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Note

De novo transcriptome analysis of needles of *Thujopsis dolabrata* var. *hondae*

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Abstract Podophyllotoxin is a starting material of the semisynthetic anticancer medicines etoposide, teniposide, and etopophos. The major plant source of podophyllotoxin is rhizomes of *Podophyllum hexandrum*, which is a Himalayan endangered species; therefore, alternative sources of podophyllotoxin or bioproduction systems have been pursued to avoid exploiting this limited natural resource. In this paper, we report de novo transcriptome analysis of *Thujopsis dolablata* var. *hondae*, which accumulates the podophyllotoxin derivatives (deoxypodophyllotoxin and *β*-peltatin A methyl ether) in its needles. We analyzed transcriptomes of the *T. dolablata* var. *hondae* young needles to obtain the sequences that putatively encode *O*-methyltransferases, cytochrome P450s, and a 2-oxoglutarate dependent dioxygenase because these protein families are responsible for podophyllotoxin-related compound formation in *P. hexandrum*. The resulting transcriptomes contained considerable numbers of coding sequences classified into the three protein families. Our results are a genetic basis for identifying genes involved in the biosynthesis of podophyllotoxin and related compounds and also for future metabolic engineering of podophyllotoxin in heterologous hosts.

Key words: 2-oxoglutarate-dependent dioxygenase, next-generation sequencer, *O*-methyltransferase, P450, podophyllotoxin.

Podophyllotoxin has been successfully used as the starting material of semisynthetic anticancer drugs, etoposide, teniposide, and etopophos. Of note, etoposide is on the World Health Organization's (WHO) list of essential medicines (Lau and Sattely 2015). Etoposide has been synthesized from podophyllotoxin via demethylation of 4′-methoxyl group on the aromatic E-ring and epimerization/acetonide-glucosylation of 7-hydroxyl group (Lata et al. 2009) (Figure 1). Currently, podophyllotoxin is mainly isolated from rhizomes of an endangered species, *Podophyllum hexandrum* (*Sinopodophyllum hexandrum*; Himalayan mayapple) (Hendrawati et al. 2012; Lata et al. 2009). In addition to *Podophyllum* spp., podophyllotoxin and related compounds are produced in other plant families including Linaceae, Apiaceae, Cupressaceae, and Hernandiaceae (Umezawa 2003a, 2003b). Therefore, much attention has been focused on biosynthesis and bioproduction of podophyllotoxin and related compounds in *Podophyllum* spp. and other plant species (Bhattacharyya et al. 2016; Kumar 2017; Kumari et al. 2014; Sasheva and Ikonkova 2017; Seegers et al. 2017; Suzuki and Umezawa 2007).

The current biosynthetic pathway of podophyllotoxin-related compounds is illustrated in Figure 2. First, Dewick et al. revealed that yatein is converted to podophyllotoxin via deoxypodophyllotoxin (=desoxypodophyllotoxin or anthricin) (Jackson and Dewick 1984; Kamil and Dewick 1986a) in *Podophyllum* plants. They stated that matairesinol is likely the branch-point compound that affords deoxypodophyllotoxin and 4′-demethylpodophyllotoxin (Broomhead et al. 1991; Dewick 1993; Kamil and Dewick 1986b). However, Kawai et al. (1994, 1999) proposed 4′-demethylyatein formation via thujaplicatin in *Thuja occidentalis*. Xia et al. (2000) showed the formation of 6-*O*-methylpodophyllotoxin via 7-hydroxymatairesinol. Kranz and Petersen (2003) reported that *β*-peltatin 6-*O*-methyltransferase activity gave *β*-peltatin A methyl ether in *Linum nodiflorum*. In

Abbreviations: 2OGD, 2-oxoglutarate-dependent dioxygenase; IIx, Genome Analyzer IIx; CYP, cytochrome P450; CAldOMT1, coniferaldehyde OMT 1; CCoAOMT, caffeoyl-CoA OMT 1; FLX, Genome Sequencer FLX; OMT, *O*-methyltransferase.

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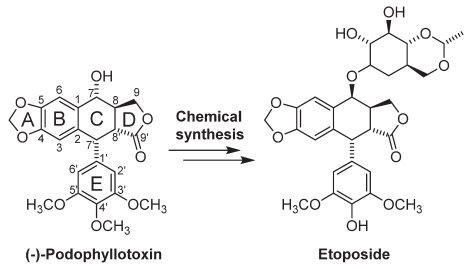

Figure 1. Chemical synthesis of etoposide from (−)-podophyllotoxin.

2003, Sakakibara et al. administered a series of isotope-labeled dibenzyl butylolactone lignans to *Anthriscus sylvestris* (Lim et al. 1999; Suzuki et al. 2002) and showed that yatein is produced from matairesinol via thujaplicatin, 4′-*O*-methylthujaplicatin, 4′,5′-di-*O*-methylthujaplicatin in this species. In contrast, Marques et al. (2013) found that a cytochrome P450 enzyme (CYP) in *P. hexandrum*, CYP719A23, catalyzes the conversion of (−)-matairesinol to (−)-pulviatolide. In 2013, Ragamustari et al. found an

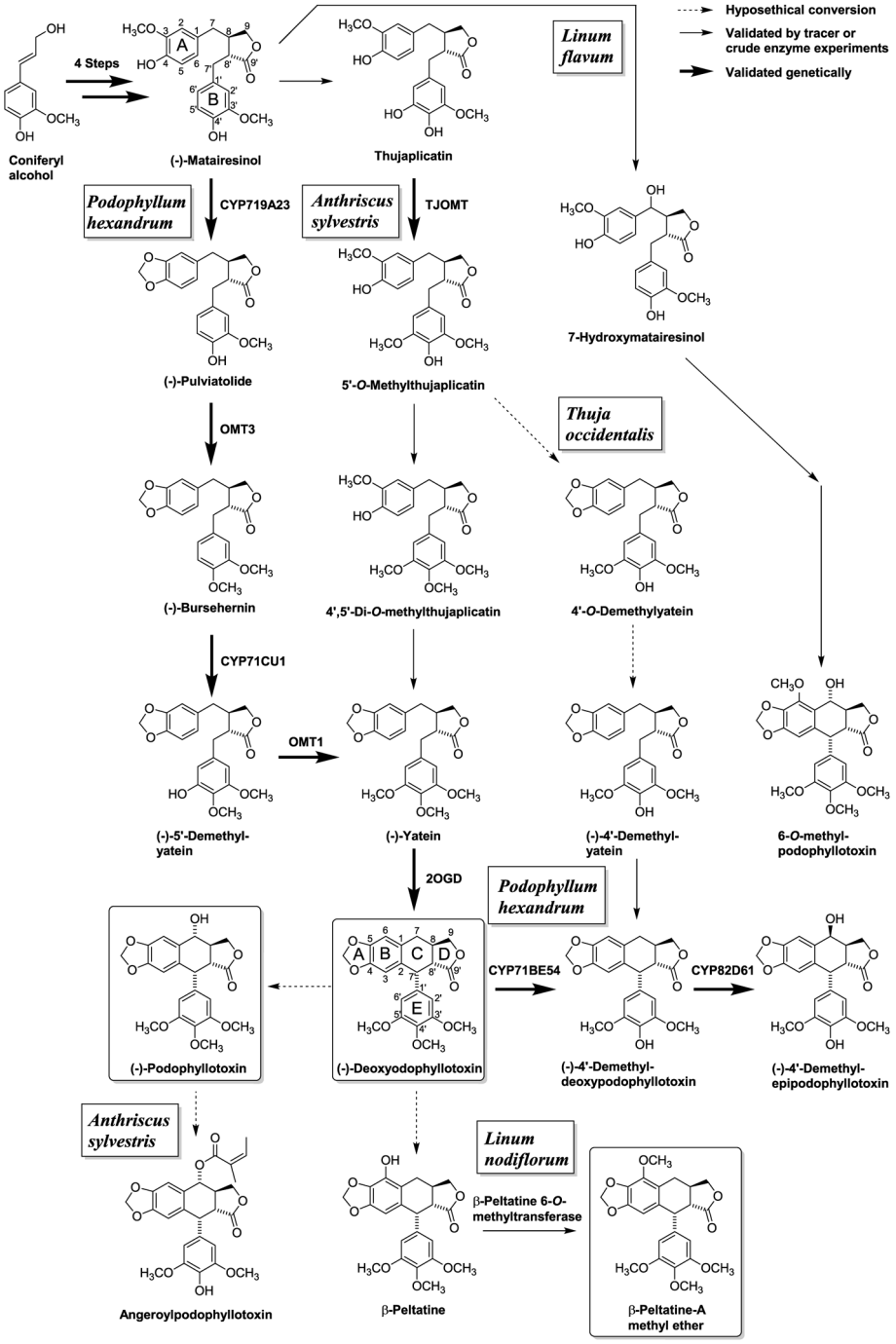

Figure 2. Current biosynthetic pathways of podophyllotoxin and related compounds.

O-methyltransferase (AsTJOMT) that catalyzes the conversion of thujaplicatin to 5′-*O*-methylthujaplicatin in *A. sylvestris*. Using *Agrobacterium*-mediated transient protein expression system in *Nicotiana benthamiana*, Lau and Sattely (2015) reported two *O*-methyltransferases (OMTs), named OMT3 and OMT1, for *O*-methylation of (−)-pulviatolide and (−)-5′-demethylyatein, two CYPs (CYP71CU1 and CYP82D61) mediating the hydroxylation of (−)-bursehernin and (−)-4′-demethylpodophyllotoxin, one CYP (CUP71BE54) demethylating 4′-methoxyl group of (−)-deoxypodophyllotoxin, and one 2-oxoglutarate-dependent dioxygenase (2OGD) named deoxypodophyllotoxin synthase that catalyzes the conversion of yatein to deoxypodophyllotoxin.

Among Cupressaceae species, *Thujopsis dolabrata* var. *hondae*, which is endemic to the northern Honshu and southern Hokkaido Islands of Japan (Kanetoshi et al. 1998), is a good source of podophyllotoxin derivatives; needles contain 1.1% of deoxypodophyllotoxin and 0.56% of *β*-peltatin-A methyl ether in the 70% acetone/water extract (Kanetoshi et al. 1999). In addition to podophyllotoxin derivative accumulation in the needles, because the wood contains the terpenoids *β*-thujaplicin (hinokitiol) and related compounds, which have high insecticidal and anti-wood-rot activities, the timber is highly valued (Inamori et al. 2006; Nakashima and Shimizu 1972). Therefore, transcriptomic analysis of *T. dolabrata* var. *hondae* could strengthen the genetic knowledgebase to unveil the biosynthetic mechanism of podophyllotoxin and related compounds and improve tree breeding.

Five-year-old *T. dolabrata* var. *hondae* trees originating from the Shimokita Peninsula, Aomori Prefecture, Japan, were obtained from Aomori Tree Seedling Commission (Aomori, Japan). The plants were planted in pots with a 2:3 ratio mixture of vermiculite and peat moss-based soil for horticulture (Hanasaki-monogatari; Akimoto-tensanbutsu, Iga, Japan). We then isolated total RNA with sufficient quality and yield from young *T. dolabrata* var. *hondae* needles near the shoot tip by using a modified cetyltrimethylammonium bromide method (Suzuki et al. 2008).

Next, we analyzed *T. dolablata* transcriptome by using two different next-generation sequencers, Genome Sequencer FLX (abbreviated hereafter as FLX; Roche Diagnostics, Basel, Switzerland) and Illumina Genome Analyzer IIx (abbreviated hereafter as IIx; Illumina, San Diego, USA). While IIx can read only about 100 bp from both 3′ and 5′ ends (paired-ends) of adapter-added cDNAs in DNA synthesis using luminescent substrates, FLX can read 200–300 bp from 3′ ends of cDNA using a pyrosequencing method. We anticipated that we could obtain complementary transcriptomic information by using two different next-generation sequencing methods.

We outsourced FLX pyrosequencing of total RNA from young *T. dolabrata* var. *hondae* needles to Takara Bio Inc., Kusatsu, Japan. The sequences were provided as a multi-FASTA (.fna) file, and the sequence quality was recorded in a .qual file from Takara Bio. Sequences were assembled using PHRED and PHRAP programs as previously described (Suzuki et al. 2011). An aliquot of RNA from *T. dolabrata* var. *hondae* young needles was used to construct a cDNA library according to the RNA-Seq protocol (Illumina). The sample was hybridized to flow cell surfaces, cluster-amplified using a cBot Cluster Generation system (Illumina), and sequenced on IIx sequencer with 100-bp paired-end reads. The obtained reads were assembled using CLC Genomics Workbench version 5.5.1 (CLC Bio Japan, Tokyo, Japan) with a minimum contig length of 200 bp and performing scaffolding. In Illumina RNA-seq analysis, Reads Per Kilobase of exon per Million mapped reads (RPKM) values (Mortazavi et al. 2008) were calculated using CLC Genomics Workbench with scaling normalization and a median value as a reference. Thus, whereas we sequenced 24,806 reads with an average 225 bp totaling approximately 5.58 Mbp using FLX sequencing, we paired and trimmed 6,412,133 reads with an average 95 bp totaling 610 Mbp using IIx. These reads have been deposited in the DNA Data Bank of Japan Sequence Read Archive (accession number DRA007617).

The reads obtained by FLX were assembled to non-redundant 3,730 contigs. The reads generated by IIx were assembled by CLC genomics workbench to give 29,925 non-redundant contigs. These obtained contigs, which will be uploaded on a server of Research Institute for Sustainable Humanosphere, Kyoto University (RISH Data Server; http://database.rish.kyoto-u.ac.jp), were individually submitted to BLASTX (Altschul et al. 1990) search against NCBI non-redundant protein sequence (nr) and Arabidopsis protein sequence (TAIR10; https://www.arabidopsis.org) databases (Suzuki et al. 2011) at the cutoff value of E-value ≤ 1e-5 and E-value ≤1, respectively. This process allowed us to determine the best-matching proteins in these public databases with the contigs at amino acid levels (Supplementary Tables S1 and S4). We then performed a keyword search against the TAIR10 annotation because many annotations tagged by BLASTX search against nr gave little information of gene functions. Using the keywords "*O*-methyltransferase", "CYP", and "2-oxoglutarate (2 OG) and Fe(II)-dependent oxygenase" enabled us to select candidate sequences encoding OMTs, CYPs, and 2OGDs (Supplementary Tables S2, S3, S5–S7).

Assembling FLX contigs and a keyword search for "*O*-methyltransferase" afforded 9 non-redundant contigs that putatively encode OMTs (Supplementary Table S2). Among them, three proteins, TdhFLX522, TdhFLX1251, and TdhFLX2512, share relatively high amino acid

identities (66%, 63%, and 75%) with a leucine carboxyl methyltransferase (AT5G42760), a protein-L-isoaspartate methyltransferase 2 (AT5G50240), and caffeoyl-CoA OMT 1 (CCoAOMT1; AT4G34050) (Do et al. 2007) in *Arabodpisis*, respectively, according to TAIR10 annotation. In contrast to FLX transcriptomic analysis, IIx reads were assembled to more contigs: 28 different IIx contigs putatively encoding OMTs (Supplementary Table S5). Among these contigs, amino acid sequences derived from *TdhIIx01261* and *TdhIIx19500* showed high identities (72%) with a putative *Arabidopsis* caffeoyl-CoA *O*-methyltransferase 6 (CCoAOMT6; AT1G67980) (Raes et al. 2003), and those derived from *TdhIIx09399* and *TdhIIx17089* shared identities with an *Arabidopsis* isoprenylcysteine carboxyl *O*-methyltransferase (AT5G59500) at 30% and 50% identities, respectively, based on TAIR10 annotation.

Plant *S*-adenosyl L-methionine dependent OMTs are roughly classified into two groups. While the group Pl-OMT I (Joshi and Chiang 1998; also named Type 2 OMT by Noel et al. 2003 and Lineage A by Lam et al. 2007) includes CCoAOMT and carboxyl OMTs, the group Pl-OMT II (Joshi and Chiang 1998; also named Type 1 OMT by Noel et al. 2003 and Lineage B by Lam et al. 2007) contains *Arabidopsis* coniferaldehyde OMT 1 (CAldOMT1) (Nakatsubo et al. 2008) and is more diverse. Previously identified lignan OMTs are all classified as Pl-OMT I (Lau and Sattely 2015; Ragamustari et al. 2013, 2014; Umezawa et al. 2013, 2019). We checked which previously identified lignan OMTs shared the highest E-value with *T. dolabrata* var. *hondae* FLX contigs by using BLASTX search, and found that *TdFLX0850* shared the highest E-value with previously identified lignan OMTs (Table 1). In IIx contigs, either of *TdhIIx05783* or *TdhIIx03595* shared the highest E-value with the previously identified lignan OMTs (Table 1). TAIR10 annotation indicated that 2 FLX contigs and 18 IIx contigs shared more or less identities with *Arabidopsis* CAldOMT1 in amino acid levels (Supplementary Tables S2 and S5). Collectively, *T. dolablata* var. *hondae* lignan OMT(s) may be encoded by some of these contigs.

Several CYPs are involved in lignan biosynthesis. For example, CYP81Q1 catalyzes the methylenedioxy bridge formation in (+)-sesamin biosynthesis (Ono et al. 2006); CYP92B14 performs oxidative rearrangement of (+)-sesamin to (+)-sesamolin and (+)-sesaminol (Murata et al. 2017); CYP719A11 mediates the conversion of (−)-matairesinol to (−)-pulviatolide (Marques et al. 2013); CYP71CU1, CYP71BE54, and CYP82D61 hydroxylate the B-ring of (−)-bursehernin, demethylate the methoxyl group at the 4′-position of (−)-deoxypodophyllotoxin, and hydroxylate the 7-position of (−)-4-demethyldeoxypodophyllotoxin in *P. hexandrum* etoposide aglycone formation, respectively (Figure 2). Therefore, we anticipated that several *CYP* genes were involved in biosynthesis of yatein and its derivatives in *T. dolablata* var. *hondae*.

Both FLX and IIx analyses afforded many contigs, 57 and 298 respectively, annotated as putative *CYP*s (Supplementary Tables S3 and S6). We performed a TBLASTN search against these contigs using the known lignan CYPs as queries. Among FLX contigs, *TdhFLX3464* was top-ranked in terms of E-value in

Table 1. The relationship between the previously identified lignan OMTs and putative *T. dolabrata* var. *hondae* OMTs.

OMT name	FLX Contig			IIx Contig			
	Name	Identity	E-value	Name	Identity	E-value	RPKM
CtMROMT	*TdFLX0850*	54%	4e-29	*TdhIIx05783*	37%	4e-79	76.42
FkMROMT	*TdFLX0850*	53%	4e-32	*TdhIIx03595*	55%	e-127	53.15
AsMROMT	*TdFLX0850*	59%	3e-30	*TdhIIx05783*	39%	4e-82	76.42
AsTJOMT	*TdFLX0850*	52%	3e-31	*TdhIIx03595*	53%	e-113	53.15
PhOMT1	*TdFLX0850*	42%	1e-23	*TdhIIx05783*	36%	2e-68	76.42
PhOMT3	*TdFLX0850*	52%	3e-32	*TdhIIx03595*	54%	e-122	53.15

Putative *T. dolabrata* var. *hondae OMTs* were selected based on the highest E-value.

Table 2. The relationship between the previously identified lignan CYPs and putative *T. dolabrata* var. *hondae* CYPs.

CYP name	FLX Contig			IIx Contig			
	Name	Identitity	E-value	Name	Identity	E-value	RPKM
CYP81Q1	*TdhFLX3464*	40%	2e-42	*TdhIIx00903*	35%	1e-96	74.34
CYP92B14	*TdhFLX3464*	38%	5e-44	*TdhIIx00903*	48%	e-173	74.34
CYP719A11	*TdhFLX3464*	33%	5e-27	*TdhIIx03868*	33%	1e-91	153.57
CYP71CU1	*TdhFLX3464*	40%	3e-45	*TdhIIx00903*	36%	e-118	74.34
CYP71BE54	*TdhFLX3464*	42%	1e-51	*TdhIIx06243*	41%	e-123	29.17
CYP82D61	*TdhFLX3464*	42%	7e-44	*TdhIIx00981*	40%	e-109	83.69

Putative *T. dolabrata* var. *hondae CYP*s were selected based on the highest E-value.

comparison with the previously identified lignan *CYP*s. *TdIIx00903*, *TdhIIx00981*, *TdhIIx03868*, and *TdhIIx06243*, were ranked as the highest E-value *CYP*s (Table 2). It is noteworthy that TdhFLX3464, TdhIIx00903, TdhIIx00981, TdhIIx03868, and TdhIIx06243 shared identities with any of CYP71B35, CYP75B1, CYP82C2, CYP71B37, and CYP75B1 in comparison with all *Arabidopsis* CYPs (Supplementary Tables S3 and S6). However, further analysis is required to reduce the number of candidate contigs, as shown in a previous study of a *P. hexandrum* (−)-podophyllotoxin inducible experimental system (Lau and Sattely 2015).

Considerable numbers of 2-oxoglutarate dependent dioxygenases (2OGDs) are involved in plant secondary metabolism (Kawai et al. 2014). In biosynthesis of podophyllotoxin-related compounds in *P. hexandrum*, a 2OGD (Phx30848; deoxypodophyllotoxin synthase) is responsible for the formation of aryltetralin structure in the enzymatic conversion of (−)-yatein to (−)-deoxypodophyllotoxin (Lau and Sattely 2015). Therefore, we searched putative *2OGD*s expressing in young needles of *T. dolablata* var. *hondae*. As a result, compared with the number of *CYP*s, fewer contigs were identified using the IIx assemble collection (105 contigs; Supplementary Table S7). However, the highest identity of *P. hexandrum* deoxypodophyllotoxin synthase with *T. dolablata* var. *hondae* 2OGD (Tdh13966) was as low as 30%, which suggests that further analyses are required to narrow down the candidates.

In summary, we established a collection of candidate gene sequences that encode OMT, CYP, and 2OGD family proteins in young *Thujopsis dolablata* var. *hondae* needles, by de novo assembly of reads generated from two different next-generation sequencers. Our analysis is the genetic basis for biochemical identification of lignan biosynthetic genes in *T. dolablata* var. *hondae*.

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