

# Dioxygenases catalyze the O-demethylation steps of morphine biosynthesis in opium poppy

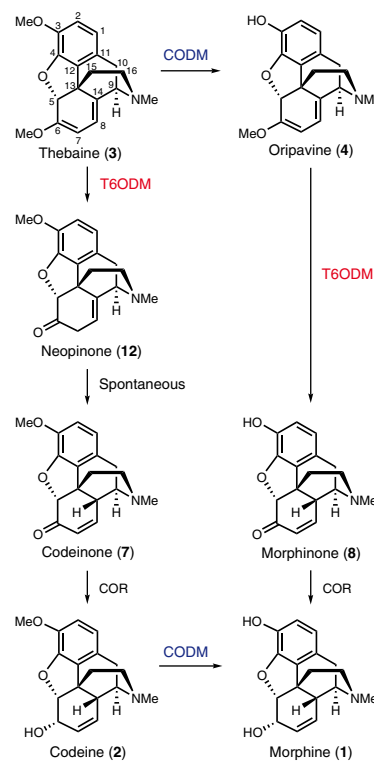
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**Two previously undetected enzymes involved in morphine biosynthesis and unique among plants to opium poppy have been identified as non-heme dioxygenases, in contrast to the functionally analogous cytochrome P450s found in mammals. We used functional genomics to isolate thebaine 6-O-demethylase (T6ODM) and codeine O-demethylase (CODM), the only known 2-oxoglutarate/Fe(II)-dependent dioxygenases that catalyze O-demethylation. Virus-induced gene silencing of T6ODM and CODM in opium poppy efficiently blocked metabolism at thebaine and codeine, respectively.**

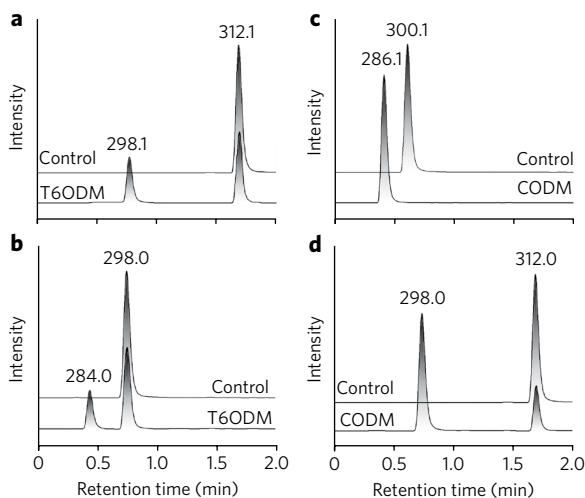
The medicinal properties of opium poppy (*Papaver somniferum* L.) have been recognized since the dawn of civilization. The licit cultivation of the plant remains the sole commercial source for several widely used pharmaceuticals, including morphine (1), codeine (2) and semisynthetic derivatives such as oxycodone. The more extensive illicit cultivation of opium poppy for the production of heroin (O,O-diacetylmorphine) continues to have profound and negative global consequences. The biosynthesis of morphine and related alkaloids in opium poppy occurs via a multistep pathway beginning with the amino acid tyrosine<sup>1</sup> (Supplementary Fig. 1). Corresponding genes encoding many of the enzymes involved in morphine biosynthesis have been isolated. However, enzymes responsible for O-demethylation at positions 6 and 3, which represent two of the three steps in the conversion of thebaine (3) to morphine (Scheme 1), have never been detected. In humans, the 3-O-demethylation of thebaine and codeine is catalyzed by CYP2D6 (refs. 2,3), and cytochrome P450s have been suggested as the enzymes responsible for the corresponding reactions in opium poppy<sup>4,5</sup>.

Avoiding the presupposition that the analogous plant enzymes are also cytochrome P450s, we used differential gene expression analysis of a mutant opium poppy chemotype to identify a candidate complementary DNA encoding thebaine 6-O-demethylase. Natural and induced mutants of opium poppy including the *top1* variety<sup>6</sup> have been reported to accumulate high levels of thebaine and oripavine (4), but not morphine or codeine<sup>7,8</sup>. The development of the *top1* variety using chemical mutagenesis was a major breakthrough for the opium poppy industry in Australia, as it allowed the efficient production of thebaine from morphine-free plants. Thebaine is the natural precursor used in the synthesis of several pharmaceuticals including oxycodone, naltrexone, naloxone and buprenorphine. Although the metabolic block in *top1* was suggested to result from a defect in the enzyme catalyzing the 6-O-demethylation of thebaine and oripavine, the biochemical basis for the phenotype was not determined<sup>6</sup>. Previously, we described an opium poppy variety (designated “T”) with a *top1* alkaloid profile<sup>8</sup>. The high-thebaine, high-oripavine, morphine- and codeine-free phenotype in T displayed Mendelian inheritance as a single, recessive locus (Supplementary Table 1). The stem transcriptome of T was then independently compared with the stem transcriptomes of three morphine-accumulating varieties (L, 11 and 40) using a cDNA

fragment-based 23,000-element microarray (Supplementary Figs. 2 and 3). Integration of all three pair-wise comparisons revealed eight candidate cDNAs exhibiting transcript levels that were lower in T compared with at least two of the high-morphine varieties. Among these, only one encoding a putative 2-oxoglutarate/Fe(II)-dependent dioxygenase (DIOX1) showed transcript levels that were lower in T compared with L, 11 and 40. This was noteworthy owing to the increased thebaine and reduced morphine content of opium poppy plants treated with the acylcyclohexanediones prohexadione



**Scheme 1 | Morphinan alkaloid biosynthesis in opium poppy, showing two routes from thebaine to morphine.** O-Demethylation at position 6 is catalyzed by T6ODM, whereas O-demethylation at position 3 is catalyzed by CODM. Thebaine can undergo O-demethylation at position 6 or position 3 to yield neopinone (12) or oripavine, respectively. Neopinone spontaneously rearranges to the more stable codeinone in aqueous solution over a wide pH range<sup>25</sup>—a process that is expedited under physiological conditions by the reduction of codeinone to codeine by codeinone reductase (COR). Codeine is demethylated by CODM to produce morphine. Demethylation of oripavine by T6ODM yields morphinone, which is reduced to morphine by COR. The opium poppy variety T used in this study is blocked at T6ODM, and accumulates thebaine and oripavine rather than morphine and codeine.



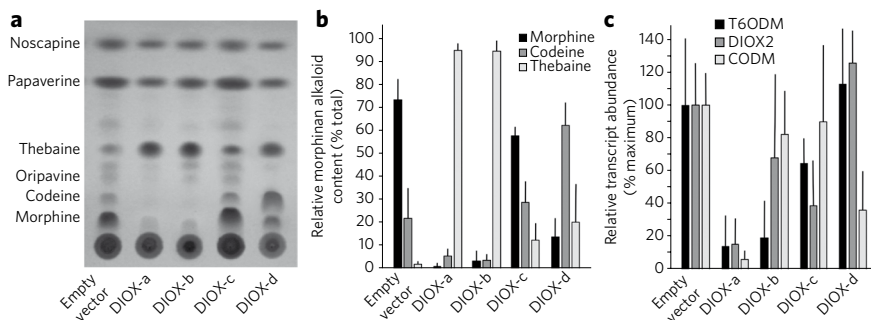
**Figure 1 | Extracted ion chromatograms showing the substrates and products of T6ODM and CODM enzyme assays.** In each panel, the upper (control) extracted ion chromatogram corresponds to an assay performed with boiled enzyme, whereas the lower (T6ODM or CODM) extracted ion chromatogram shows an assay performed with native enzyme. Reaction products were unambiguously identified using collision-induced dissociation analysis, and the resulting daughter ion mass spectra are shown in **Supplementary Figure 7**. (a) T6ODM assay with thebaine as the substrate ( $m/z$  312.1) and codeinone as the product ( $m/z$  298.1). Neopinone, which is unstable and spontaneously rearranges to codeinone in aqueous solutions<sup>25</sup>, was not detected. (b) T6ODM assay with oripavine as the substrate ( $m/z$  298.0) and morphinone as the product ( $m/z$  284.0). (c) CODM assay with codeine as the substrate ( $m/z$  300.1) and morphine as the product ( $m/z$  286.1). (d) CODM assay with thebaine as the substrate ( $m/z$  312.1) and oripavine as the product ( $m/z$  298.0). T6ODM assays were analyzed after 1 h to minimize the spontaneous formation of codeinone or morphinone adducts. CODM assays were stopped after 4 h.

calcium (5) and trinexapac-ethyl (6)<sup>9</sup>, which inhibit dioxygenases involved in the biosynthesis of gibberellin hormones.

We used the DIOX1 amino acid sequence to query our opium poppy expressed sequence tag database, which led to the identification of two highly conserved homologs (designated DIOX2 and DIOX3) (**Supplementary Methods**). A phylogenetic tree comparing several characterized and putative plant 2-oxoglutarate/Fe(II)-dependent dioxygenases placed these DIOX proteins into a distinct clade (**Supplementary Fig. 4**). Each DIOX protein had the canonical HXDX<sub>n</sub>H catalytic triad required for coordinating Fe(II), and a YX<sub>n</sub>RXS motif implicated in 2-oxoglutarate binding<sup>10</sup> (**Supplementary Fig. 5**). Recombinant His<sub>6</sub>-tagged proteins produced in *Escherichia coli* (**Supplementary Table 2**) and purified by cobalt-affinity chromatography (**Supplementary Fig. 6**) were tested for 2-oxoglutarate/Fe(II)-dependent O-demethylase activity using thebaine, oripavine or codeine as substrates. Assays consisted of Fe(II) and ascorbate as cofactors, 2-oxoglutarate and a morphinan alkaloid as substrates, and a recombinant DIOX enzyme. After incubation at 30 °C for up to 4 h, the reactions were quenched and analyzed using liquid chromatography–tandem mass spectrometry (**Fig. 1** and **Supplementary Fig. 7**).

DIOX1 catalyzed the 6-O-demethylation of thebaine and oripavine, yielding codeinone (**Fig. 1a**) and morphinone (**Fig. 1b**), respectively. Conversely, DIOX3 catalyzed the 3-O-demethylation of codeine (**Fig. 1c**) and thebaine (**Fig. 1d**), yielding morphine and oripavine, respectively. As such, DIOX1 was renamed thebaine 6-O-demethylase (T6ODM) and DIOX3 was renamed codeine O-demethylase (CODM). The enzymatic synthesis of codeinone (7) and morphinone (8) by T6ODM was accompanied by the spontaneous formation of several higher molecular weight adducts. The general instability of codeinone and morphinone in aqueous solution<sup>11,12</sup> and their reactivity with thiol-containing agents such as 2-mercaptoethanol<sup>13</sup> in the assay mixture is well documented. DIOX2 did not accept thebaine, oripavine or codeine as substrates. Notably, CODM also catalyzed the regiospecific 3-O-demethylation of the protoberberine alkaloid (*S*)-scoulerine (9) (**Supplementary Figs. 8 and 9**). Although morphinan and protoberberine alkaloids have different skeletal structures, both are members of the large and diverse group of benzyloisoquinoline alkaloids (BIAs). The substrate specificity of T6ODM, CODM and DIOX2 were further examined using an assay based on the O-demethylation–coupled decarboxylation of [1-<sup>14</sup>C]2-oxoglutarate<sup>14</sup>, which showed that other structural categories of BIAs having O-linked methyl groups were not accepted as substrates (**Supplementary Fig. 8**). Using the same assay, recombinant T6ODM produced  $K_m$  values for thebaine and oripavine of  $20 \pm 7$  and  $15 \pm 3$   $\mu\text{M}$ , respectively, whereas CODM exhibited  $K_m$  values of  $21 \pm 8$  and  $42 \pm 8$  for codeine and thebaine, respectively (**Supplementary Table 3** and **Supplementary Fig. 10**). The catalytic efficiency of CODM was lower with thebaine ( $k_{\text{cat}}/K_m = 235 \text{ s}^{-1} \text{ M}^{-1}$ ) than with codeine ( $k_{\text{cat}}/K_m = 785 \text{ s}^{-1} \text{ M}^{-1}$ ) as the substrate. These results suggest the pathway through codeinone as the preferred route in morphine biosynthesis (**Scheme 1**)<sup>15,16</sup>. The  $K_m$  values of T6ODM and CODM for 2-oxoglutarate were similar to those described for other plant 2-oxoglutarate/Fe(II)-dependent dioxygenases<sup>17</sup>. T6ODM, DIOX2 and CODM represent the first known O-demethylases in the 2-oxoglutarate/Fe(II)-dependent dioxygenase family<sup>18,19</sup>. We have deposited the corresponding gene sequences in the Entrez Gene database under accession numbers GQ500139 (T6ODM), GQ500140 (DIOX2) and GQ500141 (CODM).

Related plant enzymes such as anthocyanidin synthase, flavonol synthase and flavanone 3 $\beta$ -hydroxylase catalyze ring hydroxylation, with all but the latter leading to the introduction of double bonds.



**Figure 2 | Virus-induced gene silencing analysis.** Opium poppy seedlings were infiltrated with *Agrobacterium tumefaciens* strain GV3101 harboring pTRV1 and one of five different pTRV2 constructs. DIOX-a contained a highly conserved sequence from the coding regions of T6ODM, DIOX2 and CODM. DIOX-b, DIOX-c and DIOX-d contained gene-specific sequences from the 3' UTRs of T6ODM, DIOX2 and CODM, respectively. pTRV2 was used as the empty vector. (a) TLC of latex extracted in methanol. The  $R_f$  positions of authentic alkaloid standards are indicated in the left margin. (b) HPLC of latex extracts. Each bar represents the mean  $\pm$  s.d. for triplicate samples from 3 independent plants. (c) Real-time quantitative PCR analysis of T6ODM, DIOX2 and CODM gene transcript levels in stem samples from plants analyzed by TLC and HPLC. Each bar represents the mean  $\pm$  s.d. of 27 values (that is, 3 technical replicates on RNA samples extracted from each of 3 stem segments taken from each of 3 individual plants).

The hydroxylation of alkyl moieties is the most common reaction catalyzed by 2-oxoglutarate/Fe(II)-dependent dioxygenases. The N-demethylation of histones and nucleic acids proceeds via hydroxylation of the N-methyl moiety followed by formaldehyde elimination<sup>20</sup>. Similarly, we propose that T6ODM and CODM catalyze O-demethylation by hydroxylation of the O-methyl group. The formation of formaldehyde was confirmed using the Nash reaction<sup>21</sup> (Supplementary Fig. 11). Notably, acylcyclohexanediones did not inhibit enzyme activity *in vitro*, suggesting an indirect mode of action for the reduced metabolic flux past thebaine in plants treated with these compounds.

The metabolic functions of T6ODM and CODM were investigated *in planta* using virus-induced gene silencing (VIGS). Fragments of T6ODM, DIOX2 and CODM cDNAs (Supplementary Fig. 12) were introduced systemically into opium poppy using the tobacco rattle virus (TRV) as a vector. One pTRV2-based construct (DIOX-a) contained a conserved sequence from the coding region of T6ODM and was designed to silence all three genes simultaneously. In contrast, DIOX-b, DIOX-c and DIOX-d contained unique sequences from the 3' untranslated regions (UTRs) of the T6ODM, DIOX2 and CODM genes, respectively, and were designed to silence each gene individually. Emerging first leaves of 2-week-old to 3-week-old opium poppy seedlings were infiltrated with *Agrobacterium tumefaciens* harboring one of these four constructs, or the empty vector (pTRV2) as a control. The alkaloid content of the latex and the relative abundance of T6ODM, DIOX2 and CODM gene transcripts in the stem of infected plants were determined immediately before the onset of flowering (Fig. 2). The opium poppy variety used for the VIGS experiments accumulates an abundance of morphine, lower levels of codeine and thebaine, trace quantities of oripavine, and substantial amounts of noscapine (10) and papaverine (11). Plants treated with the empty pTRV2 vector displayed a wild-type alkaloid profile (Fig. 2a,b). In contrast, the DIOX-a and T6ODM gene-specific DIOX-b constructs resulted in a near-complete metabolic block at thebaine. The CODM gene-specific DIOX-d construct caused a substantial increase in codeine accumulation compared with morphine. Oripavine was not detected in plants treated with the DIOX-a or DIOX-d constructs as a result of silencing CODM, since the gene product converts thebaine to oripavine. The silencing of DIOX2 had no detectable effect on alkaloid content. Real-time quantitative PCR confirmed the gene-specific silencing of T6ODM, DIOX2 and/or CODM (Fig. 2c). Only partial transcript reduction was achieved for DIOX2, perhaps owing to the use of a relatively short 3' UTR fragment in the DIOX-c construct (Supplementary Fig. 12). The catalytic properties of T6ODM and CODM with respect to morphine biosynthesis were fully corroborated *in planta* using VIGS. The physiological relevance of the scoulerine 3-O-demethylase activity of each enzyme must still be determined.

Approximately 2,500 BIA structures occur naturally in members of several plant families, yet only opium poppy produces morphine and codeine. The recruitment of T6ODM and CODM from an ancestral 2-oxoglutarate/Fe(II)-dependent dioxygenase, perhaps one originally involved in protoberberine alkaloid metabolism, was a milestone evolutionary event that continues to have profound consequences, both positive and negative, for humankind. The discovery of these new enzymes could have major industrial, pharmaceutical and socioeconomic implications. Most of the licit morphine recovered from opium poppy is synthetically 3-O-methylated to yield codeine<sup>22</sup>, which is a more versatile analgesic and cough suppressant. The development of an opium poppy variety blocked at CODM would allow the direct recovery of codeine from the plant and prevent the formation of morphine, which would preclude the illicit synthesis of heroin. The extensive deployment of the *top1* variety in Australia underscores the growing demand for semisynthetic opiates, especially oxycodone, and demonstrates the commercial potential for opium poppy varieties with altered

morphinan alkaloid profiles. Our discovery of T6ODM provides a biochemical basis for the *top1* and T phenotype<sup>6</sup> and a tool to identify the genetic mechanism responsible for the specific silencing of the T6ODM gene (Supplementary Fig. 13) in opium poppy varieties blocked at thebaine. Recently, the feasibility of reconstituting BIA metabolism in yeast (*Saccharomyces cerevisiae*) has also been demonstrated<sup>23,24</sup>. Genes encoding T6ODM and CODM are essential for the production of codeine and morphine in scalable microbial systems, which could provide an alternative to conventional agriculture with respect to production cost and the regulation of controlled substances.

Received 8 September 2009; accepted 29 December 2009; published online 14 March 2010

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## Acknowledgments

We are grateful to D. Kumar (Yale University) for the pTRV1 and pTRV2 vectors, V. Irish (Yale University) for the pTRV2-PapPDS construct, the Canadian National Research Council Plant Biotechnology Institute for hosting our sequence data on their FIESTA2 annotation platform, and Sanofi-Aventis for the gift of the opium poppy varieties and the alkaloid standards used in this work. We also thank K. Zulak, R. Bourgault and J. Ziegler for technical assistance with cDNA library construction, microarray preparation and mass spectrometry, respectively. J.M.H. is the recipient of an Alberta Ingenuity Graduate Scholarship. Funding for this work was provided through a Discovery Grant from the Natural Sciences and Engineering Research Council of Canada and a Canada Research Chair in Plant Metabolic Processes Biotechnology, both awarded to P.J.F.

## Author contributions

J.M.H. and P.J.F. contributed equally to all aspects of the experimental design and execution, and the preparation of the manuscript.

## Competing financial interests

The authors declare no competing financial interests.

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