

Pathway engineering of benzyloquinoline alkaloid biosynthesis in transgenic California poppy cells with ectopic expression of tetrahydroberberine oxidase from *Coptis japonica*

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Abstract (S)-Tetrahydroberberine oxidase is the enzyme in the last step of berberine biosynthesis. While a previous report described the isolation of cDNA of tetrahydroberberine oxidase (THBO) from cultured *Coptis japonica* cells, we later found that purified THBO was heavily contaminated by triosephosphate isomerase. Here, we report the re-isolation of THBO cDNA from cultured *C. japonica* cells and its functional characterization in transgenic California poppy cells.

A cDNA clone for (S)-tetrahydroberberine oxidase was isolated from an EST library prepared from high berberine-producing cultured *C. japonica* cells based on the partial amino acid sequence of the purified enzyme. Analyses of the nucleotide sequences of the cloned cDNA inserts of 1728 base pairs revealed an open reading frame that encoded a 540-amino acid polypeptide with putative 28-amino acid signal peptides and a mature polypeptide with a molecular mass of 57,748. A protein blast search also shows that CjTHBO belongs to the FAD-containing berberine bridge enzyme oxidoreductase family. Since all attempts to produce active recombinant CjTHBO in *Escherichia coli* and *Saccharomyces cerevisiae* cells failed, we tried to express CjTHBO in California poppy (*Eschscholzia californica*) cells. When transgenic California poppy cells that ectopically expressed CjTHBO under the control of Cauliflower mosaic virus 35S promoter were established, the transgenic cells showed small but evident new alkaloid peaks, which were scarcely detected in control cells that did not express CjTHBO. LC-MS analyses showed that these peaks were coptisine and dehydrocheilanthifoline, which were expected to be generated by the reaction of CjTHBO from pathway intermediates, i.e., cheilanthifoline and stylopine. The usefulness of CjTHBO for metabolic engineering in transgenic California poppy cells is discussed.

Key words: Benzophananthridine alkaloid biosynthesis, California poppy, *Coptis japonica*, (S)-tetrahydroberberine oxidase.

Benzyloquinoline alkaloids (BIAs) are a large and diverse group of alkaloids with ~2500 defined structures (Liscombe et al. 2005). BIA biosynthesis begins with the conversion of tyrosine to both dopamine and 4-hydroxyphenylacetaldehyde by decarboxylation, ortho-hydroxylation, and deamination (Sato and Yamada 2008). Dopamine and 4-hydroxyphenylacetaldehyde are condensed by norcoclaurine synthase to yield (S)-norcoclaurine (Samanani et al. 2004; Minami et al. 2007), which is then sequentially converted to coclaurine by S-adenosyl methionine (SAM)-dependent norcoclaurine

6-O-methyltransferase (Morishige et al. 2000), to N-methylcoclaurine by coclaurine N-methyltransferase (Choi et al. 2002), to 3'-hydroxy-N-methyl coclaurine by P450 hydroxylase (Pauli and Kutchan 1998), and then to (S)-reticuline by 3'-hydroxy N-methylcoclaurine 4'-O-methyltransferase (Morishige et al. 2000). (S)-Reticuline is a central intermediate for divergent BIAs, including protoberberine, aporphin, benzophenanthridine and morphinan alkaloids.

In protoberberine biosynthesis, (S)-reticuline is converted by berberine bridge enzyme (BBE; Dittrich

Abbreviations: BBE, berberine bridge enzyme; BIAs, benzyloquinoline alkaloids; CaMV, Cauliflower Mosaic Virus; cDNA, complementary DNA; Cj, *Coptis japonica*; DTT, dithiothreitol; Ec, *Eschscholzia californica*; EST, expression sequence tag; LC-MS, liquid chromatography-mass spectrometry; P450, cytochrome P450; PVDF, polyvinylidene difluoride; RT-PCR, reverse transcriptase-polymerase chain reaction; RNAi, RNA interference; SAM, S-adenosyl methionine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; STOX, (S)-tetrahydroprotoberberine oxidase; THB, tetrahydroberberine; THBO, THB oxidase; TPI, triosephosphate isomerase.

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Authors dedicate this article to late Dr. Naosuke Okada.

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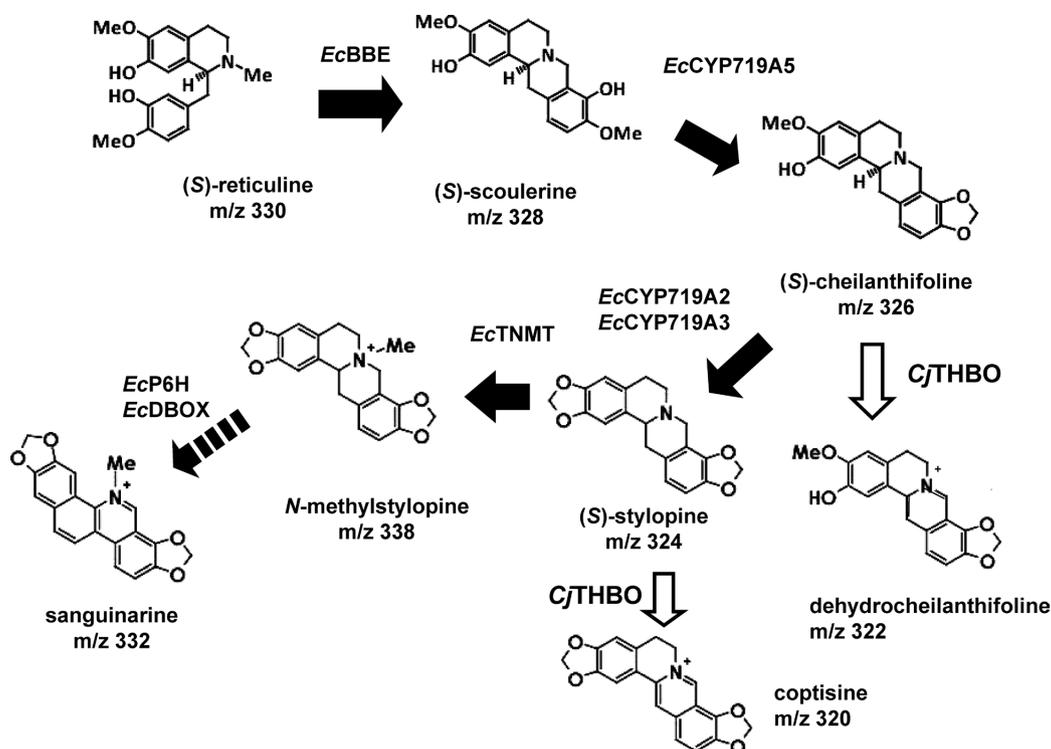


Figure 1. Metabolic pathway and predicted metabolic shift in transgenic California poppy cells that expressed *CjTHBO*. BBE, berberine bridge enzyme; CYP719A5, cheilanthifoline synthase; CYP719A2/A3, stylophine synthase; TNMT, tetrahydroprotoberberine *N*-methyltransferase; P6H, protopine 6-hydroxylase; DBOX, dihydrobenzophenanthridine oxidase.

and Kutchan 1991) to scoulerine, and then to (*S*)-tetrahydrocolumbamine by SAM-dependent scoulerine 9-*O*-methyltransferase (Takeshita et al. 1995), to tetrahydroberberine (THB; canadine) by a P450-dependent canadine synthase (Ikezawa et al. 2003), and finally to berberine by tetrahydroberberine oxidase (THBO; Okada et al. 1988). All of the cDNAs for these reactions have been isolated and functionally expressed in microbial cells to characterize their enzymological properties, except for the enzyme in the last step THBO.

While a previous report described the isolation of cDNA of THBO from cultured *Coptis japonica* cells (Okada et al. 1989), we later found that the purified THBO was contaminated by triosephosphate isomerase (TPI; Sato et al. 1990). Here, we report the re-isolation of THBO cDNA from cultured *Coptis japonica* cells and its functional characterization in transgenic California poppy (*Eschscholzia californica*) cells (Figure 1).

Materials and methods

Plant material and purification of THBO

Cultured *Coptis japonica* cells with high berberine-producing activity were maintained as described previously (Sato and Yamada 1984). Cultured cells were harvested after 3 weeks of culture and homogenized in 100 mM phosphate buffer (pH 8.0) containing 10 mM dithiothreitol (DTT) with Polyclar AT and sea sand. The homogenate was centrifuged at $10,000 \times g$ for

30 min, and then fractionated with 30–75% ammonium sulfate precipitation. The precipitates were dissolved in a minimal volume of 20 mM Tris-HCl buffer (pH 7.5) containing 2 mM DTT, and then dialyzed overnight. The dialyzed extracts were applied to SP-Toyopearl 650C to remove alkaloids. The pass-through fractions were applied to Q-Sepharose FF, BioGel HTP and Mono P columns, in which THBO active fractions were eluted by 0–1 M NaCl, 10–400 mM phosphate buffer (pH 7.0) and 25 mM Bis-Tris (pH 7.1)/10% polybuffer 74 (pH 4.0) gradients, respectively. All processes for enzyme purification were performed at 4°C.

Amino acid sequence analysis

To determine the N-terminal amino acid sequences, 0.01 mg of purified THBO was electro-transferred on a polyvinylidene difluoride (PVDF) membrane (Nihon Milipore, Yonezawa, Japan) from a gel after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (Takeshita et al. 1995). A major protein band of 58 kDa was cut from the PVDF membrane and the amino acid sequence of the peptide was determined with a protein sequencer (model 477A/120A; Applied Biosystems) as described elsewhere (Takeshita et al. 1995).

cDNA isolation

A cDNA library was constructed using poly(A)⁺ RNA isolated from 10-day-old cultured *C. japonica* cells as described previously (Choi et al. 2002). The cDNA library was randomly

sequenced and an EST library was established. We searched for the candidate cDNA by using a partial amino acid sequence (SEYEGFLEXLD) of the N-terminal amino acid of purified THBO.

Heterologous expression of THBO in microbial cells

To express THBO in *E. coli*, full-length and N-terminal 27 amino acid-truncated cDNAs were amplified by PCR and cloned into the pCold TF vector (TAKARA), and its nucleotide sequence was confirmed by DNA sequencing. Constructs were introduced in *Escherichia coli* BL21 (DE3). After cells were pre-cultured in LB medium containing 50 µg/ml ampicillin at 37°C until OD₆₀₀=0.4–0.6, recombinant protein production was induced by incubation with 0.1 mM isopropylthio-β-galactoside at 15°C for 24 h. *E. coli* cells corresponding to 5 ml of the culture were suspended in 50 mM Tris-HCl buffer (pH 7.5) containing 10% glycerol and 4 mM 2-mercaptoethanol, and disrupted by sonication. Soluble and insoluble fractions were separated by centrifugation (15,000×g) at 4°C for 15 min and used for a THBO assay or SDS-PAGE analysis.

Transformation of California poppy cells

Seeds of *Eschscholzia californica* (Takii Co., Ltd.) were sterilized and germinated on 1/2 concentration of Linsmaier-Skoog medium (Linsmaier and Skoog 1965) supplemented with 1% sucrose. Seedlings were cut into 10-mm fragments, and then used for transformation with *Agrobacterium tumefaciens* LBA4404 harboring an expression vector for *C. japonica* THBO (*CjTHBO*) in the dark at 25°C. Expression vector for full-length cDNA of *CjTHBO* was constructed under a Cauliflower Mosaic Virus (CaMV) 35S promoter in the binary vector pGWB2 as described previously (Takemura et al. 2010a). After 2 days of co-culture, transformants were selected on Linsmaier-Skoog medium containing 10 µM 1-naphthylacetic acid, 1 µM benzyladenine, 50 µg/ml hygromycin, 100 µg/ml kanamycin and 200 µg/ml cefotaxin at 25°C in the dark as described previously (Takemura et al. 2010a). After 3 months of selection culture, healthy growing cells were transferred to the liquid medium without antibiotics and cultivated at 25°C on a shaker (90 rpm) in the dark every 2 weeks. Transformants were characterized using genomic PCR as described previously (Takemura et al. 2010a) with specific primers; for THBO, Fw: 5'-ACCATGATG GGCATT TCCAGT TTTG GTA -3', Rv: 5'-TCA CTT CTT TGG CTT CTG CCA AAT ATCG -3'; for beta-actin Fw: 5'-GGT ATT GTG CTG GAT TCT GGT G -3', Rv: 5'-GTA GGA TTG CGT GGG GTA GTG -3'.

Real-time RT-PCR analysis

To quantify the mRNA expression level of *CjTHBO* genes, real-time RT-PCR was performed. cDNA was prepared from transgenic cells at day 14 of culture. cDNAs of biosynthetic genes were amplified from template cDNA (final concentration 500 pg/µl) with 5 µl DyNAmo™ HS SYBR Green Mastermix (FINNZYMES) and specific primers (actin, Fw: 5'-GGT ATT

GTG CTG GAT TCT GGT G -3', Rv: 5'-GTA GGA TTG CGT GGG GTA GTG -3', THBO, Fw: 5'-CCA ATG CCT TGA TCT TCG TT -3', Rv: 5'-CAA AGT CAT GAC CCC CAC TT -3'; final concentration 500 nM) using a DNA Engine Opticon™ System (MJ Research). After 10 min of denaturation at 95°C, PCR was performed for 40 thermal cycles (95°C for 10 s, 52°C for 20 s and 72°C for 20 s). After each run, a melting curve was obtained by heating the samples from 72 to 95°C to determine the specificity of amplification.

Metabolite analysis by LC-MS

Transgenic *E. californica* cells transformed with *CjTHBO* expression vector were sub-cultured in liquid medium for about one year to stabilize growth. Vector-control cell lines that expressed the beta-glucuronidase gene (G4, G5, G6 lines) and wild type (non-transformant; W2, W5, W6 lines) cells were similarly produced and maintained.

For the metabolite analysis, cultured cells were harvested at day 10 of subculture. About 0.25 g-fresh weight of cells were extracted in 1 ml of methanol containing 5 µl 4N HCl and 100 µM quinine as an internal standard at room temperature for overnight (Takemura et al. 2010b). Simultaneously, metabolites in culture medium were recovered with Sep-Pak C18 cartridges (Waters) and eluted with methanol. Metabolites were analyzed by an LC-10A system and LC-MS 2010 (Shimadzu) under the following conditions; column; TSK-gel ODS80Ts 4.6 mm×25 cm (TOSOH), acetonitrile/H₂O solvent gradient containing 0.05% (v/v) trifluoroacetic acid; 45% for the first 15 min, then 45 to 80% (v/v) for the next 3.5 min, hold at 80% for 6 min, and then maintain re-equilibrium at 45% for 5 min. Flow speed; 0.5 ml/min, column temperature, 40°C; analytical mode; SIM-SCAN(+), Q-array voltage; 100 V.

Berberine and chelerythrine were purchased from Wako Pure Chemicals, and sanguinarine, protopine, and allocryptopine were obtained from SIGMA-ALDRICH Co. (St. Louis, MO). (S)-Reticuline and (S)-scoulerine were gifts from Mitsui Chemicals Inc. (Tokyo, Japan). (R,S)-Tetrahydroberberine was prepared from berberine as described previously (Yamada and Okada 1985). All other chemicals were of the highest purity available.

THBO assay

THBO activity was measured in 100 mM potassium phosphate (pH 7.0), 0.4 mM (R,S)-THB and the enzyme preparation at 30°C for 60 min. After the enzyme reaction, proteins were denatured with methanol and the reaction product was analyzed by LCMS-2010 (Shimadzu).

The protein concentration was determined according to Bradford (1976) with bovine serum albumin as the standard.

Alignment analysis

The nucleotide sequence was deposited in DDBJ/GenBank™ (AB564543) and aligned using Clustal W (<http://www.genome.jp/tools/clustalw/>). The GenBank accession numbers for the sequences used are BsBBE (AF049347), AmBBE (EU881889),

recently characterized to be the said enzyme. A protein blast search also showed that STOX and our isolated putative THBO clone contained the same FAD-binding and BBE superfamily domains as other STOXs.

Expression of the recombinant polypeptide and its THBO activity

While sequence information supported the notion that our isolated cDNA would be *Cj*THBO, we tried to express recombinant *Cj*THBO protein to confirm its enzyme activity in either *E. coli* or *S. cerevisiae* cells. However, neither system was effective for producing active THBO (data not shown). In fact, even if we could produce soluble protein in *E. coli* using pCold TF vector (data not shown), large amounts of the resulting recombinant protein did not show any THBO activity. Although we made considerable effort to express enzymologically active THBO activity, we did not observe any THBO activity at all (data not shown). Thus, we finally decided to transform California poppy cells, which usually do not accumulate protoberberine alkaloids, but rather produce benzophenanthridine alkaloid through protoberberine intermediates.

Establishment of transgenic California poppy cells with the overexpression of *Cj*THBO

Expression vector for full-length cDNA of putative THBO from *C. japonica* (*Cj*THBO) was constructed under the control of CaMV 35S promoter. Next, 165 segments of California poppy seedlings were infected with *Agrobacterium tumefaciens*. After serial selection, about 40 kanamycin/hygromycin-resistant clones of *Cj*THBO transformants were established. When we analyzed 19 transgenic cell lines with genomic PCR, all of the clones clearly showed the successful integration of *Cj*THBO transgene (Figure 3). Finally, 15 clones of *Cj*THBO transformants were selected for the analysis of the expression of *Cj*THBO.

Further RT-PCR analysis of transgenic California poppy cells with *Cj*THBO transgenes clearly showed the accumulation of *Cj*THBO transcripts, whereas wild type and GUS vector-control cells did not show any transcripts of *Cj*THBO (Figure 4). Among transgenic California poppy cells with *Cj*THBO, T1, T6, T28, and T42 showed relatively high expression. To compare the effects of *Cj*THBO expression in transgenic California poppy cells, lines T3, T7 and T37 were also selected as low *Cj*THBO-expressing lines.

Alkaloid profile analysis in transgenic California poppy cells

To characterize the effects of *Cj*THBO expression on alkaloid biosynthesis, alkaloid profiles in cell extract and culture medium were compared among *Cj*THBO-expressing, vector-control and non-transformed

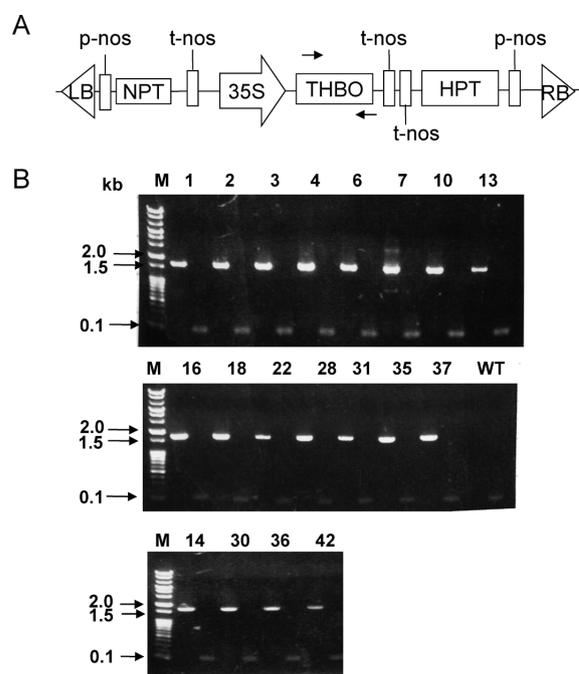


Figure 3. Genomic PCR analysis of *Cj*THBO transformants. A. Structure of expression vector. B. Genomic PCR of *Cj*THBO transformants. All *Cj*THBO transformants showed a 1.6 kb DNA amplification product, whereas non-transformant (WT) showed no product band.

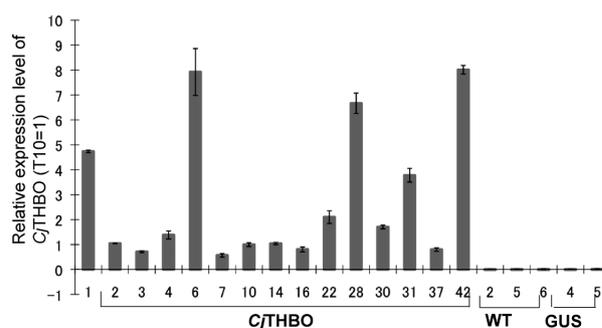


Figure 4. *Cj*THBO expression in transgenic California poppy cells. The accumulation of *Cj*THBO transcripts was evaluated with real-time PCR and their relative values were corrected by the expression level of beta-actin. The relative expression level is shown as T10=1.

California poppy cells. Whereas all of the cells and culture medium showed similar metabolite profiles, transgenic California poppy cells with *Cj*THBO showed novel peaks (peaks a and b in Figure 5A, B), in addition to peaks 1–6. Peaks 1–6 were identified by LC-MS analysis to be protopine (*m/z* 354), allocryptopine (*m/z* 370), sanguinarine (*m/z* 332), 10-hydroxychelerythrine (*m/z* 364), chelerythrine (*m/z* 348), and chelirubine (*m/z* 362) based on a comparison of their retention times and mass fragment patterns with those of reference compounds. Peaks a and b were also identified as dehydrocheilanthifoline (*m/z* 322) and coptisine (*m/z* 320) (Figure 6).

Since dehydrocheilanthifoline and coptisine can be

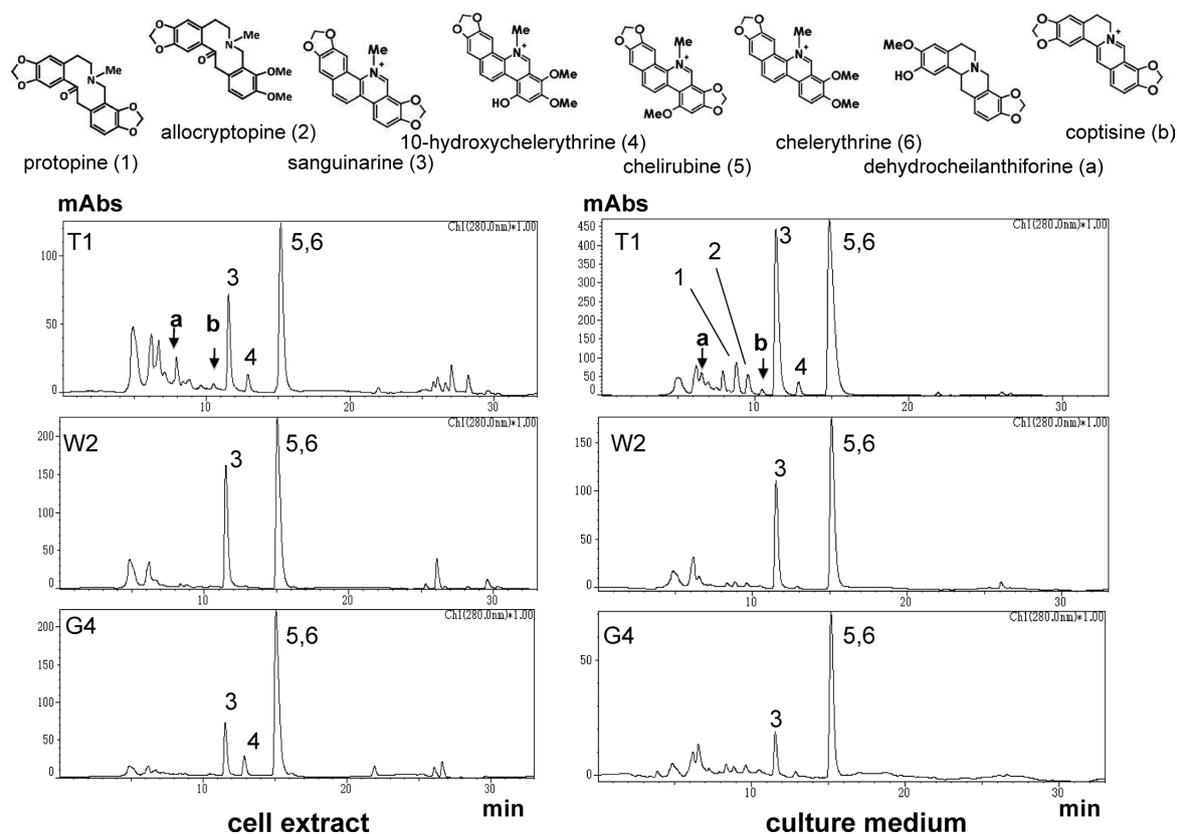


Figure 5. HPLC analysis of cell extract and culture medium of *CjTHBO* (T1), wild type (W2) and vector control line (G4). The results of representative cell lines are shown. Peaks were identified by LC-MS analysis as follows: 1, protopine; 2, allocryptopine; 3, sanguinarine; 4, 10-hydroxychelerythrine; 5, chelirubine; 6, chelerythrine. Peaks a and b were identified as dehydrocheilanthifoline and coptisine by LC-MS as shown in Figure 6.

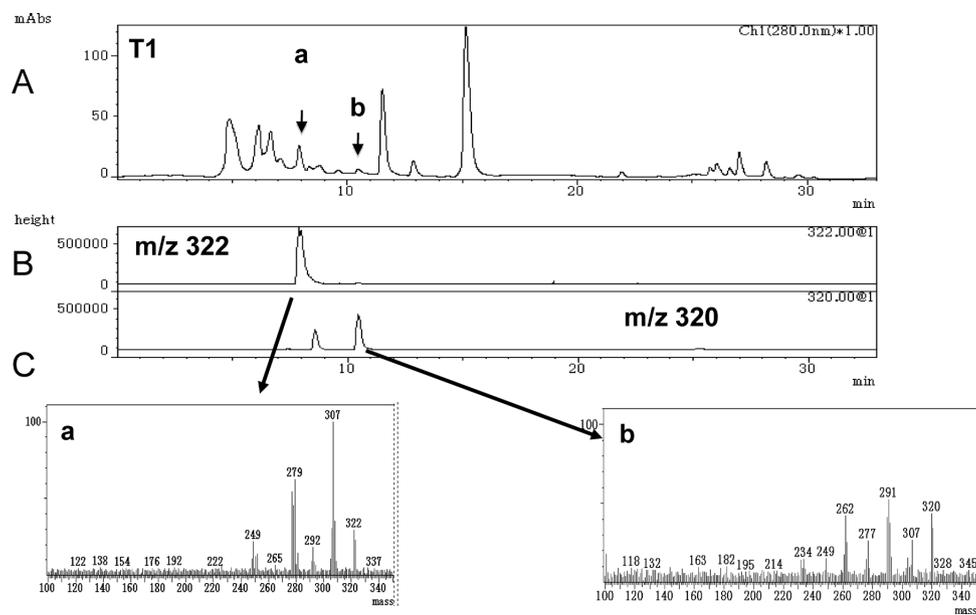


Figure 6. LC-MS analysis of novel alkaloids found in transgenic California poppy cells with *CjTHBO* expression. Mass fragment pattern of peaks a and b were identical to dehydrocheilanthifoline and coptisine.

produced by the oxidation of cheilanthifoline (m/z 326) and stylophine (m/z 324), which are intermediates of benzophenanthridine alkaloid biosynthesis in California

poppy (Figure 1), it was speculated that *CjTHBO* functioned in the alkaloid biosynthetic pathway of transgenic California poppy cells and modified the

metabolic flow. To confirm this speculation, we evaluated the effects of *CjTHBO* expression on alkaloid profiles of high (T1, T6, T28, T42) and low (T3, T7, T37) *CjTHBO*-expressing lines in comparison with those in a vector control and non-transformant.

Coptisine accumulation was mainly observed in cell extracts and culture medium of high *CjTHBO*-expressing cells (T1, T6, T28, T42). Dehydrocheilanthifoline accumulation was also mainly observed in those of high *CjTHBO*-expressing cells, whereas these two alkaloids showed different accumulation trends in high *CjTHBO*-expressing cells; T1 accumulated more dehydrocheilanthifoline than coptisine, but T42 accumulated more coptisine than dehydrocheilanthifoline (Figure 7). A low *CjTHBO*-expressing cell line (T37) showed a similar level of accumulation of coptisine as high *CjTHBO*-expressing cells, and vector control lines (G4 and G5) showed the marginal but evident accumulation of coptisine and/or dehydrocheilanthifoline. While further careful studies are needed to understand the accumulation of coptisine in vector controls, it is clear that *CjTHBO* expression is highly correlated with the accumulation of oxidation products of pathway intermediates.

Discussion

We isolated cDNA for (S)-tetrahydroberberine oxidase from *C. japonica* cells. While this enzyme cDNA was previously reported to be isolated from this plant species (Okada et al. 1989), it was triosephosphate isomerase (TPI). Recently, Chen et al. (2012) reported the functional expression of THBO isolated from *Corydalis saxicola* using a *Coptis* triosephosphate isomerase cDNA (*CjTPI*) sequence. However, we did not detect THBO activity using recombinant *CjTPI* expressed in *E. coli* (Sato et al. 1990). In fact, it is very difficult to measure THBO activity since the substrate THB is easily oxidized and THBO is very unstable. Similarly, a recent report by Gesell et al. (2011) on the functional characterization of STOX isolated from *Berberis* and *Argemone* indicated that these recombinant STOXs were also very unstable and enzyme activities could only be measured with transgenic *Spodoptera frugiperda* Sf9 insect cells without enzyme extraction.

While our unsuccessful attempt to express *CjTHBO* in microbial cells illustrates the difficulties of handling THBO *in vitro*, it also provided some additional information on the instability of STOX/THBO. When *CjTHBO* was expressed in *E. coli* cells, recombinant proteins of 58 kD were often found to exist as 28 kD fragments, which Okada et al. (1988) reported as a THBO monomer in SDS-PAGE. On the other hand, when *CjTHBO* was expressed as a soluble form using pCold TF vector, it did not show THBO activity and no

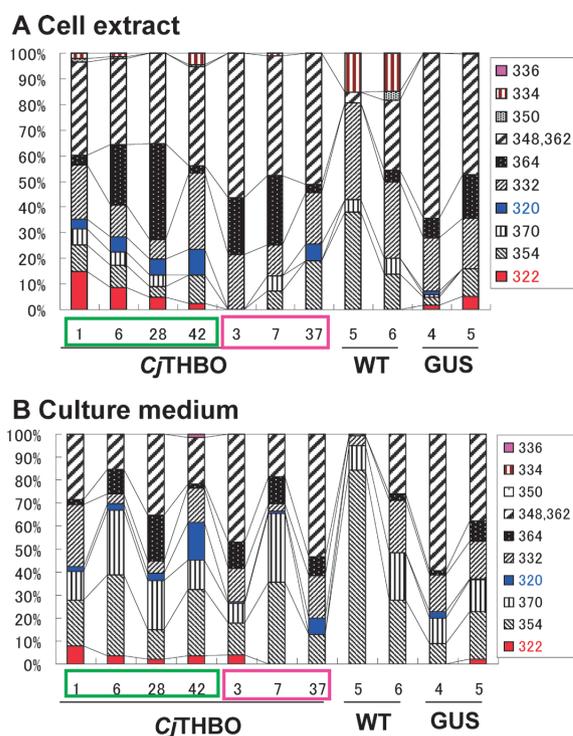


Figure 7. Alkaloid profiles in transgenic California poppy cells. A. Alkaloid profiles in cell extracts, B. Alkaloid profiles in culture media. Lines 1, 6, 28, and 42 of *CjTHBO* transformants were high *CjTHBO*-expressing, while lines 3, 7, 37 were low *CjTHBO*-expressing. Lines 5 and 6 of non-transformants (WT) and lines 4 and 5 of vector control transformants (GUS) were similarly analyzed. Alkaloid content was estimated using absorbance at 280 nm, and relative contents were calculated. *m/z* 322, dehydrocheilanthifoline (expected THBO reaction product of cheilanthifoline in Figure 6), *m/z* 354, protopine; *m/z* 370, allocryptophine; *m/z* 320, coptisine (expected THBO reaction product of stylophine in Figure 6); *m/z* 332, sanguinarine; 364, 10-hydroxychelerythrine; 348, chelerythrine; 362, chelirubine; 370, dihydrochelerythrine; *m/z* 334, dihydrosanguinarine.

specific UV spectrum for flavin, while purified STOX from *Berberis wilsoniae* had evident flavin-specific absorbance (Amann et al. 1988) and all of the deduced amino acid sequences of STOX and THBO showed the presence of FAD-binding domain. These results highlight the difficulty of expressing STOX/THBO in an *E. coli* system, while some flavin-binding enzymes have been successfully expressed in *E. coli* (e.g., YUCCA, Mashiguchi et al. 2011).

Our transgenic California poppy cells made it possible to overcome these difficulties in characterizing *CjTHBO* *in vitro*. Transgenic California poppy cells showed modified alkaloid profiles and accumulated coptisine and dehydrocheilanthifoline, oxidized products of stylophine and cheilanthifoline. While characterization of the substrate specificity of *CjTHBO* has been limited (Yamada and Okada 1985, Okada et al. 1988), it is highly probable that *CjTHBO* functions in the alkaloid pathway in California poppy and produces coptisine and dehydrocheilanthifoline. On the other hand, purified

STOX from *B. wilsoniae* showed considerably broad substrate specificities for protoberberines and simple benzyloquinoline alkaloids (Amann et al. 1988). Recombinant STOX from *B. wilsoniae* expressed in insect cells also showed a similar broad substrate specificity for canadine, tetrahydropalmatine and scoulerine (Gesell et al. 2011). The detection of limited oxidized products of protoberberines in transgenic California poppy cells indicates that CjTHBO is more substrate specific. STOX from *A. mexicana* also showed slightly different substrate specificities. These three enzymes should be useful materials for understanding the reaction specificities of STOX/THBO.

THBO is the enzyme in the last step of berberine biosynthesis. Thus, we usually think that THBO would be useless for metabolic engineering in the alkaloid pathway (Sato et al. 2007). However, our results clearly suggest that even the last step in a pathway may play a role in some other pathway if the substrates of the enzyme are available. Conversely, STOX/THBO expression in California poppy cells would inhibit the production of benzophenanthridine alkaloid, since the intermediate might be consumed. Further studies on the distribution and regulation of gene expression of STOX/THBO in BIA-producing plant species could help us to understand the evolution of the protoberberine and benzophenanthridine pathways.

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