cell suspension cultures were maintained by subculturing every 2 weeks under the same conditions.

4.3. Vector construction for RNAi

To construct RNAi vectors, Gateway[®] technology (Invitrogen, Carlsbad, CA, USA) was used. The *attB* site was incorporated by 2 PCR steps using gene-specific *attB* primers, according to the manufacturer's instructions. A 468-bp fragment (TDCi fragment) corresponding to the 5' end of the coding regions of *O. pumila* tryptophan decarboxylase gene (*TDC*) (Yamazaki et al., 2003b) was cloned into the donor vector pDONR221 using BP clonase. Similarly, a 425-bp fragment (SLSi fragment) without the conserved regions of cytochrome P450 from *O. pumila* secologanin synthase gene (*SLS*) (unpublished data) was selected and cloned into pDONR221. The gene-specific *attB* primers designed for the TDCi and SLSi fragments are as follows: TDCi-*attB*1 (AAAAAGCAGGCTATGGGCAGCATTAGTGAAAATTGTGATG) and TDCi-*attB*2 (AGAAAGCTGGGTACCATGAAACATGAAAGATTTAGGCAACTT) for the TDCi fragment; SLSi-*attB*1 (AAAAAGCAGGCTGGATGGGACGCATTCCGAGAG) and SLSi-*attB*2 (AGAAAGCTGGGTCATGGTCTCAATGGTGAGGTCC) for the SLSi fragment. The integrity of the constructs was verified by DNA sequencing. The entry clones containing the TDCi or SLSi fragments were recombined in an LR reaction with pGWB80 of the binary vector system for RNAi and selected with hygromycin. For

evaluating the RNAi effect, a control vector containing the coding regions of *Arabidopsis thaliana* β -glucuronidase (GUS) gene (Kertbundit et al., 1991) was constructed.

4.4. Induction and culture of RNAi hairy roots

The plasmids pGWB80-TDCi, pGWB80-SLSi, and pGWB80-GUSi were transferred to *Agrobacterium rhizogenes* 15834 by electroporation using 0.1-cm cuvettes. The *A. rhizogenes* 15834 clones carrying these RNAi vectors were grown at 28°C in YEB liquid medium for 1 day. After that, they were co-cultured with 1-cm cut stems of young aseptic *O. pumila* plants (Kitajima et al., 1997) in B5 liquid medium containing 2% sucrose. After 2 days, the infected stems were washed with fresh B5 liquid medium containing 2% sucrose, and then, cultured on B5 medium containing 2% sucrose and 0.2% gellan gum supplemented with 200 mg·L⁻¹ of cefotaxime (Claforan[®]) for disinfection at 25°C under dark conditions. After 3 weeks, the hairy roots that emerged from the stem fragments were excised and cultured on B5 medium containing 2% sucrose and 0.2% gellan gum supplemented with 200 mg·L⁻¹ of cefotaxime and 25 mg·L⁻¹ of hygromycin for disinfection and selection at 25°C under dark conditions. The established RNAi hairy roots, TDCi-HR, SLSi-HR, and GUSi-HR, were subcultured

once a month on B5 medium containing 2% sucrose and 0.2% gellan gum. For gene expression and metabolite analyses, RNAi hairy roots were cultured in B5 liquid medium containing 2% sucrose for 3 weeks at 25°C on a rotary shaker (80 rpm) under dark conditions.

4.5. Gene expression analysis

Total RNA was isolated from 3-week-old RNAi hairy roots using RNeasy plant mini kit (Qiagen, Hilden, Germany). Reverse transcription was carried out at 42°C in a 20-μL solution containing 1 μg of total RNA, 0.5 μg of Oligo(dT)₁₂₋₁₈ primer (Invitrogen), 10 nmol of dNTPs (Takara Bio, Shiga, Japan), 0.2 μmol of DTT (Invitrogen), and 200 units of SuperScript II Reverse Transcriptase (Invitrogen).

First-strand cDNA synthesized from 1 ng of total RNA was used for amplification of *TDC* and the control β-tubulin gene (*TUB*) (unpublished data). For semi-quantitative RT-PCR, a 24-cycle PCR reaction for *TDC* and *SLS* and a 25-cycle PCR reaction for *TUB* were carried out with ExTaq DNA polymerase (Takara Bio) and gene-specific primers. The designed gene-specific primers were as follows: TDC-F

(ATGGGCAGCATTAGTGAAAA) and TDC-R

(TTACTCAATGATATTGGTTTTTCGT) for *TDC*; SLS-F

(CCAAAGTTGCCTTTGGTAGC) and SLS-R (CAACCCCATCAGCAAATCTT) for *SLS*; TUB-F (CCAGATAACTTTGTTTTTCGG) and TUB-R (GTGAACTCCATTTTCATCCAT) for *TUB*. PCR products were separated on 1% agarose gels and stained with SYBR Green I nucleic acid gel stain (Invitrogen) for 30 min in the dark. The stained gels were washed with distilled water for 10 min in the dark and scanned with Storm 860 (GE Healthcare, Little Chalfont, Buckinghamshire, England) using the following parameters: blue fluorescence (450 nm) and photomultiplier tube (PMT) gain of 850 V. The scanned images were visualized and analyzed using Image Quant (GE Healthcare), according to the manufacturer's protocols.

4.6. Analysis of camptothecin-related alkaloids

For alkaloid extraction, 3-week-old hairy roots and cell suspension cultures were completely dried in a lyophilizer. They were disrupted using a Multi-bead shocker (Yasui Kikai, Osaka, Japan), and 1 mL of methanol was added per 10 mg of dried tissue. The mixture was sonicated for 30 min and the homogenates were stored overnight at 4°C. On the next day, the homogenates were transferred to microtubes and centrifuged at 10,000 *g* for 10 min. The clear supernatants were transferred to new microtubes and

100-fold dilution of supernatants were applied on reverse-phase HPLC using a TSK gel ODS-80TM column (4.6 mm × 150 mm; TOSOH, Tokyo, Japan) with a solvent system of methanol:H₂O (7:3). Camptothecin was monitored by its characteristic fluorescence (excitation at 365 nm and emission at 428 nm) (Asano *et al.*, 2004).

For the profiling of camptothecin-related alkaloids, the methanol extracts were analyzed by HPLC/DAD/ESI/MS, as described previously (Asano *et al.*, 2004; Yamazaki *et al.*, 2003a).

4.7. *Non-targeted metabolite analysis using infusion FTICR-MS*

High-, middle- and non-polar extracts of plant materials were subjected to FT-MS (APEX III FT-ICMS; Bruker Daltonics, Billerica, MA, USA), as described previously (Aharoni *et al.*, 2002; Hirai *et al.*, 2004, Tohge *et al.*, 2005). The fold change in each mass peak was calculated as the ratio of the signal intensity in hairy roots of *O. pumila* to that in cell suspension culture induced from hairy roots. Data mining of metabolites was based on elemental composition calculated from an accurate *m/z* value using *DISCOVArray* (Phenomenome Discoveries, Saskatoon, SK, Canada).

4.8. *Semi-quantification analysis using LC-FTICR-MS*

For LC-FTICR-MS analysis, Agilent 1100 system (Agilent, Santa Clara, CA ,
USA) coupled to Finnigan LTQ-FT (Thermo Fisher Scientific, Waltham, MA, USA)
and Xcalibur software version 2.0 (Thermo Fisher Scientific) was used (Iijima et al.,
2008, Suzuki et al., 2008). For HPLC separation, Mightysil-RP18-GP (4.6 mm × 250
mm, 5 µm; Kanto Chemical, Tokyo, Japan) was used at a flow rate of 0.6 mL/min. A
combination of pure water and 0.25% v/v acetic acid and methanol was used as the
mobile phase. Mass spectra were acquired according to a previously described method
(Iijima et al., 2008).

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Figure legends

Fig. 1. Predicted pathway for camptothecin biosynthesis from tryptophan and geraniol in *O. pumila*

Enzyme abbreviations: TDC, tryptophan decarboxylase; G10H, geraniol 10-hydroxylase; SLS, secologanin synthase; STR, strictosidine synthase.

Fig. 2. HPLC chromatograms of *O. pumila* cell cultures

(A) Hairy root cultures (B) cell suspension cultures. The peak numbers correspond to the following compounds: 1, camptothecin; 2, pumiloside; 3, 3-*O*-caffeoylquinic acid; 4, Lucidin 3-*O*- β -purimeveroside. The HPLC conditions were as follows: column, Mightysil RP-18 (4.6 $\times$ 250 mm); gradient, linear gradient from solvent A to solvent B (0–35 min), isocratic at 100% of solvent B (35–40 min); solvent A (20% methanol, 0.2% acetic acid in H₂O), solvent B (90% methanol, 0.025% acetic acid in H₂O); flow rate, 0.8 mL·min⁻¹; column temperature, 37°C.

Fig. 3. Correlation analyses between the expression of targeted genes and accumulation of camptothecin in RNAi hairy roots

The relative expression level was calculated with respect to the average expression level of the targeted genes in GUSi-HR. (A) TDCi-HR and GUSi-HR, and (B) SLSi-HR and GUSi-HR. Bars: Standard deviation (n = 3 per analysis).

Fig. 4. HPLC chromatograms of RNAi hairy roots of *O. pumila*

(A) TDCi-HR and GUSi-HR, and (B) SLSi-HR and GUSi-HR. The HPLC conditions were as described in Fig. 2.

Fig. 5. Scheme for the selection of new candidates for intermediates in camptothecin biosynthesis

Fig. 6. Correlation analyses between expression of targeted genes and accumulation of camptothecin-related alkaloids in RNAi hairy roots

The relative expression level was calculated with respect to the average expression levels of the targeted genes in GUSi-HR (n = 1).

Fig. 7. Correlation analyses between expression of targeted genes and accumulation of secologanin and its intermediates in RNAi hairy roots

The relative expression level was calculated with respect to the average expression levels of the targeted genes in GUSi-HR (n = 1)

Fig. 8. Secologanin biosynthetic pathway

Fig. 9. Possible post-strictosidine pathway and intermediates in camptothecin biosynthesis

Ex Ms is the exact mass calculated from the molecular formula.

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Fig. 1

Fig. 2

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(continued)

Fig. 7

Fig. 8

Fig. 9

Table 1. Peaks exhibiting positive correlation with *TDC* expression

Peak no.	Correlation coefficient (<i>r</i>) ^a	Retention time	Detected mass	Predicted ion form	Predicted monoisotopic mass ^b	KNapSack search		
						Formula	Exact average mass	Annotated metabolite
82	0.74	32.49–32.63	333.12341–333.12365	[M+H] ⁺	332.1162			
105	0.56	15.20–16.82	359.19667–359.19684	[M+H] ⁺	358.1895	C ₂₀ H ₂₆ N ₂ O ₄	358.1893	Gelsemicine
110-1	0.76	5.77–5.87	377.20722–377.20747					
110-2	0.65	15.08–17.38	377.20724–377.20745					
138-2	0.40	33.61–33.72	447.12873–447.12896	[M+H] ⁺	446.1216	C ₂₂ H ₂₂ O ₁₀	446.1213	Wogonin 5-glucoside, Echioidin, and many others
148	0.75	31.01–31.18	495.17646–495.17660	[M+H] ⁺	494.1693			
151	0.69	30.99–31.20	496.17976–496.17998					
171-1	0.50	18.28–18.50	505.25489–505.25514					
171-2	0.38	19.10–19.33	505.25483–505.25509					
172-1	0.49	18.29–18.49	506.25816–506.25834					
172-2	0.44	19.09–19.32	506.25804–506.25828					
206	0.62	31.04–31.23	549.18459–549.18500	[M+Na] ⁺	526.1956	C ₂₇ H ₃₀ N ₂ O ₉	526.1951	Jorumycin, antibiotic UK 2B, Lyaloside

^a Correlation coefficients were used to analyze the correlation between expression of the targeted gene and area of the selected peak.

^b Predicted ion form and predicted molecular mass matching the input *m/z* with 5-ppm tolerance were generated using elements C, H, N, and O.

Table 2. Peaks exhibiting negative correlation with *TDC* expression

Peak no.	Correlation coefficient (<i>r</i>) ^a	Retention time	Detected mass	Predicted ion form	Predicted monoisotopic mass ^b	KNAPSAcK search		
						Formula	Exact average mass	Annotated metabolite
65	-0.71	29.81–29.90	303.11133–303.11224					
128	-0.70	31.86–32.10	433.11307–433.11333	[M+H] ⁺	432.1060	C ₂₁ H ₂₀ O ₁₀	432.1057	Vitexin, Kaempferol 7-rhamnoside, and many others
130	-0.37	16.60–16.94	437.16560–437.16583	[M+H] ⁺	436.1584	C ₁₈ H ₂₈ O ₁₂	436.1581	Alpigenoside, Eleganoside C, and many others
138-1	-0.71	32.00–32.20	447.12848–447.12891	[M+H] ⁺	446.1215	C ₂₂ H ₂₂ O ₁₀	446.1213	Wogonin 5-glucoside, Echiodin, and many others
140	-0.63	24.92–25.23	457.16825–457.16843	[M+Na] ⁺	434.1791	C ₃₂ H ₂₂ N ₂	434.1783	Biscryptolepine
						C ₁₉ H ₃₀ O ₁₁	434.17881	Secologanin dimethyl acetal
141	-0.55	20.09–20.52	459.12677–459.12718	[M+Na] ⁺	436.1377	C ₂₁ H ₂₄ O ₁₀	436.1370	Phloridzin, Catechin 3- <i>O</i> - α -L-rhamnoside, and many others
180	-0.44	22.19–22.44	516.24933–516.24949					
220	-0.59	32.02–32.21	579.17112–579.17146	[M+H] ⁺	578.1640	C ₂₇ H ₃₀ O ₁₄	578.1636	Kaempferitrin, Vitexin-2''- <i>O</i> -rhamnoside, and many others
222	-0.60	32.02–32.19	580.17445–580.17474					
229	-0.60	31.97–32.24	601.15300–601.15347	[M+Na] ⁺	578.1641	C ₂₇ H ₃₀ O ₁₄	578.1636	Kaempferitrin, Vitexin-2''- <i>O</i> -rhamnoside, and many others
230	-0.55	31.95–32.25	602.15624–602.15676					

^a Correlation coefficients were used to analyze the correlation between expression of the targeted gene and area of the selected peak.

^b Predicted ion form and predicted molecular mass matching the input *m/z* with 5-ppm tolerance were generated using elements C, H, N, and O.

Graphical Abstract

Suppression of camptothecin biosynthetic genes results in metabolic modification of secondary products in hairy roots of *Ophiorrhiza pumila*

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Secondary metabolic flux was modified in the hairy roots of *Ophiorrhiza pumila* using RNAi technology to produce camptothecin, an anti-neoplastic monoterpenoid alkaloid, and related alkaloids.

Camptothecin