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# Cloning and characterization of canadine synthase involved in noscapine biosynthesis in opium poppy

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ABSTRACT

**Noscapine biosynthesis in opium poppy is thought to occur via *N*-methylcanadine, which would be produced through 9-*O*-methylation of (*S*)-scoulerine, methylenedioxy bridge formation on (*S*)-tetrahydrocolumbamine, and *N*-methylation of (*S*)-canadine. Only scoulerine 9-*O*-methyltransferase has been functionally characterized. We report the isolation and characterization of a cytochrome P450 (CYP719A21) from opium poppy that converts (*S*)-tetrahydrocolumbamine to (*S*)-canadine. Recombinant CYP719A21 displayed strict substrate specificity and high affinity ($K_m$ = 4.63 ± 0.71 µM) for (*S*)-tetrahydrocolumbamine. Virus-induced gene silencing of *CYP719A21* caused a significant increase in (*S*)-tetrahydrocolumbamine accumulation and a corresponding decrease in the levels of putative downstream intermediates and noscapine in opium poppy plants.**
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## 1. Introduction

Noscapine is an abundant benzylisoquinoline alkaloid (BIA) with a phthalideisoquinoline backbone first isolated from opium poppy (*Papaver somniferum*) by the French chemist Charles Derosne in 1803 [1,2]. Despite its pharmaceutical importance as a cough suppressant and potential anticancer drug, noscapine biosynthesis is not well established. Early radiotracer studies in opium poppy suggested that the carbon skeleton of noscapine, apart from the lactonic carbonyl group, is derived from the condensation of two tyrosine derivatives [3,4]. The lactone carbonyl carbon of noscapine was proposed to originate from the *N*-methyl group of a 1-benzylisoquinoline precursor, similar to the C-8 position in berberine, protopine, and related alkaloids [4]. The protoberberine alkaloid scoulerine was shown to be an effective precursor of noscapine [4,5]. The identification of several other secoberbine and phthalideisoquinoline alkaloids, such as macrantaldehyde, papaveroxine, and the hemiacetals egenine and narcotinehemiacetal [6–8], led to the proposal that *N*-methylation of the tetrahydroprotoberberine alkaloid (*S*)-canadine facilitated multistep oxidation to a hemiacetal that was finally dehydrogenated to the carbonyl carbon of noscapine. Beyond scoulerine, only canadine has been incorporated empirically into noscapine [9].

The recent isolation and characterization of scoulerine 9-*O*-methyltransferase (SOMT1) supported the role of (*S*)-scoulerine as a precursor to noscapine [10]. Since noscapine also possesses the same 2,3-methylenedioxy-9,10-dimethoxy substitution pattern as (*S*)-canadine, the first two steps leading to noscapine from (*S*)-scoulerine are potentially identical to those involved in the formation of the common antimicrobial alkaloid berberine [11]. Accordingly, (*S*)-scoulerine would be 9-*O*-methylated to yield (*S*)-tetrahydrocolumbamine, which would then be converted to (*S*)-canadine by the methylenedioxy bridge-forming cytochrome P450 (CYP) monooxygenase, canadine synthase (Fig. 1) [10,12,13]. Although CYP enzymes able to convert (*S*)-tetrahydrocolumbamine to (*S*)-canadine have been isolated and characterized from the berberine-producing plant, Japanese goldthread (*Coptis japonica*), such an enzyme has not been reported in opium poppy. Further support for the occurrence of canadine synthase in opium poppy was recently provided by the suppression of *CYP719A21* transcript levels using virus-induced gene silencing (VIGS) [14], which caused the accumulation of tetrahydrocolumbamine. However, the biochemical function of the enzyme has not been established.

*Abbreviations:* BIA, benzylisoquinoline alkaloid; CID, collision-induced dissociation; CPR, cytochrome P450 reductase; CYP, cytochrome P450; ESI, electrospray ionization; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LC–MS/MS, liquid chromatography–tandem mass spectrometry; SOMT, scoulerine 9-*O*-methyltransferase; TNMT, tetrahydroprotoberberine *N*-methyltransferase; TRV, tobacco rattle virus; VIGS, virus-induced gene silencing

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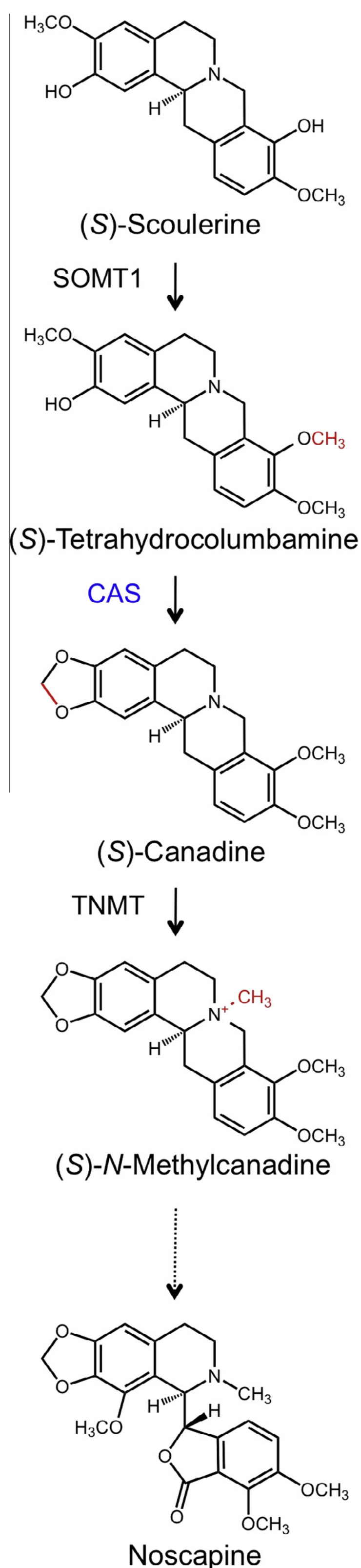


**Fig. 1.** Proposed function of CYP719A21 as canadine synthase in noscapine biosynthesis. *Abbreviations:* SOMT, scoulerine 9-*O*-methyltransferase; CAS, canadine synthase; TNMT, tetrahydroprotoperberine *N*-methyltransferase. The dashed arrow represents several uncharacterized reactions.

Several members of the CYP80, CYP82, and CYP719 families have been shown to catalyze many different reactions in benzylisoquinoline alkaloid biosynthesis, including hydroxylation, C–C or C–O coupling, and the formation of methylenedioxy bridges [11]. Enzymes in CYP80 family catalyze the hydroxylation, intramolecular C–C phenol coupling, or condensation of 1-benzylisoquinoline substrates [15–17], whereas CYP82 family members have been reported to hydroxylate or oxidize benzylisoquinoline alkaloids with protopine and protoberberine backbones [18,19]. Characterized enzymes in the CYP719 family primarily catalyze the oxidative cyclization of an *ortho*-hydroxymethoxy-substituted aromatic ring resulting in the formation of a methylenedioxy bridge [20]. Canadine synthase (CYP719A1) from *C. japonica* was the first CYP719 reported to catalyze the conversion of (*S*)-tetrahydrocolumbamine to (*S*)-canadine [13]. CYP719A2 and CYP719A3 from California poppy (*Eschscholzia californica*) convert the related protoberberine alkaloid (*S*)-cheilanthifoline to (*S*)-stylopine [21], whereas CYP719A13 from Mexican prickly poppy (*Argemone mexicana*) displays multifunctional activity, converting (*S*)-tetrahydrocolumbamine to (*S*)-canadine, (*S*)-cheilanthifoline to (*S*)-stylopine, and (*S*)-scoulerine to (*S*)-nandinine while CYP719A14 transformed only (*S*)-scoulerine to (*S*)-cheilanthifoline [22]. Recently, CYP719A23 and CYP719A24 from *Podophylum* spp. were also reported to catalyze methylenedioxy bridge formation on the non-alkaloid matairesinol in podophyllotoxin biosynthesis [23]. Although members of the CYP719A subfamily have been widely associated with the formation of berberine and sanguinarine in species such as *C. japonica*, *A. mexicana*, and *E. californica*, the occurrence of a related enzyme in opium poppy has not been reported. In this study, CYP719A21 from opium poppy was isolated by comparative transcriptomics and characterized as a noscapine biosynthetic enzyme.

## 2. Materials and methods

### *2.1. Plant material and chemicals*

Opium poppy chemotypes Bea's Choice and Veronica were cultivated as described previously [10]. (*S*)-Scoulerine was purchased from Chromadex (Chromadex Inc., Irvine, USA). (*S*)-Tetrahydrocolumbamine was prepared enzymatically from (*S*)-scoulerine using purified SOMT1 [10]. Other alkaloids were obtained as described previously [10].

### *2.2. Phylogenetic and gene expression analyses*

Amino acid alignments were performed using ClustalW [24] and a bootstrapped phylogenetic tree was constructed using Geneious (Biomatters, Newark, NJ). Gene expression analysis was performed by real-time quantitative PCR (RT-qPCR) using primers listed in Table S1 as described previously [10].

### *2.3. Cloning and expression of CYP719A21*

*CYP719A21* was amplified from stem cDNA of the opium poppy chemotype Bea's Choice using Takara Ex Taq DNA polymerase (Fisher Scientific, Ottawa, Canada) and primers listed in Table S1. For heterologous production of FLAG-tagged CYP719A21, the amplicon was ligated into the *Not*I and *Spe*I restriction site of the dual plasmid pESC-Leu2d containing cytochrome P450 reductase (CPR) from opium poppy yielding pESC-Leu2d::*CYP719A21/CPR* [19,25]. Yeast harboring pESC-Leu2d*::MSH/CPR*, which produces cytochrome P450 reductase (CPR) and methylstylopine hydroxylase [19], was used as a positive control. Yeast harboring pESC-Leu2d*::CPR* was used as the negative control.

### *2.4. Yeast culture, preparation of microsomes and immunoblot analysis*

pESC-Leu2d*::CYP719A21/CPR* was used to transform the protease-deficient *Saccharomyces cerevisiae* strain YPL 154C:Pep4. For

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