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Phloem-specific expression of the pumpkin fruit trypsin inhibitor

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Abstract. Vascular exudates of *Cucurbita maxima* (Duch.) contain a group of highly conserved serine proteinase inhibitors collectively called Pumpkin Fruit Trypsin Inhibitors (PFTIs) that prevent proteolytic activity of trypsin or chymotrypsin. Polyclonal antibodies raised against PFTIs were used to immunolocalize these low-molecular-weight proteins within the phloem tissue and to study their developmental expression. The inhibitors were translocated throughout the transport phloem and were present in vascular exudates collected from both source and sink tissues throughout the plant. During the early stages of vascular development, PFTIs accumulated specifically in sieve element–companion cell complexes of the phloem tissue. Transcripts were initially detected by reverse transcription-polymerase chain reaction in seedlings 1 d after germination and the protein detected 24 h later. Pumpkin fruit trypsin inhibitors were present in both cell types of differentiating and translocating sieve element–companion cell complexes. The inhibitors were detected in the phloem of the bicollateral vascular bundles, but the protein was most consistently localized within the cortical and bundle-associated extrafascicular phloem.

Key words: Companion cell – *Cucurbita* – Phloem protein – Sieve element – Vascular differentiation

Introduction

The long-distance transport system within the phloem tissue is composed of an intimately associated complex of two cell types, sieve elements and companion cells. During development, individual sieve elements undergo selective cytoplasmic degradation, losing their capacity for transcription and translation, to form a continuous cellular conduit throughout the plant body. Considerable molecular, developmental, and structural evidence supports the role of companion cells in providing proteins that accumulate within mature sieve elements (Thompson and Schulz 1999). The available evidence indicates that some of these proteins symplasmically traffic from the companion cells to sieve elements via pore-plasmodesmata connections (Balachandran et al. 1997) and are translocated in the assimilate stream (Golecki et al. 1998, 1999; Xoconostle-Cázares et al. 1999). More than 100 soluble proteins are found in sieve element exudates (Fisher et al. 1992; Nakamura et al. 1993; Sakuth et al. 1993), including several low-molecular-weight proteinase inhibitors.

Regulating protein turnover is a potentially important developmental and functional feature of the phloem. Although little is known about phloem-specific proteases, several different classes of proteinase inhibitors have been identified in vascular exudates from various plant species. Cysteine protease inhibitors appear to be present in exudate from *Ricinus communis* that are not immunologically detected in exudates from other species (Schobert et al. 1998). Trypsin- and chymotrypsin-type serine proteinase inhibitors and an aspartic proteinase inhibitor have been identified in vascular exudates of cucurbit fruits (MacGibbon and Mann 1986; Murray and Christeller 1995; Christeller et al. 1998; Kehr et al. 1999). In addition, a Kunitz-type serine proteinase inhibitor, commonly found in storage organs, has also been immunolocalized in sieve elements of winged bean (Habu et al. 1996). The purpose of these proteinase inhibitors in the physiology of the phloem is unknown. Defensive roles in response to insect herbivory and pathogenic infection (MacGibbon and Mann

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Abbreviations: DAG = days after germination; DAPI = 4′,6-diamidino-2-phenylindole, dihydrochloride; DIG = digoxigenin; PFTI = pumpkin fruit trypsin inhibitor; PCR = polymerase chain reaction; PP2 = phloem protein 2; P-protein = phloem protein; RT = reverse transcription

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1986) as well as key regulatory roles in sieve element differentiation (Habu et al. 1996) have been hypothesized.

Initial studies on the serine proteinase inhibitors in vascular exudates of cucurbits were undertaken to assess their potential to inhibit proteases of insects and fungal pathogens. In each study, a group of inhibitors was identified by inhibition assays using trypsin. MacGibbon and Mann (1986) identified five distinct bands by native-PAGE with trypsin-inhibitor activity in exudates collected from pumpkin fruits. Major inhibitor activity was limited to a single protein band of less than 6,000 Da on SDS-PAGE. In contrast, Chino et al. (1991) found four closely spaced bands between 46 and 50 kDa and a zone of less than 5,000 Da on negatively stained SDS-PAGE. The low-molecular-weight proteins appeared to have both trypsin and chymotrypsin inhibitor activity, whereas, the higher-molecular-weight proteins only had trypsin inhibitor activity. The low-molecular-weight pumpkin fruit trypsin inhibitor (PFTI) was purified to homogeneity, partially sequenced, and several cDNA clones were isolated by degenerate polymerase chain reaction (PCR; Murray and Christeller 1995). Two of the cDNAs (*Bm7* and *Pr10*) putatively encode chymotrypsin inhibitors, whereas a single cDNA (*Af4*) encodes a trypsin inhibitor. The 67-amino-acid deduced proteins are 92.5–94% identical and have calculated molecular weights between 7,521 and 7,622 Da. They all contain the highly conserved sequences WPEL and P_1-DxRxxRVR typical of the Proteinase Inhibitor 1 family.

The low-molecular-weight PFTIs are present in vascular exudates isolated from cucurbit fruits and seedling stems. However, proteinase inhibitors are found in many plant parts, particularly in storage organs such as seeds and fruits. In this study, we examined the tissue and cellular location of PFTIs to determine if the proteins are limited to the phloem tissue. The genes encoding PFTIs are expressed at the earliest stages of seedling development and immunolocalization experiments showed that the proteins are limited to the sieve element–companion cell complexes.

Materials and methods

Probes and standards. The PCR digoxigenin (DIG) Probe synthesis kit (Boehringer Mannheim, Indianapolis, Ind., USA) was used to generate DNA probes from *Cucurbita* cDNAs encoding phloem protein 2 (*PP2*) (Z17331; Bostwick and Thompson 1993), *PFTI* (X81447; Murray and Christeller 1995), and *actin* (Golecki et al. 1999). The PFTI cDNA (clone *Bm7*) and polyclonal antiserum were generously provided by Dr. John Christeller of the Horticulture and Food Research Institute of New Zealand.

Plant material and collection of vascular exudate. *Cucurbita maxima* Duch. cv. Big Max seeds were germinated on filter paper at 22 °C then planted in vermiculite or 1:3 perlite:soil-less potting mixture. Plants were watered with a 1:200 dilution of 20:20:20 fertilizer and grown at 20–24 °C with a 12-h photoperiod. Vascular exudate was collected from various plant parts according to specific experiments in four volumes of cold buffer [100 mM Tris-HCl, pH 8.2; 5 mM EDTA; 20 mM dithiothreitol (DTT); 1 mM phenylmethylsulfonyl fluoride (PMSF)]. Protein concentration was determined by the Bradford assay (Bio-Rad Laboratories, Hercules, Calif., USA) using bovine serum albumin (BSA) as the standard.

Analysis by SDS-PAGE and immunoblotting. Vascular exudate proteins (30 μg) were separated by SDS-PAGE in 18.5% gels (Laemmli 1970). After electrophoresis, proteins were either stained with Coomassie Brilliant Blue R250 and destained, or the proteins were transferred onto PVDF membrane (Immobilon-P; Millipore, Bedford, Mass., USA) by electroblotting in a Mini TransBlot-System (BioRad) using a Tris-glycine buffer (Towbin et al. 1979). Immunodetection procedures followed the manufacturer's instructions for chemiluminescent detection with CDP-Star (Tropix, Bedford, Mass., USA). Briefly, membranes were blocked for 1 h with blocking buffer [0.2–3.0% I-Block in phosphate-buffered saline (PBS; 137 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) with 0.1% Tween 20] and then incubated for 1 h with 1:5,000 or 1:10,000 (v/v) rabbit polyclonal anti-PFTI or rabbit non-immune serum in blocking buffer. After three washes in blocking buffer, membranes were incubated in 1:5,000 or 1:10,000 goat anti-rabbit serum conjugated to alkaline phosphatase (GAR-AP) for 1 h, washed in blocking buffer and equilibrated in assay buffer (100 mM diethanolamine, pH 10; 1 mM $MgCl_2$). Finally, blots were incubated in 0.25 mM CDP-Star in assay buffer for 5 min and signal was detected with Kodak X-Omat LS film.

Reverse transcription-PCR and gel blot analysis of RT-PCR products. Total RNA was extracted from hypocotyl tissues using the method of Carpenter and Simon (1998). Oligonucleotide primers were designed flanking the protein coding region of the PFTI clone *Bm7*: 5′ primer (5′-GAGCTCGAGATGGCAGA-AAAGTCGT-3′) and 3′ primer (5′-GCGAAGCTTTTAGC-CAATTCGAGGG-3′). Control reactions were performed using oligonucleotide primers designed from the *PP2* and *actin* sequences described in Golecki et al. (1999). One microgram of total RNA was denatured at 65 °C for first-strand synthesis using 3′ oligonucleotide primers specific for *PFTI*, *PP2*, or *actin* mRNA and the First-strand cDNA synthesis kit (Pharmacia Biotech, Piscataway, NJ, USA). Subsequent PCR amplification of the cDNA was as described by Bostwick et al. (1994). The RT-PCR products were electrophoresed in 1.5 or 2.0% agarose gels, transferred to nylon membrane (Hybond, Amersham, Arlington Heights, IL., USA) with 10 × SSC (1 × SSC = 150 mM NaCl, 15 mM sodium citrate), and probed with either DIG-labeled *PFTI*, *PP2* or *actin* probes, respectively. Blots were hybridized overnight in 5 × SSC, 1% w/v DIG-block, 0.1% N-lauroylsarcosine, 0.02% SDS at 68 °C. Blots were washed twice, 5 min, in a low-stringency wash (2 × SCC with 0.1% SDS) at room temperatue and once for 30 min in a high-stringency wash (0.1 × SSC with 0.1% SDS) at 68 °C. Blots were blocked with blocking buffer for 1 h, incubated with 1:10,000 anti-DIG-AP for 1 h, then rinsed and developed as described above for the immunoblots.

Tissue fixation for immunocytochemistry. Hypocotyl tissue was harvested at 1–6 days after germination (DAG) into fixative solution [2% w/v formaldehyde, 0.5% (v/v) glutaraldehyde in 50 mM sodium phosphate buffer pH 7.0]. Tissue was fixed for 2 h at room temperature then washed in buffer, dehydrated in an ethanol/tertiary butyl alcohol series and embedded in paraffin. Tissue was sectioned at 10 μm and adhered to slides with 100 mM poly-L-lysine. Sections were dewaxed with xylene and hydrated through an ethanol series.

Immunocytochemistry. After a brief wash in TBST (20 mM Tris-HCl, pH 7.4; 500 mM NaCl; 0.15% Tween 20), sections were incubated in blocking solution (5% goat normal serum, 2% BSA in TBST) overnight at 4 °C. Sections were then incubated in 1:500–1:1,000 anti-PFTI or non-immune serum in 0.5 × blocking solution overnight at 4 °C. After three washes in TBST, sections were incubated in 1:500–1:1,000 GAR-Cy3 (Jackson ImmunoResearch, West Grove, Pa., USA) in 0.5 × blocking solution for 2–4 h at

room temperature. To show the presence of nuclei, sections were stained in 0.4 mg/ml DAPI (4′,6-diamidino-2-phenylindole, dihydrochloride) for 5 min. Sections were analyzed and photographed using a Zeiss Axiophot microscope equipped with a Spot digital camera.

Long-distance transport of PFTIs. Seedlings of *Cucumis sativus* cv. Hoffmanns Produkta and *Cucurbita maxima* cv. Gelber Zentner were grown and grafted by the approach technique as described by Golecki et al. (1998). *Cucumis sativus* was used as the scion and *Cucurbita maxima* as the rootstock in five grafted plants. Vascular exudate samples were collected from scion and stock of 29-d-old *Cucumis sativus* and *Cucurbita maxima* approach grafts as described by Golecki et al. (1999). For immunoblot analysis, vascular exudates were diluted 1:4 in extraction buffer (0.1 M Tris, pH 8.2, 5 mM EDTA, and 20 mM DTT). Methods for protein quantification, electrophoresis, transfer, and immunological detection were as previously described. Blots were incubated overnight with *Cucurbita maxima* PFTI polyclonal antibodies (1:2500 v/v) and GAR-AP (1/5000 v/v).

Results and discussion

The pumpkin fruit trypsin inhibitors (PFTIs) are a small group of highly conserved serine proteinase inhibitors that are easily isolated from cucurbit vascular exudates. Specificity of the PFTI polyclonal antiserum was determined on immunoblots of proteins present in vascular exudates (Fig. 1A, lane 1) collected from pumpkin stems 25 DAG. Anti-PFTI serum detected proteins (Fig. 1B, lanes 4, 5) in vascular exudates comigrating with the 6.2-kDa marker, whereas, the nonimmune rabbit serum failed to cross-react with exudate proteins (Fig. 1B, lanes 2, 3). When separated in an 18.5% SDS-polyacrylamide gel, PFTIs appeared slightly smaller in size than M_r 8,100 that was determined by comigration with potato proteinase inhibitor I (Murray and Christeller 1995) or M_r 7,500 determined by mass spectroscopy (Kehr et al. 1999). In addition, there appeared to be some minor size heterogeneity among this group of highly conserved proteins. Immunoblots of proteins in vascular exudates collected from different plant parts (Fig. 1C) revealed the presence of PFTIs in all locations sampled. These proteinase inhibitors were detected in exudates from the transport phloem of hypocotyls and petioles of fully expanded leaves (Fig. 1D, lanes 6, 7); from phloem exporting from source tissues, including the main veins of the cotyledons and midribs of fully expanded leaves (Fig. 1D, lanes 8, 9); and from phloem importing to the shoot apex (Fig. 1D, lane 10). The number and quantity of the major vascular exudate proteins in all parts was similar to the typical protein profile of hypocotyl tissue (Fig. 1A, lane 1).

The presence of trypsin and chymotrypsin inhibitors in vascular exudates indicates that these serine proteinase inhibitors are soluble proteins in the translocation stream. Intergeneric approach grafts consisting of *Cucurbita maxima* rootstocks and *Cucumis sativus* scions were used to determine whether PFTIs are translocated over long distances in the plant. Vascular exudates collected from ungrafted *Cucurbita maxima* (Fig. 2A, lane 1) and *Cucumis sativus* plants (Fig. 2A, lane 4) showed the expected variation in the protein profiles when exudate proteins were separated by SDS-PAGE. Similar to other cucurbit phloem proteins, the proteinase inhibitors demonstrated substantial intergeneric divergence between *Cucurbita* and *Cucumis species*. Polyclonal antisera against *Cucurbita maxima* PFTI readily detected the proteinase inhibitor on immunoblots of total vascular exudate proteins collected from *Cucurbita maxima* control plants (Fig. 2B, lane 1), whereas the proteins were not detected among total vascular exudate proteins collected from *Cucumis sativus* control plants (Fig. 2B,

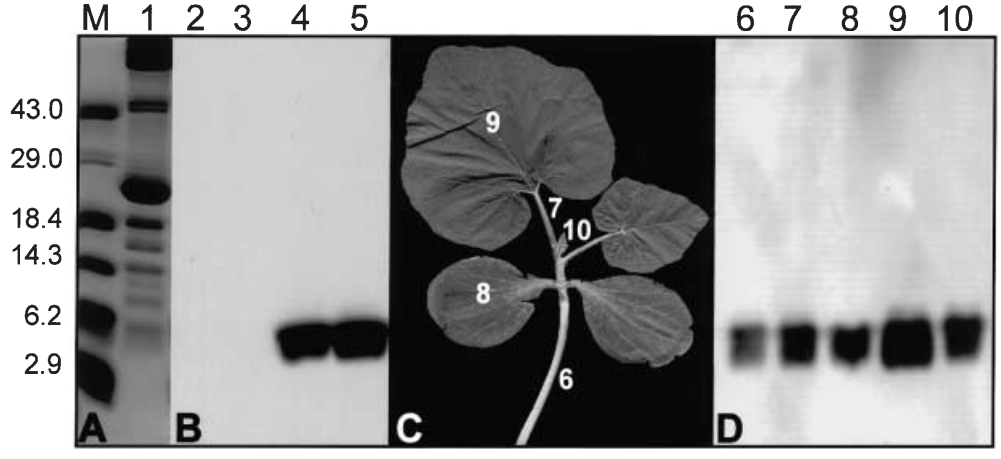


Fig. 1A–D. Pumpkin fruit trypsin inhibitors (PFTIs) were detected in vascular exudates collected throughout the plant. **A** Coomassie blue-stained SDS-polyacrylamide gel of total vascular exudate proteins (30 μg) isolated from *Cucurbita maxima* stems (25 DAG) (*lane 1*). *M* indicates molecular mass markers (kDa). **B** Immunoblot of total vascular exudate proteins (3 μg and 10 μg) reacted with rabbit nonimmune serum (*lanes 2*, *3*) and polyclonal antibodies raised against PFTI (*lanes 4*, *5*). **C** Five collection points for vascular exudates in a *Cucurbita maxima* plant: transport phloem of the hypocotyl (*6*) and petiole (*7*); transport phloem exporting from a cotyledon (*8*) and fully expanded leaf (*9*); transport phloem importing to a sink tissue (*10*). **D** Immunoblot of vascular exudates collected from the different plant parts described in **C** and probed with polyclonal antibodies raised against PFTI. The number and quantity of the proteins in all parts were similar to the protein profile of hypocotyl tissue (**A**, *lane 1*)

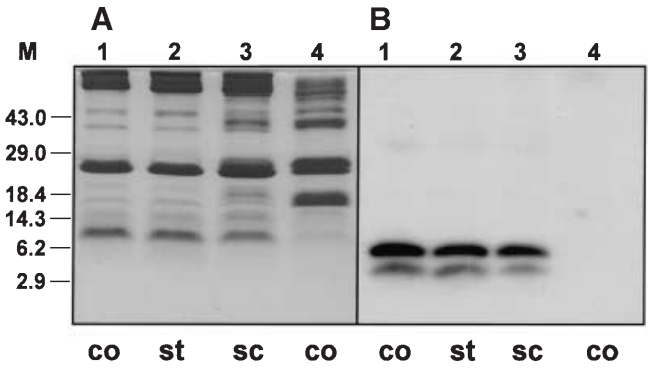


Fig. 2A,B. *Cucurbita maxima* PFTIs were translocated to *Cucumis sativus* scions in intergeneric grafted plants. **A** Coomassie blue-stained SDS-PAGE of total vascular exudate proteins isolated from the *Cucurbita maxima* ungrafted control (*co*: *lanes 1*) or rootstock (*st*: *lanes 2*) and *Cucumis sativus* scion (*sc*: *lanes 3*) or ungrafted control (*co*: *lanes 4*). *M* indicates molecular mass markers (kDa). **B** Immunoblots of a duplicate gel reacted with polyclonal antibodies raised against *Cucurbita maxima* PFTI

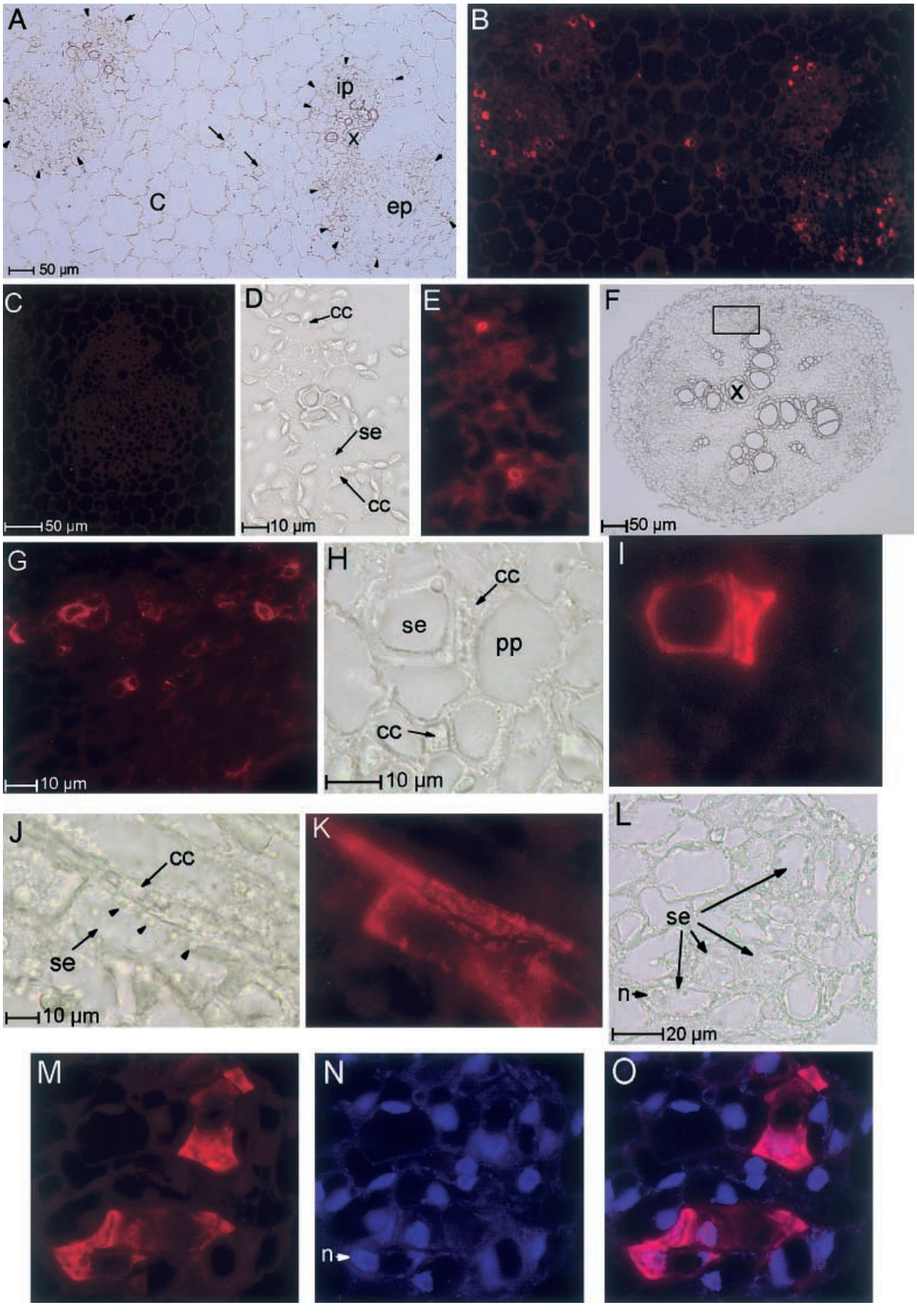
lane 4). The results from five grafted plants were identical (data shown for a single plant); the polyclonal antibodies detected the proteinase inhibitors in both the *Cucurbita maxima* stock (Fig. 2B, lane 2) and the *Cucumis sativus* scion (Fig. 2B, lane 3), providing evidence that these proteins are indeed translocated.

Immunocytochemistry was used to determine the tissue and cellular location of the proteinase inhibitors in the vegetative organs of the plant (stem/hypocotyl, leaf, and root). In stem sections, PFTIs were present in the phloem and did not occur in the xylem, cortical parenchyma, or epidermal tissues (Fig. 3A,B). Structurally, phloem in the stem occurs in four distinct areas: the internal and external fascicular phloem of the bicollateral vascular bundle and the bundle-associated and cortical extrafascicular phloem. Pumpkin fruit trypsin inhibitors were detected in the fascicular phloem as well as the bundle-associated (arrowheads) and cortical (arrows) extrafascicular phloem. Non-immune serum used as a control showed only background fluorescence (Fig. 3C). In mature leaf sections, PFTIs were detected in the phloem of major and minor veins and were present in both the adaxial and abaxial phloem of the bicollateral bundle (Fig. 3D,E). Mesophyll and epidermal tissues showed only background fluorescence. Likewise in the root, PFTIs were detected in the phloem tissue surrounding the central core of xylem (Fig. 3F,G) but not the xylem or epidermal tissues.

The phloem tissue consists of at least three cell types: sieve element, companion cell and phloem parenchyma. Within the phloem tissue of the vascular bundles, PFTIs were detected in sieve elements and companion cells but not in phloem parenchyma cells (Fig. 3H,I). Similarly, in the extrafascicular phloem, PFTIs were also detected in both cell types of the sieve element–companion cell complex (Fig. 3J,K). Although sometimes the proteinase inhibitor was localized in only one or the other cell type, within each cell type there was a characteristic staining pattern. Typically PFTIs were located within the parietal cytoplasm of the sieve element while the companion cell had signal diffusely located throughout the cytoplasm (Fig. 3H–K). Within sieve elements, fluorescence was detected coincident with the presence of phloem protein (P-protein) bodies (Fig. 3J,K). In differentiating sieve elements, both PFTIs and DAPI-staining nuclei were present showing PFTIs accumulate during early stages of development when sieve elements are nucleate prior to selective autophagy of the organelles (Fig. 3L–O).

Hypocotyls of pumpkin seedlings 1–6 DAG show a continuous developmental series from procambium through differentiated metaphloem of mature primary vascular bundles to bundles with the first secondary phloem elements (Dannenhoffer et al. 1997). Throughout the entire 6 d of development, *PFTI* mRNA was detected by RT-PCR and DNA blot hybridization in pumpkin hypocotyls (Fig. 4). In contrast, transcripts of the well-characterized phloem lectin (PP2) were not detected in hypocotyls until 3 DAG. Previous studies have shown that the initial detection of *PP2* mRNA correlates with the differentiation of metaphloem sieve element–companion cell complexes and that the protein was detected by enzyme-linked immunosorbent assay (ELISA) 1 d later (Dannenhoffer et al. 1997). Pumpkin fruit trypsin inhibitors were immunolocalized in vascular bundles of seedling hypocotyls as young as 2 DAG (Fig. 5B). The 1-d difference in the immunological detection of the proteins, PFTI and PP2, from the detection of their respective mRNAs may simply be due to the difference in sensitivity of the detection methods. Initial gene expression and protein accumulation of PFTIs was several days different than the initial gene expression and protein accumulation of the phloem

Fig. 3A–O. Pumpkin fruit trypsin inhibitors were present in sieve elements and companion cells throughout the plant. **A, B** Brightfield and fluorescent image pair of a 6-DAG hypocotyl section reacted with polyclonal antibodies raised against PFTI. Bundle phloem occurred in two patches around the xylem (*x*), as internal phloem (*ip*) and as external phloem (*ep*). Extrafascicular phloem (*arrowheads*) surrounded the bundle and cortical extrafascicular phloem (*arrows*) was dispersed throughout the cortex (*c*). Bar = 50 μm. **C** Non-immune rabbit serum control showed only background fluorescence. Bar = 50 μm. **D, E** Brightfield and fluorescent image pair of transverse section of bicollateral vein from a mature leaf. Pumpkin fruit trypsin inhibitors were present in both abaxial and adaxial phloem usually in companion cells (*cc*) and only occasionally in sieve elements (*se*). Bar = 10 μm. **F, G** Brightfield and fluorescent images of root section. Box in **F** indicates area seen in **G**. Sieve elements and companion cells of the root contained PFTIs. **F** Bar = 50 μm **G** Bar = 10 μm. **H, I** Brightfield and fluorescent image pair of transverse section of external bundle phloem. Pumpkin fruit trypsin inhibitors were present in sieve elements and companion cells but not in phloem parenchyma cells (*pp*). Bar = 10 μm. **J, K** Brightfield and fluorescent image pair of longitudinal section of extrafascicular phloem. Pumpkin fruit trypsin inhibitors occurred in sieve elements with P-protein bodies (*arrowheads*) but were not within the P-protein bodies themselves. Bar = 10 μm. **L–O** Co-localization of PFTIs and nuclei (*n*) within a single stem section that was dual-stained with anti-PFTI and DAPI. Bar = 20 μm. **L** Brightfield image. **M** Fluorescent image of PFTI staining. **N** Fluorescent image of DAPI staining. **O** Merged images of **M** and **N**. Both PFTIs and nuclei were present within sieve elements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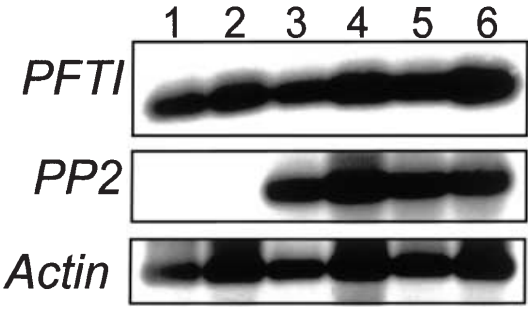

Fig. 4. Transcripts of PFTIs were detected at the earliest stages of vascular development in pumpkin seedling hypocotyls. The RT-PCR products were generated from total RNA (1 μg) with 5′ and 3′ primers flanking the protein coding sequences of genes encoding *PFTI*, *PP2*, and *actin*. *Lanes 1–6* correspond to the age (DAG) of the pumpkin seedling hypocotyls used for RNA isolation. The RT-PCR products were electrophoresed, blotted, and hybridized with digoxigenin-labeled cDNA probes corresponding to the respective mRNAs.

lectin, suggesting a difference in their expression during vascular development.

To correlate the protein accumulation with vascular bundle anatomy, PFTIs were immunolocalized in hypocotyl sections 1–6 DAG. Pumpkin fruit trypsin inhibitors were not detected in young bundles with only protophloem or in slightly older bundles with a few metaphloem elements and the beginnings of internal phloem and extrafascicular phloem (data not shown). As the bundle matured and enlarged, it developed more internal phloem and more distinct bundle-associated extrafascicular phloem. Pumpkin fruit trypsin inhibitors were first apparent within the extrafascicular phloem compared with bundle phloem (Fig. 5A,B). Once the bundle developed to the stage of forming an incipient cambium (commonly in hypocotyls about 3 DAG), PFTIs were detected in a greater percentage of the sieve element–companion cell complexes (Fig. 5C,D). Within these bundles, PFTIs were most often localized in the extrafascicular phloem surrounding the bundle, and more often localized in the external phloem than in the internal phloem. By 6 DAG and at later stages of development (24 DAG), PFTIs within the hypocotyl and stems were mainly confined to both the bundle-associated and cortical extrafascicular phloem (Fig. 3B). Such differential localization has also been noted for the phloem filament protein and phloem lectin (Clark et al. 1997; Dannenhoffer et al. 1997). In contrast to the transient expression of these proteins in the bundle phloem, the extrafascicular phloem of stems and adaxial phloem of leaves appears to be the primary site of phloem protein accumulation in cucurbits.

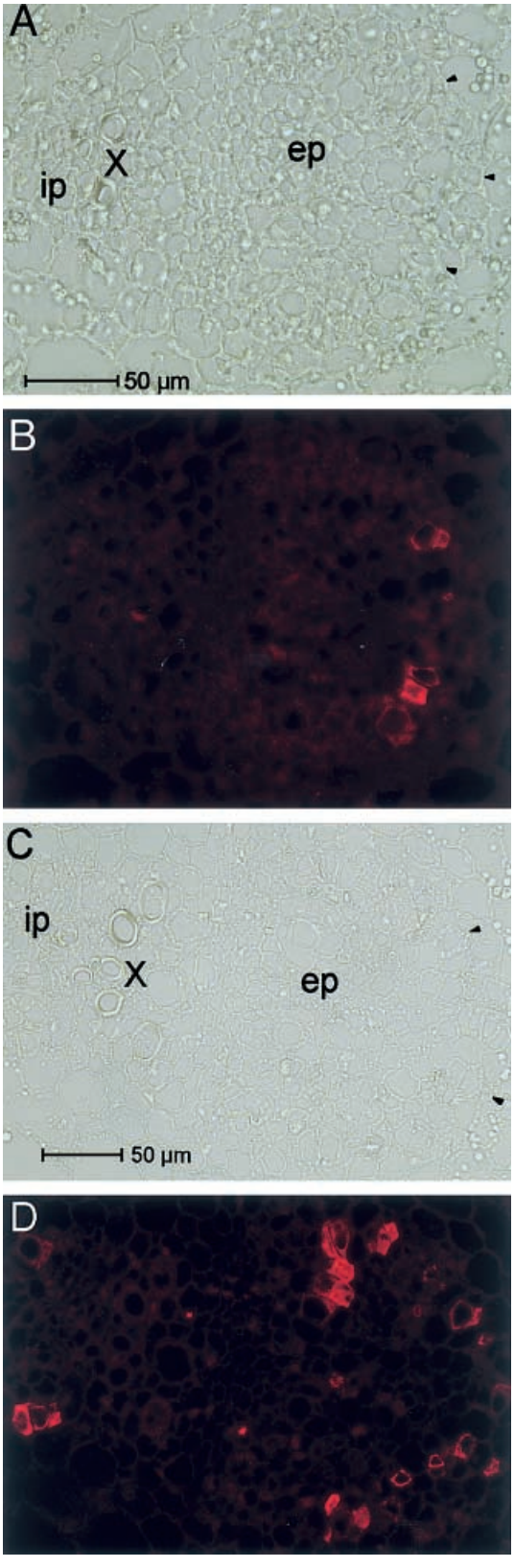

Fig. 5A–D. Developmental accumulation of PFTIs in tissue sections from hypocotyls of pumpkin seedlings. **A, B** Brightfield and fluorescent image pair of a 2-DAG hypocotyl. The bundle has maturing metaxylem (*x*), developing internal phloem (*ip*), external phloem (*ep*), and bundle-associated extrafascicular phloem (*arrowheads*). Pumpkin fruit trypsin inhibitors were rarely detected in the bundle phloem and were seen in the extrafascicular phloem. Bar = 50 μm. **C, D** Brightfield and fluorescent image pair of a 3-DAG hypocotyl. In bundles with more well-defined external phloem and extrafascicular phloem, abundant signal was detected in the phloem. Most particularly, PFTIs were present in the extrafascicular phloem bordering the bundle. Bar = 50 μm

Functional implications

The role of proteinase inhibitors in plant protection against pathogen infection or herbivory has been proposed by many authors, and genes encoding this diverse class of proteins have been investigated as a potential transgenes to generate pest resistant plants (Jouanin et al. 1998). Although the involvement of phloem-localized proteinase inhibitors in plant protection is an attractive hypothesis, contradictory results have been obtained for the effectiveness of PFTIs as defense molecules. Early studies showed that vascular exudates inhibited serine proteinases in extracts of rot-inducing fungi in pumpkins (MacGibbon and Mann 1986), but the inhibitory activity did not correspond with resistance to these fungi (Sharrock and Parkes 1990). In insect studies, Christeller and co-workers (1990, 1992, 1994) found that PFTIs from pumpkin fruit vascular exudate inhibited digestive enzymes of several chewing insects. Phloem-feeding insects rather than chewing insects would be an appropriate target for the phloem-limited PFTIs. It is widely accepted that phloem-feeding insects, such as aphids or whiteflies, utilize only free amino acids from the translocation stream and lack the proteases required to metabolize phloem proteins. However, a recent report that whiteflies can ingest and utilize plant proteins as a source of amino acids for the synthesis of insect proteins (Salvucci et al. 1998) continues to raise the possibility that PFTIs are components of a phloem-mediated defense system.

Regulating protein turnover is also a potentially important developmental and functional feature of the vascular tissue. Developmentally, proteolytic activity is high during vascular differentiation and has been shown to be coincident with programmed cell death in differentiating tracheary elements (Fukuda 1997). In both plants and animals, cysteine proteases are involved in programmed cell death, a process that can be prevented by cysteine proteinase inhibitors (Soloman et al. 1999). Unlike the conducting elements of the xylem, which are dead at functional maturity, the sieve elements of the phloem are highly modified but living at functional maturity. The partial degradation of the sieve element cytoplasm must be under tight regulatory control to inhibit the process at the appropriate developmental stage. Thus, phloem-specific proteinase inhibitors are potential candidates for the temporal control of selective autophagy during sieve element differentiation. The presence of proteinase inhibitors translocated throughout the transport phloem also suggests an active role in regulating protein turnover in the functioning sieve element–companion cell complex. Long-distance protein transport appears to be a dynamic process where specific proteins are synthesized and degraded during translocation within the transport phloem (reviewed by Thompson and Schulz 1999). While proteolytic activity was not found in cucurbit vascular exudates (Chino et al. 1991), it is likely that protein degradation is mediated in a cell-specific manner.

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