The roles of latex and the vascular bundle in morphine biosynthesis in the opium poppy, *Papaver somniferum*

Marion Weid, Jörg Ziegler, and Toni M. Kutchan*

Leibniz-Institut für Pflanzenbiochemie, Weinberg 3, 06120 Halle/Saale, Germany

Communicated by Meinhart H. Zenk, Universität Halle, Halle/Saale, Germany, August 6, 2004 (received for review May 1, 2004)

The opium poppy, *Papaver somniferum*, is one of mankind’s oldest medicinal plants. Opium poppy today is the commercial source of the narcotic analgesics morphine and codeine. Along with these two morphinans, opium poppy produces approximately eighty alkaloids belonging to various tetrahydrobenzylisoquinoline-derived classes. It has been known for over a century that morphinan alkaloids accumulate in the latex of opium poppy. With identification of many of the enzymes of alkaloid biosynthesis in this plant, biochemical data suggested involvement of multiple cell types in alkaloid biosynthesis in poppy. Herein the immunolocalization of five enzymes of alkaloid formation in opium poppy is reported: *(R,S)-3′-hydroxy-N-methylcoclaurine 4′-O-methyltransferase* central to the biosynthesis of tetrahydroisoquinoline-derived alkaloids, the berberine bridge enzyme of the sanguinarine pathway, *(R,S)-reticuline 7-O-methyltransferase* specific to laudanosine formation, and salutaridinol 7-O-acetyltransferase and codeinone reductase, which lead to morphine. In capsule and stem, both O-methyltransferases and the O-acetyltransferase are found predominantly in parenchyma cells within the vascular bundle, and codeinone reductase is localized to laticifers, the site of morphinan alkaloid accumulation. In developing root tip, both O-methyltransferases and the O-acetyltransferase are found in the pericycle of the stele, and the berberine bridge enzyme is localized to parenchyma cells of the root cortex. Laticifers are not found in developing root tip, and, likewise, codeinone reductase was not detected. These results provide cell-specific localization that gives a coherent picture of the spatial distribution of alkaloid biosynthesis in opium poppy.

The opium poppy, *Papaver somniferum*, is the source of the narcotic analgesics codeine and morphine, which accumulate in specialized internal secretory cells called laticifers (1). In the aerial parts of the plant, the laticifer cells are anastomosed, forming an articulated network (2, 3). Laticifers are found in the pericycle of the stele, and the berberine bridge enzyme is localized to parenchyma cells of the root cortex. Laticifers are not found in developing root tip, and, likewise, codeinone reductase was not detected. These results provide cell-specific localization that gives a coherent picture of the spatial distribution of alkaloid biosynthesis in opium poppy.

Nearly all enzymes of morphine biosynthesis have also been described (6). In more recent years, cDNAs encoding 10 enzymes of alkaloid biosynthesis in *P. somniferum* have been isolated and characterized as follows. On the pathway leading from L-tyrosine to the first tetrahydrobenzylisoquinoline alkaloid intermediate *(S)-norcoclaurine*, *tydc*, a cDNA encoding tyrosine/dopa decarboxylases, has been isolated (7). Transformation of *(S)-norcoclaurine* to the central isoquinoline alkaloid biosynthetic intermediate *(S)-reticuline* is understood at both the enzyme and gene level. *(S)-Norcoclaurine* is *O*-methylated by *(R,S)-norcoclaurine 6-O-methyltransferase* *(8, 9)*. *(S)-Coclaurine* is next *N*-methylated by *(R,S)-coclaurine *N*-methyltransferase* *(10)* and the cDNA encoding this enzyme has been characterized (S. Haase, J.Z., S. Frick, and T.M.K., unpublished data). *(S)-N-methylcoclaurine* is then hydroxylated by the cytochrome P450-dependent monoxygenase CYP80B1 *(S)-N-methylcoclaurine 3′-hydroxylase* *(11)*. The cDNA encoding the corresponding cytochrome P450 reductase has been isolated as well *(12)*. *(S)-3′-hydroxy-N-methylcoclaurine* is methylated to *(S)-reticuline* by *(R,S)-3′-hydroxy-N-methylcoclaurine 4′-O-methyltransferase* *(4′OMT)* *(13)*. The cDNA *(4′OMT)* has been isolated and characterized from *P. somniferum* *(J.Z., M.L. Díaz Chavez, and T.M.K., unpublished data)*.

*(S)-Reticuline* is a central intermediate of isoquinoline alkaloid biosynthesis, which leads to a plethora of alkaloidal structures. In *P. somniferum*, *(R,S)-reticuline* can be methylated by *(R,S)-reticuline 7-O-methyltransferase* *(7OMT)* *(for which the cDNA 7omt has been described)* to the tetrahydrobenzylisoquinoline laudanine *(9)*. The *N*-methyl group of *(S)-reticuline* can alternatively be oxidatively cyclized by the berberine bridge enzyme *(BBE)* to C-8 of *(S)-scoulerine* *(11, 14, 15)*. *(S)-Scoulerine* is then further converted in these plants to the antimicrobial benzo[†]phenanthridine alkaloid sanguinarine. Along the pathway on which *(S)-reticuline* is specifically converted to morphine, cDNAs encoding two biosynthetic enzymes have been identified. Salutaridinol 7-O-acetyltransferase *(SalAT)*, encoded by *salat*, transfers an acetyl moiety from acetyl-CoA to the 7-hydroxy group of salutaridinol *(16, 17)*. Codeinone reductase *(COR)* is encoded by *cor* and catalyzes the penultimate step in morphine biosynthesis, the NADPH-dependent reduction of the keto moiety of codeinone to the 6-hydroxy group of codeine *(18, 19)*.

The combined results of the enzymological and molecular genetic work suggested a spatial distribution of alkaloid biosynthesis in poppy. It has also been suggested, based on the failure to produce morphine in undifferentiated *P. somniferum* cell cultures, that morphine accumulation is related to cytodifferentiation, noting that laticifers are absent from these cultures but present in tissue cultures *(20, 21)*. We now address the question of cell-specific localization of the enzymes of alkaloid biosynthesis and the site of gene transcript accumulation. The results of these localization experiments provide us with an insight into the multiple levels of regulation involved in *P. somniferum* alkaloid biosynthesis. The necessity of understanding the cell-specific expression of alkaloid biosynthetic genes is also central to choosing proper gene transcriptional promoters for the design of meaningful metabolic engineering experiments with *P. somniferum*.

Herein, fluorescence immunocytological localization is carried out with the 4′OMT *(reticuline pathway)*, the 7OMT...
Animals were inoculated three times at 4-week intervals according to the company’s standard protocol. Two weeks after the final immunization, the animals were bled. For immunolocalization experiments, two guinea pigs were inoculated three times with MLP 15; the second immunization followed the first by 14 days, and the third was 4 weeks later. After an additional 2 weeks, the animals were bled.

**Western Blot Analysis.** For the preparation of protein gel blots, plant material of *P. somniferum* was frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle. The powder was extracted with 1.5 volumes (wt/vol) 50 mM Tris-HCl, pH 7.5, containing 20 mM DTT, 0.1% Triton X-100, and 2% polyvinylpyrrolidone. Cellular debris was removed by centrifugation at 10,000 × g for 10 min at 4°C. For electrophoretic resolution, 10–25 μg of protein was subjected to SDS/PAGE in a 12% acrylamide gel (24). The resolved proteins were transferred to a nitrocellulose filter according to ref. 25. The filter was incubated at room temperature for 1 h in 20 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 0.5% Tween 20, and 3% powdered milk. Antibody-containing serum was diluted 1:200 to 1:500 in fresh 20 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 0.5% Tween 20, and 3% powdered milk and added to the filter for 1 h at room temperature. The filter was then washed with the same buffer four times each for 10 min at room temperature. Secondary antibody (anti-rabbit IgG alkaline phosphatase conjugate for anti-guinea pig alkaline phosphatase conjugate, Sigma) was diluted 1:5,000 in fresh 20 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 0.5% Tween 20, and 3% powdered milk and added to the filter for 2–3 h at room temperature. The filter was then washed with the same buffer four times each for 10 min at room temperature. Sites of secondary antibody binding were visualized after addition of 0.4 mM nitro blue tetrazolium chloride and 0.4 mM 5-bromo-4-chloro-3-indolyl phosphate in 20 mM Tris-HCl, pH 9.5, containing 150 mM NaCl and 5 mM MgCl₂ to the filter. Color development times were typically 20 min.

**Immunolocalization.** Immunolocalization of the *P. somniferum* alkaloid biosynthetic enzymes and of MLP 15 was carried out according to ref. 26, with the exception that 3–5% BSA in PBS was used as the blocking and antibody-containing solution. Anti-MLP 15, anti-COR, anti-SalAT, anti-4′OMT, and anti-
BBI antibodies were diluted 1:500, and anti-7OMT antibody was diluted 1:1000. Anti-callose antibody (Biosupplies, Parkville, Australia) was used at a final concentration of 10 µg/ml. The following secondary antibodies were used in a 1:500 dilution: Alexa 488 goat anti-rabbit IgG, Alexa 568 goat anti-guinea pig IgG, and Alexa 350 goat anti-mouse IgG (Molecular Probes). The immunostained sections were sealed in a citifluor anti-fadent mounting medium (Citifluor, Leicester, U.K.). Staining with aniline blue was carried out according to ref. 27.

**In Situ Hybridization.** In situ hybridization was carried out according to ref. 28. For the generation of sense and antisense RNA, 7OMT and cor were subcloned into pGEM-T Easy (Promega). In vitro transcription was performed with digoxigenin-UTP (Roche Molecular Biochemicals) for 7OMT and with fluorescein-UTP (Roche Molecular Biochemicals) for cor initiated from either the T7 or SP6 promoter according to the manufacturer’s instructions. Hybridizing transcript was visualized either with anti-digoxigenin antibody alkaline phosphatase conjugate or with anti-fluorescein antibody alkaline phosphatase conjugate diluted 1:1,000 in 1% blocking reagent (Roche Molecular Biochemicals) and 1% acetylated BSA (Biotrend, Cologne, Germany) in Tris-buffered saline (100 mM Tris-HCl, pH 7.4, containing 150 mM NaCl).

**Microscopy.** Bright field microscopy and fluorescence microscopy were performed by using an Axioskop microscope (Zeiss). For fluorescence detection, the appropriate filter combination was used. Photographs were made with a Fuji video camera HZ300, and for fluorescence microscopy a Sony charge-coupled device camera was used.

**Results and Discussion**

**Organ-Specific Localization of Enzymes.** Polyclonal antibodies raised against 4’OMT, SalAT, 7OMT, COR, BBE, and MLP 15 were initially evaluated by hybridization to protein gel blots that contained crude protein extracts from *P. somniferum* capsule, stem, leaf, and root. In general, the antibody preparations crossreacted with a single band of appropriate size blotted from a SDS/PAGE gel (Fig. 2). The MLP family of *P. somniferum* contains at least eight genes divided into two subfamilies (29). It was therefore not surprising that anti-MLP 15 antibody reacted with more than one low-molecular-weight protein. With the exception of BBE, the richest source of alkaloid biosynthetic enzymes and MLP 15 was capsule tissue followed by stem tissue. Anti-BBE antibody reacted with protein from stem and root. Benzocaliclike alkaloid and low levels of morphinan alkaloids were found in root tissue (data not shown) and the enzyme levels detected on the protein gel blot were correspondingly low. Based on this Western blot analysis and previous knowledge of distribution of enzyme activities in the plant (6, 16–18), capsule, stem, and root material was used for subsequent immunolocalization experiments.

**Tissue-Specific Localization of Enzymes.** The capsule (fruit) of opium poppy is a rich source of latex and, therefore, of morphinan alkaloids. The capsule dissected in cross section revealed the parietal placenta with attached ovules. Tissue containing the large- and small-vascular bundle was excised and fixed (Fig. 5 A and B, which is published as supporting information on the PNAS web site). The reddish-brown discoloration that forms upon excision is a typical indicator of the oxidation (polymerization of phenolics) of exuded latex. Immunolocalization of the alkaloid biosynthetic enzymes and MLP 15 in cross sections prepared from this area of capsule indicate that two cell types are involved (Fig. 5). Localized (green fluorescence) to parenchyma cells that surround laticifers within the large- and small-vascular bundle (Fig. 3 A and B), was 4’OMT, which participates in the central isooquinoline alkaloid biosynthetic pathway leading to (S)-reticuline (Fig. 1). The red fluorescence shows the position of the latex-specific MLP 15 and is indicative of a laticifer. Likewise, SalAT, an enzyme specific to morphine biosynthesis, is localized to the same cell type as 4’OMT (Fig. 3 D and E, green fluorescence) within the vascular bundle. In contrast, COR, which is the penultimate enzyme of morphine biosynthesis occurring downstream of SalAT, is clearly colocalized with MLP 15 to laticifers, demonstrated by the yellow fluorescence, which results from an overlay of red and green fluorescence (Fig. 3 G and H). In capsule, COR was also found to a small extent in parenchyma cells, a phenomenon that was not observed in stem (Fig. 6, which is published as supporting information on the PNAS web site). Shown by green fluorescence in Fig. 3 J–L is 7OMT, which lies on the biosynthetic pathway to laudanine (Fig. 1) but not to morphine and is localized to parenchyma cells within the vascular bundle but, in contrast to 4’OMT and SalAT, only to those cells distal to laticifers Fig. 3 J and K, red fluorescence). The preimmune serum controls for each antibiosynthetic enzyme antibody are shown in Fig. 3 C, F, I, and L. Fig. 3 E and G nicely demonstrates the presence of fused laticifer cells (fluorescing red in E and yellow in G) that form an articulated network in capsule.

Stem tissue that lies within 2–4 cm of the capsule is also a rich source of latex. In longitudinal-sections of upper stem, sieve elements with their sieve plates and vesiculated laticifers can be readily identified (Fig. 5C). Immunolocalization of the alkaloid biosynthetic enzymes and MLP 15 in longitudinal-sections prepared from this area of stem also indicate that two cell types are involved in alkaloid biosynthesis in *P. somniferum* (Fig. 4). In these experiments, single sections were irradiated with different excitation wavelengths to visualize alkaloid biosynthetic enzyme, callose of sieve plates, or MLP 15 of laticifer cells. In Fig. 4 A, 4’OMT (green arrow, green fluorescence) has been localized to cells adjacent to a laticifer (Fig. 4C, red arrow and red fluorescence). The sieve element identified by the presence of a sieve plate (Fig. 4B, blue arrow and blue fluorescence) is spatially separated from the cells containing biosynthetic enzyme and also shows no green fluorescence. SalAT, as in capsule, is localized similarly to 4’OMT (Fig. 4D). Again, the distinctly
different position of a laticifer and sieve element are indicated by red and blue arrows and shown respectively also in Fig. 4F and E. COR, on the other hand, is localized only to laticifers (Fig. 4G, green fluorescence). The same cells in Fig. 4G and I are labeled with anti-COR and anti-MPL 15 antibody. The sieve element indicated by blue arrows in Fig. 4G and H is devoid of green or red fluorescence. Also consistent with those results obtained in capsule, 7OMT is localized to the vascular bundle but not to laticifers or sieve elements (Fig. 4J–L).

Finally, developing root tips were sectioned, and the immunolocalization of 7OMT, BBE, and SalAT was carried out (Figs. 7A and 8, which are published as supporting information on the PNAS web site). The 7OMT and SalAT were found in the pericycle within the stele. Laticifers were not found in root tip, and likewise, COR was not immunologically detected. BBE localized to the largest part of the root cortex that consists of thin-walled parenchyma cells, which often have large intercellular spaces. This tissue proved to be quite fragile and severed when treated with anti-BBE antibody. BBE was not detected in aerial parts of the plant by fluorescence immunocytology. In the P. somniferum variety used in this study, (S)-scoulerine, the product of the reaction catalyzed by BBE, was below the limits of detection by liquid chromatography MS, suggesting that too little of this vesicular enzyme may be present for detection by

![Image of immunolabeling of alkaloid biosynthetic enzymes in cross sections of capsule of P. somniferum.](image-url)
fluorescence immunocytological methods in single cells. The vesicular localization of BBE has recently been demonstrated by ImmunoGold labeling of shoot tissue of *E. californica* and *P. somniferum* (30).

## Roles of the Vascular Bundle and Laticifers in Alkaloid Biosynthesis

Tyrosine/dopa decarboxylase participates in the very early stages of tetrahydrobenzylisoquinoline alkaloid biosynthesis, acting at the interface between primary and secondary metabolism (6). *Tydc* transcript has been reportedly detected in phloem in *P. somniferum* aerial plant parts (31). This finding is consistent with the results reported herein. Transcripts of *7omt* and *cor* were detected in phloem, and *cor* transcript was localized to laticifer cells (Fig. 7B and Fig. 9, which is published as supporting information on the PNAS web site). Based on the results obtained herein, early stages of isoquinoline alkaloid biosynthesis in *P. somniferum* occur in parenchyma cells associated with the vascular bundle and surrounding laticifers in aerial plant parts. Although cDNAs encoding each biosynthetic enzyme have not yet been identified, it appears that beginning from the primary metabolite L-tyrosine, enzymatic transformations through to the branch point intermediate (S)-reticuline occur in parenchyma associated with laticifers. Diversification into laudanine then takes place only in those parenchyma cells that lie

---

<table>
<thead>
<tr>
<th>Gene</th>
<th>Localization</th>
<th>Enzyme</th>
<th>Image</th>
</tr>
</thead>
<tbody>
<tr>
<td>4OMT</td>
<td>A</td>
<td>biosynthetic enzyme</td>
<td>Fig. 4A</td>
</tr>
<tr>
<td>SaAT</td>
<td>D</td>
<td>biosynthetic enzyme</td>
<td>Fig. 4D</td>
</tr>
<tr>
<td>COR</td>
<td>G</td>
<td>biosynthetic enzyme</td>
<td>Fig. 4G</td>
</tr>
<tr>
<td>7OMT</td>
<td>J</td>
<td>biosynthetic enzyme</td>
<td>Fig. 4J</td>
</tr>
<tr>
<td>MLP 15</td>
<td>C</td>
<td>anti-MLP 15</td>
<td>Fig. 4C</td>
</tr>
<tr>
<td>MLP 15</td>
<td>L</td>
<td>anti-MLP 15</td>
<td>Fig. 4L</td>
</tr>
<tr>
<td>MLP 15</td>
<td>F</td>
<td>anti-MLP 15</td>
<td>Fig. 4F</td>
</tr>
</tbody>
</table>

*Fig. 4.* Immunolabeling of alkaloid biosynthetic enzymes, callose, and MLP 15 in longitudinal sections of stem of *P. somniferum*. Each row of micrographs represents a single section irradiated with a different excitation wavelength to visualize alkaloid biosynthetic enzyme (A, D, G, and J), callose of sieve plates (B, E, H, and K), or MLP 15 of laticifer cells (C, F, I, and L). The green arrows indicate the position of anti-biosynthetic enzyme antibody; the red arrows indicate the position of anti-MLP 15 antibody and is indicative of laticifer cells; the blue arrows indicate the position of anti-callose antibody and is indicative of sieve plates. (A) Localization of 4OMT. (D) Localization of SaAT. (G) Localization of COR. (J) Localization of 7OMT. (B, E, H, and K) Callose of sieve elements. (C, F, I, and L) MLP 15 in laticifers. Single sections were probed with three primary antibodies (anti-biosynthetic enzyme, anti-MLP, and anti-callose) and three secondary antibodies. Green (excitation, 488 nm; emission long-pass filter, 520 nm), blue (excitation, 568 nm; emission long-pass filter, 590 nm), and red (excitation, 365 nm; emission long-pass filter, 420 nm) fluorescence images are presented individually. la, laticifer; sp, sieve plate.
distal to laticifers. Further transformation of (S)-reticuline into 

morphine is probably spatially located in the parenchyma that 

surround laticifers at least up until the 7-O-acetylation of 

salutaridinol. Thebaine, the subsequent intermediate in co-

surround laticifers at least up until the 7-

morphine is probably spatially located in the parenchyma that 

can be determined. The third branch in alkaloid biosynthesis 

immunolocalization of COR herein and the known site of 

reduced in the laticifer into codeine, consistent with both the 

ation of thebaine into neopinone is not known because an in vitro 

enzyme assay has not yet been established. Codeinone is then 

reduced in the laticifer into codeine, consistent with both the 

immunolocalization of COR herein and the known site of 

codeine accumulation in latex (5). The demethylation of codeine 

into morphine, as for the demethylation of thebaine into neopin-

ome, still requires more study before a cell-specific localization 

can be determined. The third branch in alkaloid biosynthesis 

occurring after (S)-reticuline, which has been investigated herein, 

that leads to benzylphenanthridines appears to proceed in 

thin-walled parenchyma of root cortex, based on the cellular 

immunolocalization of BBE. 

Two cell types, laticifers and phloem parenchyma, have been 

identified herein for the biosynthesis of isoquinoline alkaloids in 

P. somniferum. It has been recently reported that sieve elements 

are the alkaloid biosynthetic cell of P. somniferum, based on 

immunolocalization of CYP80B1, BBE, and COR (32), an 

observation that we never made in our own studies, whether 

immunolocalization of CYP80B1, BBE, and COR (32), an 

immunolocalization of in vitro enzymatic, molecular genetic, or immunocytological. This find-

ing is also inconsistent with the endoplasmic reticulum mem-

brane localization of the cytochrome P450-dependent CYP80B1 and the necessity for an endoplasmic reticulum membrane-

bound cytochrome P450 reductase for enzyme activity that such an enzyme likely be located in the cytosol of a sieve element (33). In addition, BBE is enzymatically active at pH 9–10 and com-

pletely inactive at neutral or acidic pH. BBE is located in planta 
in smooth vesicles with a basic pH as demonstrated both 

biochemically and by ImmunoGold labeling, in which experi-

ments BBE was immunolocalized to smooth vesicles in idioblasts of 
P. somniferum (14, 30). Likewise, it is unlikely to be functional in 
the cytosol of a sieve element. Based on the low quality of the 

fluorescence micrographs presented in that work and the general 
inability to clearly discern cells types in those pictures, we can 
only conclude that the localization of all three investigated 

alkaloid biosynthetic enzymes to sieve elements represents a 

misinterpretation of the micrographs. This misinterpretation 

may also have resulted from the use of a commercial low-

morpheine variety (P. somniferum cv. Marianne) in the Bird et al. (32) study that further hampered specific detection of signals from low-level biosynthetic enzymes. 

In conclusion, two cell types, parenchyma within the vascular 

bundle and laticifers, are sites of the biosynthesis of isoquinoline alkaloids in P. somniferum. The early stages of morphine bio-

synthesis occur in parenchyma cells surrounding laticifers and 

then at late stages, possibly at the level of either salutaridinol-

7-O-acetate or thebaine, moves into the laticifer, which is the 

storage site of the morphinans thebaine, codeine, and morphine. 

The role of intercellular transporters of alkaloidal intermediates 

as well as intracellular transport into vesicles within laticifers 

adds a potential level of regulation to morphine biosynthesis that 

still needs to be investigated.

This paper is dedicated to Professor Detlef Gröger (Leibniz-Institut für Pflanzenbiochemie), on the occasion of his 75th birthday. We thank Dr. Bettina Hause (Leibniz-Institut für Pflanzenbiochemie) and Professor Gerhard Wanner (Ludwig-Maximilians-Universität, Munich) for helpful discussions that allowed us to develop the techniques necessary to carry this project through to fruition. This work was supported by the Deutsche Forschungsgemeinschaft and Fonds der Chemischen Industrie.