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# Expression of the phloem lectin is developmentally linked to vascular differentiation in cucurbits

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**Abstract.** The conducting elements of phloem in angiosperms are a complex of two cell types, sieve elements and companion cells, that form a single developmental and functional unit. During ontogeny of the sieve element/companion cell complex, specific proteins accumulate forming unique structures within sieve elements. Synthesis of these proteins coincides with vascular development and was studied in *Cucurbita* seedlings by following accumulation of the phloem lectin (PP2) and its mRNA by RNA blot analysis, enzyme-linked immunosorbent assay, immunocytochemistry and in situ hybridization. Genes encoding PP2 were developmentally regulated during vascular differentiation in hypocotyls of *Cucurbita maxima* Duch. Accumulation of PP2 mRNA and protein paralleled one another during hypocotyl elongation, after which mRNA levels decreased, while the protein appeared to be stable. Both PP2 and its mRNA were initially detected during metaphloem differentiation. However, PP2 mRNA was detected in companion cells of both bundle and extrafascicular phloem, but never in differentiating sieve elements. At later stages of development, PP2 mRNA was most often observed in extrafascicular phloem. In developing stems of *Cucurbita moschata* L., PP2 was immunolocalized in companion cells but not to filamentous phloem protein (P-protein) bodies that characterize immature sieve elements of bundle phloem. In contrast, PP2 was immunolocalized to persistent P-protein bodies in sieve elements of the extrafascicular phloem. Immunolocalization of PP2 in mature wound sieve elements was similar to that in bundle phloem. It appears that PP2 is synthesized in companion cells, then transported into differentiated sieve elements where it is a component of P-protein filaments in bundle phloem and persistent P-protein bodies in extrafascicular phloem. This differential accumulation in bundle and extrafascicular elements may result from different functional roles of the two types of phloem.

**Key words:** Companion cell – *Cucurbita* (phloem lectin) – Lectin (PP2) – Phloem protein – Sieve element – Vascular differentiation

## Introduction

A perplexing feature of the phloem of most angiosperms is the appearance of distinct proteinaceous structures in the translocating cells. Since the nucleus and ribosomes degenerate during sieve element maturation, synthesis of these proteins must occur either in immature sieve elements prior to organelle degeneration or in companion cells from which they would then be transported, presumably via plasmodesmata, into sieve elements. Ultrastructural studies have shown that phloem proteins (P-proteins) accumulate early in phloem ontogeny and persist in senescent sieve elements. Deposition of P-proteins into distinctive structures, bodies and filaments, occurs during sieve element differentiation and appears to be a dynamic process that involves protein-protein interactions (Cronshaw and Esau 1968a; Read and Northcote 1983a). Although the structural appearance and biochemical nature of several P-proteins have been described, our understanding of their developmental expression and accumulation in specific cell types is limited.

Cucurbits are an attractive system for P-protein studies because of their well-defined vascular anatomy

Dedicated to Prof. Dr. R. Kollmann on the occasion of his 65th birthday

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Abbreviations: DAG = days after germination; ELISA = enzyme-linked immunosorbant assay; LM = light microscopy; MS = Murashige and Skoog; PP1 = phloem protein 1; PP2 = phloem protein 2; P-protein = phloem protein; SE/CC = sieve element/companion cell complex(es)

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and abundant protein present in vascular exudates. Fischer (1884) and Crafts (1932) described two types of phloem in cucurbits, fascicular and extrafascicular, that differ in their ontogeny and position in stems. The fascicular or bundle phloem is derived from the procambium and is located internal and external to the xylem, forming the characteristic bicollateral vascular bundles. In contrast, the extrafascicular phloem, including commissural sieve elements, is derived from ground tissue and is composed of small groups of sieve element/companion cell complexes (SE/CC) located in the cortex and in arcs bordering the internal and external bundle phloem (Crafts 1932; Blyth 1958). Long-distance transport of photoassimilates occurs in sieve elements of the bundle phloem, while the transport function of the extrafascicular phloem is unresolved (Smith et al. 1987). Phloem protein bodies accumulate in immature sieve elements in both types of phloem. Aggregates of P-protein in sieve elements of the bundle phloem disperse forming filaments during the later stages of differentiation, but remain as aggregates in mature sieve elements of the extrafascicular phloem (Cronshaw and Esau 1968b; Evert et al. 1973).

An abundant P-protein in cucurbits is the 48-kDa dimeric lectin (PP2) that specifically binds poly(β–1,4-*N*-acetylglucosamine) (Sabnis and Hart 1978; Allen 1979). PP2 is encoded by a small gene family of two to eight genes that are highly conserved among *Cucurbita* species (Bostwick et al. 1994). In *C. maxima,* PP2 is encoded by two identical genes indicating that it is a homodimer (Bostwick et al. 1994). Moreover, PP2 is a soluble cytoplasmic protein and a component of phloem filaments due to covalent linkage with polymers of the phloem filament protein (PP1) (Kleinig et al. 1975; Read and Northcote 1983a, 1983b). Anatomical observations, combined with the physical characteristics of isolated P-proteins, have led investigators to suggest that P-protein might serve as a wound-sealing mechanism to prevent the loss of assimilates from disrupted sieve elements (Eschrich 1975; Schulz 1986).

Localization studies have shown that the phloem lectin accumulates in sieve elements and companion cells of both bundle and extrafascicular phloem. Nuske and Eschrich (1976) applied $^{14}$C-labeled amino acids to the cotyledons of *C. maxima* seedlings and recovered from vascular exudates labeled basic proteins with molecular weights similar to PP1 and PP2. Microautoradiography showed label concentrated in the companion cells of the mature metaphloem in both internal and external bundle phloem. Using fresh tissue sections of *C. maxima*, Smith et al. (1987) immunocytochemically localized PP2 to SE/CC of the mature phloem, although antibodies bound predominantly to P-protein bodies of the extrafascicular phloem. Ultrastructural pre-embedding immunolocalization of PP2 showed gold particles in close association with P-protein filaments and the sieve element reticulum lining the plasma membrane. We have previously shown by in situ hybridization that PP2 transcripts accumulated specifically in the companion cells of mature bundle and extrafascicular phloem in hypocotyls of *C. maxima* (Bostwick et al. 1992). These studies suggest that the phloem lectin and its mRNA are localized in very specific cell types in mature phloem tissue.

To investigate the dynamics of P-protein deposition, we have analyzed accumulation of PP2 mRNA and protein during vascular differentiation and related these events to early stages of phloem development. Evidence is presented that shows PP2 is synthesized in companion cells during phloem development, but differentially accumulates within the P-protein bodies in sieve elements of the bundle and extrafascicular phloem. Our results also suggest that PP2 traffics between companion cells, the exclusive site of synthesis, and sieve elements, the primary site of deposition.

## Materials and methods

*Probes and standards.* The DECAPrime radioactive labeling kit (Ambion, Inc., Austin, Tex., USA) was used to generate DNA probes from cPC20 (PP2 cDNA) (Bostwick et al. 1992) [α-$^{32}$P]dATP (111 TBq/mmol) was obtained from DuPont NEN Research Products (Boston, Mass., USA). In vitro transcripts of cPC20 were made with the MEGAscript kit (Ambion). PP2 was affinity-purified on a chitotriose-agarose column (Sigma Chemical Co., St. Louis, Mo, USA) eluted with 0.1 M glycine buffer (pH 3.0). Antibodies used for enzyme-linked immunosorbent assay (ELISA) were raised against PP2 in New Zealand White rabbits by the Iowa State University Cell & Hybridoma Facility, Ames, Iowa, USA. Immunoglobulin G was purified on a protein A column (Sigma) according to Harlow and Lane (1988). Antibodies used in the immunolocalization experiments were generously donated by Dr. M.C. Alosi.

*Isolation of RNA and dot blot analysis.* Pumpkin seedlings (*Cucurbita maxima* Duch. cv Big Max) were grown in a growth chamber at 25 °C with a 16-h photoperiod. Total RNA was isolated by a guanidinium-HCl method (Cathala et al. 1983). A standard curve was developed from in vitro transcripts to quantitate levels of PP2 mRNA. For dot blot analysis, 250 ng of total RNA was loaded into each well and transferred to nylon membrane (MagnaGraph, MSI, Westboro, Mass., USA). The blots were washed twice with 6 × saline sodium citrate (SSC; 0.9 M NaCl, 0.09 M $C_6H_5Na_3O_7$-$2H_2O$), UV-crosslinked and baked for 1 h at 80 °C. Blots were hybridized with cDNA probes, washed according to Sambrook et al. (1989) and exposed to X-ray film. Signals were quantitated by laser densitometry or directly with the Betascope blot analyzer (Betagene, Waltham, Mass., USA). Hybridization with ribosomal DNA probes verified equal loading of total RNA.

*Protein quantitation (ELISA).* Total protein was extracted by grinding 0.2 g of pumpkin hypocotyl in 0.8 mL of extraction buffer [100 mM Tris-HCl, pH 8.2; 5 mM EDTA; 20 mM dithiothreitol (DTT); 2 mM phenylmethylsulfonyl fluoride (PMSF)] and cleared by centrifugation. Protein concentration was determined by the Bradford assay (Bio-Rad Laboratories, Hercules, Calif., USA) using bovine serum albumin (BSA) standards. The PP2 standards and protein extracts were diluted in carbonate coating buffer (CCB; 15 mM $Na_2CO_3$, 35 mM $NaHCO_3$, pH 9.6), loaded into wells of Immulon 4 microtiter plates, then allowed to adsorb at 4 ° C overnight. Wells were washed with Tween-Tris buffered saline (TTBS; 500 mM NaCl; 20 mM Tris, pH 7.5; 0.15% Tween 20), then PP2 antibodies (1:20 000, v/v) were added and incubated for 2 h. The wells were washed again with TTBS, then alkaline phosphatase-conjugated goat anti-rabbit antibodies (1:5000, v/v) (Jackson ImmunoResearch Laboratories, West Grove, Pa., USA) were added and incubated for 1 h. Color was developed with Sigma 104 Phosphatase Substrate and read on a Dynatech MR5000 plate reader.

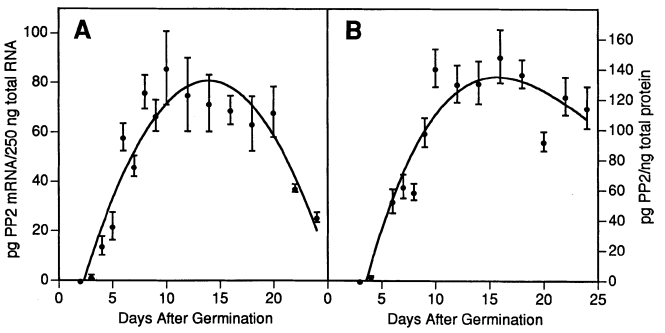



**Fig. 1A,B.** The accumulation of PP2 and its mRNA in developing pumpkin hypocotyls. **A** Amount of PP2 mRNA (in pg) detected in total RNA extracted from hypocotyls of pumpkin seedlings from 2 to 24 DAG. **B** Accumulation of PP2 detected by ELISA in total protein extracted from the same hypocotyls. Three to four hypocotyls were pooled for RNA and protein extraction at each time point. Quantitation of RNA and protein levels are an average of three replicates. Vertical bars represent the standard error of the mean. Regression curves were fitted to the average value for each time point

*Pulse-labeling of phloem exudate proteins.* The roots of pumpkin seedlings 10 days after germination (DAG) were cut under water to approx. 5 mm in length. Seedlings were immediately transferred to 150 µL of an aqueous solution of 1.85 MBq $^{35}SO_4$ (50 pmol $SO_4^{-2}$, 80.29 MBq/mL, 37 TBq/mmol; Amersham Life Science Inc., Arlington Heights, Ill., USA) and incubated for 2 h at 25 °C with continuous fluorescent light. The roots were rinsed twice with water, blotted dry then placed in 3 mL of Murashige and Skoog (MS) salts (4.5 µmol $SO_4^{-2}$; Life Technologies, Inc., Gaithersburg, Md., USA) and incubated for an additional 6, 12, 24, 48, or 72 h. Vascular exudate was collected from cut hypocotyls of two seedlings at each time point and diluted in 80 µL of extraction buffer. The experiment was repeated three times. Proteins were separated by SDS-PAGE and fluorography was performed using Entensify (DuPont NEN).

*In situ hybridization.* Pumpkin hypocotyl tissue was fixed in 2% glutaraldehyde in 50 mM $KPO_4$ buffer, then dehydrated in ethanol and tertiary butyl alcohol before embedding in paraffin. For light microscopy (LM), sections (7–10 µm) were stained with toluidine blue or a combination of safranin, orange G, tannic acid, and iron alum (Sharman 1943). Digoxigenin-labeled sense and antisense riboprobes were synthesized by in vitro transcription using PP2 cDNA templates. Probe preparation and hybridization protocols were described by Bostwick et al. (1992).

*Immunolocalization.* PP2 was immunolocalized in developing internodes of squash seedlings (*Cucurbita moschata* L. cv Butternut) 14 DAG. Wound phloem was induced in hypocotyl tissue and prepared for microscopy according to Schulz (1990). Longitudinal hand-cut sections of developing internodes were fixed and embedded in LR White resin (London Resin Co., Basingstoke, Hampshire, UK) according to Schulz et al. (1989). For LM, 1-µm sections were either stained with 0.05% crystal violet or incubated with PP2 antibodies (1:100 or 1:600, v/v) for 1 h, then washed and incubated with goat anti-rabbit-5 nm gold (1:25, v/v) (Amersham Buchler, Braunschweig, Germany) for 1 h. Sections were washed in phosphate-buffered saline (PBS; 137 mM NaCl, 3 mM KCl, 10 mM $Na_2HPO_4$, 2 mM $KH_2PO_4$, pH 7.4) and distilled water, then incubated for 10–15 min with Intense M (Amersham) for silver enhancement. For electron microscopy, ultrathin sections were collected on gold grids, hydrated in PBS and incubated with primary and secondary antibodies as described for LM. For controls, the primary antibody was replaced with 3% BSA or preimmune serum. The sections were poststained with 0.5% $OsO_4$, saturated uranyl acetate and lead citrate, then examined in Philips EM301 and CM 10 electron microscopes.

## Results

*Accumulation of PP2 mRNA and protein.* Steady-state levels of PP2 mRNA in hypocotyls of developing pumpkin seedlings were determined by dot blot analysis of total RNA. Hybridization was compared with

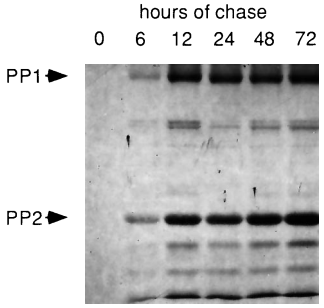



**Fig. 2.** Pulse-labeling of vascular exudate proteins synthesized in vivo. The roots of intact pumpkin seedlings were exposed to 50 pmol $^{35}SO_4^{-2}$ in water for 2 h and then incubated in MS salts (4.5 µmol $SO_4^{-2}$) for periods up to 72 h. Vascular exudates were collected, and equal amounts of total exudate protein (25 µg) were separated by SDS-PAGE and visualized by fluorography. *Lanes 0, 6, 12, 24, 48,* and *72* show proteins extracted after 0, 6, 12, 24, 48, and 72 h of chase, respectively. The positions of PP1 and PP2 are indicated by *arrows*

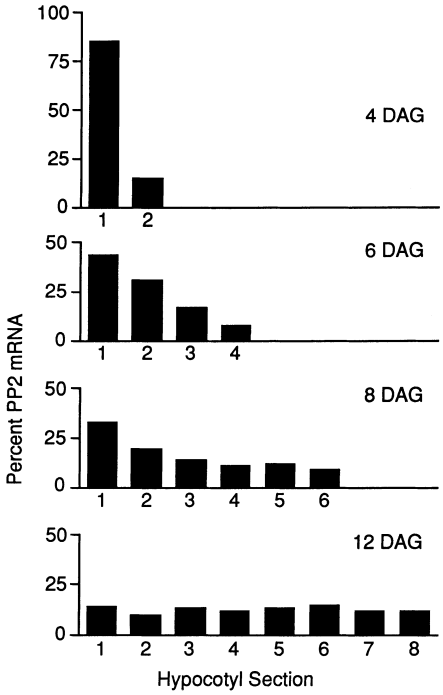



**Fig. 3.** PP2 mRNA accumulates differentially in developing pumpkin hypocotyls. Bars show the percentage of PP2 mRNA that was detected by dot blot analysis in 1-cm sections of developing pumpkin hypocotyls. At each developmental stage the bars cumulatively equal 100% of the PP2 mRNA detected in the entire hypocotyl. Hypocotyl section *1* was adjacent to the root. Hypocotyls of seedlings at 4, 6, 8, and 12 DAG were approx. 2, 4, 6 and 8 cm in length, respectively. By 12 DAG, elongation of the hypocotyl has ceased

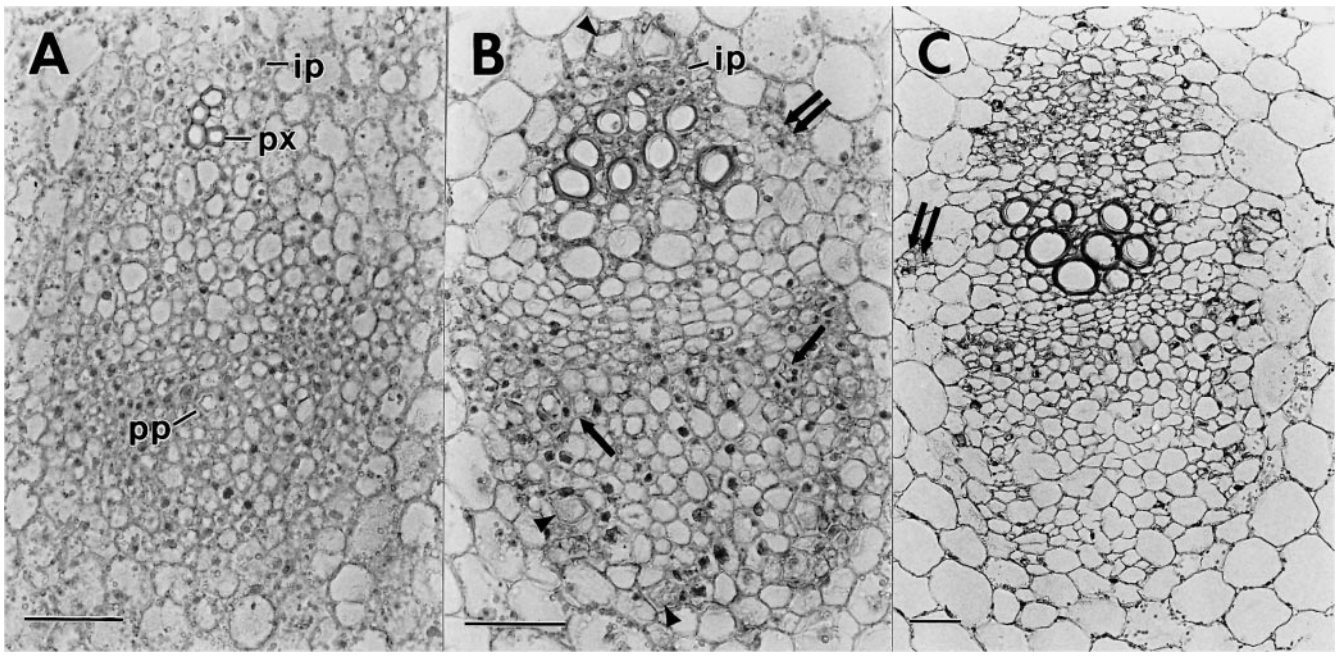


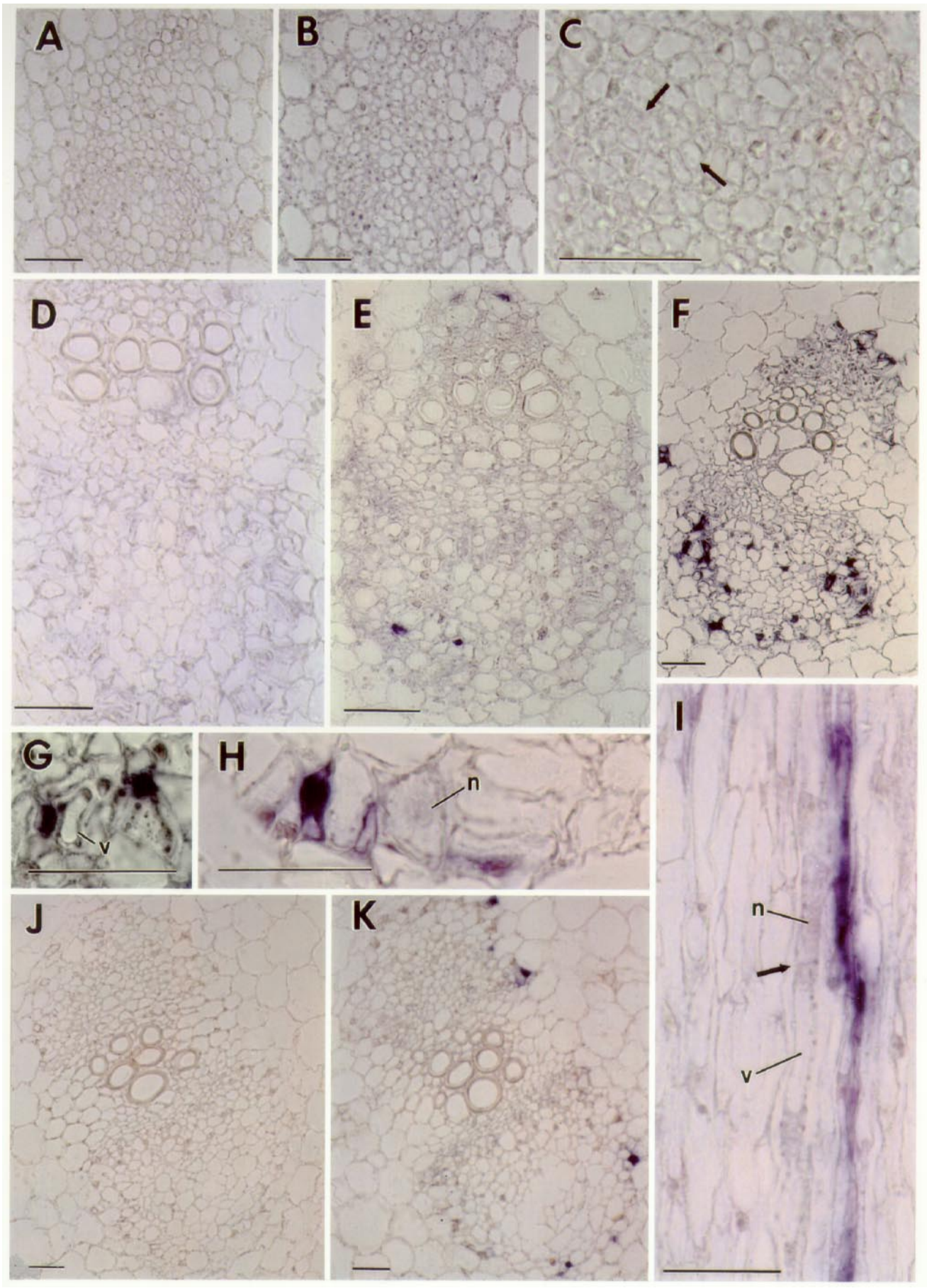

**Fig. 4A–C.** Light micrographs of vascular bundles in developing pumpkin hypocotyls. **A** Bundle of hypocotyl 1 DAG. Bundle is mostly procambial cells with several protoxylem elements (*px*) and developing protophloem elements (*pp*) in the external bundle phloem. Internal bundle phloem (*ip*) consists of several small cells. **B** Bundle of hypocotyl 3 DAG. Internal (*ip*) and external phloem portions of the vascular bundle have increased in size. Metaphloem sieve elements (often with P-protein bodies) and companion cells are differentiating within the external phloem (*arrows*). Extrafascicular phloem next to the bundle (*arrowheads*) have differentiating SE/CC but cortical extrafascicular strands lag behind in development (*double arrow*). **C** Bundle of hypocotyl 6 DAG. The bundle has large regions of internal and external bundle phloem and is bordered by extrafascicular phloem. Extrafascicular vascular strands occur within the cortex (*double arrow*). Bars = 50 μm

standard curves generated from in vitro-synthesized transcripts. The PP2 mRNA was initially detected at 3 DAG and increased over the next 7 d to a maximum of approx. 0.04% of total RNA at 10 DAG (Fig. 1A). At 10 DAG, hypocotyls had reached approx. 90% of their total length. Steady-state PP2 mRNA levels declined after 14 DAG; the amount of PP2 mRNA at 24 DAG was 30% of the maximal level.

The amount of PP2 in extracts from pumpkin hypocotyls was determined by comparing ELISA results with standard curves of purified PP2. The accumulation of PP2 paralleled the increase in its mRNA during the first 10 to 14 d of seedling development (Fig. 1B). Protein was initially detected at 4 DAG, lagging behind the appearance of the mRNA by approx. 1 d. In contrast to the decreasing levels of mRNA that were observed at 14 DAG, PP2 remained at maximal levels until 18 DAG followed by a 16% reduction during the last 6 d of the experiment.

The protein level that was maintained during the period of decreasing mRNA was indicative of protein stability. We examined the relative stability of P-proteins by SDS-PAGE of the pulse-labeled proteins in vascular exudates isolated from 10-DAG pumpkin seedlings. Radiolabeled proteins were not detected immediately following the pulse period, but could be detected within 4–6 h following the pulse (Fig. 2). Radioactivity incorporated into PP1 and PP2 accumulated during 12–24 h following the pulse and did not decrease during subsequent time points up to 72 h.

**Fig. 5A–I.** In situ hybridization of PP2 mRNA with digoxigenin-labeled PP2 sense and antisense riboprobes. **A–C** Hypocotyls 1–2 DAG. **A** Sense riboprobe control. **B** Antisense riboprobe. Transverse section of a whole bundle. **C** Antisense riboprobe. Transverse section of the external bundle phloem. At 1–2 DAG, PP2 mRNA was not detected in differentiating sieve elements (*arrows*). **D-F** *Hypocotyls 3 DAG showing two different developmental stages.* **D** Sense riboprobe control. **E** Antisense riboprobe. In a less developed bundle, PP2 mRNA was detected in a few companion cells within the internal bundle phloem and the extrafascicular phloem. **F** Antisense riboprobe. In more developed bundles, PP2 mRNA was detected in many companion cells of developing SE/CC. PP2 transcripts were detected within the external metaphloem, the internal phloem and the extrafascicular phloem both bordering the bundle and in the cortex. **G-I** *Hypocotyls 3 DAG, companion cell-specific PP2 mRNA accumulation.* **G** Antisense riboprobe. Transverse section of two SE/CC showing PP2 mRNA accumulation within the companion cells but not immature sieve elements. Both sieve elements have P-protein bodies; one has a degenerating nucleus and the other an intact vacuole (*v*). **H** Antisense riboprobe. Transverse section showing cortical extrafascicular phloem with labeled companion cells of SE/CC and sieve element with an intact nucleus (*n*). **I** Antisense riboprobe. Longitudinal section showing labeled companion cells and an immature sieve tube with individual elements with a nucleus (*n*) and vacuole (*v*). Arrow indicates developing sieve plate. **J, K** *Hypocotyls 6 DAG.* **J** Sense riboprobe control. **K** Antisense riboprobe. The bundle has abundant internal and external phloem and well-developed extrafascicular phloem. PP2 mRNA accumulates within the companion cells of SE/CC of all phloem types, most often in extrafascicular phloem bordering the bundle and within the cortex. Bars = 50 μm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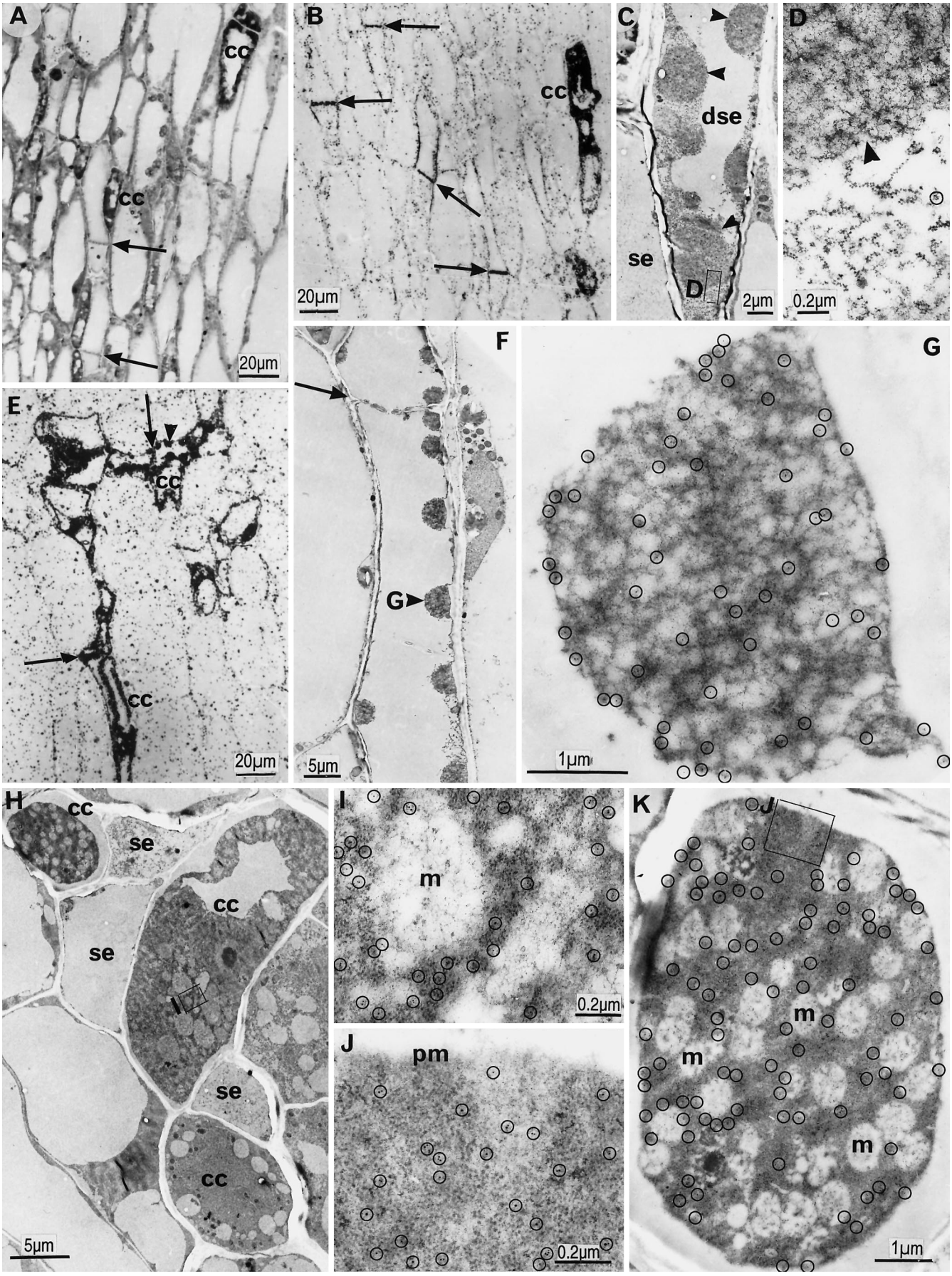
*Correlation of P-protein expression and phloem development.* Observations on expanding hypocotyls showed spatial differences in the developmental accumulation of PP2 mRNA (Fig. 3). Total RNA isolated from 1-cm segments of pumpkin hypocotyls at 4, 6, 8, and 12 DAG was probed with $^{32}$P-labeled PP2 cDNA and the relative percentage of PP2 mRNA in each segment was determined. In hypocotyls 4 DAG, PP2 mRNA was disproportionate in the segments, with the most transcripts occurring in the lowest segment where vascular tissue matures first. As hypocotyl elongation continued (6–8 DAG), the distribution of PP2 mRNA in the segments was skewed reflecting developmental differences in the vascular tissue from base to apex. PP2 mRNA levels in all segments were similar when hypocotyls had fully elongated (12 DAG).

To relate PP2 expression with phloem development in hypocotyls, we examined vascular differentiation in developing pumpkin seedlings from 0 to 24 DAG. The major stages of primary growth and bundle development occurred within the first 6 DAG. At 2 DAG when PP2 mRNA was not detectable, the vascular tissue consisted of procambial bundles containing few differentiated protoxylem and protophloem elements (Fig. 4A). By 3 DAG, when PP2 mRNA was first detected, the vascular bundles had increased in size and were structurally similar to all later stages (Fig. 4B). Within external bundle phloem, differentiating metaphloem SE/CC were apparent and often contained prominent P-protein bodies. The internal bundle phloem had become larger and contained differentiated SE/CC**.** Bundle-associated extrafascicular phloem was also differentiating; however, development of the cortical extrafascicular elements lagged behind. By 6 DAG, when PP2 mRNA levels had increased substantially, the vascular bundles were well developed with abundant metaphloem elements (Fig. 4C). Bundle-associated and cortical extrafascicular elements also had differentiated SE/CC. Maximum PP2 mRNA accumulation in the hypocotyl occurred about 10–12 DAG when the primary phloem was fully mature and the secondary phloem was differentiating.

*Companion cell-specific expression in SE/CC.* To determine the overall pattern of PP2 gene expression in the phloem tissue and specific patterns in individual SE/CC, PP2 mRNA was localized in pumpkin hypocotyl tissue by in situ hybridization during early periods of vascular development. PP2 transcripts were not detected in differentiating sieve elements of hypocotyls 1–2 DAG (Fig. 5A–C). By 3 DAG, PP2 mRNA was detected in companion cells of bundle and extrafascicular phloem, occurring more often in the bundle-associated extrafascicular phloem than in the bundle phloem (Fig. 5D–E). In hypocotyls with a slightly more developed vasculature, PP2 mRNA was detected in companion cells of the internal and external phloem, as well as the bundle-associated and cortical extrafascicular phloem (Fig. 5F). Immature sieve elements of the bundle phloem containing P-protein bodies did not accumulate PP2 mRNA. Immature sieve elements lacked PP2 transcripts even at early stages of differentiation when the vacuole and nucleus were still present (Fig. 5G–I). The vascular bundles in hypocotyls 6 DAG had developed abundant internal and external phloem with prominent bundle-associated and cortical extrafascicular sieve elements. PP2 mRNA accumulated specifically in the companion cells of SE/CC in all phloem types (Fig. 5J, 5K), although its distribution most often resembled later developmental stages where PP2 transcripts were most prevalent in the extrafascicular phloem (Bostwick et al. 1992).

*Immunolocalization of PP2 in SE/CC.* Immunolocalization of PP2 in squash stems showed different patterns of lectin accumulation in SE/CC of the bundle phloem compared with the extrafascicular phloem. In LM sections, sieve elements of the bundle phloem were identified by sieve plates and their association with companion cells (Fig. 6A). Immunolabeling of PP2 in bundle phloem showed light labeling at sieve plates and in a parietal position of mature sieve elements and dense labeling of companion cells in developing SE/CC (Fig. 6B). PP2 was not detected in the P-protein bodies or cytoplasm of immature sieve elements in the bundle phloem (Fig. 6C,D). In contrast, PP2 was detected in both sieve elements and companion cells of mature extrafascicular phloem. In addition to the cytoplasm of companion cells, sieve plates and the persistent P-protein bodies typical of extrafascicular sieve elements were densely labeled (Fig. 6E). In the extrafascicular phloem, PP2 was primarily limited to the persistent P-protein

**Fig. 6A–K.** Immunolocalization of PP2 in bundle, extrafascicular, and wound phloem. **A–D** *Bundle phloem.* **A** Light micrograph of bundle phloem stained with crystal violet. Sieve plates (*arrows*) and companion cells (*cc*) are shown. **B** Light micrograph of bundle phloem immunolabeled with PP2 antiserum visualized by silver-enhanced colloidal gold. Sieve plates (*arrows*) and parietal regions of mature sieve elements are lightly labeled and companion cells (*cc*) densely labeled. **C** Electron micrograph of a mature sieve element (*se*) with dispersed P-protein filaments and developing sieve element (*dse*) with P-protein bodies (*arrowheads*). **D** Higher magnification of the developing sieve element in C. Immunolabeling with 5-nm colloidal gold was not observed in either the cytoplasm or P-protein body (*arrowhead*) with the exception of a small amount of background (*circle*). **E-G** *Extrafascicular phloem.* **E** Light micrograph of sieve plates (*arrows*) and persistent P-protein bodies (*arrowhead*) of extrafascicular sieve elements and their companion cells (*cc*) densely labeled by PP2 antiserum. **F** Electron micrograph of mature extrafascicular sieve elements with sieve plate (*arrow*) and persistent P-protein bodies. The arrowhead labeled *G* indicates the P-protein body shown in panel **G. G** Colloidal gold particles (*circles*) labeled the filaments of the persistent P-protein body. **H-K** *Wound phloem 3 d after induction.* **H** Electron micrograph of wound phloem with irregularly shaped and sized sieve elements (*se*) and companion cells (*cc*) of wound phloem. **I** Higher magnification of the large companion cell in **H.** Colloidal gold particles (*circles*) are distributed over the cytoplasm with numerous ribosomes, but do not appear over the mitochondrion (*m*) or small vacuoles. **J** High magnification of a smaller companion cell (from K). Lectin is localized between numerous ribosomes, but not outside the plasma membrane (*pm*). **K** Upper companion cell from **H** with numerous mitochondria (*m*). Dense labeling (*circles*) of the cell except for mitochondria and cell wall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bodies (Fig. 6F,G). To examine PP2 accumulation in newly formed SE/CC, young wound-sieve elements were induced (Fig. 6H). Electron microscopic immunolocalization showed dense labeling of PP2 in the cytoplasm of companion cells between numerous ribosomes, while mitochondria and vacuoles were not labeled (Fig. 6I–K). Phloem protein filaments were dispersed in mature wound-sieve elements and were lightly labeled with PP2 antiserum (data not shown).

## Discussion

Phloem tissue in angiosperms has a unique cellular composition in which the conducting elements are a complex of two cell types that are developmentally related and functionally dependent upon one another. Structural details of SE/CC ontogeny have been well characterized for several decades, yet little is known about specific physiological interactions between these cells. Mature enucleate sieve elements lack ribosomes and apparently require companion cell cooperation for protein synthesis and cell viability. However, it is uncertain when these events occur. Traditionally, it was thought that proteins present during sieve element differentiation are synthesized within sieve elements rather than transported from companion cells. Transport from companion cells to sieve elements would occur as a secondary function for long-term maintenance of mature sieve elements following selective autophagy. This idea is supported by the findings of Fisher et al. (1992) who suggested that $^{35}$S-labeled proteins in wheat moved between companion cells and mature sieve elements along the translocation pathway. Alternatively, protein transport between differentiating sieve elements and companion cells could occur as plasmodesmatal connections are established. Either model supports the idea that these two cell types share intricate cell-to-cell communication throughout development and at maturity.

Synthesis and deposition of the phloem lectin in SE/CC provided an effective tool with which to investigate the interdependency of these two cell types. Expression of genes encoding PP2 appears to be developmentally regulated in the phloem of pumpkin hypocotyls. The pattern of PP2 gene expression we observed during the first 10–15 DAG was similar to that previously reported by Sham and Northcote (1987). During the period of hypocotyl elongation, PP2 accumulation paralleled the appearance of its mRNA, both reaching maximum levels at the end of hypocotyl elongation. Levels of PP2 mRNA in sequential 1-cm hypocotyl sections collected during early stages of elongation (4–6 DAG) correlated with seedling development, where the basal portion of the hypocotyl was the most developmentally advanced (Maksymowych and Orkwiszewski 1993). By the end of rapid seedling growth, PP2 mRNA levels were equally distributed throughout the hypocotyl. In contrast to Sham and Northcote's (1987) report of rapid decreases in PP2 mRNA within 3 d after maximal accumulation, our analyses showed PP2 mRNA gradually declined following maximal accumulation. As steady-state levels of PP2 mRNA decreased, protein concentrations remained high, alluding to the stability of these proteins.

P-proteins are generally considered to be stable, structural proteins (Sabnis and Sabnis 1995) and therefore, good candidates with which to study protein distribution in SE/CC. However, Nuske and Eschrich (1976) suggested that P-proteins were continually synthesized in companion cells of mature metaphloem and consequently had a relatively high turn-over rate. Hypocotyls of 14-d-old *C. maxima* seedlings were labeled in vivo during developmental stages where both PP2 mRNA and protein were present at very high concentrations. However, the extended labeling period (12 h) and lack of a chase period in the Nuske and Eschrich experiments precluded assessment of protein stability. The results of our pulse-labeling experiments showed no reduction in labeled proteins during the 24- to 72-h chase periods, demonstrating stability of the major P-proteins consistent with their designation as structural proteins (Sabnis and Sabnis 1995).

Expression of phloem lectin genes was tightly linked to vascular differentiation in pumpkin hypocotyls. At the earliest stages of seedling development (1–2 DAG), vascular bundles in hypocotyls were primarily procambial cells with several differentiating protophloem elements. We were unable to detect PP2 mRNA by in situ hybridization, dot blot analysis or reverse-transcriptase polymerase chain reaction (data not shown) at these developmental stages. Thus, genes encoding the phloem lectin do not appear to be transcribed in either determined meristematic tissue or protophloem sieve elements. As the bundle phloem continued to develop and metaphloem began to differentiate, PP2 mRNA was detected in companion cells, but not in immature sieve elements. Since protophloem sieve elements generally lack companion cells, the companion cell-specific pattern of gene expression observed at all subsequent developmental stages could explain why PP2 mRNA was not detected in the earliest differentiated vascular elements.

Coincident with differentiation of bundle metaphloem, the extrafascicular phloem began to differentiate, initially in the arcs bordering the bundle and, slightly later, in the cortex. During rapid hypocotyl elongation (3–6 DAG), PP2 mRNA was easily detected within internal and external bundle phloem and extrafascicular phloem. This was a time of active metaphloem differentiation. However, as hypocotyl development continued, hybridization of PP2 mRNA within the bundle phloem became more rare while it was easily detected in the extrafascicular phloem. The apparent decrease in the number of cells of the bundle phloem where PP2 mRNA was detected could be related to the onset of translocation in mature sieve elements. Recently, Toyama et al. (1995) showed rapid decreases in mRNA levels of a PP2-like lectin in etiolated cucumber cotyledons in response to cytokinin applications, suggesting that lectin gene expression may be down-regulated in response to hormonal cues. Regulatory molecules translocated in mature sieve elements of the bundle phloem, not encountered in the extrafascicular

phloem, could differentially affect transcription in the two phloem types.

PP2 mRNA was limited to companion cells at all stages of vascular development in pumpkin hypocotyls. This is in contrast to the traditional view of P-protein synthesis where the entire process was thought to occur in immature sieve elements or in companion cells of mature SE/CC (Cronshaw and Esau 1968a; Nuske and Eschrich 1976). Immunolocalizing PP2 to companion cells in developing bundle phloem demonstrated that initial protein accumulation also occurs in companion cells. Inducing wound phloem provided an additional avenue to examine PP2 accumulation in SE/CC. The urgent need to reconnect disrupted vascular bundles should result in expression of essential proteins, possibly at reduced concentrations due to abbreviated developmental periods (Schulz 1990). Less filamentous P-protein was observed in wound-sieve elements of squash as compared with either bundle or extrafascicular sieve elements; however, PP2 labeling in companion cells appeared as dense as the bundle phloem. Similar to PP2 expression in developing hypocotyls, the presence of PP2 in the companion cells of the wound phloem appeared to be correlated with early events in vascular development.

Although PP2 synthesis is restricted to companion cells, the protein appears to be ultimately deposited within mature sieve elements of both the bundle and extrafascicular phloem (Smith et al. 1987). Read and Northcote (1983b) presented a structural model for P-protein filaments based on relatively simple interactions between the phloem lectin and the phloem filament protein. However, the different PP2 localization patterns that we observed in the bundle and extrafascicular phloem suggest that these interactions are more complex. Typically, the large fibrillar P-protein bodies disperse forming filaments in maturing bundle sieve elements, whereas P-protein bodies in differentiated extrafascicular sieve elements remain aggregated (Cronshaw and Esau 1968b; Evert et al. 1973). Although companion cells of the immature bundle phloem were heavily labeled with the PP2 antibody, the large filamentous P-protein bodies in developing sieve elements of the bundle phloem were not immunolabeled. The lack of immunolabel in immature bundle phloem suggests that PP2 is not a component of dispersing P-protein bodies. However, in the extrafascicular phloem, PP2 was easily detected in both companion cells and persistent P-protein bodies of the sieve elements. These differences may reflect different physiological functions of the two types of phloem. The dispersal of P-protein bodies to form phloem filaments appears to be a significant event that is coordinated with the functional maturity of sieve elements and onset of long-distance translocation of assimilates within bundle phloem (Cronshaw and Esau 1968b). The phloem lectin may accumulate within companion cells prior to the disruption of P-protein bodies at which time the protein is transported into the maturing sieve element where PP2 cross-links with existing phloem filaments. In extrafascicular phloem, PP2 cross-linking phloem filaments in P-protein bodies might prevent their dispersal.

Current models for macromolecular trafficking between cells suggest that intercellular protein movement via plasmodesmata is a highly regulated process (Lucas 1995). The data presented here indicate temporal and spatial separation of the phloem lectin from phloem filament proteins during differentiation of the SE/CC in bundle phloem. Elucidation of the mechanism of phloem lectin movement from companion cells to sieve elements during sieve element maturation may be key to understanding the coordinate development of these two cell types. Deposition of P-protein during phloem development is clearly a dynamic process that may involve novel regulatory mechanisms of protein trafficking between companion cells and sieve elements.

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## References

Allen AK (1979) A lectin from the exudate of the fruit of the vegetable marrow (*Cucurbita pepo*) that has a specificity for a β-1,4-linked *N*-acetylglucosamine oligosaccharide. Biochem J 183: 133–137

Bostwick DE, Dannenhoffer JM, Skaggs MI, Lister RM, Larkins BA, Thompson GA (1992) Pumpkin phloem lectin genes are specifically expressed in companion cells. Plant Cell 4: 1539–1548

Bostwick DE, Skaggs MI, Thompson GA (1994) Organization and characterization of *Cucurbita* phloem lectin genes. Plant Mol Biol 26: 887–897

Blyth A (1958) Origin of primary extraxylary stem fibers in dicotyledons. Univ Cal Berkeley Publ Bot 30: 145–232

Cathala G, Savouret JF, Mendez B, West BL, Karin M, Martial JA, Baxter JD (1983) Laboratory methods: a method for isolation of intact translationally active ribonucleic acid. DNA 2: 329–335

Crafts AS (1932) Phloem anatomy, exudation and transport of organic nutrients in cucurbits. Plant Physiol. 7: 183–225

Cronshaw J, Esau K (1968a) P-Protein in the phloem of *Cucurbita.* I. The development of P-protein bodies. J Cell Biol 38: 25–39

Cronshaw J, Esau K (1968b) P-Protein in the phloem of *Cucurbita.* II. The P-protein of mature sieve elements. J Cell Biol 38: 292–303

Eschrich W (1975) Sealing systems in phloem. In: Zimmerman MH, Milburn JA (eds) Encyclopedia of plant physiology, NS vol 1: Transport in plants, 1. Phloem transport. Springer, Berlin, pp 39–56

Evert RF, Eschrich W, Eichhorn SE (1973) P-protein distribution in mature sieve elements of *Cucurbita maxima*. Planta 109: 193–210

Fischer A (1884) Untersuchungen über das Siebröhrensystem der Cucurbitaceae. Gebrüder Borntraeger, Berlin

Fisher DB, Wu Y, Ku MSB (1992) Turnover of soluble proteins in the wheat sieve tube. Plant Physiol 100: 1433–1441

Harlow E, Lane D (1988) Antibodies: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp 309–311

Kleinig M, Thones J, Dorr I, Kollmann R (1975) Filament formation in vitro of sieve tube protein from *Cucurbita maxima* and *Cucurbita pepo*. Planta 127: 163–170

Lucas WJ (1995) Plasmodesmata: intercellular channels for macromolecular transport in plants. Curr Opin Cell Biol 7: 673–680

Maksymowych R, Orkwiszewski JA (1993) The role of nodal regions in growth of *Xanthium* (Compositae) stem. Am J Bot 80: 1318–1322

Nuske J, Eschrich W (1976) Synthesis of P-protein in mature phloem of *Cucurbita maxima*. Planta 132: 109–118

Read SM, Northcote DH (1983a) Subunit structure and interactions of the phloem proteins of *Cucurbita maxima* (pumpkin). Eur J Biochem 134: 561–569

Read SM, Northcote DH (1983b) Chemical and immunological similarities between the phloem proteins of three genera of *Cucurbitaceae.* Planta 158: 119–127

Sabnis DD, Hart JW (1978) The isolation and some properties of a lectin (haemagglutinin) from *Cucurbita* phloem exudate. Planta 142: 97–101

Sabnis DD, Sabnis HM (1995) Phloem proteins: structure, biochemistry and function. In: Iqbal M (ed) The cambial derivatives. Gebrüder Borntraeger, Berlin, pp 271–292

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, 2nd edn Cold Spring Harbor, NY

Schulz A (1986) Wound phloem in transition to bundle phloem in primary roots of *Pisum sativum* L. II. The plasmatic contact between wound-sieve tubes and regular phloem. Protoplasma 130: 27–40

Schulz A (1990) Wound-sieve elements. In: Behnke H-D, Sjolund RD (eds) Sieve elements: comparative structure, induction and development. Springer, Berlin, pp 199–217

Schulz A, Alosi MC, Sabnis DD, Park RB (1989) A phloem-specific, lectin-like protein is located in pine sieve-element plastids by immunocytochemistry. Planta 179: 506–515

Sham M-H, Northcote DH (1987) Transcription and translation of phloem protein (PP2) during phloem differentiation in *Cucurbita maxima.* Planta 170: 392–399

Sharman BC (1943) Tannic acid and iron alum with safranin and orange G in studies of the shoot apex. Stain Technol 18: 105–111

Smith LM, Sabnis DD, Johnson RPC (1987) Immunochemical localisation of phloem lectin from *Cucurbita maxima* using peroxidase and colloidal-gold labels. Planta 170: 461–470

Toyama T, Teramoto H, Takeba G, Tsuji H (1995) Cytokinin induces a rapid decrease in the levels of mRNAs for catalase, 3-hydroxy-3-methylglutaryl CoA reductase, lectin and other unidentified proteins in etiolated cotyledons of cucumber. Plant Cell Physiol 36: 1349–1359