A Tale of Three Cell Types: Alkaloid Biosynthesis Is Localized to Sieve Elements in Opium Poppy

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Opium poppy produces a diverse array of pharmaceutical alkaloids, including the narcotic analgesics morphine and codeine. The benzylisoquinoline alkaloids of opium poppy accumulate in the cytoplasm, or latex, of specialized laticifers that accompany vascular tissues throughout the plant. However, immunofluorescence labeling using affinity-purified antibodies showed that three key enzymes, (S)-N-methylcoclaurine 3’-hydroxylase (CYP80B1), berberine bridge enzyme (BBE), and codeinone reductase (COR), involved in the biosynthesis of morphine and the related antimicrobial alkaloid sanguinarine, are restricted to the parietal region of sieve elements adjacent or proximal to laticifers. The localization of laticifers was demonstrated using antibodies specific to the major latex protein (MLP), which is characteristic of the cell type. In situ hybridization showed that CYP80B1, BBE, and COR gene transcripts were found in the companion cell paired with each sieve element, whereas MLP transcripts were restricted to laticifers. The biosynthesis and accumulation of alkaloids in opium poppy involves cell types not implicated previously in plant secondary metabolism and dramatically extends the function of sieve elements beyond the transport of solutes and information macromolecules in plants.

INTRODUCTION

The opium poppy is an ancient medicinal plant and the only commercial source for the narcotic analgesics morphine and codeine. Global production of morphine for licit pharmaceutical applications is ~150 tons annually (United Nations, 2002a). However, the estimated illicit production of morphine for the synthesis of heroin is at least 10-fold higher and contributes to numerous social and political problems throughout the world (United Nations, 2002b). Despite the widespread significance of opium poppy, many basic aspects of morphine and codeine metabolism are poorly understood, including the cellular localization of their biosynthesis in the plant.

Morphine is a major component of the alkaloid-rich latex in opium poppy. Latex is the cytoplasm of specialized cells, or laticifers, that form an internal secretory system associated with phloem tissues of the vascular system throughout the plant (Thureson-Klein, 1970). The manual lancing of unripe seed capsules is the traditional method for the collection of opium poppy latex. The air-dried latex, or opium, then is extracted with solvents to isolate morphine. When damaged, laticifers release copious volume of latex because their cellular contents are under positive turgor pressure similar to sieve elements of the phloem. Moreover, despite the compound origin of opium poppy laticifers, perforations develop between the lateral walls of adjacent cells, ensuring a contiguous network of latex vessels (Nessler and Mahlberg, 1977). Opium poppy latex is characterized by an abundance of major latex proteins (MLPs), which constitute a family of highly conserved, low molecular weight polypeptides found exclusively in laticifers (Nessler et al., 1985).

Morphine and codeine are members of the large and diverse group of benzylisoquinoline alkaloids, of which >2500 different structures have been identified in plants. Alkaloids are considered secondary metabolites because they are not essential for normal plant development but often play important ecophysiological roles. Morphine is most abundant in the latex of aerial organs, whereas the antimicrobial agent sanguinarine is the major alkaloid in opium poppy roots (Facchini and De Luca, 1995). Morphine and sanguinarine share a common biosynthetic pathway (Figure 1), beginning with the condensation of two L-Tyr derivatives to produce the central precursor (S)-norcoclaurine (Facchini, 2001). Specific O- and N-methyltransferases convert (S)-norcoclaurine to (S)-N-methylcoclaurine. A cytochrome P450-dependent monoxygenase [(S)-N-methylcoclaurine 3’-hydroxylase (CYP80B1)] catalyzes the 3’-hydroxylation of (S)-N-methylcoclaurine (Pauli and Kutchan, 1998). The subsequent 4’-O-methylation of (S)-3’-hydroxy-N-methylcoclaurine yields (S)-reticuline, the last common intermediate in the biosynthesis of both sanguinarine and morphine. Berberine bridge enzyme (BBE) catalyzes the conversion of (S)-reticuline to (S)-scoulerine, the first committed step in the sanguinarine pathway (Facchini et al., 1996). Alternatively, (S)-reticuline can be isomerized to its (R)-epimer as the first step in the formation of morphine. The NADPH-dependent enzyme codeinone reductase (COR) converts (–)-codeinone to (–)-codeine as the penultimate step in morphine biosynthesis (Unterlinner et al., 1999).

The accumulation of morphine and related secondary metabolites in the large, membranous vesicles of opium poppy latex has contributed to the long-standing assumption that alkaloids are synthesized in laticifers (Fairbairn and Wassel, 1964; Fairbairn et al., 1968; Wilson and Coscia, 1975; Roberts et al., 1983). How-
ever, several key enzymes involved in morphine biosynthesis have not been detected in latex (Gerardy and Zenk, 1993a, 1993b), suggesting that although alkaloids accumulate in laticifers, their synthesis occurs elsewhere. Using immunofluorescence labeling and in situ hybridization to identify the cellular localization of alkaloid biosynthesis in opium poppy, we show here that key alkaloid biosynthetic enzymes and gene transcripts are found in sieve elements and companion cells, respectively. These two phloem cell types have not been implicated previously in plant secondary metabolism. The implication of sieve elements in the biosynthesis of complex alkaloids dramatically extends the function of the phloem beyond the transport of solutes and information macromolecules in plants.

RESULTS

Key Alkaloid Biosynthetic Enzymes Generally Are Located in All Plant Organs

Polyclonal antibodies were raised against recombinant CYP80B1, BBE, and COR in both mice and rabbits. Immunoblot analysis using affinity-purified IgG fractions demonstrated the specificity of the antibodies. One band was detected in each lane of an immunoblot containing crude protein extracts from different opium poppy organs using affinity-purified mouse IgGs raised against CYP80B1, BBE, and COR (Figure 2). Identical results were obtained using affinity-purified rabbit IgGs (data not shown). The immunoreactive proteins were consistent with the expected molecular masses of CYP80B1 (54 kD), BBE (57 kD), and COR (36 kD). All three enzymes were present in each organ except for BBE, which was not detected in the carpel (Figure 2). The highest level of each protein was found in roots. No signals were detected on immunoblots probed with preimmune IgG fractions.

Immunolocalization Identifies a Distinct Cell Type Involved in Alkaloid Biosynthesis

Immunofluorescence labeling using resin-embedded cross-sections of various opium poppy organs showed the colocalization of CYP80B1, BBE, and COR to a specific cell type associated with vascular tissue throughout the secondary phloem, which surrounds a core of secondary xylem (Figure 3A). The use of affinity-purified antibodies showed that CYP80B1 (Figure 3B), BBE (Figure 3C), and COR (Figure 3D) were localized to the same cells in the root vascular tissue. Colocalization using affinity-purified mouse anti-COR and rabbit anti-MLP IgGs, which specifically labeled latex proteins, showed that laticifers were adjacent or proximal to cells containing CYP80B1, BBE, and COR (Figure 3D).

Identical results were obtained using sections of stem (Figures 3E to 3H), leaf (Figures 3I to 3L), and carpel (Figures 3M to 3P) from opium poppy. CYP80B1, BBE, and COR antibodies were colocalized to the cells adjacent or proximal to laticifers, which were identified clearly in stem (Figure 3H), leaf (Figure 3J), and carpel (Figure 3P) using the MLP antibodies. No signals were detected in tissues probed with preimmune IgG fractions. In stems, laticifers generally were larger than those in roots and located closer to the cortex than sieve elements and companion cells (Figure 3F). Similarly, the large laticifers in leaves were abaxial to other phloem tissues (Figure 3I). A cross-section of a vascular bundle in the carpel shows the extensive anastomosis that often occurs between adjacent laticifers (Figure 3M). The size, angular shape, spatial distribution, and ubiquitous occurrence of cells labeled with the CYP80B1, BBE, and COR antibodies are consistent with their identification as sieve elements.
The localization of CYP80B1, BBE, and COR to the cytoplasm of sieve elements was confirmed by counterstaining with calcifluor white, which binds specifically to cellulose and thus demarcates cell walls. All three enzymes were localized to the parietal region of the sieve element cytoplasm, whereas MLP was found dispersed throughout the cytoplasm of laticifers (Figure 6). Immunofluorescence labeling clearly was not associated with cell walls.

### DISCUSSION

#### A Tale of Three Cell Types

We have shown that the biosynthesis and accumulation of alkaloids in opium poppy involves three cell types of the phloem, two of which have not been implicated previously in plant secondary metabolism. Three key alkaloid biosynthetic genes are expressed in companion cells of the phloem, as shown by the accumulation of the gene transcripts in this cell type throughout the plant (Figures 4 and 5). The relative abundance of CYP80B1, BBE, and COR transcript accumulation (Facchini et al., 1996; Unterlinner et al., 1999; Huang and Kutchan, 2000) is out the plant (Figures 4 and 5). The relative abundance of CYP80B1, BBE, and COR transcript accumulation (Facchini et al., 1996; Unterlinner et al., 1999; Huang and Kutchan, 2000) is consistent with the level of each protein in various organs of opium poppy (Figure 2). The localization of the CYP80B1, BBE, and COR enzymes to sieve elements (Figure 3) implies that the corresponding gene transcripts are translated in companion cells and that the proteins are transported to adjacent sieve elements. Mature angiosperm sieve elements lack a variety of cellular organelles, including a nucleus and ribosomes, and thus are incapable of basic transcriptional and translational processes. As a result, a sieve element depends on its paired companion cell for survival. Companion cell–specific gene expression and translation of mRNAs that encode proteins found in sieve elements, such as lectins, are well established (Bostwick et al., 1992). The movement of fluorescently labeled phloem proteins from companion cells to sieve elements has been demonstrated (Balachandran et al., 1997).
Symplastic connections among adjoining sieve elements create the contiguous sieve tubes that allow solutes to be transported systemically throughout the plant. The bulk flow of phloem sap in these conductive sieve tubes produces considerable shear forces (Fisher, 1990); thus, alkaloid biosynthetic enzymes and other proteins must be anchored to the parietal region of sieve elements to prevent dislodging and translocation. Many sieve element proteins, such as P proteins, are not translocated along the solute stream (Knoblauch and van Bel, 1998), probably because they are anchored to the sieve element reticulum (SER) (Oparka and Turgeon, 1999). Ultrastructural observations suggest that small protein anchors immobilize the parietal SER and other cellular organelles, forming a channel adjacent to the plasma membrane (Ehlers et al., 2000). Sieve element proteins have been suggested to reside in this channel and along the parietal SER. Immunofluorescence labeling of CYP80B1, BBE, and COR along the cellular periphery supports the localization of alkaloid biosynthesis to the parietal layer of sieve elements (Figures 3, 5, and 6).

The localization of CYP80B1, BBE, and COR gene transcripts and enzymes to companion cells and sieve elements, respectively, is consistent with the association of Tyr/dopa decarboxylase (TYDC) to vascular tissues in opium poppy (Facchini and De Luca, 1995; El-Ahmady and Nessler, 2001). Although TYDC is involved in other biochemical processes in addition to catalyzing the first steps in alkaloid formation and thus is not neces-
sarily a direct marker for morphine and sanguinarine biosynthesis, it is notable that TYDC gene expression was not detected in laticifers (Facchini and De Luca, 1995; El-Ahmady and Nessler, 2001). It also is notable that low levels of TYDC mRNAs were found in xylem parenchyma (Facchini and De Luca, 1995), as were CYP80B1, BBE, and COR transcripts. Although CYP80B1, BBE, and COR represent only three of many biosynthetic enzymes, we suggest that alkaloid formation is restricted to sieve elements. This notion is supported by the positions in the alkaloid biosynthetic pathway of CYP80B1 at a common early step, BBE at the branch point in the sanguinarine pathway, and COR at the penultimate stage in morphine biosynthesis. The colocalization of CYP80B1, BBE, and COR to the same sieve elements shows that both morphine and sanguinarine biosynthesis occurs in the same cell type and implies that different alkaloids accumulate in the same laticifers. Because morphine and sanguinarine biosynthesis requires a common pathway intermediate, (S)-reticuline, the localization of both branch pathways to the same cell has regulatory implications with respect to the relative accumulation of each alkaloid in the plant.

The involvement of multiple, adjacent cell types in alkaloid biosynthesis and accumulation in opium poppy raises intriguing questions about the transport of products from sieve elements to laticifers. Recently, a multidrug-resistance-type, ATP binding cassette (ABC) protein from Coptis japonica (CjMDR1) was shown to transport the benzylisoquinoline alkaloid berberine (Shitan et al., 2003). In situ hybridization showed that CjMDR1 transcripts were most abundant in rhizome xylem tissues; thus, the transporter could function in the translocation of berberine from sites of synthesis to the rhizome, a major site of alkaloid accumulation in C. japonica. A membrane-bound ABC transporter also might reside at the interface between sieve elements and laticifers in opium poppy and participate in the transport of alkaloids between these cell types. It is noteworthy that an ABC transport protein transcript was identified in rice phloem (Asano et al., 2002). However, symplastic transport of alkaloids also must be considered, although plasmodesmata connecting the two cell types have not been reported.

Figure 4. Alkaloid Biosynthetic Gene Transcripts Are Localized to the Companion Cells Paired with Sieve Elements in Opium Poppy.

(A) to (D) In situ hybridization using DIG-labeled antisense probes for CYP80B1 (A), BBE (B), COR (C), and MLP (D) performed on stem [(A) and (B)] and carpel [(C) and (D)] sections.

(E) and (F) In situ hybridization using DIG-labeled sense probes for CYP80B1 (E) and MLP (F) performed on stem (E) and carpel (F) sections.

Asterisks and arrowheads show the locations of several laticifers and labeled companion cells, respectively. Bars = 25 μm.

The Plant Cell
Alkaloid Biosynthesis in Plants Involves Diverse Cell Types

Plant secondary metabolism and accumulation are associated with a diverse array of cell types, which are well represented in the biosynthesis of several distinct alkaloids. Particularly surprising are the alkaloid biosynthetic pathways in which individual enzymes reside in different cell types. Early enzymes of monoterpene indole alkaloid formation are localized to leaf epidermis in *Catharanthus roseus*, whereas late enzymes are found several cell layers away in laticifers and idioblasts, in which the products accumulate.

**Figure 5.** Colocalization of MLP, Biosynthetic Enzymes or Gene Transcripts, and Callose Confirms the Role of Sieve Elements and Companion Cells in Alkaloid Biosynthesis.

(A) Immunofluorescence localization of COR (blue), MLP (yellow), and callose (red) in a serial overlay of LR White-embedded, longitudinal root sections (0.3 μm thick) of opium poppy. Callose was localized using a β-1,3-linked glucan monoclonal antibody.

(B) and (C) In situ hybridization using a DIG-labeled antisense probe for CYP80B1 (B) and localization of callose using aniline blue (C) in a root longitudinal section. Closed arrowheads point to two sieve plates, and the open arrowhead shows a DIG-labeled companion cell.

(D) and (E) In situ hybridization using a DIG-labeled antisense probe for BBE (D) and localization of callose using aniline blue (E) in a root cross-section. Arrowheads show the locations of a sieve plate and pit fields. Bars = 15 μm.

**Figure 6.** Immunofluorescence Localization of Alkaloid Biosynthetic Enzymes to the Parietal Layer of Sieve Elements.

Root cross-sections were counterstained with calcofluor white.

(A) CYP80B1 (red) and MLP (yellow).

(B) BBE (green) and MLP (yellow).

(C) COR (blue) and MLP (yellow). Bar = 25 μm.
accumulate (St-Pierre et al., 1999). The first committed and last enzymes in tropane alkaloid biosynthesis in *Atropa belladonna* and *Hyoscyamus niger* occur in the pericarp, whereas an intermediate enzyme is found in the adjacent endodermis (Hashimoto et al., 1991; Nakajima and Hashimoto, 1999; Suzuki et al., 1999). Homospermidine synthase, the first step of the pyrrolizidine pathway in *Senecio vernalis*, is restricted to distinct groups of endodermis and neighboring cortical cells located opposite the phloem (Moll et al., 2002). Although their biosynthetic enzymes are restricted to roots, tropane and pyrrolizidine alkaloids are translocated systemically and accumulate in the cells of other organs (Hartmann et al., 1989; Hashimoto et al., 1991). Despite the evolutionary independence of these alkaloid biosynthetic pathways, the emerging paradigm clearly implicates multiple cell types and the intercellular translocation of pathway intermediates or products.

The involvement of multiple cell types and differential sites of product formation and accumulation also are features of benzylisoquinoline alkaloid biosynthesis in opium poppy. However, the localization of alkaloid biosynthetic enzymes to sieve elements and their corresponding gene transcripts to companion cells is unique among plant secondary metabolic pathways. No other biosynthetic pathway has been localized to sieve elements, which have been shown to possess only a limited number of enzymes. Sieve element cytoplasm typically includes only several hundred polypeptides, few of which have been identified (Kehr et al., 1999). Metabolites and enzymes associated with sieve elements include ascorbate and monodehydroascorbate reductases, which help to maintain an antioxidative environment (Walz et al., 2002), and glutathione, glutaredoxin, and glutathione reductases, which help to maintain an antioxidative environment (Walz et al., 2002), and glutathione, glutaredoxin, and glutathione reductase (Zamski et al., 1996), and other NAD(P)H-dependent enzymes. Sieve element cytoplasm typically includes only several hundred polypeptides, few of which have been identified (Kehr et al., 1999). Metabolites and enzymes associated with sieve elements include ascorbate and monodehydroascorbate reductases, which help to maintain an antioxidative environment (Walz et al., 2002), and glutathione, glutaredoxin, and glutathione reductase, which suggest the capacity for glutathione-dependent thiol reduction (Alosi et al., 1988; Szederkenyi et al., 1997). The ability of sieve elements to harbor a complex metabolic pathway has not seriously been considered. Nevertheless, the isolation of glutathione reductase (Alosi et al., 1988), mannitol dehydrogenase (Zamski et al., 1996), and other NAD(P)H-dependent enzymes (Walz et al., 2002) supports the catalytic functionality of COR in sieve elements.

The localization of benzylisoquinoline alkaloid biosynthesis to sieve elements in opium poppy demonstrates the unexpected metabolic competence of this unusual cell type. Our results extend the fundamental physiological role of sieve elements beyond the transport of solutes and information macromolecules. It is interesting to speculate on whether or not benzylisoquinoline alkaloid biosynthesis, in general, is localized to sieve elements or if other secondary metabolic pathways display similar cell type–specific localization. A more general role for these cell types could emerge as additional pathways are localized at the cellular level.

**METHODS**

**Plant Material**

Opium poppy (Papaver somniferum cv Marianne) plants were maintained in a growth chamber at 23°C with a photoperiod of 14 h. Plant organs were harvested 2 to 3 d after anthesis except for carpels, which were collected to 3 d after anthesis.

**Heterologous Expression and Purification of Proteins**

CYP80B1 (Huang and Kutchan, 2000) and BBE1 (Facchini et al., 1996) open reading frames were inserted in frame into pET29 (Novagen, Madison, WI), and the constructs were introduced into *Escherichia coli* strain BL21(DE3). The COR (Unterlinner et al., 1999) open reading frame was inserted in frame into pRSET, and the constructs were introduced into *E. coli* strain ER2566 (New England Biolabs, Boston, MA). Heterologous expression was performed according to the pET29 manual. Briefly, 1 L of NZY broth (86 mM NaCl, 20 mM MgSO4, 5 mM MgCl2 yeast extract, and 10 mg/L casein hydrolysate) containing 50 mg/L kanamycin (pET29-BBE) or 25 mg/L ampicillin (pRSET-CYP80B1 and pRSET-COR) was inoculated with 5 mL of overnight bacterial culture and incubated at 37°C. At a density of OD600 = 0.5, the cultures were induced for 4 h with 400 μM isopropyl-β-D-thiogalactopyranoside. Cells were pelleted, resuspended in homogenization buffer (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 10 μM phenylmethylsulfonyl fluoride [PMSF], and 5 mM 2-mercaptoethanol), and ruptured using a French press (Spectronic Instruments, Rochester, NY). Cell debris and protein inclusion bodies were recovered by centrifugation. The rinsed pellet was solubilized in homogenization buffer containing 6 M urea, and the solution was passed through a 0.20-μm filter. Recombinant proteins were affinity purified using a Ni2+-charged HiTrap column according to the manufacturer’s instructions (Pharmacia Biotech).

**Preparation of Antibodies**

Antibodies were prepared from purified antigens using repeated subcutaneous injections as described by Harlow and Lane (1988). Antigen proteins were dialyzed against 146 mM NaCl, resuspended at a concentration of 400 μg/mL, emulsified 1:1 with Freund’s complete adjuvant, and injected into mice (100 μL) or rabbits (500 μL). Preimmune sera were collected from each animal, and IgG fractions were purified using an Affi-Gel Protein A MAPSII Kit (Bio-Rad). Booster injections were performed every 3 weeks until a sufficient titer was achieved. Antibodies against BBE, CYP80B1, and COR were affinity-purified using purified protein immobilized on nitrocellulose membranes (Smith and Fisher, 1984). Sera were incubated with the immobilized antigen for 3 h, rinsed in TBST (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% [v/v] Tween 20), and eluted with 50 mM Gly buffer, pH 2.3. Purified IgGs were neutralized in 1 M Tris-HCl, pH 8.8, dialyzed against TBS (20 mM Tris-HCl, pH 7.5, and 150 mM NaCl) containing 0.2% (w/v) sodium azide, and concentrated using Centricon YM10 spin columns (Millipore, Bedford, MA).

**Immunoblot Analysis**

Plant tissues were frozen in liquid nitrogen and ground to a fine powder in the presence of 100 mg/g (fresh weight) polyvinyl polypyrollidone. Tissues were suspended in extraction buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 5 μM PMSF, and 5 mM 2-mercaptoethanol) and incubated on ice, and the supernatant was collected by centrifugation. Soluble proteins (25 μg) were fractionated by SDS-PAGE (Laemmli, 1970) and transferred to nitrocellulose membranes. Protein blots were incubated with 10% nonfat milk (Bio-Rad). Membranes were washed in TBST and developed in AP buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, and 5 mM MgCl2) containing 0.7% (w/v) sodium azide, and concentrated using Centricon YM10 spin columns (Millipore, Bedford, MA).
20 μM nitroblue tetrazolium and 20 μM 5-bromo-4-chloro-3-indolyl phosphate as substrates (Sambrook et al., 1989).

**Tissue Fixation and Embedding for Immunocytochemical Localization**

Tissue fixation and immunocytochemical localization were performed as described previously (Voznesenskaya et al., 1999). Briefly, tissues were immersed in fixation buffer (50 mM Pipes, pH 7.0, 1.25% [v/v] glutaraldehyde, 2% [v/v] paraformaldehyde, and 5 μM PMSF), cut with a razor blade into 1.5- to 2-mm sections, fixed for 2 h, and rinsed in 50 mM Pipes, pH 7.0, containing 5 μM PMSF. The tissues were dehydrated using a 30 to 100% [v/v] ethanol series with a 2-h incubation in each solution. After dehydration, LR White resin (London Resin Company, London, UK) was introduced into the ethanol series at an initial ratio of 1:4 [v/v] and gradually increased to 1:3, 1:2, 1:1, 2:1, and 3:1 [v/v]. Finaly, tissues were immersed in pure resin, cast into 1-mL gelatin capsules, and incubated at 60°C for 16 h. Sections were cut 1 μm thick using a Reichert-Jung Ultracut E microtome (Leica Microsystems, Wetzlar, Germany).

**Tissue Fixation and Embedding for In Situ Hybridization**

Organs were immersed in FAA (50% [v/v] ethanol, 5% [v/v] acetic acid, and 3.7% [v/v] formaldehyde), cut with a razor blade into 2- to 5-mm segments, and fixed overnight at 4°C. Tissues were dehydrated using an ethanol/tertiary butanol (t-butanol) series (4:1, 5:2:3, 5:3:1.5, 4.5:5:5.0, 2.5:7.5:0, and 0:1 ethanol:t-butanol:water) with a 2-h incubation in each solution except for the final step, which was overnight. Paraplast Plus (Oxford Labware, St. Louis, MO) was added to a paraffin infiltration series (1:1, 6.7:3.3, and 1:0 wax:t-butanol) with overnight incubations for each step. Embedded tissues were cut into 10-μm sections using an American Optical 620 microtome (Buffalo, NY). Sections were placed onto aminopropyltriethoxysilane-coated slides and incubated overnight at 37°C to promote the firm adhesion of sections to the slides.

**Immunocytochemical Localization**

Affinity-purified anti-BBE, anti-CYP80B1, and anti-COR IgGs were used at concentrations of 20 μg/mL, 45 μg/mL, and 35 μg/mL, respectively. The anti-MLP (Griffith and Nessler, 1983) IgG fraction was purified using the Affi-Gel Protein A MAPSS1 Kit (Bio-Rad) and used at a concentration of 20 μg/mL. A mouse monoclonal antibody specific to callose (Biosupplies, Parkville, Australia) was used at a concentration of 15 μg/mL. Tissue sections were incubated with primary antibodies for 2 h and rinsed three times in TBS containing 1% [w/v] BSA (BSA Fraction V; Roche Diagnostics, Mannheim, Germany) and twice in TBS for 10 min. Sections were incubated for 1 h with either Alexa 488–conjugated goat anti-mouse IgG or Alexa 594–conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR) and then rinsed in TBS and water. Slides were sealed using Aquaperm (ThermoShandon, Pittsburgh, PA).

**In Situ Hybridization**

In situ hybridization was performed as described by St-Pierre et al. (1999). Briefly, CYP80B1, BBE, COR, and MLP (Nessler and Vonder Haar, 1990) open reading frames in pBluescript SK+ (Stratagene) served as templates for the synthesis of sense and antisense digoxigenin (DIG)-labeled RNA probes using T3 and T7 RNA polymerases. DIG-labeled probes were hydrolyzed at 60°C in 40 mM sodium carbonate buffer, pH 10, to produce fragments 200 to 400 nucleotides in length. The pH was neutralized using 10% [v/v] acetic acid, and the RNA was resuspended in 50 μL of deionized water. Sections were deparaffinized and rehydrated using an ethanol series (1:0, 1:0, 9.5:0.5, 7:3, and 1:1 ethanol/water) with a 5-min incubation in each solution. Sections were incubated in prehybridization buffer (100 mM Tris-HCl, pH 8.0, and 50 mM EDTA) containing 5 μg/mL proteinase K (Roche Diagnostics) for 30 min and then blocked in TBS (10 mM Tris-HCl, pH 7.5, and 150 mM NaCl) containing 2 mg/mL Gly. Sections were postfixed in 3.7% [v/v] formaldehyde in PBS (100 mM sodium phosphate buffer, pH 7.2, and 140 mM NaCl), incubated in 100 mM triethanolamine buffer, pH 8.0, containing 0.25% [v/v] acetic anhydride, and finally rinsed in TBS. The slides were inverted onto 100 μL of hybridization buffer (10 mM Tris-HCl, pH 6.8, 10 mM sodium phosphate buffer, pH 6.8, 40% [v/v] deionized formamide, 10% [w/v] dextran sulfate, 300 mM NaCl, 5 mM EDTA, 1 mg/mL yeast tRNA, 500 ng/mL DIG-RNA, and 0.8 unit/mL RNase inhibitor [Invitrogen, Carlsbad, CA]) spread over a cover slip. Slides were sealed in a Petri dish lined with filter paper soaked in 50% (v/v) formamide and incubated overnight at 50°C.

Slides were immersed in 2× SSC (1× SSC = 300 mM NaCl and 30 mM sodium citrate, pH 7.0) at 37°C until the cover slips fell off. Sections were incubated in 50 mg/mL RNase A (Roche Diagnostics) in 500 mM NaCl, 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA for 30 min at 37°C. Slides were washed in 2 L of the following solutions for 1 h: 2× SSC and 1× SSC at room temperature and 0.1× SSC at 60°C. Slides were rinsed in TBST and blocked for 1 h in TBST containing 2% [w/v] BSA. Slides were inverted onto cover slips carrying 100 μL of goat anti-DIG-AP conjugate (Roche Diagnostics) diluted 1:200 in TBST containing 1% [w/v] BSA and incubated for 2 h in sealed Petri dishes lined with filter paper soaked in TBST. After incubation, the slides were rinsed in TBST and AP buffer. Colorimetric development was performed in AP buffer containing 400 μM 5-bromo-4-chloro-3-indolyl phosphate and 428 μM nitroblue tetrazolium for 2 to 24 h.

**Aniline Blue, Toluidine Blue O, and Calceofluor White Staining**

Deparaffinized and rehydrated tissue sections were stained in 67 mM phosphate buffer, pH 8.5, containing 0.05% [w/v] aniline blue to detect callose. Sections were stained in 0.5% [w/v] calceofluor white to localize cell walls. For general anatomy, LR White sections were stained in benzoyl fluoride (10 mM sodium benzoylate, pH 4.4) containing 0.1% [w/v] toluidine blue O.

**Fluorescence and Light Microscopy**

Immunofluorescence labeling was viewed using a Leica DM RXA2 microscope (Leica Microsystems, Wetzlar, Germany), and images were acquired with a Retiga EX digital camera (QImaging, Burnaby, British Columbia, Canada). Alexa 488 and Alexa 594 fluorescent labels were detected using Leica L5 and TX2 filters, respectively. Aniline blue and calceofluor white were detected using a Leica A1 filter. Deconvolution and false-color imaging was performed using Open Lab version 2.09 (Improvision, Coventry, UK). Light microscopy images were captured using the Leica microscope and the Retiga camera mounted with a RGB color liquid crystal filter (QImaging).

Upon request, materials integral to the findings of this publication will be made available in a timely manner to all investigators on similar terms for noncommercial research purposes. To obtain materials, please contact P.J. Facchin, pfacchin@ualgyc.ca.

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