PERGAMON

Phytochemistry 53 (2000) 555–564

PHYTOCHEMISTRY

www.elsevier.com/locate/phytochem

Distribution of morphinan and benzo[*c*]phenanthridine alkaloid gene transcript accumulation in *Papaver somniferum*

Fong-Chin Huang, Toni M. Kutchan*

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

Received 12 October 1999; accepted 10 November 1999

Abstract

The opium poppy *Papaver somniferum* L. produces the antimicrobial benzo[*c*]phenanthridine alkaloid sanguinarine and the narcotic analgesic morphinan alkaloid morphine. Transcripts of three genes of alkaloid biosynthesis in *P. somniferum* in developing seedlings, mature plants and plant cell suspension culture were monitored for temporal/spatial or for methyl jasmonate-induced accumulation by RNA gel blot analysis. These genes encoded (*S*)-*N*-methylcoclaurine 3′-hydroxylase (CYP80B1) that is common to morphine and sanguinarine biosynthesis, the berberine bridge enzyme (BBE) that lies on the pathway to sanguinarine, and codeinone reductase (COR) the penultimate enzyme of morphine biosynthesis. In developing *P. somniferum* seedlings, the morphine precursor thebaine was present throughout the first twenty days of germination. In contrast, sanguinarine was present in detectable quantities only after day five after germination and continued to increase at least until day twenty. Accumulation of *cyp80b1*, *bbe1* and *cor1* gene transcripts paralleled these differences. In the mature poppy plant, *cyp80b1, bbe1* and *cor1* gene transcripts were detected in the root, the stem, the leaf lamina and the leaf mid rib. Only *cyp80b1* and *cor1*, however, were found in the flower bud and the capsule. Consistent with the fact that sanguinarine accumulation, but not that of morphine, can be induced in opium poppy cell suspension culture by addition of methyl jasmonate to the culture medium, *cyp80b1* and *bbe1*, but not *cor1* transcript accumulated in response to elicitor treatment.

Keywords: *Papaver somniferum* L; Papaveraceae; Opium poppy; Gene transcript distribution; Morphine; Sanguinarine; Berberine bridge enzyme (*bbe1*); Codeinone reductase (*cor1*); (*S*)-*N*-methylcoclaurine 3′-hydroxylase (*cyp80b1*)

1. Introduction

Alkaloid-containing plants are part of mankind's original materia medica. Many are still in use today as sources of prescription drugs. One of the most widely used medicinal alkaloids is the antitussive and analgesic codeine from the opium poppy *Papaver somniferum* L. In fact, the search for useful drugs of defined structure from plants began with the isolation of morphine from dried latex, or opium, of *P. somniferum* in 1806 (Sertürner, 1806). *P. somniferum* is currently one of the most important renewable resources for pharmaceutical alkaloids and produces more than a 100 different alkaloids that are derived from the amino acid L-tyrosine and have the tetrahydrobenzylisoquinoline alkaloid (*S*)-reticuline as a common intermediate (Kutchan, 1998). In addition to the narcotic analgesic alkaloid morphine, this plant also produces the benzo[*c*]phenanthridine alkaloid sanguinarine. The benzo[*c*]phenanthridines have antimicrobial properties and are thought to be part of the chemical defense system of *P. somniferum* (Dzink & Socransky, 1985; Cline & Coscia, 1988). Although each of the known enzymes of morphine biosynthesis has been detected in both *P. somniferum* plants and cell suspension culture, plant

Abbreviations: BBE, berberine bridge enzyme; COR, codeinone reductase; CYP80B1, (*S*)-*N*-methylcoclaurine 3′-hydroxylase; TYDC, tyrosine/dopa decarboxylase.

* Corresponding author. Tel.: +49-345-5582-258; fax: +49-345-5582-173.

E-mail address: kutch@ipb.uni-halle.de (T.M. Kutchan).

0031-9422/00/$ - see front matter
PII: S0031-9422(99)00600-7

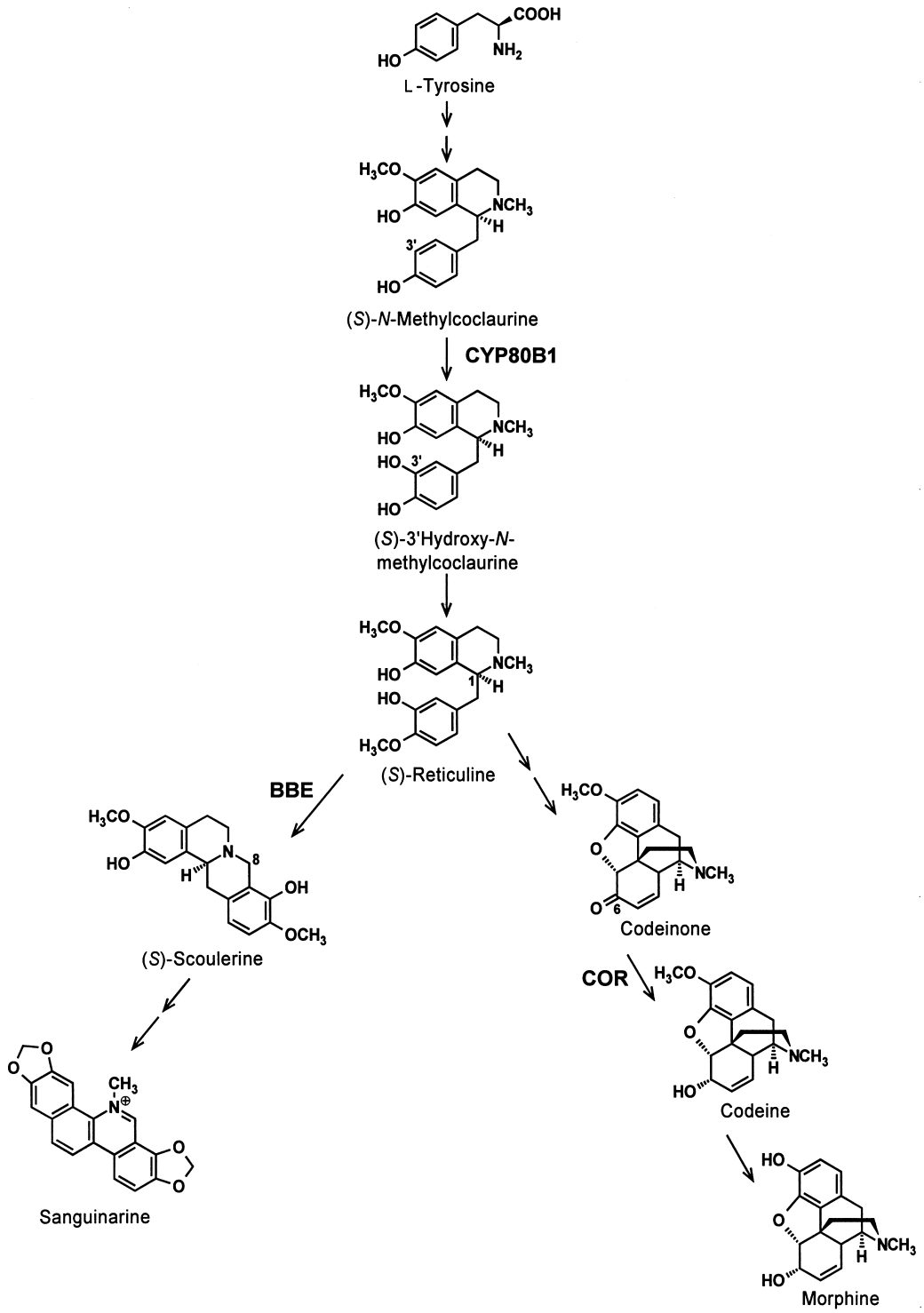

Fig. 1. Biosynthetic pathway from L-tyrosine to sanguinarine and morphine. The positions of the three enzymes CYP80B1, BBE and COR, investigated in this study are indicated. CYP80B1 is common to both sanguinarine and morphine biosynthesis, while BBE participates only in the formation of alkaloids derived from (*S*)-scoulerine, such as sanguinarine, and COR only in morphine synthesis.

cell cultures have never been shown to accumulate morphine (Kutchan, 1998). *P. somniferum* cell suspension cultures do however, upon elicitation with fungal elicitors, accumulate sanguinarine (Eilert, Kurz, & Constabel, 1985; Cline & Coscia, 1988). This response to elicitation by accumulation of benzo[*c*]phenanthridine alkaloids is similar to that observed with cell suspension cultures of the California poppy *Eschscholzia californica* (Kutchan & Zenk, 1993; Kutchan, 1993).

Morphine and sanguinarine biosynthesis is very well understood at the enzyme level from the results of the laboratory of M.H. Zenk (reviewed in Kutchan, 1998). It is known that morphine and other alkaloidal intermediates such as dopamine accumulate in smooth vesicles within the *P. somniferum* laticifer cells (Roberts, McCarthy, Kutchan & Coscia, 1983; Kutchan, Rush & Coscia, 1986). These vesicles are thought to bud out from the smooth endoplasmic reticulum, although there is no direct evidence for this. In the mature plant, laticifers form a reticulated system that extends throughout the aerial portions of the plant. Exuded latex is the cytoplasm of these reticulated laticifer cells. The latex has been shown to contain certain enzymes such as polyphenolase, acid phosphatase and dopa decarboxylase (Roberts et al., 1983). Cell differentiation to laticifers appears to be related to opium alkaloid accumulation (Nessler & Mahlberg, 1977, 1978; Roberts et al., 1983; Kutchan et al., 1985, 1986; Rush, Kutchan & Coscia, 1985), but the cellular localization of morphine alkaloid biosynthesis is not yet completely understood. Although the tyrosine/dopa decarboxylase (TYDC) gene family has been investigated at the molecular genetic level, the enzyme is involved in multiple biochemical processes in this plant and is not necessarily a direct marker for alkaloid biosynthesis (Facchini & De Luca, 1994; Maldonado-Mendoza, López-Meyer, Galef, Burnett & Nessler, 1996). Results of in situ hybridization, however, suggest that gene transcripts accumulate differentially throughout the plant. For example, *tydc1* transcript is abundant in roots whereas *tydc2* transcript is predominant in stems (Facchini & De Luca, 1995).

We have isolated cDNAs[1] several enzymes of tetrahydrobenzylisoquinoline alkaloid biosynthesis from *P. somniferum*. These are specifically the cytochrome P-450-dependent monooxygenase (*S*)-*N*-methylcoclaurine 3′-hydroxylase (CYP80B1) that hydroxylates (*S*)-*N*-methylcoclaurine to (*S*)-3′-hydroxy-*N*-methylcoclaurine on the pathway to the branchpoint isoquinoline alkaloid intermediate (*S*)-reticuline shown in Fig. 1 (Pauli & Kutchan, 1998). This enzyme is common to both the morphine and sanguinarine biosynthetic pathways. Specific to the sanguinarine pathway is the berberine bridge enzyme (BBE) that oxidatively cyclizes the *N*-methyl moiety of (*S*)-reticuline to the bridge carbon C-8 of (*S*)-scoulerine (Dittrich & Kutchan, 1991; Facchini, Penzes, Johnson & Bull, 1996; Hauschild, Pauli & Kutchan, 1998) and specific to morphine biosynthesis is codeinone reductase (COR) the penultimate enzyme of the morphine pathway that reduces codeinone to codeine (Unterlinner, Lenz & Kutchan, 1999). We have used these cDNAs herein to analyze the expression pattern of sanguinarine and morphine biosynthetic gene transcript accumulation in *P. somniferum* plants and plant cell suspension culture and relate this information to the site of alkaloid accumulation.

2. Results

2.1. Isolation and characterization of cyp80b1 from P. somniferum

The cDNA encoding (*S*)-*N*-methylcoclaurine 3′-hydroxylase should occur in all plants that produce benzylisoquinoline-derived alkaloids (Pauli & Kutchan, 1998). In order to isolate *cyp80b1* from *P. somniferum*, an amino acid sequence comparison was made between CYP80B1 and CYP80A1. CYP80A1 is the cytochrome P-450-dependent oxidase berbamunine synthase of bisbenzylisoquinoline alkaloid biosynthesis of *Berberis stolonifera*. Both CYP80A1 and CYP80B1 oxidatively transform the substrate *N*-methylcoclaurine, so it was reasoned that a comparison should reveal conserved regions necessary for substrate binding. Oligonucleotides were designed for RT-PCR based on this comparison and were used as primers to generate a partial *cyp80b1* cDNA using RNA isolated from *P. somniferum* stem tissue as template. The sequence of this partial clone was similar in sequence to *cyp80b1* from *E. californica* and was then used to screen a *P. somniferum* cDNA library prepared in λ-ZAPII. The cDNA that was isolated was missing the first six amino acids as compared to CYP80B1 from *E. californica* (Fig. 2). CYP80B1 from *P. somniferum* is 77% identical to CYP80B1 from *E. californica* and 43% identical to CYP80A1 from *B. stolonifera*.

The six missing amino acids were added by PCR and the putative full-length cDNA was subcloned into pFastBac1 for functional expression in insect cell culture (Pauli & Kutchan, 1998). Active expression verified that the cDNA encoded an enzyme with (*S*)-*N*-methylcoclaurine 3′-hydroxylase activity. The physical characteristics and substrate specificity of CYP80B1 from *P. somniferum* were similar to the *E. californica* enzyme. The pH optimum was 8.0, the temperature

[1] The cDNA sequence reported herein has been deposited in the GenBank database under the GenBank Accession Number AF134590 (cyp80b1).

optimum was ranged between 25 and 30°C, with a reproducible reduction in activity at 20°C. The K_m for the substrate (*S*)-*N*-methylcoclaurine was 20 μM. The values for the *E. californica* hydroxylase were pH 7.5, 35°C and 15 μM, respectively (Pauli & Kutchan, 1998). The enzymes from both species were found to hydroxylate only (*S*)-*N*-methylcoclaurine and could utilize NADPH, but not NADH as cofactor. This same hydroxylase from *P. somniferum*, however, showed an absolute requirement for the presence of a plant cytochrome P-450 reductase for functional heterologous expression. The cDNA encoding a cytochrome P-450 reductase from *E. californica* was co-expressed herein with *P. somniferum* hydroxylase in insect cell culture to produce active hydroxylase (Rosco, Pauli, Priesner & Kutchan, 1997). The hydroxylase from *E. californica* was active in insect cells in the absence of plant P-450 reductase (Pauli & Kutchan, 1998). Genomic DNA blot analysis indicates that there are two *cyp80b1* genes in the *P. somniferum* genome (Fig. 3).

```
          1                                                  50
PsoCYP80B1  ......SLVA VVITTFLYLI FRDSSPKGLP PGPKPWPIVG NLLQLGEK..
EcaCYP80B1  MEVVTVA-I- -I-SSI---L -GG-GH-N-- ---------- --------..
BstCYP80A1  .MDYIVGF-S ISLVAL--FL LFKPKHTN-- -S-PA----- H-PD-IS-NS

          51                                                 100
PsoCYP80B1  P..HSQFAQL AETYGDLFSL KLGSETVVVA STPLAASEIL KTHDRVLSGR
EcaCYP80B1  -..-A---E- -Q----I-T- -M-T------ --SS------ -----I--A-
BstCYP80A1  -PFLDYMSNI -QK--P-IH- -F-LHSSIF- --KE--M-V- Q-N-K-----

          101                                                150
PsoCYP80B1  YVFQSFRVKE HVENSIVWSE CNETWKKLRK VCRTDLFTQK MIESQAEVRE
EcaCYP80B1  ---------G ---------D -T----N--- ----E----- ------H---
BstCYP80A1  QPLPC--I-P -IDY--L--D S-SY---G-- ILH-EI-S-- -LQA-EKN--

          151                                                200
PsoCYP80B1  SKAMEMVEYL KKNVGNEVKI AEVVFGTLVN IFGNLIFSQN IFKLGDESSG
EcaCYP80B1  K-CE------ M-KQ-E---- V--I------ ---------- --E---PN--
BstCYP80A1  RV-GNL-NFI MTK--DV-EL RSWL--CAL- VL-HVV--KD V-EYS-.Q--

          201                                                250
PsoCYP80B1  SVEMKEHLWR MLELGNSTNP ADYFPFLGKF DLFGQSKDVA DCLQGIYSVW
EcaCYP80B1  -S-F--Y--- ---------- -----M---- -----R-E-- E--K---AI-
BstCYP80A1  E-G-DKLIHG --MT-GDFDV -S---V-AR- --H-LKRKMD EQFKLLIKI-

          251                                                300
PsoCYP80B1  GAMLKESKIA KQHNNSK.KN DFVEILLDSG LDDQQINALL MEIFGAGTET
EcaCYP80B1  ----Q-R-L- -KVDGY-S-- ---DVC---- -N-Y------ --L-------
BstCYP80A1  EGEV....L- RRA-RNPEPK -MLDV-IAND FNEH----MF --T--P-SD-

          301                                                350
PsoCYP80B1  SASTIEWALS ELTKNPQVTA NMRLELLSVV GKR.PVKESD IPNMPYLQAF
EcaCYP80B1  --------MT ------KI-- KI-S-IQT-- -E-.S----- F--L---E-T
BstCYP80A1  NSNI-----A Q-I---DKL- KL-E--DR-- -RSST----H FSEL-----C

          351                                                400
PsoCYP80B1  VKETLRLHPA TPLLLPRRAL ETCKVLNYTI PKECQIMVNA WGIGRDPKRW
EcaCYP80B1  ---------P ---------- ---TI----- --D------- --------T-
BstCYP80A1  ----M--Y-P ISIMI-H-CM ---QVMG--- --GMDVH--- HA------D-

          401                                                450
PsoCYP80B1  TDPLKFSPER FLNSSIDFKG NDFELIPFGA GRRICPGVPL ATQFISLIVS
EcaCYP80B1  ----T----- -----V--R- ---S------ -------L-I -N---A-L-A
BstCYP80A1  K-----Q--- --D-D-EYN- KQ-QF----S -------R-- -VRI-P-VLA

          451                                                493
PsoCYP80B1  SLVQNFDWGL PKGMDPSQLI MEEKFGLTLQ KEPPLYIVPK TRD
EcaCYP80B1  TF---L--C- -N--SVDH-- V--------- -----F---- S-V
BstCYP80A1  ---HA-G-E- -D-VPNEK-D ---L-T-S-C MAK--RVI-- V-I
```

Fig. 2. Amino acid sequence comparison of CYP80B1 from *P. somniferum* (PsoCYP80B1) and *E. californica* (*Eca*CYP80B1) (Pauli & Kutchan, 1998) and CYP80A1 (berbamunine synthase, BstCYP80A1) from *B. stolonifera* (Kraus & Kutchan, 1995). Only those amino acid residues that differ from CYP80B1 from *P. somniferum* are shown. Short stretches of similarity between EcaCYP80B1 and BstCYP80A1 facilitated the isolation of PsoCYP80B1.

2.2. Differential regulation of cyp80b1, bbe1 and cor1 during seedling development

In previous studies conducted on developing *Papaver* seedlings, sanguinarine and thebaine were found to increase in level during the days following germination (Rush et al., 1985; Wieczorek, Nagakura, Sund, Jendrzejewaki & Zenk, 1986; Facchini et al., 1996). We have again characterized our experimental variety of *P. somniferum* herein under our standard laboratory/greenhouse conditions in order to correlate alkaloid accumulation with biosynthetic gene expression using cDNAs that were not available at the time of these earlier publications. Sanguinarine is absent from germinating seedlings until day five after emergence of the radical (Fig. 4A). The concentration of sanguinarine continued to increase from approximately 0.1 pmole/mg fresh tissue weight to 0.65 pmole/mg fresh weight throughout the 20 day period investigated. In contrast, thebaine was already present on day two after germination at a 1500-fold higher concentration (150 pmole/mg fresh weight) than sanguinarine and its level remained high throughout the first 20 days after germination (Fig. 4B). These differences were reflected in the relative amounts of gene transcript detected by RNA gel blot analysis over the same time period of 2 to 20 days post germination (Fig. 4C). The transcript of *bbe1*, encoding the enzyme BBE that directs the central intermediate (*S*)-reticuline into the sanguinarine biosynthetic pathway, is almost absent at germination. This correlates well with the absence of sanguinarine in germinating seedlings. The transcript of *cor1*, specific to the morphine pathway, is present on day two after germination. This is in agreement with the

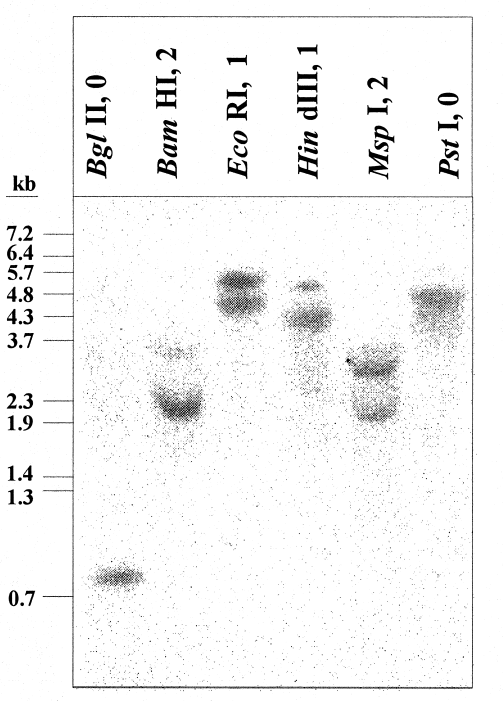

Fig. 3. Genomic DNA gel blot analysis. *P. somniferum* genomic DNA was hybridized to a nearly full length cDNA encoding CYP80B1. The numbers following the restriction endonuclease names indicate the number of recognition sites that occur within the reading frame.

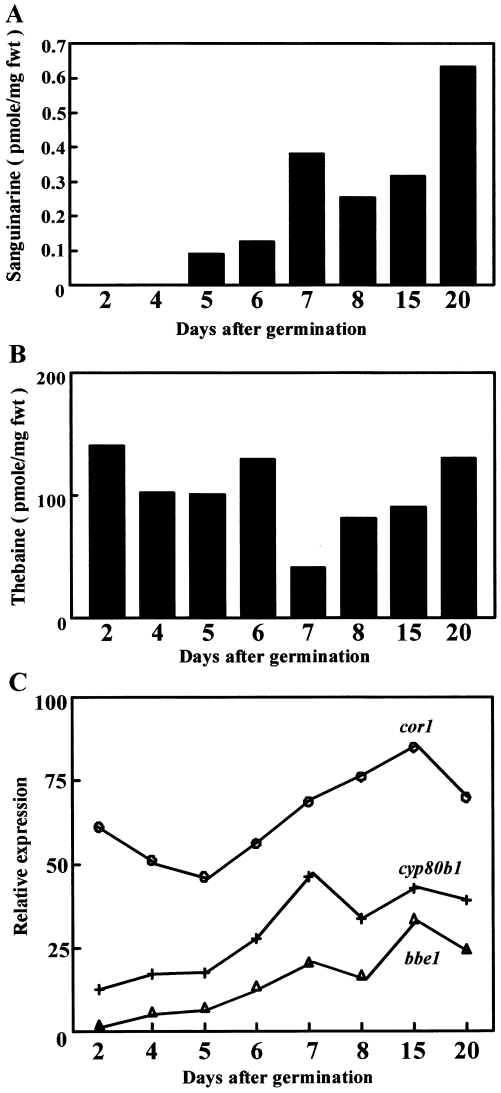

Fig. 4. Temporal accumulation of alkaloids and biosynthetic gene transcript in developing *P. somniferum* seedlings. (A) Graphic presentation of the results of HPLC analysis of the benzo[*c*]phenanthridine alkaloid sanguinarine and (B) of the morphine biosynthetic precursor thebaine from 2 to 20 days after germination. (C) Graphic presentation of relative transcript accumulation for the alkaloid biosynthetic genes *cyp80b1, bbe1* and *cor1* as determined by RNA gel blot analysis. RNA was isolated from developing seedlings on days 2, 4, 5, 6, 7, 8, 15 and 20 after germination. The blot was hybridized to cDNAs encoding *cyp80b1*, common to both sanguinarine and morphine biosynthesis, *bbe1*, which participates only in the formation of sanguinarine and *cor1*, involved only in morphine synthesis. All values were normalized to hybridization to actin transcript.

presence of the morphinan alkaloid thebaine at that time. The transcript of *cyp80b1*, which participates in both morphine and sanguinarine biosynthesis, is also detectable on day two after germination and increases in level following a pattern similar to *bbe1*.

2.3. Expression pattern of cyp80b1, bbe1 and cor1 in the mature plant

RNA gel blot analysis of gene transcript accumulation in various organs of the mature *P. somniferum* plant indicates that *cyp80b1* transcript is present predominantly in the root, the stem and the leaf, with higher levels in the leaf mid rib than the lamina (Fig. 5). It is present, however, at lower levels in the flower bud and the capsule. Similarly, *bbe1* transcript is predominant in the root, the stem and the leaf mid rib, with less in the leaf lamina, but absent in the flower bud and the capsule. We define leaf lamina here as a leaf tissue from which the mid rib has been removed. In contrast to *cyp80b1* and *bbe1*, *cor1* transcript is predominant in the capsule, the stem, the leaf mid rib, with less in the leaf lamina. Lower levels are present in the root and the flower bud. This correlates with the accumulation of morphine in reticulated laticifers associated with vascular cells.

2.4. Preferential induction of cyp80b1 and bbe1 in cell suspension culture

There are no reproducible reports in the literature of cell suspension cultures of *P. somniferum* that produce morphine. It has, however, been well established that upon elicitation, *P. somniferum* cell suspension cultures produce sanguinarine rather than morphine (Eilert, Kurz & Constabel, 1985). Likewise, cell suspension cultures of *P. bracteatum* respond to elicitor addition with production of sanguinarine rather than thebaine (Cline & Coscia, 1988). This response to elicitor addition with the accumulation of benzo[*c*]phenanthridine rather than morphinan alkaloid accumulation is reflected in an RNA gel blot analysis of the cell suspension culture elicited by the addition of 100 μM methyl jasmonate to the culture medium (Fig. 6). Transcripts of *cyp80b1* and *bbe1*, both essential to sanguinarine biosynthesis, rapidly increase in levels after elicitor addition, reaching initial maxima 6 h after elicitation. The levels of both transcripts decrease over the following 6 h, then begin to increase again up until at least 24 h. This is a pattern that is similar to results obtained with other systems (Dittrich & Kutchan, 1991; Kutchan & Zenk, 1993). In contrast, *cor1* transcript maintains a relatively constant level throughout the 24 h period investigated.

2.5. Expression of cyp80b1, bbe1 and cor1 in various Papaver species

Many species of *Papaver* produce tetrahydrobenzylisoquinoline- derived alkaloids. *P. rhoeas* contains alkaloids such as rhoeadine, *P. orientale* accumulates oripavine and *P. bracteatum* produces thebaine, but none of these species accumulate morphine (Southon & Buckingham, 1989). All of these species should contain CYP80B1 necessary for synthesis of the central

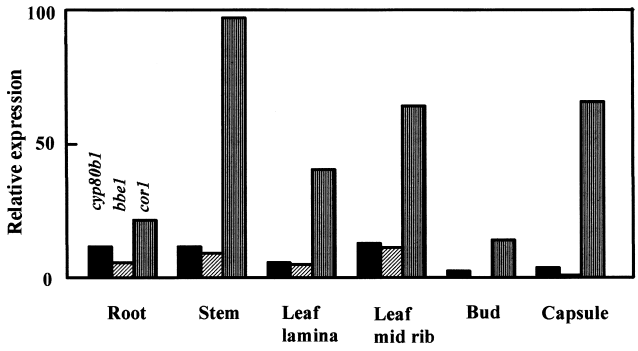

Fig. 5. Spatial distribution of alkaloid biosynthetic gene transcript in the mature *P. somniferum* plant. Graphic presentation of relative transcript accumulation for the alkaloid biosynthetic genes *cyp80b1, bbe1* and *cor1* as determined by RNA gel blot analysis in various organs of the mature *P. somniferum* plant. All values were normalized to hybridization to actin transcript. *cyp80b1* is common to both sanguinarine and morphine biosynthesis, while *bbe1* participates only in the formation of sanguinarine and *cor1* only in morphine synthesis.

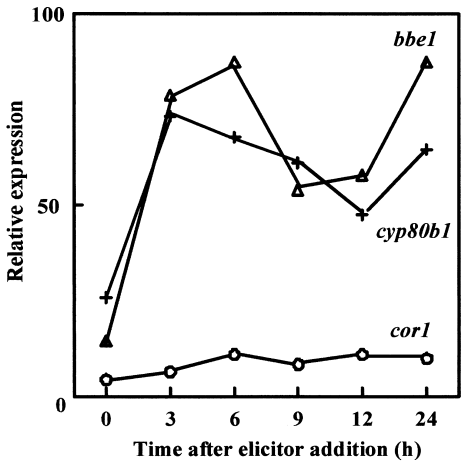

Fig. 6. Response of alkaloid biosynthetic gene transcript accumulation to elicitation in cell suspension cultures of *P. somniferum*. Graphic representation of an RNA gel blot prepared from methyl jasmonate-induced cell suspension cultures. RNA was isolated at 0, 3, 6, 9, 12 and 24 h after addition of elicitor to the culture medium. The blot was hybridized to three alkaloid biosynthetic cDNAs *cyp80b1, bbe1* and *cor1*. All values were normalized to hybridization to actin transcript. *cyp80b1* is common to both sanguinarine and morphine biosynthesis, while *bbe1* participates only in the formation of sanguinarine and *cor1* only in morphine synthesis.

tetrahydrobenzylisoquinoline alkaloid intermediate (*S*)-reticuline, those that accumulate (*S*)-scoulerine-derived alkaloids should also have BBE and only *P. somniferum* should express *cor1*. Leaf RNA gel blot analysis of these three transcripts in four *Papaver* species indicated that, as expected, *cyp80b1* transcript is present in *P. rhoeas*, *P. orientale, P. bracteatum* and *P. somniferum*, with the highest relative amount in *P. somniferum* (Fig. 7). Transcript of *bbe1* was also detectable in all four *Papaver* species, with the highest relative levels in *P. orientale* and *P. somniferum*. The morphine biosynthesis-specific transcript *cor1* was surprisingly present in all four species. The highest relative amounts of *cor1* transcript were present in *P. bracteatum* and *P. somniferum*. The high stringency wash conditions (65°C) that were used in the northern analysis should exclude cross-hybridization with other members of the gene family, such as *cor2* (Unterlinner et al., 1999).

3. Discussion

We examined herein the expression pattern of three genes of alkaloid biosynthesis in the opium poppy *P. somniferum*. These genes participate in the biosynthesis of two alkaloids representative of two different classes of isoquinoline alkaloids, the benzo[*c*]phenanthridine sanguinarine and the morphinan morphine. The gene *bbe1* is specific to sanguinarine synthesis and *cor1* is specific to morphine synthesis. Both alkaloids arise from a bifurcation in the central tetrahydrobenzylisoquinoline pathway after (*S*)-reticuline (Fig. 1). The gene *cyp80b1* participates in (*S*)-reticuline biosynthesis, occurring before the pathway bifurcation and is, therefore, common to both sanguinarine and morphine biosynthesis.

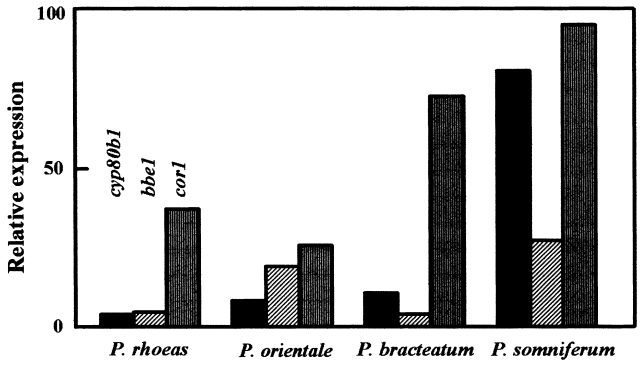

Fig. 7. Distribution of alkaloid biosynthetic gene transcript in various *Papaver* species. Graphic representation of an RNA gel blot prepared from mature leaf material from *P. rhoeas, P. orientale, P. bracteatum* and *P. somniferum*. The blot was hybridized to three alkaloid biosynthetic cDNAs *cyp80b1, bbe1* and *cor1*. Transcript for *cyp80b1* should be present in all species that produce tetrahydrobenzylisoquinoline-derived alkaloids. Transcript for *bbe1* should be present in all species that produce (*S*)- scoulerine-derived alkaloids. Transcript for *cor1* should be present only in species that synthesize morphine.

In our previous work, we established that benzo[*c*]-phenanthridines such as sanguinarine accumulate during development of *P. bracteatum* seedlings (Rush et al., 1985). We have herein analyzed developing seedlings of *P. somniferum* for sanguinarine accumulation and have correlated it to the accumulation of transcripts of the two biosynthetic genes *cyp80b1* and *bbe1*. Our results for the continuous accumulation of *bbe1* through the first 15 days after germination are at variance with those reported by Facchini et al. (1996). In that study, *bbe* transcript was detected only transiently on day 3 and day 6 after imbibition. The results reported herein demonstrate that *cyp80b1* and *bbe1* transcript accumulation parallels that of sanguinarine accumulation.

Thebaine levels in germinating seedling were high compared to those of sanguinarine. Even at the highest level measured for sanguinarine on day 20, the thebaine level was 200-fold higher. *Cor1* transcript was present at all time intervals analyzed even though morphine was below HPLC detection limits in seedlings during the first twenty days after germination. Morphine has been shown by radioimmunoassay, however, to begin to accumulate in developing seedlings seven days after germination (Wieczorek et al., 1986). As observed for *cor1* transcript, COR enzyme activity is also present in seedlings at relatively constant levels throughout development, and cannot therefore be the rate limiting enzyme of morphine biosynthesis (Unterlinner et al., 1999).

The site of morphine biosynthesis in *P. somniferum* is a long- standing question. Transcript of *cor1* was detected herein in each plant organ analyzed, with the highest relative amounts in the stem, the leaf mid rib and the capsule consistent with the latex rich aerial organs. This correlates, in part, to the in situ hybridization results with cDNAs encoding TYDC, an enzyme that occurs at a very early step in (*S*)-reticuline biosynthesis (Facchini & De Luca, 1995). Transcripts encoding *tydc* were found associated with vascular tissue in mature roots and stems. Transcript accumulation appeared restricted to the metaphloem and the protoxylem in the vascular bundles of aerial organs. Transcripts for *tydc1* and *tydc2* were not detected in the capsule, but for another allele, *tydc5*, the transcript was detected by RNA gel blot analysis to 1.2% of that level found in the root (Maldonado-Mendoza et al., 1996).

Transcript of *cyp80b1* was detected in all plant organs analyzed, with the highest levels in the root, the stem and the leaf mid rib, but also detectable in the flower bud and the capsule. Transcript of *bbe1* was

detected at approximately equal levels in the root, the stem and the leaf mid rib, but not in the flower bud or the capsule. This is at variance with previously reported results where *bbe1* was not detected in the leaf (Facchini et al., 1996). Under field conditions, *P. somniferum* synthesizes sanguinarine in leaf (Fisinger & Zenk, unpublished), which is consistent with the *bbe1* transcript distribution that we found.

It is not clear why cell cultures of *P. somniferum* cannot produce morphine. All of the known enzymes of morphine biosynthesis have been detected in cell suspension culture (reviewed in Kutchan, 1998). Accumulation of morphinan alkaloids is thought to correlate to the appearance of laticifer cells in the developing plant and in differentiating plant cell culture (Rush et al., 1985; Kutchan et al., 1985). As for COR enzyme activity (Lenz & Zenk, 1995), *cor1* transcript is found in cell suspension cultures of *P. somniferum*, but does not increase in response to addition of the elicitor substance methyl jasmonate to the culture medium. This supports the result that morphine accumulation is not elicitor inducible, but does not explain the complete lack of morphine in cell culture.

Consistent with the induction of sanguinarine accumulation in culture (Eilert et al., 1985; Cline & Coscia, 1988; Facchini et al., 1996), transcripts for the sanguinarine biosynthetic genes *cyp80b1* and *bbe1* are induced. The time course that we observe is consistent with that reported for the TYDC allele *tydc2* (Facchini et al., 1996).

With morphine biosynthetic genes now in hand, we believe that we can begin to address the question why only *P. somniferum* produces morphine, while other *Papaver* species such as *P. rhoeas, P. orientale* and *P. bracteatum* do not. It was expected that all four *Papaver* species investigated herein by RNA gel blot analysis would yield positive results for hybridization to *cyp80b1*. Each of the species *P. rhoeas, P. orientale, P. bracteatum* and *P. somniferum* synthesize tetrahydrobenzylisoquinoline-derived alkaloids. The gene *cyp80b1* must, therefore, be expressed in each plant. Our results are consistent with this hypothesis. Likewise, *bbe1* transcript accumulation was detected in each species. Alkaloids in which BBE lies along the biosynthetic pathway also occur in each species. For example, norsanguinarine in *P. rhoeas, P. orientale* and *P. bracteatum* and sanguinarine in *P. somniferum* (Southon & Buckingham, 1989). Unexpectedly, *cor1* transcript was present to some degree in all four species. A review of the literature revealed no alkaloids reported in *P. rhoeas* for which COR should participate in the synthesis. Similarly, *P. orientale* accumulates the alternate morphine biosynthetic precursor oripavine, but COR is not involved in the biosynthesis of oripavine, acting rather after this alkaloid along the biosynthetic pathway to morphine (Brochmann-Hanssen, 1984). *P. bracteatum* produces the morphine precursor thebaine as a major alkaloid. As for oripavine in *P. orientale*, COR would act after thebaine formation on the pathway to morphine. *P. bracteatum* has been reported to contain neopine as a minor alkaloid (Southon & Buckingham, 1989), but COR has not yet been demonstrated to reduce neopinone directly to neopine. From the results of our northern analysis, it appears that the fact that *P. rhoeas, P. orientale* and *P. bracteatum* do not produce morphine is not related to the absence of transcript of the morphine biosynthesis-specific gene *cor1*. The expression of *cor1* could simply be an evolutionary remnant in these species.

A fascinating but still unanswered question in biology concerns the origin of species-specific secondary metabolite accumulation in plants. As we identify more genes from alkaloid biosynthetic pathways such as those for morphine, we should be able to analyze a broad range of taxonomically related species and provide insight into whether specialization is a result of loss of gene expression or rather a consequence of the appearance of new genes in response to selection pressure.

4. Experimental

4.1. General experimental procedures

Total RNA was isolated and RNA gels were run and blotted on nylon membranes as previously described (Pauli & Kutchan, 1998). The same blots were washed and re-used for hybridization to all cDNAs. All blots were normalized to hybridize to actin transcript to account for differences in gel loading and blotting. Genomic DNA was isolated and DNA gels were run and blotted according to (Bracher & Kutchan, 1992). cDNA clones were labeled by random-primed labeling with [α-^{32}P]dCTP and oligodeoxynucleotides were end-labeled with [γ-^{32}P]ATP. Hybridized RNA on northern blots and DNA on Southern blots were visualized with a Raytest BAS-1500 phosphorimager. The entire nucleotide sequence on both DNA strands of full-length cDNA clones in pGEM-T was determined by dideoxy cycle sequencing using internal DNA sequences for the design of deoxyoligonucleotides as sequencing primers. Results of enzyme assays are the means of at least two separate experiments performed in duplicate.

4.2. Plant material

Cell suspension cultures of the opium poppy *Papaver somniferum* L. were provided by the cell culture laboratory of the Lehrstuhl für Pharmazeutische Biologie, Universität München, Germany. Cultures were

routinely grown in either 1 l conical flasks containing 400 ml of Linsmaier–Skoog medium (Linsmaier & Skoog, 1965) over 7 days at 23°C on a gyratory shaker (100 rpm) in diffuse light (750 lux). Differentiated opium poppy plants were grown outdoors in Upper Bavaria. Seedlings were grown on a substrate from 7 to 56 days in a greenhouse at 20°C, 65% relative humidity and continuous light. Elicitation of *P. somniferum* cell suspension cultures was achieved by aseptic addition of methyl jasmonate to a final concentration of 100 μM to the culture medium (Gundlach, Müller, Kutchan & Zenk, 1992).

4.3. Isolation and characterization of clones encoding CYP80B1 and BBE

Partial cDNAs encoding cytochromes P-450 from *P. somniferum* were generated by PCR using cDNA produced by reverse transcription of total RNA isolated from the stem material of mature plants. DNA amplification was performed under the following conditions: Primer sequences, 5′-CAA ATC AAT GCC/A T/ATG T/CTT/C ATG GAA-3′ and 5′-CCT TCT T/GCC TGC/A ACC AAA TGG TAT-3′; cycle, 2 min 94°C, 35 cycles of 94°C, 45 sec; 50°C, 45 sec; 72°C, 2 min; then 72°C, 10 min. At the end of 35 cycles, the reaction mixture was cooled to 4°C. The amplified DNA was then resolved by agarose gel electrophoresis, the band of approximately the correct size (400 bp) was isolated and subcloned into pGEM-T (Promega) prior to nucleotide sequence determination. A partial clone that was similar in nucleotide sequence to *cyp80b1* from *E. californica* was used to screen a cDNA library in λ-ZAPII (Stratagene) prepared from RNA isolated from *P. somniferum* leaf tissue from mature plants. A near full-length cDNA, lacking the first 17 nucleotides of the reading frame, but putatively encoding CYP80B1 was thus isolated. After determination of the nucleotide sequence on both strands, the full length reading frame, free of the 5′- and 3′-flanking sequences, was generated by PCR. Six codons were added to the sense primer to replace the six missing amino acids M E V V T V (as determined from the sequence of *E. californica* CYP80B1) so that the cDNA could be functionally expressed in insect cell culture as described in (Pauli & Kutchan, 1998).

DNA encoding *bbe1* was generated by PCR using genomic DNA isolated from cell suspension culture. The gene *bbe1* contains no introns and could therefore be used to generate DNA suitable for functional heterologous expression (Facchini et al., 1996; Hauschild et al., 1998). DNA amplification was performed under the following conditions: primer sequences, 5′-GGATCC ATG ATG TGC AGA AGC TTA-3′ and 5′-GAATTC CTA CAA TTC CTT CAA CAT-3′ using the same amplification conditions as for *cyp80b1* given above. The amplified DNA was then resolved by agarose gel electrophoresis, the band of approximately the correct size (1600 bp) was isolated and subcloned into pGEM-T prior to nucleotide sequence determination. The *Bam*HI/*Eco*RI restriction fragment from pGEM-T/BBE1 was ligated into *Bam*HI/*Eco*RI digested pFastBac1 (Life Technologies, Eggenstein). pFastBac/BBE1 was transposed into baculovirus DNA in the *Escherichia coli* strain DH10BAC (Life Technologies, Eggenstein) 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 the procedure followed by Kutchan, Bock & Dittrich (1994) and Kraus & Kutchan (1995). Identity of the clone as that encoding BBE was ascertained by the presence of enzyme activity measured as done by Kutchan et al. (1994).

4.4. Alkaloid analysis

Morphinan and benzo[*c*]phenanthridine alkaloids were extracted and quantitated exactly as described by Blechert et al. (1997). Individual alkaloids were analyzed by HPLC using the following gradient: [column, Knauer Vertex Eurospher 100-C18, 5 μm (4 × 250 mm); solvent system, (A) 97.99% (v/v) H_2O, 2% CH_3CN, 0.01% (v/v) H_3PO_4, (B) 1.99% (v/v) H_2O, 98% CH_3CN, 0.01% H_3PO_4; gradient for morphinans: 0–25 min 0–46% B, 25–26 min 46–100% B, 26–30 min 100–0% B, 30–32 min 0% B; gradient for benzo[*c*]phenanthridines: 0–5 min 25–40% B, 5–20 min 40–70% B, 20–25 min 70–100% B, 25–27 min 100% B, 27–30 min 100–25% B; flow 1 ml/min] with detection at 284 nm using authentic alkaloids as reference materials.The distribution of RNA transcription of several genes encoding morphine and sanguinarine biosynthetic enzymes in *P. somniferum* have been analyzed.

Acknowledgements

This work was supported by SFB 369 of the Deutsche Forschungsgemeinschaft, Bonn and Fonds der Chemischen Industrie, Frankfurt.

References

Blechert, S., Bockelmann, C., Brümmer, O., Füßlein, M., Gundlach, H., Haider, G., Hölder, S., Kutchan, T. M., Weiler, E. W., & Zenk, M. H. (1997). Structural separation of biological activities of jasmonates and related compounds. *J. Chem. Soc. Perkin. Trans*, *1*, 3549–3560.

Bracher, D., & Kutchan, T. M. (1992). Strictosidine synthase from

Rauvolfia serpentina: Analysis of a gene involved in indole alkaloid biosynthesis. *Arch. Biochem. Biophys*, *294*, 717–723.

Brochmann-Hanssen, E. (1984). A second pathway for the terminal steps in the biosynthesis of morphine. *Planta Med*, *50*, 343–345.

Cline, S. D., & Coscia, C. J. (1988). Stimulation of sanguinarine production by combined fungal elicitation and hormonal deprivation in cell suspension cultures of *Papaver bracteatum*. *Plant Physiol*, *86*, 161–165.

Dittrich, H., & Kutchan, T. M. (1991). Molecular cloning, expression and induction of berberine bridge enzyme, an enzyme essential to the formation of benzophenanthridine alkaloids in the response of plants to pathogenic attack. *Proc. Natl. Acad. Sci. USA*, *88*, 9969–9973.

Dzink, J.L., & Socransky, S.S. (1985). Comparative in vitro activity of sanguinarine against oral microbial isolates. *Antimicrobial Agents and Chemotherapie*, 663-665.

Eilert, U., Kurz, W. G. W., & Constabel, F. (1985). Stimulation of sanguinarine accumulation in *Papaver somniferum* cell cultures by fungal elicitors. *J. Plant Physiol*, *119*, 65–76.

Facchini, P. J., & De Luca, V. (1994). Differential and tissue-specific expression of a gene family for tyrosine/dopa decarboxylase in opium poppy. *J. Biol. Chem*, *269*, 26684–26690.

Facchini, P. J., & De Luca, V. (1995). Phloem-specific expression of tyrosine/dopa decarboxylase genes and the biosynthesis of isoquinoline alkaloids in opium poppy. *Plant Cell*, *7*, 1811–1821.

Facchini, P. J., Penzes, C., Johnson, A. G., & Bull, D. (1996). Molecular characterization of berberine bridge enzyme genes from opium poppy. *Plant Physiol*, *112*, 1669–1677.

Gundlach, H., Müller, M. J., Kutchan, T. M., & Zenk, M. H. (1992). Jasmonic acid is a signal transducer in elicitor-induced plant cell cultures. *Proc. Natl. Acad. Sci. USA*, *89*, 2389–2393.

Hauschild, K., Pauli, H. H., & Kutchan, T. M. (1998). Isolation and analysis of a gene *bbe1* encoding the berberine bridge enzyme from the California poppy *Eschscholzia californica*. *Plant Mol. Biol*, *36*, 473–478.

Kraus, P. F. X., & Kutchan, T. M. (1995). Molecular cloning and heterologous expression of a cDNA encoding berbamunine synthase, a C-O phenol-coupling cytochrome P-450 from the higher plant *Berberis stolonifera*. *Proc. Natl. Acad. Sci. USA*, *92*, 2071–2075.

Kutchan, T. M., Ayabe, S., & Coscia, C. J. (1985). Cytodifferentiation and Papaver alkaloid accumulation. In J. D. Phillipson, M. F. Roberts, & M. H. Zenk, *The chemistry and biology of isoquinoline alkaloids* (pp. 281–294). Berlin: Springer-Verlag.

Kutchan, T. M., Rush, M. D., & Coscia, C. J. (1986). Subcellular localization of alkaloids and dopamine in different vacuolar compartments of *Papaver bracteatum*. *Plant Physiol*, *81*, 161–166.

Kutchan, T. M. (1993). 12-Oxo-phytodienoic acid induces accumulation of berberine bridge enzyme transcript in a manner analogous to methyl jasmonate. *J. Plant Physiol*, *142*, 502–505.

Kutchan, T. M., & Zenk, M. H. (1993). Enzymology and molecular biology of benzophenanthridine alkaloid biosynthesis. *J. Plant Res*, *Special Issue 3*, 165–173.

Kutchan, T. M., Bock, A., & Dittrich, H. (1994). Heterologous expression of strictosidine synthase and berberine bridge enzyme in insect cell culture. *Phytochemistry*, *35*, 353–360.

Kutchan, T. M. (1998). Molecular genetics of plant alkaloid biosynthesis. In G. Cordell, *The alkaloids*, *vol. 50* (pp. 257–316). San Diego: Academic Press.

Linsmaier, E. M., & Skoog, F. (1965). Organic growth factor requirements of tobacco tissue cultures. *Physiol. Plant*, *18*, 100–127.

Lenz, R., & Zenk, M. H. (1995). Purification and properties of codeinone reductase (NADPH) from *Papaver somniferum* cell cultures and differentiated plants. *Eur. J. Biochem*, *233*, 132–139.

Maldonado-Mendoza, I. E., López-Meyer, M., Galef, J. R., Burnett, R. J., & Nessler, C. L. (1996). Molecular analysis of a new member of the opium poppy tyrosine/3,4-dihydroxyphenylalanine decarboxylase gene family. *Plant Physiol*, *110*, 43–49.

Nessler, C. L., & Mahlberg, P. G. (1977). Ontogeny and cytochemistry of alkaloidal vesicles in laticifers of *Papaver somniferum* L. (Papaveraceae). *Amer. J. Bot*, *64*, 541–551.

Nessler, C. L., & Mahlberg, P. G. (1978). Laticifer ultrastructure and differentiation in seedlings of *Papaver bracteatum* L., Population Arya II (Papaveraceae). *Amer. J. Bot*, *65*, 978–983.

Pauli, H. H., & Kutchan, T. M. (1998). Molecular cloning and functional heterologous expression of two alleles encoding (*S*)-*N*-methylcoclaurine 3'-hydroxylase (CYP80B1), a new methyl jasmonate-inducible cytochrome P-450 dependent monooxygenase of benzylisoquinoline alkaloid biosynthesis. *Plant J*, *13*, 793–801.

Roberts, M. F., McCarthy, D., Kutchan, T. M., & Coscia, C. J. (1983). Localization of enzymes and alkaloidal metabolites in *Papaver* latex. *Arch. Biochem. Biophys*, *222*, 599–609.

Rosco, A., Pauli, H. H., Priesner, W., & Kutchan, T. M. (1997). Cloning and heterologous expression of cytochrome P450 reductases from the Papaveraceae. *Arch. Biochem. Biophys*, *348*, 369–377.

Rush, M. D., Kutchan, T. M., & Coscia, C. J. (1985). Correlation of the appearance of morphinan alkaloids and laticifer cells in germinating *Papaver bracteatum* seedlings. *Plant Cell Rep*, *4*, 237–240.

Sertürner, F. W. A. F. (1806). Darstellung der reinen Mohnsäure (Opiumsäure) nebst einer chemischen Untersuchung des Opiums mit vorzüglicher Hinsicht auf einen darin neu entdeckten Stoff und die dahin gehörigen Bemerckungen. *J. Pharm. Ärzte Apoth. Chem*, *14*(1), 47–93.

Southon, I. W., & Buckingham, J. (1989). *Dictionary of alkaloids*. New York: Chapman and Hall.

Unterlinner, B., Lenz, R., & Kutchan, T. M. (1999). Molecular cloning and functional expression of codeinone reductase — the penultimate enzyme in morphine biosynthesis in the opium poppy *Papaver somniferum*. *Plant J*, *18*, 465–475.

Wieczorek, U., Nagakura, N., Sund, C., Jendrzejewski, S., & Zenk, M. H. (1986). Radioimmunoassay determination of six opium alkaloids and its application to plant screening. *Phytochemistry*, *25*, 2639–2646.