Molecular Genetic Analysis of an *O*-Methyltransferase of the Opium Poppy *Papaver somniferum*

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Abstract

Isoquinoline alkaloids are a large class of compounds derived from the amino acid L-tyrosine containing many physiologically active members. Among the isoquinoline alkaloids, morphine is one of the pharmaceutically important members that is still derived from the plant that produces it, the opium poppy *Papaver somniferum*. *P. somniferum* produces over 80 alkaloids derived from L-tyrosine. We have isolated cDNAs encoding several enzymes of tetrahydrobenzylisoquinoline-derived alkaloid biosynthesis from this plant. The first enzyme in the biosynthetic pathway for which we have isolated a cDNA is norcoclaurine 6-*O*-methyltransferase. The next is the cytochrome P-450-dependent monooxygenase (*S*)-*N*-methylcoclaurine 3'-hydroxylase. These enzymes are common to the morphine, noscapine and sanguinarine biosynthetic pathways. Specific to the sanguinarine pathway is the berberine bridge enzyme that oxidatively cyclizes the *N*-methyl moiety of (*S*)-reticuline to the bridge carbon C-8 of (*S*)-scoulerine. Finally, specific to morphine biosynthesis are salutaridinol 7-*O*-acetyltransferase and codeinone reductase the penultimate enzyme of the morphine pathway that reduces codeinone to codeine. Given the number of cDNAs specific to various alkaloid biosynthetic pathways that we now have, attempts at metabolic engineering of *P. somniferum* can be made. We describe herein details of the isolation and biochemical characterization of a cDNA encoding the *P. somniferum O*-methyltransferase OMTPS3.

INTRODUCTION

Alkaloids are pharmacologically active, nitrogen-containing, basic compounds originally believed to be only of plant origin. Since the isolation of the first alkaloid morphine, more than 12,000 alkaloids have been defined. Approximately 20% of flowering plants produce alkaloids. Each species accumulates alkaloids in a unique and defined patterned. The role of alkaloids in plants has been a longstanding question, but a picture emerges that supports an ecochemical function for these compounds. Alkaloid-containing plants were also mankind's original *materia medica*. Many of these plants are still used today as sources of prescription drugs, for example the analgesics morphine and codeine are isolated from the opium poppy *Papaver somniferum*. Alkaloid biosynthetic pathways are attractive targets for molecular biology because of their role in plant chemical ecology and the biotechnological potential for the production of commercially important compounds.

S-Adenosyl-L-methionine-dependent methyltransferases are involved in the *O*-methylation of many plant natural products. For several alkaloids of the isoquinoline type, the complete biosynthetic pathway has been elucidated at the enzyme level, which provides us with insightful information into the substrate specificity of the various biosynthetic enzymes (reviewed in Kutchan, 1998). The biosynthesis of the central isoquinoline alkaloid intermediate (*S*)-reticuline requires the action of two *O*-methyl-transferases, norcoclaurine 6-*O*-methyltransferase and 3'-hydroxy-*N*-methylcoclaurine 4'-*O*-methyltransferase (Rüffer et al., 1983; Frenzel and Zenk, 1990; Sato et al., 1994; Hara et al., 1995). Partial purification and characterization of these enzymes indicated that they might have relatively strict substrate specificity. It was thought that only minor

variations to the presumed in vivo substrate could be tolerated by the enzymes. This was found not only to be true for alkaloid biosynthetic pathways, but for other types of natural products as well. Regiospecific oxygen methylation significantly contributes to the vast metabolic diversity of plant secondary metabolism. *O*-methyltransferase encoding genes are tentatively identified and annotated based upon sequence similarity to those of other proteins (Ibrahim et al., 1998; Joshi and Chiang, 1998; Schröder et al., 2002). Understanding gene function, however, requires more substantial biochemical characterization. An example of how we approach this problem with respect to methyltransferases is provided herein.

MATERIALS AND METHODS

Plant Material

P. somniferum seedlings were routinely grown aseptically on Gamborg B5 medium (Gamborg et al., 1968) containing 0.8% agar in a growth chamber at 22°C, 60% relative humidity under cycles of 16 h light/8 h dark with a light intensity of 85 µmol sec^{-1} m^{-2} per µA. Differentiated *P. somniferum* plants were grown either outdoors in Saxony-Anhalt or in a greenhouse at 24°C, 18 h light and 50% humidity.

Generation of Partial cDNAs from *P. somniferum*

Partial cDNAs encoding *O*-methyltransferases from *P. somniferum* were produced by PCR using cDNA generated by reverse transcription of mRNA isolated from floral stem. DNA amplification using either Taq or Pfu polymerase was performed under the following conditions: 3 min at 94°C, 35 cycles of 94°C, 30 s; 50°C, 30 s; 72°C, 1 min. At the end of 35 cycles, the reaction mixtures were incubated for an additional 7 min at 72°C prior to cooling to 4°C. The amplified DNA was resolved by agarose gel electrophoresis, the band of approximately correct size (400 bp) were isolated and subcloned into pGEM-T Easy (Promega) prior to nucleotide sequence determination.

Generation of Full-Length cDNAs

The complete nucleotide sequence was generated in two steps using one *O*-methyltransferase-specific PCR primer and one RACE-specific primer as specified by the manufacturer. The 5'- and 3'-RACE-PCR experiments were carried out using a SMART cDNA amplification kit (Clontech). RACE-PCR was performed using the following PCR cycle: 3 min at 94°C, 25 cycles of 94°C, 30 s; 68°C, 30 s; 72°C, 3 min. At the end of 25 cycles, the reaction mixtures were incubated for an additional 7 min at 72°C prior to cooling to 4°C. The amplified DNA was resolved by agarose gel electrophoresis, the band of the expected size were isolated and subcloned into pGEM-T Easy prior to sequencing.

The full-length clone was generated in one piece using primers specific for the open reading frame for PCR with *P. somniferum* floral stem cDNA as template. The final primers used for cDNA amplification contained recognition sites for the restriction endonucleases *Bam*HI and *Not*I, appropriate for subcloning into pFastBac HTa (Life Technologies) for functional expression. DNA amplification was performed under the following conditions: 3 min at 94°C, 35 cycles of 94°C, 30 s; 60°C, 30 s; 72°C, 2 min. At the end of 35 cycles, the reaction mixtures were incubated for an additional 7 min at 72°C prior to cooling to 4°C. The amplified DNA was resolved by agarose gel electrophoresis, the band of approximately correct size was isolated and subcloned into pCR4-TOPO (Invitrogen) prior to nucleotide sequence determination.

Heterologous Expression and Enzyme Purification

The full-length cDNA generated by RT-PCR was ligated into pFastBac HTa that had been digested with restriction endonucleases *Bam*HI and *Not*I. The recombinant plasmid was transposed into baculovirus DNA in the *Escherichia coli* strain DH10BAC (Life Technologies) and then transfected into *Spodoptera frugiperda* Sf9 cells according to the manufacturer's instructions. The insect cells were propagated and the recombinant

virus was amplified according to (Kutchan et al., 1995). INSECT-XPRESS serum-free medium (Bio Whittaker) was used in the enzyme expression experiments. After infection of 20 ml suspension grown insect cells had proceeded for 3-4 days at 28°C and 130 rpm, the cells were removed by centrifugation under sterile conditions at 900 x g for 5 min at 4°C. All subsequent steps were performed at 4°C. The pellet was discarded and to the medium was added 0.73 g NaCl, 2.5 ml glycerol and 50 µl mercaptoethanol. The pH was adjusted to 7.0 with 1.0 M NaOH. The His-tagged *O*-methyltransferase was then purified by affinity chromatography using a cobalt resin (Talon, Clontech) according to the manufacturer's instructions.

RESULTS AND DISCUSSION

We attempted to isolate cDNAs encoding methyltransferases of (*S*)-reticuline biosynthesis by RT-PCR using conserved regions in methyltransferases that are believed to be involved in binding of the co-substrate S-adenosylmethionine (Frick and Kutchan, 1999). A partial clone generated by RT-PCR with methyltransferase specific primers was used to isolate a full-length cDNA by RACE-PCR. The translation of the nucleotide sequence of this cDNA *omtps3* is given in Fig. 1. The amino acid sequence did not show a remarkable similarity to known methyltransferase sequences. The conserved regions indicative of identity as a methyltransferase are shaded in this figure.

The biosynthesis of the central isoquinoline alkaloid intermediate (*S*)-reticuline requires the action of two *O*-methyltransferases, norcoclaurine 6-*O*-methyltransferase and 3'-hydroxy-*N*-methylcoclaurine 4'-*O*-methyltransferase, and one *N*-methyltransferase. As for alkaloid biosynthesis, *O*-methyltransferases are central also to flavonoid biosynthesis. A common structural feature, a catechol moiety, is found between the substrate of early isoquinoline alkaloid biosynthesis, norcoclaurine, and the substrate of early phenylpropanoid biosynthesis, caffeic acid. A series of forty potential substrates were tested with the heterologously expressed, recombinant methyltransferases OMTPS3 of *P. somniferum*. We have found in the past that *O*-methyltransferases are surprisingly permissive with respect to the broad range of substrates that can be methylated (Frick and Kutchan, 1999; Frick et al., 2001). The structures tested varied from simple catechols, such as catechol and guaiacol, to complex tetrahydrobenzylisoquinoline alkaloids. The exact list of substrates tested in this study is as follows: catechol, protocatechuic acid, dopamine, caffeic acid, guaiacol, isovanillic acid, vanillic acid, (*R,S*)-norcoclaurine, (*S*)-coclaurine, (*R,S*)-isococlaurine, (*R,S*)-4'-*O*-methylcoclaurine, (*R,S*)-nororientaline, (*R*)-norprotosinomenine, (*S*)-norprotosinomenine, (*R*)-norreticuline, (*S*)-norreticuline, (*R*)-7-dehydroxy-norreticuline, (*S*)-7-dehydroxy-norreticuline, (*R,S*)-*N*-methylcoclaurine, (*R,S*)-6-*O*-methyllaudanosoline, (*S*)-4'-*O*-methyllaudanosoline, (*R*)-reticuline, (*S*)-reticuline, (*R,S*)-orientaline, (*R*)-protosinomenine, (*R,S*)-isoorientaline, (*R,S*)-laudanidine, (*R,S*)-codamine, (*S*)-scoulerine, (*S*)-coreximine, morphine, codeine, quercetin, quercetin-3-methylether, quercetin-7-methylether, luteolin, morin, esculetin, cyanidin and salutaridine. Of these forty substrates, OMTPS3 was able to methylate only catechol (2.0 pmol/sec/mg protein) and esculetin (1.2 pmol/sec/mg protein). The kinetic parameters for these two *O*-methylation reactions were determined and are presented in Table 1. There is no substantial difference in the efficieny of methylation of these two substrates as determined by the ratio k_{cat}/K_m.

RNA gel blot analysis using *omtps3* as hybridization probe with total RNA isolated from bud, capsule, leaf, root and stem of *P. somniferum* indicated that *omtps3* is most expressed strongly in stem tissue and root tissue (Fig. 2). Taken together with the substrate specificity of the enzyme, this idicates that OMTPS3 may be involved in phenolic metabolism *in planta* and could be involved in the chemical defense system of *P. somniferum*. Further studies will clearly be necessary to more completely characterize the methyltransferases of *P. somniferum* and to elucidate their functional roles in vivo.

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Tables

Table 1. Kinetic parameters (K_m and V_{max}) of the *O*-methyltransferase OMTPS3 for substrates and co-substrate AdoMet derived from Lineweaver-Burk plot analysis.

Enzyme	Substrates	K_m for AdoMet (µM)	V_{max} for AdoMet (pmol/s)	K_m (µM)	V_{max} (pmol/s)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}.mM^{-1}$)
OMTPS3	Catechol	100	1.66	14.56	0.48	0.7×10^{-2}	0.55
	Esculetin	127	2.25	16.72	1.06	1.7×10^{-2}	1.05

Figures

MGSIRNVDEDIQACRYAMELASASVPVMVLKTAIELDILEIIKKPGPGTQ
ISVSEIVSQIQNLKNPDAPVMLDR-MLRLLASYNILTCSLKDGGNDDKVE
RLYGLAPVCKFLTKNEAGCSMSALLLMNQDKVLMESWYHLKDAILDGGIP
FNKAYGVHAFEYHGKDLRFNKVFNKGMSDHTTITMNKILETYKGFEGLNS

motif A **motif J** **motif K**
IVDVGGGVGATVSMIISKYPTIQGINFDLPHVIQDAPSFPGIKHVGGDMF

motif B **motif C**
ASVPNADAIFMKWILHDWSDEHSVKILKNCYDAL--PKNGKVIIVECIIP

motif L
EVSDSSVAGHGVFHLDNIMLAHNPGGKERSLKQFENLAKDTGFTDFQVVC

SAYDTYVMEFYKN

Fig. 1. Amino acid sequence of the *O*-methyltransferase OMTPS3 isolated from *P. somniferum*. The shaded motifs are conserved regions indicative of plant methyltransferases.

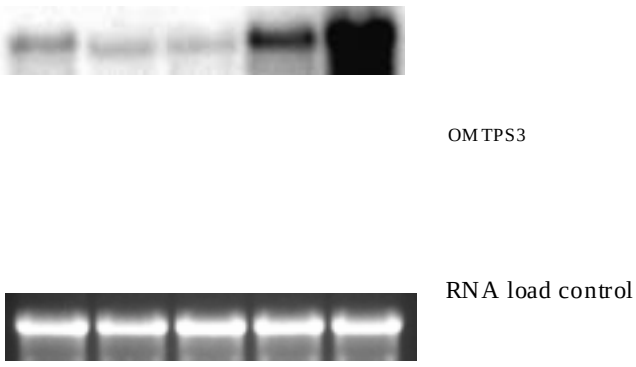

Fig. 2. Spatial distribution of *O*-methyltransferase gene transcript in the *P. somniferum* plant. Total RNA was isolated from bud, capsule, leaf, root and stem and analyzed by RNA gel blotting (50 µg total RNA per lane). RNA load control was visualized under UV light after ethidium bromide staining. The gel was then blotted onto a nylon membrane and the blot was hybridized to the cDNA encoding OMTPS3.