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Phytochemistry 64 (2003) 177–186
PHYTOCHEMISTRY
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Developmental and inducible accumulation of gene transcripts involved in alkaloid biosynthesis in opium poppy

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Received 16 January 2003; received in revised form 26 March 2003

Dedicated to the memory of Professor Jeffrey B. Harborne

Abstract

Opium poppy (*Papaver somniferum*) produces a large number of benzylisoquinoline alkaloids, including morphine and sanguinarine, derived from tyrosine via the branch-point intermediate (*S*)-reticuline. Molecular clones for the three methlytransferases involved in (*S*)-reticuline biosynthesis, (*S*)-norcoclaurine-6-*O*-methyltransferase (6OMT), (*S*)-3′-hydroxy-*N*-methylcoclaurine-4′-*O*-methyltransferase (4′OMT), and (*S*)-coclaurine *N*-methyltransferase (CNMT), were isolated from opium poppy and shown to share extensive homology with the corresponding cDNAs from Japanese goldthread (*Coptis japonica*). These cDNAs were used together with previously isolated clones for tyrosine/dopa decarboxylase (TYDC), (*S*)-*N*-methylcoclaurine-3′-hydroxylase (CYP80B1), berberine bridge enzyme, (BBE), (*7S*)-salutaridinol 7-*O*-acetyltransferase (SAT), and codeinone reductase (COR), to compare the accumulation of gene transcripts encoding eight alkaloid biosynthetic enzymes in opium poppy. Transcript levels generally increased in developing seedlings and were consistently high in stems and flower buds, but were more variable in roots and leaves of mature plants. The accumulation of each transcript, with the exception of COR, showed a marked induction in response to elicitor treatment or wounding of cultured cells. Specific gene transcript levels often correlated with the accumulation of morphine or sanguinarine, with notable exceptions. Our data suggest some degree of coordination in the developmental and inducible regulation of alkaloid biosynthetic genes in opium poppy.

Keywords: Benzylisoquinoline alkaloid; Developmental gene expression; Elicitor-induced gene expression; Methyltransferase; Opium poppy; *Papaver somniferum*; Papaveraceae; Wound-induced gene expession

1. Introduction

Benzylisoquinoline alkaloids are a large and diverse group of natural products consisting of more than 2500 defined structures found mainly in five plant families, including the Papaveraceae (Facchini, 2001). Opium poppy (*Papaver somniferum*; Papaveraceae) produces more than 100 different benzylisoquinoline alkaloids, several of which are pharmaceutically important including the analgesics morphine **1** and codeine **2**, the muscle relaxant papaverine, the anti-tumorigenic drug noscapine, and the antimicrobial agent sanguinarine **3**. The cultivation of opium poppy for the commercial production of morphine **1** and codeine **2** is an important part of the agricultural landscape in many parts of the world. However, the illicit production of morphine **1** for the synthesis of heroin via a simple *O,O*-diacetylation reaction accounts for almost 90% of the global output of opium poppy.

Benzylisoquinoline alkaloid biosynthesis begins with the conversion of L-tyrosine **4** to dopamine **5** and 4-hydroxyphenylacetaldehyde **6** by a lattice of decarboxylation, *ortho*-hydroxylation, and deamination reactions (Fig. 1). Tyrosine/dopa decarboxylase (TYDC) catalyzes the conversion of L-tyrosine **4** and L-dopa **7** to tyramine **8** and dopamine **5**, respectively. Dopamine **5** and 4-hydroxyphenylacetaldehyde **6** condense to form (*S*)-norcoclaurine **9**, the central precursor to all benzylisoquinoline alkaloids in plants (Facchini, 2001; Samanani and Facchini, 2001). (*S*)-Coclaurine **10** is produced from (*S*)-norcoclaurine **9** by a 6-*O*-methyltransferase (6OMT; Morishige et al., 2000), and subsequently converted to (*S*)-*N*-methylcoclaurine **11** by a *N*-methyltransferase (CNMT; Choi et al., 2002). A P450 hydroxylase (CYP80B1; Pauli and Kutchan, 1998) converts (*S*)-*N*-methylcoclaurine to (*S*)-3′-hydroxy-*N*-methylcoclaurine **12**, from which (*S*)-reticuline **13** is

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doi:10.1016/S0031-9422(03)00292-9

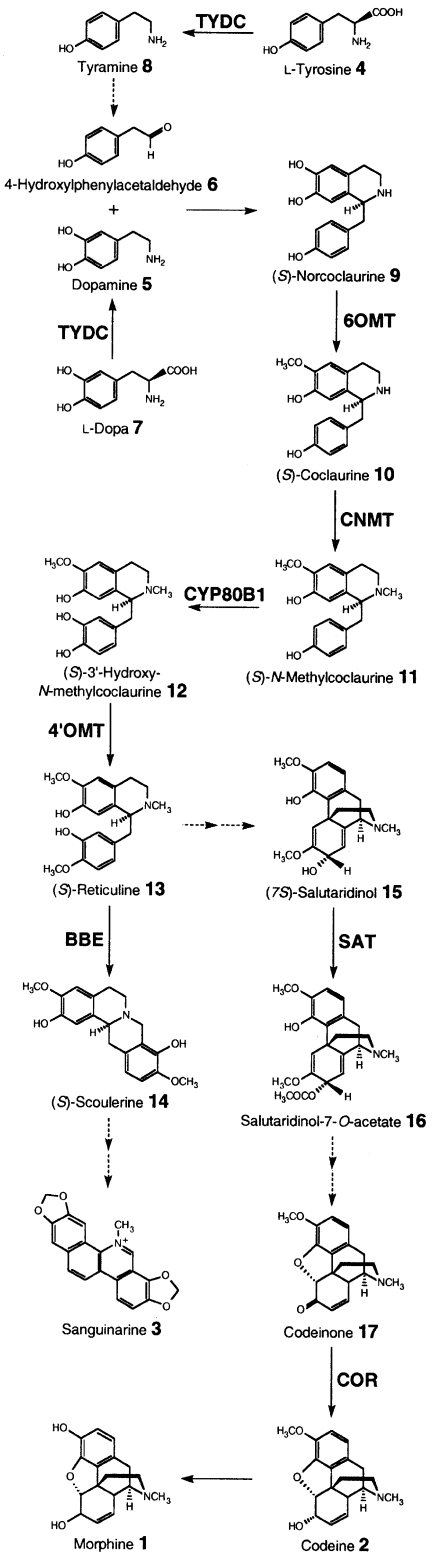

Fig. 1. Biosynthetic pathway leading to sanguinarine **3** and morphine **3** showing enzymes for which corresponding opium poppy cDNAs have been isolated. Enzyme abbreviations: TYDC, tyrosine/dopa decarboxylase; 6OMT, (*S*)- norcoclaurine-6-*O*-methyltransferase; CNMT, (*S*)- coclaurine *N*-methyltransferase; CYP80B1, (*S*)-*N*-methylcoclaurine-3′-hydroxylase; 4′OMT, 3′-hydroxy-(*S*)-*N*-methylcoclaurine-4′-*O*-methyltransferase; BBE, berberine bridge enzyme; SAT, (7S)-salutaridinol 7-*O*-acetyltransferase; COR, codeinone reductase.

formed via a 4′-*O*-methyltransferase (4′OMT; Morishige et al., 2000). (*S*)-Reticuline **13** is the key branch-point intermediate in the formation of morphine **1** and sanguinarine **3** (Fig. 1). The first committed step in sanguinarine biosynthesis is the conversion of (*S*)-reticuline **13** to (*S*)-scoulerine **14** by the berberine bridge enzyme (BBE; Facchini et al., 1996a). (*S*)-Scoulerine **14** is then converted to (*S*)-stylopine by two P450-dependent oxidases resulting in the formation of two methylenedioxy groups (Bauer and Zenk, 1989,1991). Sanguinarine (**3**) is produced from (*S*)-stylopine by the successive action of an *N*-methyltransferase (Rueffer et al., 1990), two additional P450-dependent enzymes, a spontaneous intramolecular rearrangement (Rueffer and Zenk, 1987; Tanahashi and Zenk, 1990), and an oxidase (Arakawa et al., 1992). In contrast, the conversion of (*S*)-reticuline **13** to (*R*)-reticuline (De-Eknamkul and Zenk, 1992) followed by intramolecular carbon–carbon phenol coupling to form salutaridine (Gerardy and Zenk, 1993a), are the first steps in morphine **1** biosynthesis (Fig. 1). Salutaridine is reduced to (7*S*)-salutaridinol **15** (Gerardy and Zenk, 1993b), which is converted to salutaridinol-7-*O*-acetate **16** by (7*S*)-salutaridinol-7-*O*-acetyltransferase (SAT) via closure of an oxide bridge (Grothe et al., 2001). In the final steps, codeinone **17** is produced from salutaridinol-7-*O*-acetate **16** and reduced to codeine **2** by codeinone reductase (COR; Unterlinner et al., 1999), which is demethylated to yield morphine **1**.

Several cDNAs or genes encoding enzymes involved in benzylisoquinoline alkaloid biosynthesis have been isolated from opium poppy including TYDC (Facchini and De Luca, 1994), CYP80B1 (Huang and Kutchan, 2000), BBE (Facchini et al., 1996a), SAT (Grothe et al., 2001) and COR (Unterlinner et al., 1999). In addition, cDNAs encoding 6OMT, 4′OMT (Morishige et al., 2000) and CNMT (Choi et al., 2002) have been isolated from Japanese goldthread (*Coptis japonica*; Ranunculaceae). This growing collection of molecular tools creates an unprecedented opportunity to study the regulation of benzylisoquinoline alkaloid pathways.

Alkaloid biosynthesis is controlled by both developmental and inducible factors in opium poppy (Facchini, 2001). Although the specific cell types involved in alkaloid production are not known, many alkaloids accumulate in large vesicles contained in the articulated laticifers that accompany vascular tissues throughout the plant. Some alkaloids, such as morphine **1**, are found in all plant organs, whereas others, such as sanguinarine (**3**), are more abundant in roots. These alkaloids are also found in developing seedlings immediately after seed germination (Facchini et al., 1996a; Huang and Kutchan, 2000). Opium poppy cell cultures also produce sanguinarine, but not morphine **1**, in response to treatment with certain fungal elicitors (Facchini et al., 1996b). Gene transcripts encoding some biosynthetic enzymes have been linked to the spatial accumulation of

specific alkaloids in the plant, with some notable exceptions (Facchini and De Luca, 1994, 1995; Facchini et al., 1996a; Huang and Kutchan, 2000). Similarly, TYDC, BBE, and CYP80B1 (Facchini et al., 1996a,b; Park et al., 1999; Huang and Kutchan, 2000) display transcriptional induction in response to elicitor treatment or wounding of opium poppy cell cultures in a manner temporally consistent with the accumulation of sanguinarine (**3**).

In this paper, we report the isolation of molecular clones encoding 6OMT, CNMT, and 4′OMT from opium poppy. These cDNAs are used together with previously isolated clones for TYDC, CYP80B1, BBE, SAT, and COR to show the comparative developmental and inducible accumulation of multiple mRNAs involved in benzylisoquinoline alkaloid biosynthesis in opium poppy plants and cell cultures. This study represents the most extensive survey to date of alkaloid biosynthetic gene transcript accumulation in opium poppy.

2. Results and discussion

2.1. Isolation and characterization of 6OMT, CNMT, and 4′OMT from opium poppy

Full-length cDNAs for 6OMT, CNMT, and 4′OMT from *C. japonica* (Morishige et al., 2000; Choi, 2002) were used to screen an opium poppy cDNA library at moderately high stringency. Screening with Cj6OMT resulted in the isolation of one full-length cDNA (Ps6OMT) and several partial clones representing the same gene. The CjCNMT probe also led to the isolation of a single full-length cDNA (PsCNMT). In contrast, screening with Cj4′OMT produced two different full-length cDNAs (Ps4′OMT1 and Ps4′OMT2) and a number of partial clones representing the same two genes.

The 1176-bp Ps6OMT cDNA contained an open reading frame of 1038 bp encoding a predicted translation product of 346 amino acids with a molecular mass of 38.5 kDa. The 1,363-bp Ps4′OMT1 and 1,342-bp Ps4′OMT2 cDNAs contained open reading frames of 1062 and 1071 bp, respectively. The predicted PsOMT1 translation product consisted of 354 amino acids with a molecular mass of 39.4 kDa, whereas the deduced PsOMT2 protein was composed of 357 amino acids with a molecular mass of 39.6 kDa. The 1286-bp PsCNMT cDNA contained an open reading frame of 1053 bp encoding a predicted translation product of 351 amino acids with a predicted molecular mass of 41.0 kDa. Sequences for these clones have been deposited in the GenBank database under the accession numbers AY217335 (Ps6OMT), AY217336 (PsCNMT), AY217333 (Ps4′OMT1), and AY217334 (Ps4′OMT2).

Ps4′OMT1 and Ps4′OMT2 share 81% identity (90% homology) with each other, and 48 and 50% identity (70 and 73% homology), respectively, with Ps6OMT (Fig. 2). In contrast, PsCNMT shares only 26% about identity with any of the opium poppy *O*-methyltransferases. Comparison of the predicted translation product from each opium poppy cDNA with proteins in the GenBank database using the BLAST search program revealed extensive homology with the corresponding *O*- or *N*-methyltransferase from *C. japonica* (Fig. 2). Alignment of Ps4′OMT1 and Ps4′OMT2 with Cj4′OMT revealed 65 and 69% identity (80 and 83% homology), respectively (Fig. 2). Similarly, alignment of Ps6OMT with Cj6OMT showed 68% identity (81% homology; Fig. 2), whereas PsCNMT and CjCNMT were found to share 63% identity (80% homology; Fig. 3). A large number of other *O*-methyltransferases, including some involved in other alkaloid biosynthetic pathways, share less than 40% identity with opium poppy 6OMT and 4′OMTs. Although the sequence similarity among available NMTs is relatively low (Choi et al., 2002) opium poppy CNMT exhibits 35% identity with cyclopropane-fatty acyl phospholipid synthase from *Mesorhizobium loti*, 27% idenity with phosphoethanolamine *N*-methyltransferase from *Lycopersicon esculentum* (Solanaceae), and 51% identity with a hypothetical protein from *Arabidopsis thaliana* (Brassicaceae). The *O*- and *N*-methyltransferases involved in (*S*)-reticuline **13** formation appear to be highly conserved in plants that synthesize benzylisoquinoline alkaloids, as shown previously for BBE and CYP80B1 (Facchini et al., 1996a; Huang and Kutchan, 2000).

2.2. Developmental expression of alkaloid biosynthetic genes in opium poppy

RNA gel-blot hybridization analysis showed many conserved, but also some differential, aspects of alkaloid biosynthetic gene transcript accumulation in opium poppy plants. Although gene transcripts for all eight biosynthetic enzymes were detected in every organ of the plant, mRNA levels were generally highest in stems and flower buds and lowest in leaves (Fig. 4). In roots, *6OMT* and *CNMT* transcripts were detected at moderate to high levels, whereas 4′OMT mRNA levels were comparatively low. The spatial accumulation of *CYP80B1*, *BBE*, *SAT*, and *COR* transcripts was similar with the highest levels in stems, followed by flower buds and roots. Overall TYDC transcript levels were highest in roots; however, TYDC mRNAs detected in roots were predominantly of lower molecular weight than those found in aerial organs. The low- and high-molecular weight bands correspond to transcripts that hybridized to the individual TYDC1 and TYDC2 probes, respectively. These data are consistent with those reported previously for the expression of specific *TYDC* genes in opium poppy (Facchini and De Luca, 1994, 1995).

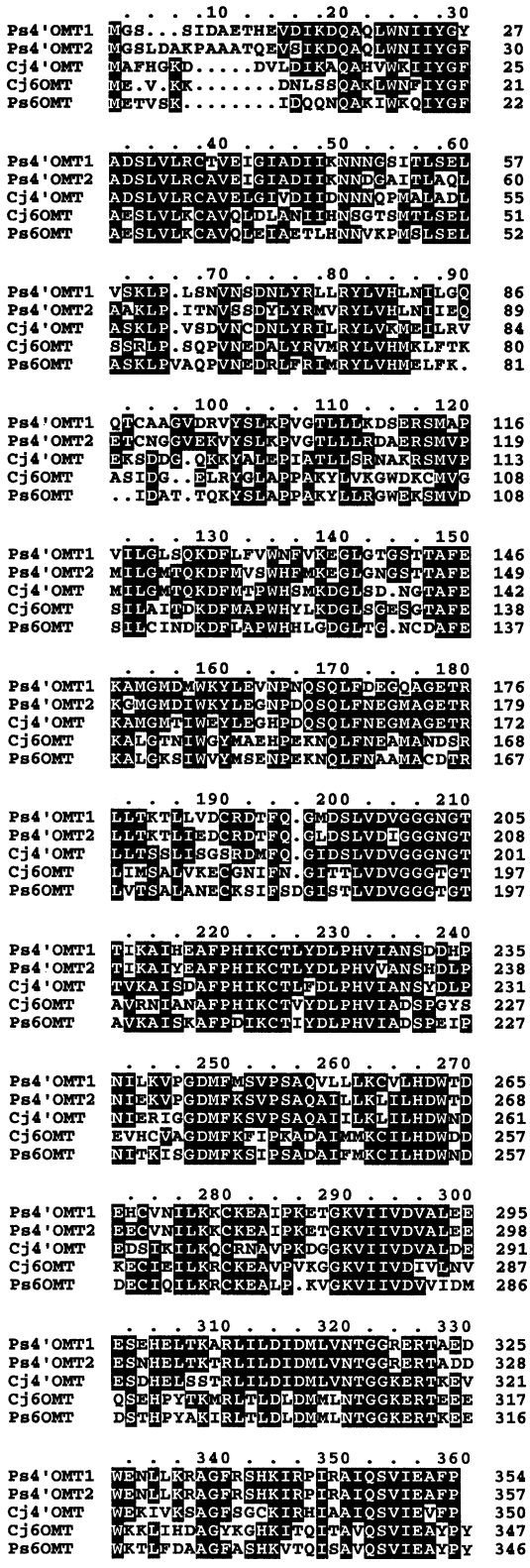

Fig. 2. Alignment of deduced amino acid sequences for Ps4′OMT1, Ps4′OMT2, and Ps6OMT from opium poppy with 3′-hydroxy-*N*-methylcoclaurine-4′-*O*-methyltransferase (Cj4′OMT) and norcoclaurine-6-*O*-methyltransferase (Cj6OMT) from *Coptis japonica*. Shaded boxes indicate residues that are identical in at least 60% of the aligned proteins. Gaps introduced into sequences to maximize alignments are shown by dots.

Transcripts of differential molecular weight were also detected in gel blots probed with the COR cDNA. Genomic DNA gel blot analysis showed the presence of at least six *COR* genes in opium poppy (Unterlinner et al., 1999); thus, the occurrence of at least two distinct mRNAs is not unexpected. Although detection of *COR* mRNAs in roots, stems, and leaves was shown previously, the transcripts were reported only at low levels (Unterlinner et al., 1999), which is at variance with the relative abundance of *COR* transcripts shown in Fig. 4. These variations suggest that the level of some transcripts could vary in different opium poppy varieties or the expression of certain genes might be influenced by environmental factors and growth conditions. Similarly, we were previously unable to detect *BBE* transcripts in the leaves of opium poppy cultivar 'Marianne' (Facchini

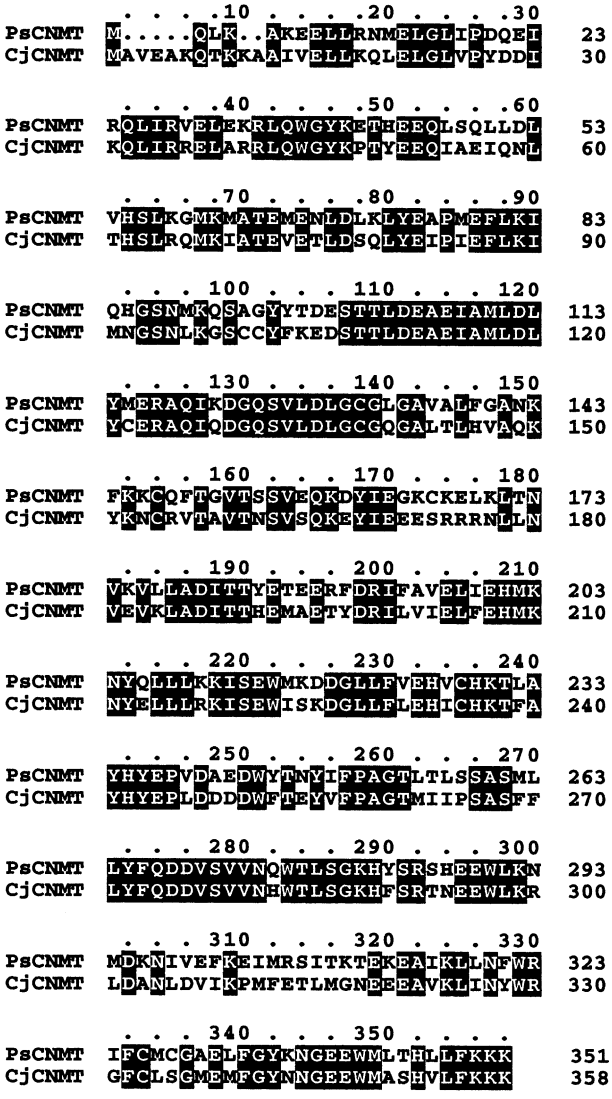

Fig. 3. Alignment of deduced amino acid sequences for PsCNMT from opium poppy with coclaurine *N*-methyltransferase (CjCNMT) from *Coptis japonica*. Shaded boxes indicate residues that are identical in both of the aligned proteins. Gaps introduced into sequences to maximize alignments are shown by dots.

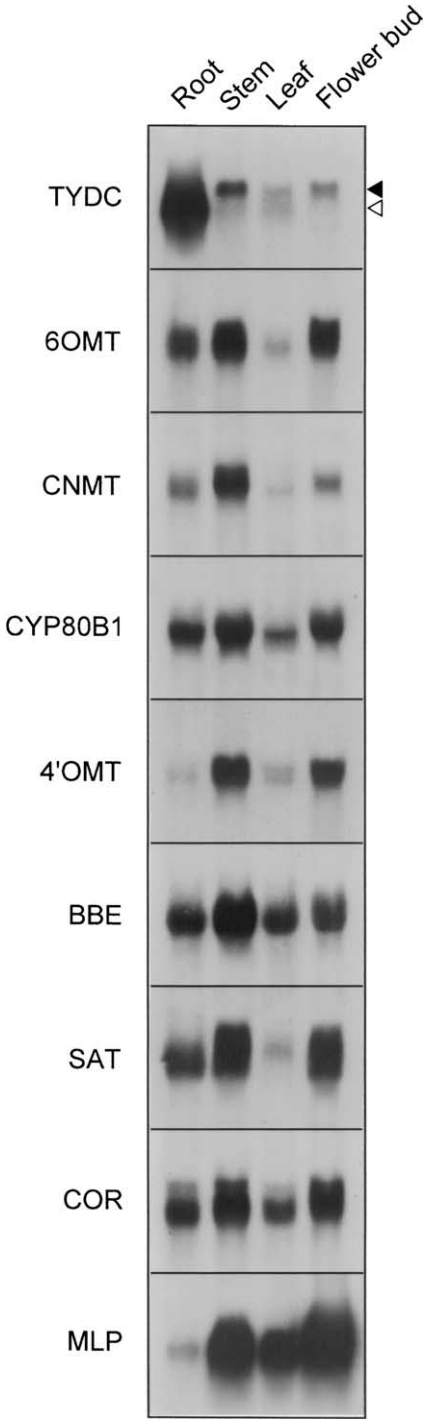

Fig. 4. Accumulation of alkaloid biosynthetic gene transcripts in different organs of mature opium poppy plants. Total RNA was extracted and fractionated on formaldehyde-agarose gels, transferred to nylon membranes, and hybridized at high stringency with ^{32}P-labeled probes. The TYDC probe was synthesized from an equal mixture of TYDC1 and TYDC2 cDNAs. *Open arrowheads* and *closed arrowheads* in Figs. 4–7 show transcripts hybridized to the TYDC1 and TYDC2 probes, respectively. Other probes were prepared from the corresponding opium poppy cDNA. Gels were stained with ethidium bromide prior to blotting to ensure equal loading. Results are representative of duplicate experiments with consistent results. Abbreviations in Figs. 4–7 are the same as in Fig. 1 except: MLP, major latex protein.

et al., 1996a). In the present study, however, BBE mRNAs were abundant in leaves (Fig. 4) in agreement with the results of Huang and Kutchan (2000) obtained using a different opium poppy cultivar. We also found *BBE* transcripts in flower buds (Fig. 4), but not in isolated carpels or seed capsules (data not shown).

Many of the observations concerning the spatial distribution of gene transcript accumulation in opium poppy plants are also applicable to developing seedlings (Fig. 5). The level of each transcript increased after seed imbibition, although considerable temporal and quantitative variation was apparent. *6OMT*, *CNMT*, and *4′OMT* transcript levels were transiently induced from 4 to 7 days after imbibition, and then returned to near baseline levels (Fig. 5). Although similar results were observed for *TYDC2* transcripts, *TYDC1* transcript levels increased sharply only on day 7, but declined rapidly by day 10. CYP80B1, BBE, and SAT mRNA levels were induced for 4–7 days after imbibition and remained high for the duration of the experiment. A similar induction time-course was shown for 1,2-dehydroreticuline reductase, which catalyzes the synthesis of (*R*)-reticuline, in developing opium poppy seedlings (De-Eknamkul and Zenk, 1992). In contrast, *COR* transcript levels increased more slowly with a maximum on day 16 (Fig. 5). Our data for *CYP80B1*, *BBE*, and *COR* transcript accumulation in seedlings are in agreement with those reported previously (Huang and Kutchan, 2000). Comparison of mRNA profiles from mature organs and whole seedlings suggests that the expression of alkaloid biosynthetic genes continues to change throughout the growth and development of the plant (Figs. 4 and 5). Although the proportion of various tissues is different in a mature plant compared to a seedling, the general abundance of several biosynthetic gene transcripts show a substantial increase in certain organs during development.

Our data suggest at least some degree of coordination in the developmental regulation of alkaloid biosynthetic genes in opium poppy. However, despite the common spatial and temporal accumulation of many gene transcripts in various plant organs and in developing seedlings, it is not known whether these mRNAs occur in the same cell type. Only TYDC transcripts have been localized by in situ hybridization and shown to be associated with phloem tissues (Facchini and De Luca, 1995). However, the cell type-specific localization of gene transcripts and enzymes involved in alkaloid biosynthesis is poorly understood. Despite the role of laticifers in alkaloid accumulation, the nature of the relationship between latex and alkaloid biosynthesis is not known. The accumulation of major latex protein (MLP) transcripts provides a general indication of latex and, thus, laticifer abundance in each organ (Fig. 4; Nessler and Vonder Haar, 1990). Although alkaloid accumulation appears restricted to the laticifers of

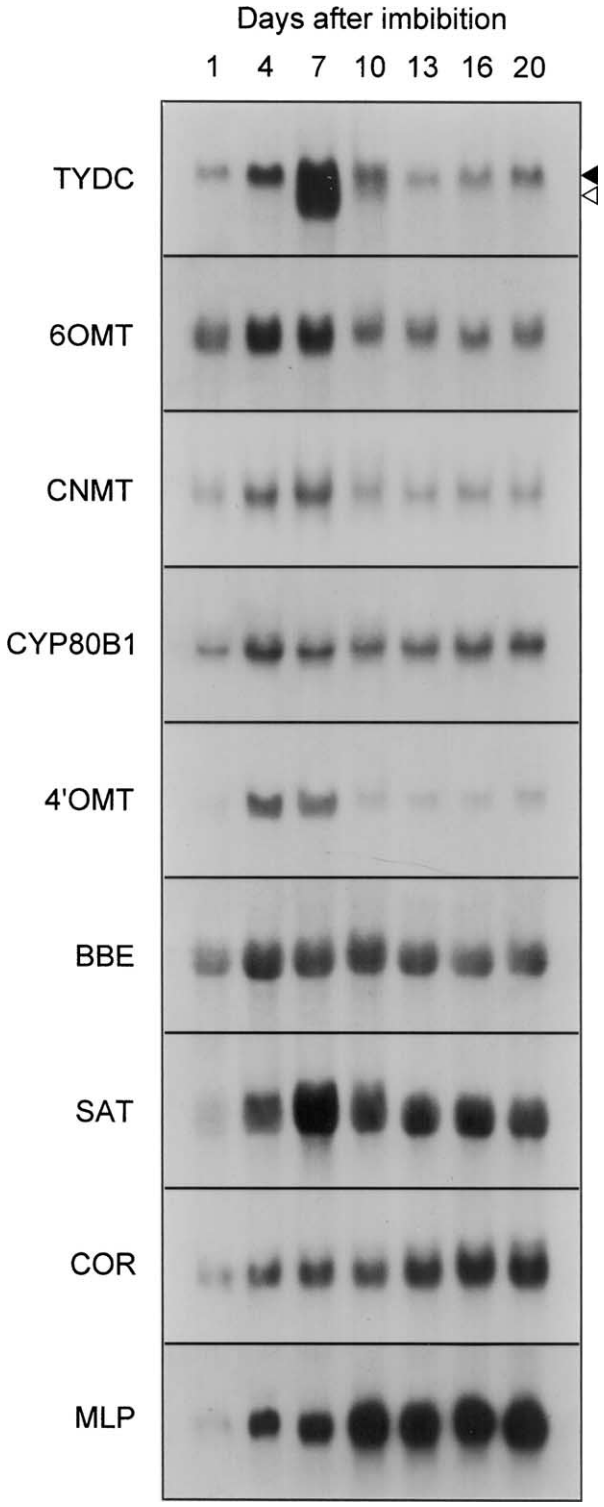

Fig. 5. Accumulation of alkaloid biosynthetic gene transcripts in whole opium poppy seedlings on successive days after imbibition. Total RNA was extracted and fractionated on formaldehyde-agarose gels, transferred to nylon membranes, and hybridized at high stringency with ^{32}P-labeled probes. The TYDC probe was synthesized from an equal mixture of TYDC1 and TYDC2 cdnas. other probes were prepared from the corresponding opium poppy cDNA. Gels were stained with ethidium bromide prior to blotting to ensure equal loading. Results are representative of duplicate experiments with consistent results.

opium poppy, the differential accumulation of gene transcripts encoding MLP and several alkaloid biosynthetic enzymes suggests that a distinct cell type other than laticifers might be involved in alkaloid formation. The involvement of multiple cell types has been demonstrated for the biosynthesis and accumulation of several other groups of alkaloids (reviewed by De Luca and St-Pierre, 2000). However, it is not possible to rule out the independent regulation of genes encoding MLP and alkaloid biosynthetic enzymes in the same cell.

The abundance of most gene transcripts generally correlates with the spatial and temporal accumulation of specific alkaloids, such as sanguinarine **3** and thebaine, in opium poppy plants and seedlings (Facchini and De Luca, 1995; Facchini et al., 1996a; Huang and Kutchan, 2000). However, the detection of *BBE* transcripts in some aerial organs was unexpected since sanguinarine **3** accumulates predominantly in roots (Facchini and De Luca, 1995). The possible root-specific expression of genes encoding enzymes downstream of BBE could account for the lack of sanguinarine **3** in other organs. Moreover, sanguinarine **3** levels only begin to increase in developing seedlings 5 days after imbibition (Facchini et al., 1996a; Huang and Kutchan, 2000) despite an earlier abundance of BBE mRNAs (Fig. 5). These data support the role of enzymes other than BBE in the control of sanguinarine (**3**) biosynthesis. Certainly, other molecular, biochemical, and cellular factors influence metabolic flux through alkaloid pathways despite apparent correlations between transcript levels and product accumulation in certain tissues.

2.3. Inducible expression of alkaloid biosynthetic genes in opium poppy

Treatment of opium poppy cell suspension cultures with a fungal elicitor caused the marked and rapid induction of seven of eight alkaloid biosynthetic gene transcripts (Fig. 6). The increases in transcript abundance were consistent with the time-course for sanguinarine (**3**) accumulation (Facchini et al., 1996b). Only COR mRNAs levels did not increase to the same extent, although a marginal and transient induction was detected in elicitor-treated cells (Fig. 6). A marginal increase in *MLP* transcript levels was also observed (Fig. 6). The minor induction in COR transcripts is consistent with a previous study (Huang and Kutchan, 2000). The low abundance of *MLP* transcripts in opium poppy cell cultures has also been reported (Nessler and Vonder Haar, 1990), although the induction of *MLP* mRNAs after elicitor treatment was not tested.

Elicitor-induced transcript accumulation for *TYDC* (Facchini et al., 1996b), *CYP80B1* (Huang and Kutchan, 2000), and *BBE* (Facchini et al., 1996a) genes has been well documented. However, the concomitant induction of *6OMT, CNMT, 4′OMT,* and *SAT*

transcripts was unexpected since enzyme activity measurements in elicitor-treated California poppy (*Eschscholzia californica*; Papaveraceae) cell cultures showed that only membrane-associated, but not soluble

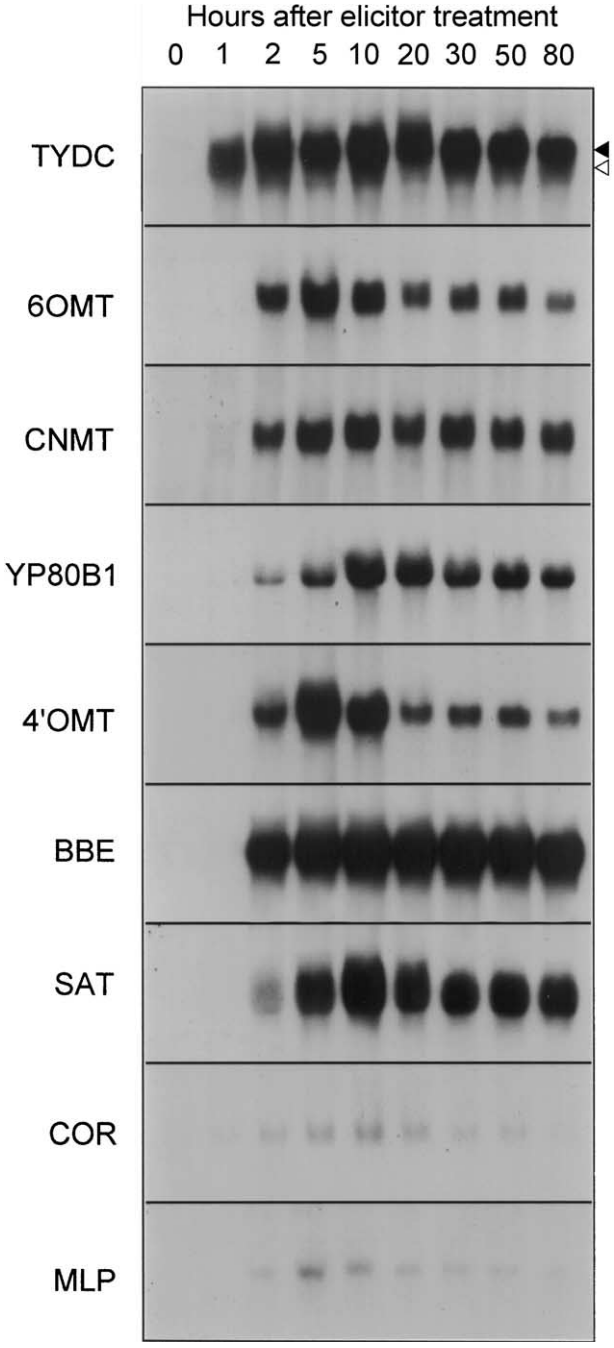

Fig. 6. Accumulation of alkaloid biosynthetic gene transcripts in opium poppy cell suspension cultures at different times after treatment with a fungal elicitor. Total RNA was extracted and fractionated on formaldehyde-agarose gels, transferred to nylon membranes, and hybridized at high stringency with ^{32}P-labeled probes. The TYDC probe was synthesized from an equal mixture of TYDC1 and TYDC2 cDNAs. Other probes were prepared from the corresponding opium poppy cDNA. Gels were stained with ethidium bromide prior to blotting to ensure equal loading. Results are representative of duplicate experiments with consistent results.

enzymes of sanguinarine **3** biosynthesis were induced (Blechert et al., 1995). Moreover, the accumulation of sanguinarine **3** is induced by methyl jasmonate in California poppy, but not opium poppy cell cultures (Gündlach et al., 1992; Facchini et al., 1996b). These data suggest the differential regulation of some sanguinarine (**3**) biosynthetic genes in opium poppy and California poppy cell cultures.

Differences among the induction time-courses for each transcript were also evident (Fig. 6). TYDC mRNAs were detected within one hour after the addition of elicitor. A temporal delay in *TYDC2* transcripts accumulation after a more rapid, but transient, induction of TYDC1 mRNAs (Fig. 6) is consistent our previous work (Facchini et al., 1996b). The induction of most other gene transcripts was remarkably similar to that of *TYDC2*. The strong induction of *SAT* transcripts was unexpected since dedifferentiated opium poppy cell suspension cultures generally do not accumulate morphine **1** or pathway intermediates. Only *COR* transcripts were differentially regulated in response to elicitor treatment, although this cannot account for the lack of morphine **1** in cultured opium poppy cells. Indeed, all known enzymes of the morphine **1** pathway have been detected in cultured cells (De-Eknamkul and Zenk, 1992; Gerardy and Zenk, 1993a,b; Grothe et al., 2001; Unterlinner et al., 1999). In addition to the possible absence of a currently unknown enzyme, the general developmental or cellular competency of dedifferentiated cultures to synthesize and store certain alkaloids must be questioned.

The accumulation of alkaloid biosynthetic gene transcripts in wounded opium poppy cells is similar to their induction in elicitor-treated cultures (Fig. 7). All gene transcripts with the exception of those encoding COR and MLP, which increased only marginally, showed a marked induction in response to wounding. TYDC1 mRNAs were abundant only on the first day after wounding, whereas elevated levels all other transcripts were detected beginning on day 2 (Fig. 7).

Our data suggest at least partially conserved molecular mechanisms for the elicitor- and wound-induced expression of alkaloid biosynthetic genes in opium poppy cell cultures. Unfortunately, little is known about the molecular nature of gene regulation involved in benzylisoquinoline alkaloid biosynthesis. In *E. californica*, alkaloid biosynthesis can be induced at elicitor concentrations below the threshold required to activate the hypersensitive response suggesting that alkaloid formation is coupled to a signal transduction pathway not mediated by reactive oxygen species (Roos et al., 1999). A transient decrease in cytosolic pH caused by an efflux of protons from the vacuole and triggered by a G-protein-controlled redox-dependent plasma membrane protein was also implicated in the elicitor-mediated activation of alkaloid biosynthesis (Roos et al., 1998,

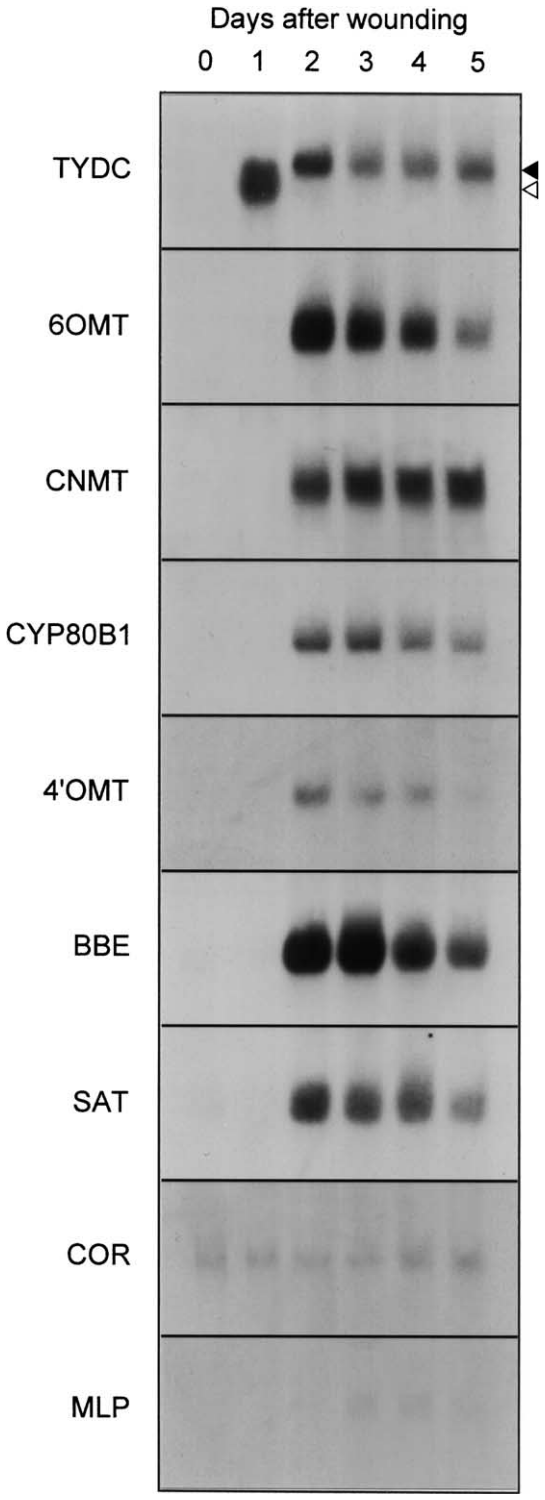

Fig. 7. Accumulation of alkaloid biosynthetic gene transcripts in opium poppy cell suspension cultures at different times after wounding. Total RNA was extracted and fractionated on formaldehyde-agarose gels, transferred to nylon membranes, and hybridized at high stringency with ^{32}P-labeled probes. The TYDC probe was synthesized from an equal mixture of TYDC1 and TYDC2 cDNAs. Other probes were prepared from the corresponding opium poppy cDNA. Gels were stained with ethidium bromide prior to blotting to ensure equal loading. Results are representative of duplicate experiments with consistent results.

1999) suggesting common components in the regulation of alkaloid biosynthetic genes. G-proteins and Ca^{2+} as a second messenger appear to function in the induction of benzylisoquinoline alkaloid biosynthesis in bloodroot (*Sanguinaria canadensis*; Papaveraceae) cell cultures (Mahady and Beecker, 1994; Mahady et al., 1998). Similar studies have not been performed on opium poppy cell cultures. Moreover, common promoter elements involved in wound-induced gene expression could not be identified in opium poppy *TYDC* and *BBE* promoters (Park et al., 1999). The role of a common transcriptional regulator controlling the expression of multiple biosynthetic genes has been demonstrated for monoterpenoid indole alkaloid pathways in Madagascar periwinkle (*Catharanthus roseus*; Apocyanaceae; Van Der Fits and Memelink, 2000). The coordinated accumulation of several gene transcripts in opium poppy plants, seedlings, and cell cultures suggests that master transcriptional control factors also function in the regulation of benzylisoquinoline alkaloid biosynthesis.

3. Concluding remarks

This study represents the most extensive survey of benzylisoquinoline alkaloid biosynthetic gene transcript accumulation to date. A considerable degree of coordination was detected in the developmental and inducible accumulation of gene transcripts representing eight enzymes involved in alkaloid biosynthesis in opium poppy. Our results suggest the presence of at least partially conserved mechanisms of gene regulation. However, some differences were also found in the temporal and spatial accumulation of specific mRNAs under various conditions. Most notable were the low levels of certain gene transcripts in roots and leaves, the temporal divergence in transcript accumulation in developing seedlings, and the induction of SAT, but not COR, mRNAs in response to elicitor treatment or wounding. Although some aspects of alkaloid biosynthesis and accumulation in opium poppy are clearly controlled at the transcriptional level, our data suggest that regulation of the overall pathway involves a complex and interdependent set of molecular, biochemical, and cellular factors.

4. Experimental

4.1. Maintenance of plants and cell suspension cultures

Opium poppy (*Papaver somniferum* L. cv. Marianne) plants were cultivated at 20 °C/18 °C (light/dark) in a growth chamber (Conviron, Winnipeg, Canada) with a photoperiod of 16 h. A combination of fluorescent (Cool White, Sylvania, Mississauga, ON, Canada) and

incandescent lighting was used. Seedlings were grown at 23 °C in sterile Petri plates (100×15 mm) on Phytagar (Gibco, Burlington, ON, Canada) containing Gamborg B5 salts and vitamins, under a photoperiod of 16 h using wide-spectrum fluorescent tubes (Gros-Lux Wide Spectrum, Sylvania) with a fluence rate of 35 μmol m^{-2} s^{-1}. Before germination, the seeds were surface sterilized with 20% (v/v) sodium hypochlorite for 15 min, and thoroughly rinsed with sterile water. Opium poppy (*P. somniferum* cv. Marianne) cell suspension cultures were maintained in the dark at 23 °C on 1B5C medium consisting of Gamborg B5 salts and vitamins plus 550 μM *myo*-inositol, 1 g l^{-1} hydrolyzed casein, 60 mM sucrose, and 4.5 μM 2,4-D. Cells were subcultured every 6 days using a 1:4 dilution of inoculum to fresh medium.

4.2. Elicitor treatment and wounding of cell suspension cultures

Fungal elicitor was prepared from *Botrytis* sp. according to Facchini et al. (1996b). A section (1 cm^2) of mycelia cultured on potato dextrose agar was grown in 50 ml 1B5C medium, including supplements but lacking 2,4-D, on a gyratory shaker (120 rpm) at 22 °C in the dark for 6 days. Mycelia (ca. 10 g fresh weight) and remaining medium (ca. 40 ml) were homogenized at maximum speed for 10 min with a Polytron (Brinkmann, Westbury, NY, USA), autoclaved (121 °C) for 20 min, and subsequently centrifuged under sterile conditions with the supernatant serving as elicitor. Elicitor treatments were initiated by the addition of 0.5 ml of fungal homogenate to 50 ml of cultured cells in rapid growth phase (2–3 days after subculture). Wounding of cell suspension cultures was performed by collecting cells in sterile Petri plates (50×10 mm), withdrawing most of the medium, and repeatedly cutting into the cellular mass with a sharp scalpel (Park et al., 1999). Cells were collected by vacuum filtration and stored at -80 °C.

4.3. RNA isolation and gel blot hybridization analysis

Total RNA was isolated according to Logemann et al. (1987), and poly(A)$^+$ RNA was selected by oligo(dT) cellulose chromatography. For gel blot analysis, 15 μg of total RNA was fractionated on 1.0% (w/v) agarose gels, containing 7% (v/v) formaldehyde, before transfer to nylon membranes. Blots were hybridized to random-primer-[^{32}P]-labeled full-length cDNAs at 65 °C in 0.25 M sodium phosphate, pH 8.0, 7% (w/v) SDS, 1% (w/v) BSA, and 1 mM EDTA. Blots were washed at 65 °C, twice with 2X SSC containing 0.1% (w/v) SDS, and twice with 0.2X SSC containing 0.1% (w/v) SDS (1X SSC = 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0). The blots were autoradiographed for 18 h on Kodak X-OMAT film at −80 °C.

4.4. cDNA library construction and screening

A unidirectional oligo(dT)-primed cDNA library was constructed in λUni-ZAPII XR, according to the manufacturers instructions (Stratagene, La Jolla, CA, USA), using poly(A)$^+$ RNA isolated from opium poppy cell suspension cultures treated for 10 h with fungal elicitor. The primary library, containing 1×10^7 phage, was screened by membrane hybridization with [^{32}P]-labeled probes prepared from cDNAs for norcoclaurine-6-*O*-methyltransferase (Cj6OMT), coclaurine *N*-methyltransferase (CjCNMT), and 4′OMT, 3′-hydroxy-*N*-methylcoclaurine-4′-*O*-methyltransferase (Cj4′OMT) from *Coptis japonica* (Morishige et al., 2000; Choi et al., 2002). Hybridization conditions were identical to those described for RNA gel blot hybridization analysis except that the membranes were hybridized and washed at 60 °C. Plasmids were rescued from phage that produced a positive signal using the R408 helper phage. Double-stranded cDNAs were sequenced using the dideoxynucleotide chain-termination method. The ClustalX software package was used to generate basic sequence alignments, which were then optimized manually.

Acknowledgements

We thank Craig Nessler for the MLP cDNA clone and Nailish Samanani for technical assistance. P.J.F is the Canada Research Chair in Plant Metabolic Processes Biotechnology. S.-U.P. is the recipient of a Postdoctoral Research Associateship offered through the Alberta Ingenuity Fund. This work was supported by Natural Sciences and Engineering Research Council of Canada Discovery and Strategic grants to PJF.

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