

# Translocation of Structural P Proteins in the Phloem

Bettina Golecki,<sup>a</sup> Alexander Schulz,<sup>a</sup> and Gary A. Thompson<sup>b,1</sup>

<sup>a</sup> Botanisches Institut, Universität Kiel, Olshausenstrasse 40, D-24098 Kiel, Germany

<sup>b</sup> Department of Plant Sciences, University of Arizona, Tucson, Arizona 85721

Phloem-specific proteins (P proteins) are particularly useful markers to investigate long-distance trafficking of macromolecules in plants. In this study, genus-specific molecular probes were used in combination with intergeneric grafts to reveal the presence of a pool of translocatable P protein subunits. Immunoblot analyses demonstrated that *Cucurbita* spp P proteins PP1 and PP2 are translocated from *Cucurbita maxima* stocks and accumulate in *Cucumis sativus* scions. *Cucurbita maxima* or *Cucurbita ficifolia* PP1 and PP2 mRNAs were not detected in *Cucumis sativus* scions by either RNA gel blot analysis or reverse transcription-polymerase chain reaction, indicating that the proteins, rather than transcripts, are translocated. Tissue prints of the *Cucumis sativus* scion, using antibodies raised against *Cucurbita maxima* PP1 or PP2, detected both proteins in the fascicular phloem of the stem at points distal to the graft union and in the petiole of a developing leaf, suggesting that the proteins move within the assimilate stream toward sink tissues. *Cucurbita maxima* PP1 was immunolocalized by light microscopy in sieve elements of the extrafascicular phloem of *Cucumis sativus* scions, whereas *Cucurbita maxima* PP2 was detected in both sieve elements and companion cells.

## INTRODUCTION

The long-distance movement of macromolecules in vascular tissues can impact profoundly normal plant growth and development. The importance of long-distance signaling in response to wounding as well as systemic infections by plant pathogens, such as viruses, has been well documented (Narváez-Vásquez et al., 1995; Schaller and Ryan, 1995; Nelson and Van Bel, 1998). However, little is known about the mechanisms or effects of translocating the numerous proteins that are known to be expressed specifically within the phloem tissue. The phloem of most angiosperms contains proteinaceous structures, collectively called P proteins (phloem proteins), that accumulate in differentiating sieve elements and persist in translocating sieve elements. The P protein is deposited initially into ultrastructurally distinct polymorphous or crystalline bodies during sieve element differentiation (reviewed in Cronshaw, 1975; Cronshaw and Sabnis, 1990; Sabnis and Sabnis, 1995). P protein bodies either persist or more often disperse, forming a filamentous network in the parietal cytoplasm that is thought to be immobilized through interactions with the appressed endomembrane system (Smith et al., 1987). Disruption of sieve elements that occurs during wounding results in the accumulation of P protein filaments at the sieve plate, ostensibly blocking translocation by forming P protein plugs.

P protein filaments in *Cucurbita maxima* (pumpkin) are composed of two very abundant proteins: phloem protein 1

(PP1), a 96-kD phloem filament protein, and phloem protein 2 (PP2), a 48-kD dimeric lectin that specifically binds poly( $\beta$ -1,4-*N*-acetylglucosamine) (Beyenbach et al., 1974; Sabnis and Hart, 1978; Allen, 1979; Read and Northcote, 1983b). Analysis of soluble phloem filaments present in phloem exudates of cucurbits indicated that PP1 monomers and PP2 dimers were covalently cross-linked via disulfide bonds, forming high molecular weight polymers (Read and Northcote, 1983a, 1983b). The phloem filament protein and phloem lectin have been localized immunocytochemically to both sieve elements and companion cells (Smith et al., 1987; Clark et al., 1997; Dannenhoffer et al., 1997). However, in situ hybridization experiments in hypocotyls of *Cucurbita maxima* seedlings established that PP1 and PP2 mRNAs accumulate only in companion cells in both immature and differentiated sieve element-companion cell complexes (Bostwick et al., 1992; Clark et al., 1997; Dannenhoffer et al., 1997). Thus, PP1 and PP2 apparently are synthesized in companion cells and subsequently transported into sieve elements via pore-plasmodesma contacts. High-resolution immunolocalization studies of differentiating sieve element-companion cell complexes of the bundle phloem suggest that PP1 accumulates in the dispersive P protein bodies of developing sieve elements; PP2 appears to be retained in companion cells before the period of selective autophagy and then moves into sieve elements where the lectin cross-links and anchors dispersed PP1 polymers with appressed endomembranes (Smith et al., 1987; Dannenhoffer et al., 1997). Both proteins accumulate within the persistent P protein bodies of the extrafascicular phloem of cucurbits, possibly cross-linking, which prevents dispersal of the P protein bodies.

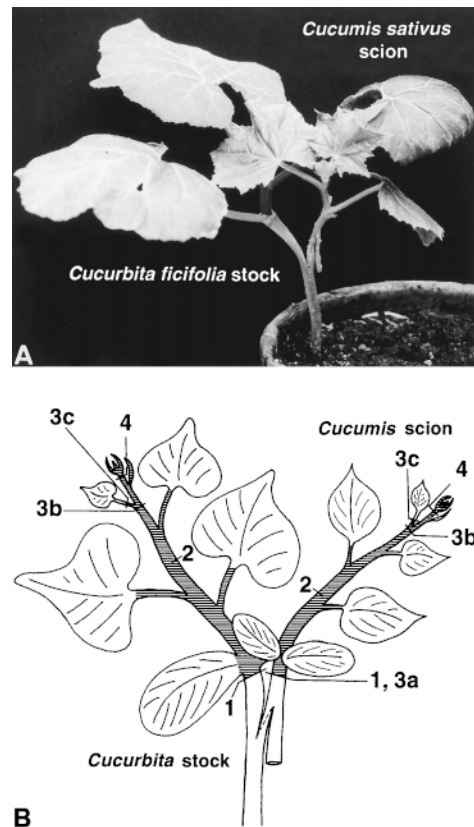
<sup>1</sup>To whom correspondence should be addressed. E-mail garyt@u.arizona.edu; fax 520-621-7186.

In contrast to the incorporation of P proteins into polymerized structures, several lines of evidence suggest the existence of a pool of unpolymerized PP1 and PP2 subunits within sieve element–companion cell complexes. In their analysis of phloem filament structure, Read and Northcote (1983a) estimated that as much as 43% of PP1 and 18% of PP2 were present as free monomers or dimers in phloem exudates of *Cucurbita maxima*. Alosi et al. (1988) questioned whether P protein filament formation or stabilization by disulfide linkages is possible when the reducing environment of the phloem sap is considered. The existence of a pool of unpolymerized P protein subunits is supported further by the apparent translocation of genus-specific P proteins or their precursors in intergeneric grafts between members of the Cucurbitaceae (Tiedemann and Carstens-Behrens, 1994; Golecki et al., 1998). These observations are possible because SDS-PAGE profiles of phloem exudate proteins collected from different cucurbit genera show considerable size heterogeneity (Sabnis and Hart, 1976, 1979). Additional proteins with molecular weights typical of *Cucurbita* spp P proteins were observed in exudate samples collected from *Cucumis sativus* (cucumber) scions when grafted onto *Cucurbita* spp stocks. Moreover, subsequent developmental analysis demonstrated that the appearance of the additional proteins in *Cucumis sativus* scions was strongly correlated to the establishment of intergeneric sieve element connections in the graft union (Golecki et al., 1998).

P proteins share functional similarities among genera of the Cucurbitaceae but are sufficiently divergent with regard to their protein and nucleic acid sequences so that genus-specific probes can be used to determine their origin in intergeneric grafts (A.M. Clark and G.A. Thompson, unpublished results). In this study, we used the intergeneric divergence of PP1 and PP2 to demonstrate that these proteins are capable of long-distance movement in the phloem of grafted plants. Evidence is presented that *Cucurbita* spp PP1 and PP2 are translocated from *Cucurbita maxima* or *Cucurbita ficifolia* stocks to *Cucumis sativus* scions via phloem bridges formed at the graft union. Our results also demonstrate that PP2 exits from sieve elements and accumulates in companion cells of the extrafascicular phloem of the *Cucumis sativus* scion. The implications for long-distance movement of macromolecules and intercellular interactions between sieve elements and companion cells at a distance from the point of protein synthesis are discussed.

## RESULTS

We have exploited the intergeneric divergence of the two major P proteins in cucurbits to determine whether the phloem filament protein PP1 and phloem lectin PP2 are translocated over long distances in the transport phloem. Intergeneric approach grafts consisting of *Cucurbita maxima* or *Cucurbita ficifolia* stocks and *Cucumis sativus* scions



**Figure 1.** Phloem Exudate and Tissue Collection Points from a *Cucurbita* spp and *Cucumis sativus* Approach Graft.

**(A)** Typical approach-grafted plant consisting of a *Cucurbita ficifolia* stock and *Cucumis sativus* scion 15 days after grafting.

**(B)** Phloem exudate samples for SDS-PAGE and immunoblot analyses were collected from the basal end of the stem at site 1. Shoot tissue for RNA analyses was collected from the stem axis including the petioles (site 2; shaded). Tissue prints were made from transverse cuts at sites 3a, 3b, and 3c, and tissues fixed for immunolocalization using cryosections were collected from sites 4.

were used in this study (Figure 1A). We have shown previously that additional proteins in phloem exudates of *Cucumis sativus* scions grafted to *Cucurbita* spp stocks were detected easily within 9 to 11 days after grafting (Golecki et al., 1998). Figure 1B diagrammatically shows a grafted plant and the points at which samples were collected.

### *Cucurbita maxima* PP1 and PP2 Accumulate in *Cucumis sativus* Scions

To avoid cross-contamination of *Cucumis sativus* tissue with *Cucurbita maxima* phloem sap, the first transverse cut across the scion hypocotyl below the cotyledons always separated the *Cucumis sativus* scion from the *Cucurbita*

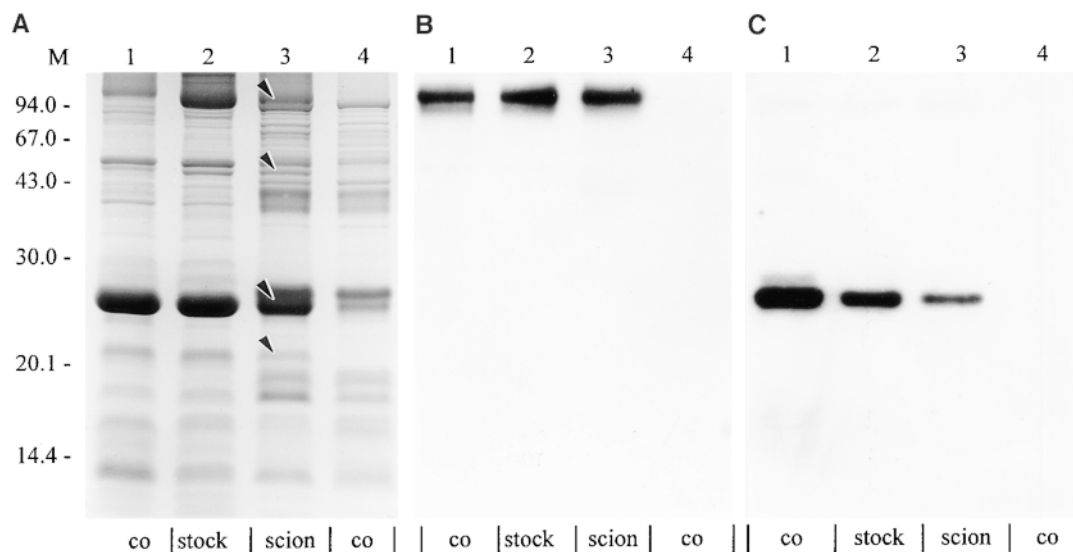
*maxima* stock. Phloem exudates immediately were collected from the basal end of the stem below the cotyledons (Figure 1B, site 1) for SDS-PAGE and immunoblot analysis. Phloem exudates collected from ungrafted *Cucurbita maxima* (Figure 2A, lane 1) and *Cucumis sativus* plants (Figure 2A, lane 4) show variation in the protein profiles when phloem exudate proteins were separated by SDS-PAGE. In grafted plants, at least four additional proteins that appear to originate from the *Cucurbita maxima* stock (Figure 2A, lane 2) were detected readily in phloem exudates collected from the *Cucumis sativus* scion (Figure 2A, lane 3). The very abundant *Cucurbita maxima* phloem filament protein PP1 and phloem lectin PP2 seem to be among these additional proteins.

Polyclonal antisera against *Cucurbita maxima* PP1 and PP2 were used to verify the identity of these proteins in phloem exudates of grafted *Cucumis sativus* scions by immunoblot analysis. Divergence in the primary sequence of these proteins in *Cucurbita maxima* and *Cucumis sativus* allowed us to use these antibodies to distinguish definitively between P protein homologs in the *Cucumis sativus* scion when grafted on *Cucurbita maxima* rootstock. Indeed, both the phloem filament protein (Figure 2B, lane 1) and the phloem lectin (Figure 2C, lane 1) were detected readily on immunoblots of total phloem exudate proteins collected from *Cucurbita maxima* control plants, whereas neither pro-

tein was detected among total phloem exudate proteins collected from *Cucumis sativus* control plants (Figures 2B and 2C, lanes 4). In grafted plants, the respective polyclonal antibodies detected both proteins in the *Cucurbita maxima* stock (Figures 2B and 2C, lanes 2) and the *Cucumis sativus* scion (Figures 2B and 2C, lanes 3). These results show that two of the additional proteins in the *Cucumis sativus* scion are identical to PP1 and PP2 of the *Cucurbita maxima* stock. This could be due to either translocation of PP1 and PP2 proteins across the graft union or translocation of mRNA encoding these proteins, allowing for de novo protein synthesis in the *Cucumis sativus* scion.

### PP1 and PP2 mRNAs Are Not Translocated

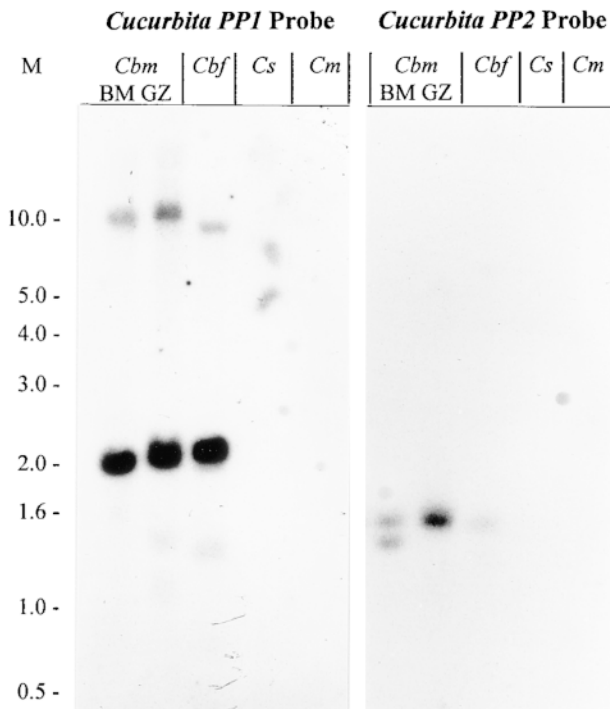
The ability of the *Cucurbita maxima* PP1 and PP2 nucleic acid probes to discriminate between genes encoding P proteins in *Cucurbita* spp and *Cucumis* spp was demonstrated initially by genomic DNA gel blot analyses. Hybridization patterns were identical to those reported previously for HindIII-digested genomic DNA from *Cucurbita maxima* cv Big Max when probed with the <sup>32</sup>P-labeled protein coding sequences of PP1 and PP2 (Figure 3, lanes *Cbm* BM) genes isolated from the same pumpkin cultivar (Bostwick et al., 1994; Clark et al., 1997). Genes encoding the phloem lectin



**Figure 2.** SDS-PAGE and Immunoblot Analyses of *Cucurbita* PP1 and PP2 in Phloem Exudates of 13-Day-Old Intergeneric Grafts of *Cucumis sativus* on *Cucurbita maxima*.

(A) Coomassie blue-stained SDS-polyacrylamide gel of total phloem proteins isolated from the *Cucurbita maxima* ungrafted control (co) (lane 1) or stock (lane 2) and *Cucumis sativus* scion (lane 3) or ungrafted control (lane 4). Arrowheads in lane 3 show the position of additional proteins in exudates from the *Cucumis sativus* scion. M indicates molecular mass markers (in kilodaltons).

(B) and (C) Immunoblots of duplicate gels reacted with polyclonal antibodies raised against the phloem filament protein PP1 (B) and the phloem lectin PP2 (C) of *Cucurbita maxima* cv Big Max. Lanes are as given in (A).



**Figure 3.** Genomic DNA Gel Blot Analyses Demonstrate the Specificity of the *Cucurbita maxima* PP1 and PP2 Nucleic Acid Probes.

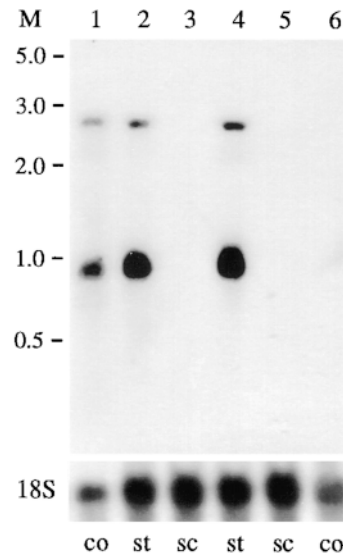
Genomic DNA isolated from *Cucurbita maxima* (Cbm) cvs Big Max (BM) and Gelber Zentner (GZ), *Cucurbita ficifolia* (Cbf), *Cucumis sativus* (Cs), and *Cucumis melo* (Cm) was restricted with HindIII, separated in a 0.9% agarose gel, blotted, and hybridized with  $^{32}\text{P}$ -labeled probes corresponding to the protein coding sequences of either PP1 or PP2 of *Cucurbita maxima* cv Big Max. M indicates molecular length markers (in kilobases).

and the phloem filament protein also were detected in the genomes of *Cucurbita maxima* cv Gelber Zentner (Figure 3, lanes Cbm GZ) and *Cucurbita ficifolia* (Figure 3, lanes Cbf). However, homologous genes were not detected with these probes in genomic DNA isolated from either *Cucumis sativus* or *Cucumis melo* (Figure 3, lanes Cs and Cm, respectively).

To distinguish between P protein translocation or de novo protein synthesis as a result of mRNA translocation from the *Cucurbita maxima* stock to *Cucumis sativus* scion, we investigated the presence of PP1 and PP2 mRNAs in *Cucumis sativus* scions grafted onto *Cucurbita maxima* or *Cucurbita ficifolia* rootstocks by RNA blot analysis and reverse transcription-polymerase chain reaction (RT-PCR) with *Cucurbita maxima*-specific probes and primers, respectively. Total RNA isolated from the shoot axis of *Cucumis sativus* scions and *Cucurbita maxima* stocks, respectively (Figure 1B, sites 2), was subjected to RNA gel blot hybridization with  $^{32}\text{P}$ -labeled probes corresponding to *Cucurbita maxima* PP1 and

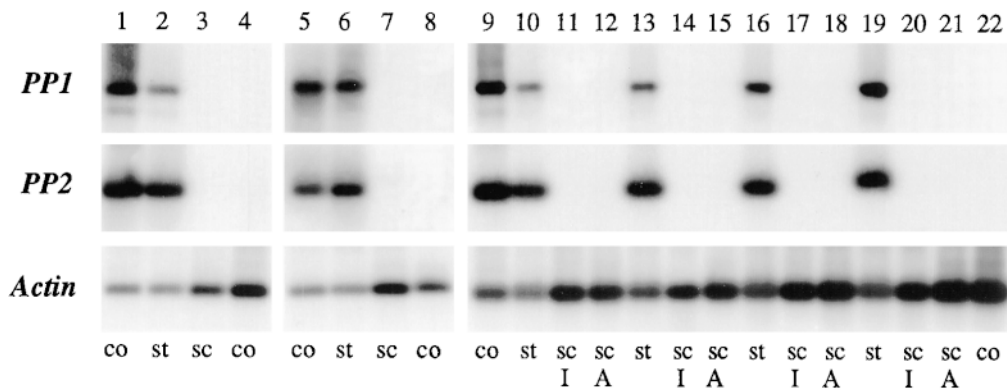
PP2 protein coding regions. The two expected mRNAs of ~2500 (PP1) and 1000 (PP2) nucleotides were observed in the *Cucurbita maxima* control and stocks (Figure 4, lanes 1, 2, and 4) but were absent in *Cucumis sativus* scions (Figure 4, lanes 3 and 5) and the *Cucumis sativus* ungrafted control (Figure 4, lane 6).

RT-PCR of total RNA confirmed the results of the RNA gel blot analysis. PCR primers flanking the protein coding regions of *Cucurbita maxima* PP1 and PP2 were used for first-strand synthesis from mRNA templates and amplification of PP1 (2430 bp) and PP2 (657 bp) cDNAs. The amplification products were verified by DNA gel blot hybridization with radiolabeled *Cucurbita maxima*-specific probes. PCR-generated PP1 and PP2 cDNAs from *Cucurbita maxima* control plants and the *Cucurbita maxima* stocks hybridized with both *Cucurbita maxima* PP1 and PP2 probes (Figure 5, lanes 1, 2, 9, 10, 13, 16, and 19), respectively. Identical results were obtained when *Cucurbita ficifolia* was used as the stock (Figure 5, lanes 5 and 6). In contrast, hybridization was not detected in blots of RT-PCR products from *Cucumis sa-*



**Figure 4.** RNA Gel Blot Analyses of *Cucurbita maxima* PP1 and PP2 mRNAs from Intergeneric Grafts of *Cucumis sativus* Scions on *Cucurbita maxima* Stocks.

Total RNA (10  $\mu\text{g}$  per lane) isolated from stems and petioles of grafted and ungrafted plants (Figure 1, sites 2) was separated by electrophoresis in an agarose-glyoxyl gel. The RNA was transferred onto a Magna Charge membrane and hybridized to  $^{32}\text{P}$ -labeled DNA probes corresponding to the protein coding sequences of PP1 and PP2 of *Cucurbita maxima* cv Big Max. Lane 1 contains 1.25  $\mu\text{g}$  of total RNA isolated from a *Cucurbita maxima* control (co) plant; lanes 2 and 4, *Cucurbita maxima* stocks (st); lanes 3 and 5, *Cucumis sativus* scions (sc); and lane 6, a *Cucumis sativus* control plant. The blot at bottom shows hybridization with the 18S ribosomal gene probe. M indicates molecular length markers (in kilobases).



**Figure 5.** RT-PCR Gel Blot Analyses of *Cucurbita* PP1 and PP2 mRNAs from Intergeneric Grafts of *Cucumis sativus* Scions on *Cucurbita maxima* or *Cucurbita ficifolia* Stocks.

RT-PCR products generated from total RNA with 5' and 3' primers that flank the protein coding sequences of genes encoding PP1 and PP2 in *Cucurbita maxima* were separated by agarose gel electrophoresis, blotted, and hybridized to corresponding  $^{32}\text{P}$ -labeled DNA probes (the PP1 open reading frame [ORF] is 2430 bp and the PP2 ORF is 657 bp). PP1- and PP2-specific probes hybridized with PCR-generated cDNA from RNA templates isolated from *Cucurbita maxima* ungrafted control (co) plants or *Cucurbita maxima* stocks (st) from intergeneric grafted plants either 13 days after grafting (lanes 1 and 2) or 28 days after grafting (lanes 9, 10, 13, 16, and 19). Similarly, lanes 5 and 6 show hybridization with cDNAs generated by RT-PCR of RNA templates isolated from *Cucurbita ficifolia* control plants or *Cucurbita ficifolia* stocks from intergeneric grafts 13 days after grafting. *Cucurbita* primers failed to amplify either PP1 or PP2 cDNAs from RNA templates isolated from *Cucumis sativus* scions (sc) (lanes 3, 7, 11, 12, 14, 15, 17, 18, 20, and 21) grafted onto either of the *Cucurbita* spp stocks and ungrafted *Cucumis sativus* control plants (lanes 4, 8, and 22). Tissue from either the distal internode (I) or apex (A) of *Cucumis sativus* scions of intergeneric grafted plants was analyzed at 28 days after grafting. Actin was amplified, blotted, and hybridized to the  $^{32}\text{P}$ -labeled *Cucumis sativus* actin gene probe as a control.

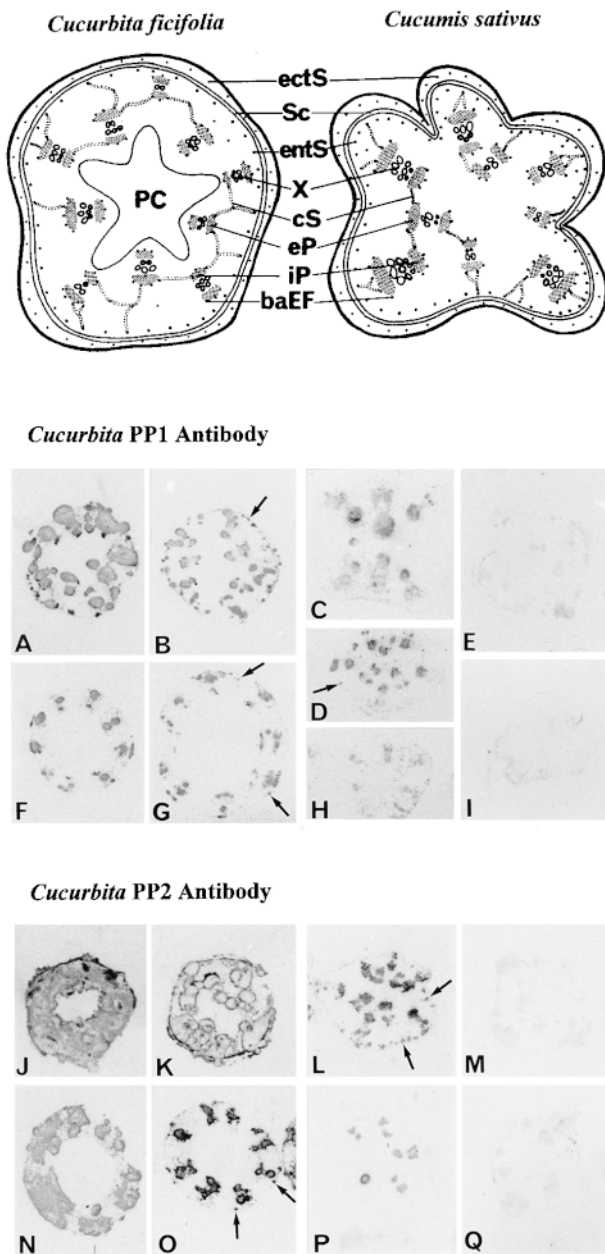
*tivus* ungrafted control plants (Figure 5, lanes 4, 8, and 22) or *Cucumis sativus* scions grafted for 13 days on either *Cucurbita maxima* stocks (Figure 5, lane 3) or *Cucurbita ficifolia* stocks (Figure 5, lane 7). Furthermore, hybridization was not detected in blots of RT-PCR products from internodal (Figure 5, lanes 11, 14, 17, and 20) or apical (Figure 5, lanes 12, 15, 18, and 21) tissues of *Cucumis sativus* scions grafted onto four different *Cucurbita maxima* stocks for 28 days, demonstrating that PP1 and PP2 mRNAs are not present in the *Cucumis sativus* scions. The absence of *Cucurbita* PP1 and PP2 mRNA in *Cucumis sativus* scions after grafting on both *Cucurbita* spp disproves the hypothesis of mRNA transport across the graft union and de novo protein synthesis in the *Cucumis sativus* phloem.

#### Immunolocalization of *Cucurbita ficifolia* PP1 and PP2 in the Bundle Phloem of the *Cucumis sativus* Scion

Immunoblot analyses clearly demonstrated the specificity of the polyclonal antibodies raised against purified *Cucurbita maxima* PP1 and PP2 for the detection of these proteins in phloem exudates collected from *Cucumis sativus* scions. To follow the route of long-distance movement of *Cucurbita ficifolia* P proteins and to localize their accumulation in the phloem tissue of *Cucumis sativus* scions, these antibodies were used in immunolocalization studies of stems and petioles of young leaves. The phloem anatomy of stems close to

the shoot apex in *Cucurbita* spp and *Cucumis* spp is shown in Figure 6. The transport phloem in stems and petioles consists of two types of phloem, the fascicular or bundle phloem and the extrafascicular phloem (Fischer, 1884; Crafts, 1932). The fascicular phloem is located internally and externally to the xylem, forming the characteristic bicollateral vascular bundle. The extrafascicular phloem consists of longitudinal strands of sieve element-companion cell complexes bordering the internal and external fascicular phloem (bundle-associated extrafascicular phloem) and as an anastomosing network in the cortex (cortical extrafascicular phloem). The cortical extrafascicular phloem can be subdivided further into entocyclic and ectocyclic phloem strands and commissural sieve tubes that form the lateral connections between the longitudinal strands. Transverse sections of stems in *Cucurbita* spp can be distinguished easily from those of *Cucumis sativus* because of the large pith cavity that is absent in *Cucumis sativus* (Figure 6) and its rather round shape, which is much more irregular in *Cucumis sativus*.

Tissue prints were made from transverse cuts of the hypocotyl ~1 cm above the graft union directly below the cotyledons (Figure 1B, site 3a), from the stem ~10 cm above the graft union (Figure 1B, sites 3b), and from petioles of young leaves ~10 cm above the graft union (Figure 1B, site 3c). Specific immune responses with both antibodies were obtained in stem and petiole tissue prints of the *Cucumis sativus* scion (Figures 6C, 6D, 6H, 6L, and 6P) as well as the *Cucurbita ficifolia* control (Figures 6A, 6F, 6J, and 6N) and



**Figure 6.** Tissue Printing with *Cucurbita*-Specific PP1 and PP2 Polyclonal Antibodies.

Line drawings depict the vascular anatomy of a transverse section in stems of *Cucurbita* spp and *Cucumis sativus*. Bicolateral vascular bundles are composed of internal (iP) and external (eP) fascicular phloem flanking the xylem (X) on two sides. The extrafascicular phloem consists of entocyclic (entS) and ectocyclic (ectS) phloem strands separated by a sclerenchyma (Sc) ring in the cortex and commissural sieve elements (cS) forming lateral connections between the longitudinal phloem strands. Bundle-associated extrafascicular phloem (baEF) is located in arcs bordering the internal and external bundle phloem. A pith cavity (PC) is present in *Cucurbita* spp stems.

stock (Figures 6B, 6G, 6K, and 6O). *Cucurbita ficifolia* PP1 and PP2 were not detected on tissue prints of ungrafted *Cucumis sativus* control plants (Figures 6E, 6I, 6M, and 6Q). Immunolabeling of the internal and external bundle phloem in the *Cucumis sativus* scion stem 1 cm above the graft union (Figure 6C) and 10 cm above the graft union (Figures 6D and 6L) indicates that *Cucurbita ficifolia* PP1 and PP2 are present throughout the translocating phloem of the *Cucumis sativus* scion. Furthermore, both proteins were immunolocalized in the bundle phloem of a young leaf petiole (~20% expansion) (Figures 6H and 6P), which is consistent with the view that these structural proteins are translocated within the normal assimilate stream from source (*Cucurbita ficifolia* rootstock) to sink (shoot apex and young leaf of *Cucumis sativus* scion). In addition, PP1 and PP2 were immunolocalized in entocyclic and commissural sieve tubes outside of the bundle phloem in stems and petioles of the *Cucurbita ficifolia* stock (Figures 6B, 6G, and 6O). A similar immune response outside the bundle phloem of the scion stem (Figures 6D and 6L) suggests that the extrafascicular phloem of the *Cucumis sativus* scion also accumulates *Cucurbita ficifolia* P proteins.

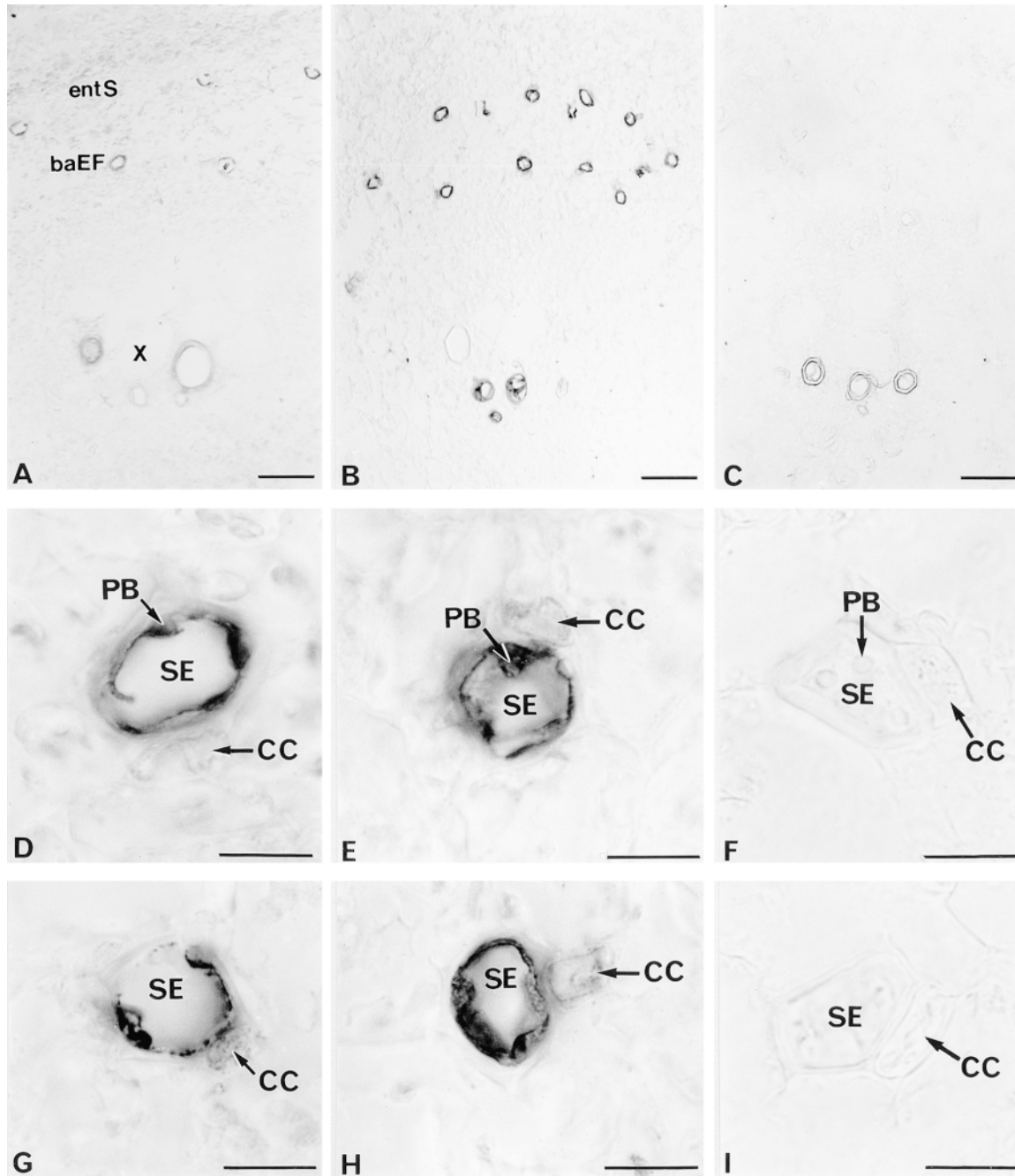
#### Immunolocalization of *Cucurbita maxima* PP1 and PP2 in Extrafascicular Phloem of the *Cucumis sativus* Scion

The accumulation of PP1 and PP2 in the extrafascicular phloem was shown clearly in immunolocalization studies of cryosections prepared from stem segments collected 0.5 to 1.0 cm below the shoot apex (Figure 1B, sites 4) of 19-day-old grafted plants consisting of a *Cucumis sativus* scion on a *Cucurbita maxima* stock. Both P proteins accumulated in the bundle-associated and entocyclic extrafascicular phloem of the *Cucurbita maxima* stock (Figures 7A and 8A) and *Cucumis sativus* scion (Figures 7B and 8B). Higher magnification

(A) to (I) *Cucurbita maxima* PP1 antibodies reacted with tissue prints from (A) *Cucurbita ficifolia* ungrafted control stem; (B) *Cucurbita ficifolia* stock stem; (C) *Cucumis sativus* scion hypocotyl 1 cm above the graft union; (D) *Cucumis sativus* scion stem 10 cm above the graft union; (E) *Cucumis sativus* ungrafted control stem; (F) *Cucurbita ficifolia* ungrafted control petiole; (G) *Cucurbita ficifolia* stock petiole; (H) *Cucumis sativus* scion petiole of a young leaf; and (I) *Cucumis sativus* ungrafted control petiole.

(J) to (Q) *Cucurbita maxima* PP2 antibodies reacted with tissue prints from (J) *Cucurbita ficifolia* ungrafted control stem; (K) *Cucurbita ficifolia* stock stem; (L) *Cucumis sativus* scion stem 10 cm above the graft union; (M) *Cucumis sativus* ungrafted control stem; (N) *Cucurbita ficifolia* ungrafted control petiole; (O) *Cucurbita ficifolia* stock petiole; (P) *Cucumis sativus* scion petiole; and (Q) *Cucumis sativus* ungrafted control petiole.

Arrows in (B), (D), (G), (L), and (O) indicate extrafascicular phloem.



**Figure 7.** Immunolocalization of *Cucurbita maxima* PP1 in Stems of Intergenic Grafts of *Cucumis sativus* Scions on *Cucurbita maxima* Stocks.

Transverse cryosections of stem tissue were reacted with *Cucurbita*-specific antiserum raised against the phloem filament protein PP1. baEF, bundle-associated extrafascicular phloem; CC, companion cell; entS, entocyclic phloem strands; PB, P protein body; SE, sieve element.

(A) *Cucurbita maxima* stock.

(B) *Cucumis sativus* scion.

(C) *Cucumis sativus* ungrafted control.

(D) *Cucurbita maxima* stock.

(E) *Cucumis sativus* scion.

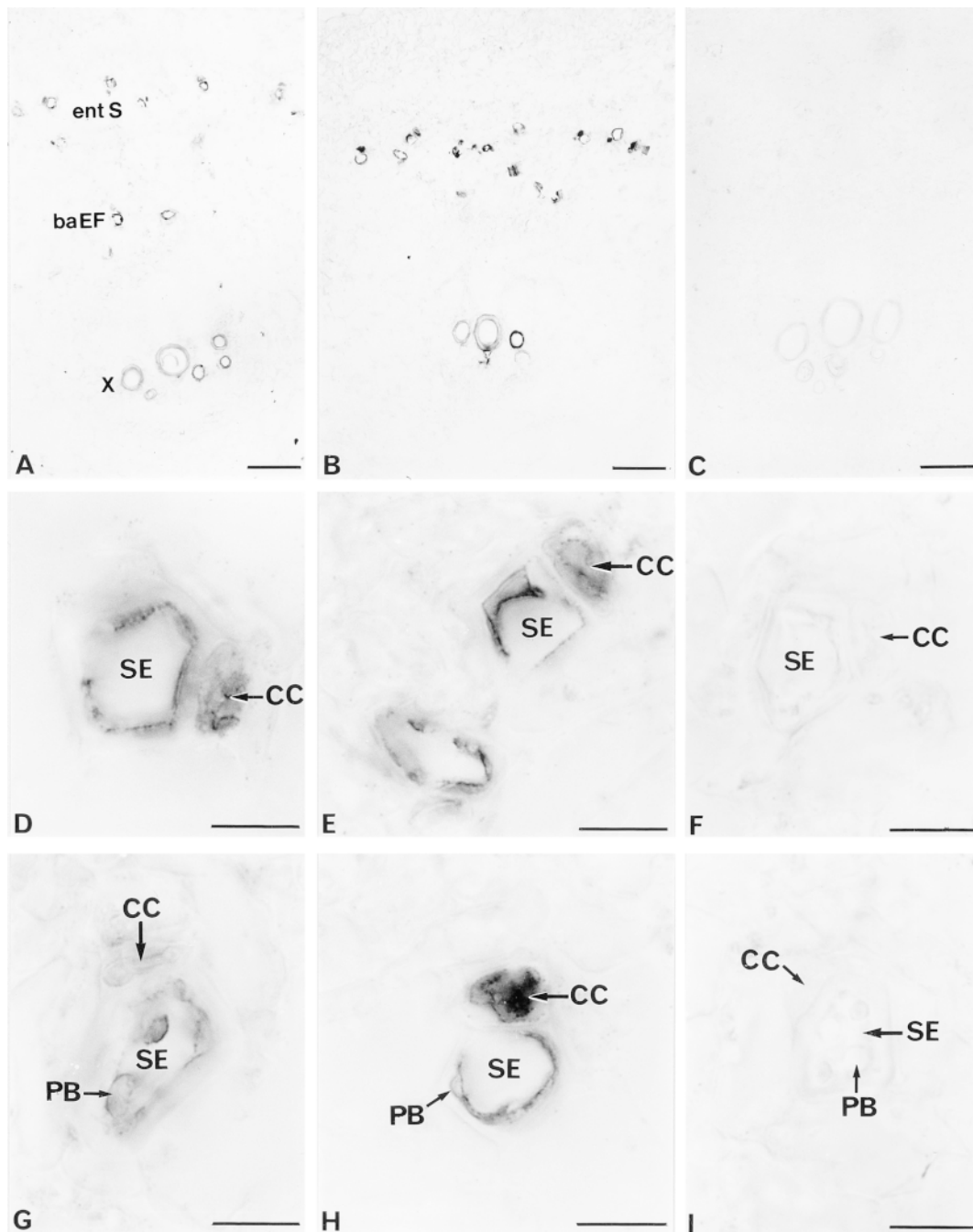
(F) *Cucumis sativus* ungrafted control.

(G) *Cucurbita maxima* ungrafted control.

(H) *Cucumis sativus* scion.

(I) *Cucumis sativus* ungrafted control.

Bars in (A) to (C) = 50  $\mu$ m; bars in (D) to (I) = 10  $\mu$ m.



**Figure 8.** Immunolocalization of *Cucurbita maxima* PP2 in Stems of Intergeneric Grafts of *Cucumis sativus* Scions on *Cucurbita maxima* Stocks.

Transverse cryosections of stem tissue were reacted with *Cucurbita*-specific antiserum raised against the phloem lectin PP2. baEF, bundle-associated extrafascicular phloem; CC, companion cell; entS, entocyclic phloem strands; PB, P protein body; SE, sieve element.

(A) *Cucurbita maxima* stock.

(B) *Cucumis sativus* scion.

(C) *Cucumis sativus* ungrafted control.

(D) *Cucurbita maxima* stock.

(E) *Cucumis sativus* scion.

(F) *Cucumis sativus* ungrafted control.

(G) *Cucurbita maxima* ungrafted control.

(H) *Cucumis sativus* scion.

(I) *Cucumis sativus* ungrafted control.

Bars in (A) to (C) = 50  $\mu$ m; bars in (D) to (I) = 10  $\mu$ m.



showed the detection of PP1 and PP2 in the sieve element–companion cell complexes of the *Cucurbita maxima* stock (Figures 7D and 8D), *Cucurbita maxima* ungrafted control (Figures 7G and 8G), and *Cucumis sativus* scions (Figures 7E and 7H, and 8E and 8H). These immune responses were specific for *Cucurbita maxima* PP1 and PP2 in the *Cucumis sativus* scion because neither of the *Cucurbita maxima* P proteins was detected in the phloem tissue and surrounding cells of the ungrafted *Cucumis sativus* control (Figures 7C, 7F, and 7I, and 8C, 8F, and 8I). P proteins were not detected in the immature fascicular phloem (Figures 7A and 7B, and 8A and 8B). Nonspecific immunolabeling of the lignified walls of xylem elements was observed (Figures 7A to 7C, and 8A to 8C).

Interestingly, high magnifications of the extrafascicular phloem in the *Cucumis sativus* scion showed differences in the accumulation of the 96-kD phloem filament protein and the 48-kD (24.5-kD subunits) dimeric phloem lectin within the sieve element–companion cell complex. *Cucurbita maxima* PP1 was localized with intense signals in parietal regions and persistent P protein bodies of sieve elements and with very weak signals in companion cells (Figures 7E and 7H). In sharp contrast, *Cucurbita maxima* PP2 was localized with intense signals in both cell types (Figures 8E and 8H). These observations provide evidence that subsequent to long-distance movement within sieve tubes, P proteins move from sieve elements into companion cells via connecting pores–plasmodesma contacts.

## DISCUSSION

P proteins were defined initially as ultrastructurally distinct proteinaceous filaments or aggregates that accumulate within differentiating sieve elements (Esau and Cronshaw, 1967). The present observations on the long-distance movement of P proteins across a graft union are in contrast to the traditional concept that these proteins form only immobilized polymeric structures within individual sieve tube members (Smith et al., 1987; Fisher et al., 1992). Immunoblot analyses of *Cucurbita maxima* P proteins in phloem exudate from intergeneric grafts clearly illustrated that the phloem filament protein PP1 and the phloem lectin PP2, both originating from the stock, accumulated within the *Cucumis sativus* scion. In a previous study, it was confirmed that functional sieve elements bridging the graft union must be established before additional vascular proteins in *Cucumis sativus* scions could be observed by using SDS-PAGE at 10 days after grafting (Golecki et al., 1998). Furthermore, transport of carboxyfluorescein from the stock to the scion verified that newly formed vascular bridges were functional at that time. Thus, at 13 days after grafting, we can exclude the possibility that the polymerized P protein was dislodged from its parietal position in sieve elements of the stock plant during the grafting process. In addition, the method of exudate collec-

tion from the *Cucumis sativus* scion excluded any contamination with P protein from the stock at the time of sampling. Although *Cucurbita maxima* P proteins were identified easily, their respective transcripts were undetectable in the *Cucumis sativus* scion, indicating that the proteins, rather than their mRNAs, move within the assimilate stream. This contrasts with recent reports of mRNA trafficking between the companion cell and sieve element (Kühn et al., 1997) but supports our previous findings that PP1 and PP2 mRNA accumulates exclusively in companion cells (Bostwick et al., 1992; Clark et al., 1997; Dannenhofer et al., 1997).

## Transport Form of P Proteins

Transport of P proteins across the graft union suggests that they are in a mobile form incapable of blocking translocation within sieve elements. Although we cannot rule out the long-distance movement of small polymers, the most likely form of translocated P protein is either PP1 monomers or PP2 dimers. In vivo labeling of soluble phloem proteins of wheat, rice, and castor bean indicated that between 100 and 200 soluble polypeptides are present in the translocation stream (Fisher et al., 1992; Nakamura et al., 1993; Sakuth et al., 1993). Because these phloem-mobile proteins range in size from 10 to 70 kD, the 96-kD phloem filament protein of cucurbits is the largest phloem protein for which long-distance movement has been demonstrated. Based on observations of <sup>35</sup>S-labeled phloem-mobile proteins in wheat, Fisher et al. (1992) presented a model for long-distance transport of proteins that essentially followed the normal assimilate source-to-sink translocation pathway. Phloem exudates collected at several points along the source-to-sink pathway in wheat showed similar protein concentrations and compositions. *Cucurbita ficifolia* PP1 and PP2 were detected in tissue prints of the stems of *Cucumis sativus* scions near (1 cm) and at a distance from (10 cm) the graft union as well as in the petioles of sink leaves. These data suggest that structural P proteins also are translocated in the assimilate stream to sink tissues and provide direct evidence of specific protein translocation in sieve elements.

Our previous studies of protein movement in intergeneric grafts also indicated that similar quantities of translocated protein from *Cucurbita* spp stocks are distributed throughout the *Cucumis sativus* scion (Golecki et al., 1998). Thus, the soluble P protein appears to be maintained at a steady-state level in the phloem throughout the entire vascular system. This would concur with previous reports of little or no differences in SDS-PAGE and isoelectric focusing protein patterns in phloem exudates collected from the following developmental states: 5-day-old seedlings and mature plants; young petioles and the oldest stem internodes; dark-grown and light-grown seedlings; and vegetative and flowering plants (Sabnis and Hart, 1976, 1978, 1979; Smith et al., 1987).

The relationship between mobile P protein subunits and microscopic observations of proteinaceous structures in sieve elements is unclear. The myriad of ultrastructural studies that show the P protein deposited into filaments and bodies in differentiating and translocating sieve elements strongly support the presence of polymerized P protein structures. Recent confocal laser scanning imaging of functional sieve elements in intact fava bean leaves (Knoblauch and Van Bel, 1998) indicates that previous ultrastructural observations cannot simply be ignored as artifacts generated during tissue preparation. However, the presence of mobile P protein filaments in the sieve tube lumen *in vivo* is unlikely and would hinder assimilate translocation. Alosi et al. (1988) observed that pure phloem exudate does not polymerize rapidly; they suggested that diluting the P protein during collection induces conformational changes, exposing sulfhydryl residues that can cross-link rapidly. Filaments in diluted samples analyzed to date are probably due to rapid oxidation of P protein subunits that occurs during exudate collection. Thus, P proteins of cucurbits appear to be present in two forms: (1) individual PP1 and PP2 subunits that are translocatable and (2) polymerized filaments composed of PP1 and PP2 subunits that are immobilized within individual sieve tube members. The interrelationship of the two forms remains to be determined.

#### Intercellular Trafficking and Accumulation of P Proteins

Differential accumulation of *Cucurbita maxima* PP1 and PP2 within sieve element–companion cell complexes of the extrafascicular phloem in *Cucumis sativus* scions raises several enigmatic questions regarding P protein trafficking and accumulation. *Cucurbita maxima* PP2 was immunolocalized in both sieve elements and companion cells (Figure 7), whereas *Cucurbita maxima* PP1 was limited primarily to sieve elements (Figure 8) in both the stock and scion. Two scenarios can be envisioned to explain these data in mature sieve element–companion cell complexes: either PP2 readily moves between the two cell types, while PP1 is physically retained within the sieve element, or both proteins traffic from sieve elements to companion cells, where PP1 is rapidly degraded and PP2 accumulates, possibly to be recycled. Regulation of macromolecular trafficking within the sieve element–companion cell complex certainly involves the numerous symplasmic connections, or pore–plasmodesma contacts, that form between sieve elements and companion cells (reviewed in Van Bel and Kempers, 1997). Although size exclusion limits (SELs) of plasmodesmata between mesophyll cells range from 0.75 to 1.0 kD (Terry and Robards, 1987; Wolf et al., 1989; Robards and Lucas, 1990), the SELs of pore–plasmodesma contacts in translocating sieve element–companion cell complexes are much larger. Microinjection of either companion cells or sieve elements with a series of fluorescein isothiocyanate (FITC)-labeled conjugates demonstrated a minimum SEL of 3 kD for the

pore–plasmodesma contacts in the extrafascicular phloem of *Cucurbita maxima* and up to 25 kD in the fascicular phloem of fava bean (Kempers et al., 1993; Kempers and Van Bel, 1997). Coincidentally, the Stokes radius of the globular PP2 dimer (2.9 nm; Anantharam et al., 1986) is similar in size to the 10-kD dextran conjugates (2.3 nm) that readily moved from sieve elements to companion cells in fava bean (Kempers and Van Bel, 1997). The increased SEL of pore–plasmodesma contacts shown by Kempers and Van Bel (1997) might allow unrestricted movement of *Cucurbita* spp PP2 dimers or monomers between sieve elements and companion cells in the *Cucumis sativus* scion, while limiting the considerably larger 96-kD phloem filament protein to the sieve element.

*Cucurbita maxima* PP1 and PP2 have the capacity to increase the SEL of plasmodesmata and mediate cell-to-cell movement in parenchymatic tissue. Balachandran et al. (1997) demonstrated extensive cell-to-cell movement of the phloem lectin after microinjecting FITC-labeled PP2 into mesophyll cells of *C. maxima* cotyledons. In coinjection studies with dextrans, very low concentrations of PP2 directed movement of 20-kD, but not 40-kD, FITC-dextrans. Microinjection of mesophyll cells with an 80- to 100-kD fraction isolated from *C. maxima* phloem exudate composed primarily of PP1 also allowed movement of 20-kD FITC-dextrans. Thus, both proteins could interact with pore–plasmodesma contacts to increase the SEL and move from sieve elements into companion cells. Schobert et al. (1995) identified several chaperones in phloem exudates from castor bean seedlings and speculated about their involvement in unfolding proteins to facilitate symplasmic movement from companion cells to sieve elements. Unidirectional movement of the large 96-kD phloem filament protein soon after its synthesis in companion cells (Clark et al., 1997) could be mediated by chaperones or other “movement-related” proteins in the companion cell that are either developmentally expressed or lacking in the assimilate stream. Alternatively, Fisher et al. (1992) hypothesized that phloem-associated proteins targeted for degradation in companion cells are selectively removed from sieve elements in the transport phloem.

#### Developmental and Functional Implications of P Protein Translocation

Protein and mRNA localization patterns provide convincing evidence that PP1 and PP2 are synthesized in companion cells of differentiating sieve element–companion cell complexes within the transport phloem (Smith et al., 1987; Clark et al., 1997; Dannenhoffer et al., 1997). Based on ultrastructural studies of developing vascular tissues, polymerized forms of the P protein (i.e., tubules, bodies, or filaments) appear to accumulate during differentiation. However, P protein mRNA also can be detected readily in companion cells of mature sieve element–companion cell complexes, especially in the extrafascicular phloem (Bostwick et al., 1992;

Dannenhoffer et al., 1997). Furthermore, microautoradiography of in vivo-labeled proteins led Nuske and Eschrich (1976) to conclude that P proteins are synthesized continually in the companion cells of mature metaphloem. In bundle phloem, in situ hybridization revealed that *PP2* mRNA was easier to detect in early stages of primary growth than later, suggesting that P protein synthesis in the bundle phloem was of limited duration after the onset of translocation (Dannenhoffer et al., 1997). Given a finite developmental period for the accumulation of the polymerized P protein, the continued synthesis of PP1 and PP2 in mature sieve element-companion cell complexes could be the origin of mobile P protein subunits, especially in the extrafascicular phloem where P protein mRNA is most often detected in mature sieve element-companion cell complexes.

Functional continuity of bundle and extrafascicular phloem was demonstrated by immunodetection of *Cucurbita* spp PP1 and PP2 in the entocyclic phloem of young *Cucumis sativus* scion stems (Figures 7 and 8). The anastomosing network of extrafascicular sieve elements with the bundle phloem would allow a steady state level of mobile P protein to be obtained throughout the vascular system. This model suggests that mobile P protein continually enters the assimilate stream throughout the translocation pathway rather than being loaded in source tissues. However, the fate of mobile P protein in the translocation conduit seems to be determined by the direction and intensity of assimilate transport. Tiedemann and Carstens-Behrens (1994) showed by using SDS-PAGE of vascular exudates from intergeneric grafts that *Cucurbita ficifolia* PP1 and PP2 appear in fruit of the *Cucumis sativus* scion, a strong sink organ. The mechanisms of P protein transport appear remarkably similar to the long-distance movement of phloem-distributed viruses that move to all sink tissues including young leaves (Roberts et al., 1997; Santa Cruz et al., 1998).

In contrast, reports of viral movement out of the importing phloem of class III veins by cell-to-cell transport are not paralleled by P protein movement. In transport phloem of stems, neither PP1 nor PP2 was detected outside the sieve element-companion cell complexes by light or electron microscopy immunocytochemistry (Clark et al., 1997; Dannenhoffer et al., 1997), even though plasmodesmata link companion cells with neighboring parenchyma cells (Kempers et al., 1998). Although microinjection studies have shown that PP1 and PP2 can gate plasmodesmata to mediate cell-to-cell movement in nonvascular tissue (Balachandran et al., 1997), P proteins do not seem able to gate the plasmodesmata linking companion cells with phloem parenchyma to move beyond the sieve element-companion cell complex.

The functional role of P proteins and the interrelationship between mobile P protein subunits and polymerized structures remain unresolved. However, P protein deposition appears to be much more dynamic than originally thought, and the existence of translocatable P protein subunits suggests that P protein interactions can occur at a distance from the site of synthesis. It appears likely that polymerized

and unpolymerized P proteins exist in dynamic equilibrium within sieve elements, where the concentration of each is responsive to physiological changes within the vascular system. Change in the redox state of the phloem sap has been proposed as one mechanism to regulate gel-sol transitions between P protein subunits and filaments (Alosi et al., 1988). Blockage of sieve tubes by P protein filaments plugging the sieve plate pores (Evert, 1982; Schulz, 1986) and oxidative cross-linking of P protein subunits at wound surfaces (Read and Northcote, 1983a) are consistent with the interpretation that the redox state in the sieve tube sap plays a regulatory role in aggregating P protein filaments. Intergeneric grafting studies also indicate that divergent P proteins from two genera can coexist without dramatic effects on the plant phenotype. The ability to follow genus-specific P proteins in both transgenic and intergeneric grafted plants will provide greater insights into the functional significance of transporting these proteins throughout the plant.

## METHODS

### Plant Cultivation and Grafting

Seedlings of *Cucumis sativus* cv Hoffmanns Produkta, *Cucurbita maxima* cv Gelber Zentner, and *Cucurbita ficifolia* cv Clevia were grown and grafted by the technique described by Golecki et al. (1998). *Cucumis sativus* was used as the scion and *Cucurbita maxima* or *Cucurbita ficifolia* as rootstocks. *Cucurbita maxima* cv Big Max and *Cucumis melo* cv Charentais were used only as ungrafted control plants.

### Isolation of Phloem Exudate Proteins

Phloem exudate samples were collected from scion and stock of 13-day-old *Cucumis sativus* and *Cucurbita maxima* approach grafts. In all cases, the first transverse cut across the scion hypocotyl below the cotyledons separated the *Cucumis sativus* scion from the *Cucurbita maxima* stock to avoid cross-contamination of *Cucumis sativus* tissue with *Cucurbita maxima* phloem sap. Afterward, exudate from the scion and the stock was taken immediately from the basal end of the hypocotyl below the cotyledons and from a subsequent cut below the next leaf (Figure 1, site 1). For immunoblot analysis, phloem exudates were diluted 1:4 in extraction buffer (0.1 M Tris, pH 8.2, 5 mM EDTA, and 20 mM DTT). Total protein concentration of each sample was determined according to Lowry et al. (1951) or by the Bradford assay (Bio-Rad), using BSA as a standard.

### SDS-PAGE and Immunoblot Analysis

Reduced phloem exudate proteins were separated by SDS-PAGE in 15% acrylamide gels (Laemmli, 1970). After electrophoresis, the gels were either stained with Coomassie Brilliant Blue R 250 or the proteins were transferred from the gel to Immobilon-P membrane (Millipore,

Bedford, MA) by electroblotting in a TransBlot apparatus (Bio-Rad) by using a Tris-glycine buffer (Towbin et al., 1979). Polyclonal antibodies against *Cucurbita maxima* PP1 and PP2 were raised in New Zealand white rabbits, and IgG was purified on protein A columns as described by Dannenhoffer et al. (1997) and Clark et al. (1997). Blots were incubated overnight with either *Cucurbita maxima* PP1 (1:500,000 [v/v]) or PP2 (1:500,000 [v/v]) polyclonal antibodies. Alkaline phosphatase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) was diluted 1:10,000 for the secondary antibody reaction. The immunoblotting procedure and the detection with the chemiluminescent substrate were adapted from the Tropix Western-Light kit protocol (Tropix, Bedford, MA).

### RNA and DNA Isolation

Total RNA and genomic DNA were extracted from various organs by using the method of Gustincich et al. (1991) as modified by Clark et al. (1997). RNA was isolated from hypocotyls, stems, and petioles of individual *Cucumis sativus* scions and their respective *Cucurbita* spp rootstocks (Figure 1B, site 2), as well as from ungrafted *Cucumis* spp and *Cucurbita* spp control plants. Genomic DNA was isolated from young leaves of *Cucumis* spp and *Cucurbita* spp control plants.

### RNA Gel Blot Analysis

Ten micrograms of total RNA was electrophoresed in a 1.2% agarose-glyoxal gel. The *Cucurbita maxima* hypocotyl control sample was intentionally underloaded (1.25 µg of total RNA) to allow the blot to be overexposed during autoradiography. RNA was transferred onto nylon membrane (Magna Charge; Micron Separations Inc., Westboro, MA) and probed with <sup>32</sup>P-labeled *Cucurbita maxima* PP1 and PP2 DNA probes. DNA probes for all nucleic acid hybridizations were generated by polymerase chain reaction (PCR) amplification of the protein coding sequences of their respective genes (see Bostwick et al. [1994] for PP2 primers; PP1 5' primer 5'-GCGAATTCATGAGTTTGCAG-3' and 3' primer 5'-GCGCTCGAGTCAACACTTCCTTTGC-3') and labeled using the RadPrime DNA Labeling System (Gibco BRL). Hybridization and wash conditions were as described by the membrane manufacturer. Blots were prehybridized at 65°C in prehybridization solution (5 × SSPE [1 × SSPE is 0.18 M NaCl, 10 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.7, and 1 mM EDTA], 50% formamide, 0.1% SDS, 100 µg/mL denatured salmon sperm DNA, and 5 × Denhardt's solution [1 × Denhardt's solution is 0.02% Ficoll, 0.02% PVP, and 0.02% BSA]) and hybridized at 65°C in hybridization solution (5 × SSPE, 10% dextran sulfate, 0.5% SDS, 100 µg/mL denatured salmon sperm DNA, and 5 × Denhardt's solution). After hybridization, blots were washed several times at 65°C for 30 min each in 2 × SSPE and 0.5% SDS and rinsed with 2 × SSPE. After autoradiography, the blot was stripped and rehybridized with a <sup>32</sup>P-labeled 18S ribosomal gene probe, as previously described.

### Reverse Transcription-PCR and Gel Blot Analysis of Reverse Transcription-PCR Products

Oligonucleotide primers (see RNA gel blot analysis) flanking the protein coding sequences of *Cucurbita maxima* PP1 and PP2 genes

(Bostwick et al., 1992; Clark et al., 1997) were used for first-strand synthesis and amplification of mRNA templates. Control reactions were performed using the 5' primer (5'-GGIACTGGAATGGTIAAGG-3') and 3' primer (5'-GIGATCTCCTTGCTCATACTG-3') designed from the sequence of a pea actin cDNA (Genbank accession number X67666). One microgram of total RNA was denatured for 3 min at 65°C and added to the reverse transcription reaction mix (final concentrations: 1 mM 3' primer, 5 mM DTT, 1 mM each deoxynucleotide triphosphate, 1 × reverse transcriptase buffer [Gibco BRL], and 150 units Moloney murine leukemia virus reverse transcriptase in a total volume of 20 µL). Samples were incubated at 37°C for 60 min, heated to 95°C for 5 min, and cooled to 10°C for 15 min. The cDNA was amplified by PCR as described by Bostwick et al. (1992). The reverse transcription-PCR (RT-PCR) products were electrophoresed in a 1.0% agarose gel, transferred onto nitrocellulose membrane (Gibco BRL), and probed with either <sup>32</sup>P-labeled *Cucurbita maxima* PP1 or PP2 DNA probes or a *Cucumis sativus* actin probe. Blots were hybridized overnight in 2 × phosphate saline solution (Sambrook et al., 1989) at 65°C and washed three times in a low-stringency solution (2 × SSC [1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate] and 0.1% SDS) and for 30 min in a high-stringency solution (0.1% SSC and 0.1% SDS) at 65°C.

### Genomic DNA Blot Analysis

Genomic DNA was digested to completion with the restriction endonuclease HindIII and electrophoresed in a 0.9% agarose gel. DNA was transferred onto Optitran nitrocellulose membrane (Schleicher & Schuell). Blots were prehybridized at 65°C in prehybridization solution (6 × SSC, 0.5% SDS, 100 µg/mL denatured salmon sperm DNA, and 5 × Denhardt's solution) and hybridized at 65°C in prehybridization solution containing <sup>32</sup>P-labeled *Cucurbita maxima* PP1 or PP2 DNA probes. Blots were then washed under low-stringency (twice for 5 min each in 7 × SSPE and 0.1% SDS at room temperature and twice for 5 min each in 1 × SSPE and 0.5% SDS at 37°C) and high-stringency (1 hr in 0.1 × SSPE and 1% SDS at 65°C) conditions, as described by the membrane manufacturer.

### Tissue Printing

PP1 and PP2 were immunolocalized on tissue prints of 17- and 15-day-old *Cucumis sativus* and *Cucurbita ficifolia* approach grafts. Corresponding ungrafted plants were used as controls. Tissue prints were obtained from transverse-cut hypocotyls of the *Cucumis sativus* scion (Figure 1B, site 3a), transverse-cut stems of the scion and rootstock (~10 cm above the graft union; Figure 1B, sites 3b), and transverse-cut petioles of young leaves (~10 cm above the graft union; Figure 1B, sites 3c). Immediately after cutting, the exposed tissue was blotted on paper towel before printing on nitrocellulose (Protan BA 85; Schleicher & Schuell). Tissue prints were blocked for 1 hr in Tris-buffered saline (TBS; 500 mM NaCl and 20 mM Tris, pH 7.5) plus 3% dry milk for PP1 and 5% (w/v) dry milk for PP2 immunolocalization. Then, either PP1 (1:5000 [v/v]) or PP2 antibodies (1:2000 [v/v]) were added and incubated for 3 hr. Tissue prints were washed with TBS and then incubated for 1 hr with 10-nm gold-labeled goat anti-rabbit IgGs (1:50 [v/v]; Amersham Life Science). After washing with TBS, TTBS (TBS with 0.15% [v/v] Tween 20), and distilled water, the prints were incubated for 20 min with Intense M (Amersham Life Science) for silver enhancement.

### Immunolocalization

*Cucurbita maxima* PP1 and PP2 were immunolocalized in 19-day-old *Cucumis sativus* and *Cucurbita maxima* approach grafts. Ungrafted plants were used as controls. Hand-cut stem sections (Figure 1B, sites 4) were postfixed at room temperature in 2% paraformaldehyde–0.1 M cacodylate buffer, pH 7.3, for 30 min, washed twice in PBS (137 mM NaCl, 3 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, and 2 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.2), covered with tissue-freezing medium (Jung, Leica Instruments GmbH, Nussloch, Germany), and frozen at –30°C for 30 to 60 sec in the freeze station of a Reichert-Jung Frigocut 2800E cryotome (Leica Instruments). Transverse 14- $\mu$ m sections were mounted on poly-L-lysine-coated slides and incubated overnight (17 hr) with *Cucurbita maxima* PP1 (1:2500 [v/v]) or PP2 (1:4000 [v/v]) polyclonal antibodies. Sections were then washed and incubated with goat anti-rabbit 10 nm gold (1:50 [v/v]; Amersham Life Science) for 1 hr, followed by subsequent washes in PBS and distilled water, and incubated for 15 to 25 min with Intense M for silver enhancement.

### ACKNOWLEDGMENTS

This research was supported by Grant No. Schu 617/4-1 from the Deutsche Forschungsgemeinschaft, Germany; Grants No. IBN-9422615 and No. 9727626 from the National Science Foundation Integrative Plant Biology Program; and a Collaborative Research Grant from the North Atlantic Treaty Organization International Scientific Exchange Programmes, Belgium.

Received May 13, 1998; accepted October 26, 1998.

### REFERENCES

- Allen, A.K. (1979). A lectin from the exudate of the fruit of the vegetable marrow (*Cucurbita pepo*) that has a specificity for a  $\beta$ -1,4-linked *N*-acetylglucosamine oligosaccharide. *Biochem. J.* **183**, 133–137.
- Alosi, M.C., Melroy, D.L., and Park, R.B. (1988). The regulation of gelation of phloem exudate from *Cucurbita* fruit by dilution, glutathione, and glutathione reductase. *Plant Physiol.* **86**, 1089–1094.
- Anantharam, V., Patanjali, S.R., Swamy, M.J., Sanadi, A.R., Goldstein, I.J., and Suroliya, A. (1986). Isolation, macromolecular properties, and combining site of chito-oligosaccharide-specific lectin from the exudate of ridge gourd (*Luffa acutangula*). *J. Biol. Chem.* **261**, 14621–14627.
- Balachandran, S., Xiang, Y., Schober, C., Thompson, G.A., and Lucas, W.J. (1997). Phloem sap proteins from *Cucurbita maxima* and *Ricinus communis* have the capacity to traffic cell to cell through plasmodesmata. *Proc. Natl. Acad. Sci. USA* **94**, 14150–14155.
- Beyenbach, J., Weber, C., and Kleinig, H. (1974). Sieve-tube proteins from *Cucurbita maxima*. *Planta* **119**, 113–124.
- Bostwick, D.E., Dannenhoffer, J.M., Skaggs, M.I., Lister, R.M., Larkins, B.A., and Thompson, G.A. (1992). Pumpkin phloem lectin genes are specifically expressed in companion cells. *Plant Cell* **4**, 1539–1548.
- Bostwick, D.E., Skaggs, M.I., and Thompson, G.A. (1994). Organization and characterization of *Cucurbita* phloem lectin genes. *Plant Mol. Biol.* **26**, 887–897.
- Clark, A.M., Jacobsen, K.R., Dannenhoffer, J.M., Skaggs, M.I., and Thompson, G.A. (1997). Molecular characterization of a phloem-specific gene encoding the filament protein, phloem protein 1 (PP1), from *Cucurbita maxima*. *Plant J.* **12**, 49–61.
- Crafts, A.S. (1932). Phloem anatomy, exudation and transport of organic nutrients in cucurbits. *Plant Physiol.* **7**, 183–225.
- Cronshaw, J. (1975). P-proteins. In *Phloem Transport*, S. Aronoff, J. Dainty, P.R. Gorham, L.M. Srivastava, and C.A. Swanson, eds (New York: Plenum Press), pp. 79–147.
- Cronshaw, J., and Sabnis, D.D. (1990). Phloem proteins. In *Sieve Elements, Comparative Structure, Induction and Development*, H.-D. Behnke and R.D. Sjolund, eds (Berlin: Springer-Verlag), pp. 257–283.
- Dannenhoffer, J.M., Schulz, A., Skaggs, M.I., Bostwick, D.E., and Thompson, G.A. (1997). Expression of the phloem lectin is developmentally linked to vascular differentiation in cucurbits. *Planta* **201**, 405–414.
- Esau, K., and Cronshaw, J. (1967). Tubular components in cells of healthy and tobacco mosaic virus-infected *Nicotiana*. *Virology* **33**, 26–35.
- Evert, R.F. (1982). Sieve-tube structure in relation to function. *Bio-science* **32**, 789–795.
- Fischer, A. (1884). Untersuchungen über das Siebröhrensystem der Cucurbitaceen. (Berlin: Gebrüder Borntraeger).
- Fisher, D.B., Wu, Y., and Ku, M.S.B. (1992). Turnover of soluble proteins in the wheat sieve tube. *Plant Physiol.* **100**, 1433–1441.
- Golecki, B., Schulz, A., Carstens-Behrens, U., and Kollmann, R. (1998). Evidence for graft transmission of structural phloem proteins or their precursors in heterografts of Cucurbitaceae. *Planta* **206**, 630–640.
- Gustincich, S., Manfioletti, G., del Sal, G., and Schneider, C. (1991). A fast method for high quality genomic DNA extraction from whole human blood. *BioTechniques* **11**, 298–302.
- Kempers, R., and Van Bel, A.J.E. (1997). Symplasmic connections between sieve elements and companion cell in the stem phloem of *Vicia faba* L. have a molecular exclusion limit of at least 10 kDa. *Planta* **201**, 195–201.
- Kempers, R., Prior, D.A.M., Van Bel, A.J.E., and Oparka, K.J. (1993). Plasmodesmata between sieve element and companion cell of extrafascicular stem phloem of *Cucurbita maxima* permit passage of 3 kDa fluorescent probes. *Plant J.* **4**, 567–575.
- Kempers, R., Ammerlaan, A., and Van Bel, A.J.E. (1998). Symplasmic constriction and ultrastructural features of the sieve element/companion cell complex in the transport phloem of apoplasmically and symplasmically phloem-loading species. *Plant Physiol.* **116**, 271–278.
- Knoblauch, M., and Van Bel, A.J.E. (1998). Sieve tubes in action. *Plant Cell* **10**, 35–50.
- Kühn, C., Franceschi, V.R., Schulz, A., Lemoine, R., and Frommer, W.B. (1997). Macromolecular trafficking indicated by localization

- and turnover of sucrose transporters in enucleate sieve elements. *Science* **275**, 1298–1300.
- Laemmli, U.K.** (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J.** (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**, 265–275.
- Nakamura, S., Hayashi, H., Mori, S., and Chino, M.** (1993). Protein phosphorylation in the sieve tubes of rice plants. *Plant Cell Physiol.* **34**, 927–933.
- Narváez-Vásquez, J., Pearce, G., Orozco-Cardenas, M., Franceschi, V.R., and Ryan, C.A.** (1995). Autoradiographic and biochemical evidence for the systemic translocation of systemin in tomato plants. *Planta* **195**, 593–600.
- Nelson, R.S., and Van Bel, A.J.E.** (1998). The mystery of virus trafficking into, through and out of vascular tissue. *Progr. Bot.* **59**, 476–533.
- Nuske, J., and Eschrich, W.** (1976). Synthesis of P-protein in mature phloem of *Cucurbita maxima*. *Planta* **132**, 109–118.
- Read, S.M., and Northcote, D.H.** (1983a). Subunit structure and interactions of the phloem proteins of *Cucurbita maxima* (pumpkin). *Eur. J. Biochem.* **134**, 561–569.
- Read, S.M., and Northcote, D.H.** (1983b). Chemical and immunological similarities between the phloem proteins of three genera of Cucurbitaceae. *Planta* **158**, 119–127.
- Robards, A.W., and Lucas, W.J.** (1990). Plasmodesmata. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **41**, 369–419.
- Roberts, A.G., Santa Cruz, S., Roberts, I.M., Prior, D.A.M., Turgeon, R., and Oparka, K.J.** (1997). Phloem unloading in sink leaves of *Nicotiana benthamiana*: Comparison of a fluorescent solute with a fluorescent virus. *Plant Cell* **9**, 1381–1396.
- Sabnis, D.D., and Hart, J.W.** (1976). A comparative analysis of phloem exudate proteins from *Cucumis melo*, *Cucumis sativus* and *Cucurbita maxima* by polyacrylamide gel electrophoresis and isoelectric focusing. *Planta* **130**, 211–218.
- Sabnis, D.D., and Hart, J.W.** (1978). The isolation and some properties of a lectin (haemagglutinin) from *Cucurbita* phloem exudate. *Planta* **142**, 97–101.
- Sabnis, D.D., and Hart, J.W.** (1979). Heterogeneity in phloem protein complements from different species: Consequences to hypotheses concerned with P-protein functions. *Planta* **145**, 459–466.
- Sabnis, D.D., and Sabnis, H.M.** (1995). Phloem proteins: Structure, biochemistry and function. In *The Cambial Derivatives*, M. Iqbal, ed (Berlin: Gebrüder Borntraeger), pp. 271–292.
- Sakuth, T., Schobert, C., Pecsvaradi, A., Eichholz, A., Komor, E., and Orlich, G.** (1993). Specific proteins in the sieve-tube exudate of *Ricinus communis* L. seedlings: Separation, characterization and in-vivo labeling. *Planta* **191**, 207–213.
- Sambrook, J., Fritsch, E.F., and Maniatis, T.** (1989). *Molecular Cloning: A Laboratory Manual*, 2nd ed. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).
- Santa Cruz, S., Roberts, A.G., Prior, D.A.M., Chapman, S., and Oparka, K.J.** (1998). Cell-to-cell transport and phloem-mediated transport of potato virus X: The role of virions. *Plant Cell* **10**, 495–510.
- Schaller, A., and Ryan, C.A.** (1995). Systemin—A polypeptide defense signal in plants. *Bioessays* **18**, 27–33.
- Schobert, C., Grossmann, P., Gottschalk, M., Komor, E., Pecsvaradi, P., and Nieden, U.Z.** (1995). Sieve-tube exudate from *Ricinus communis* L. seedlings contains ubiquitin and chaperones. *Planta* **196**, 205–210.
- Schulz, A.** (1986). Wound phloem in transition to bundle phloem in primary roots of *Pisum sativum* L. II. The plasmic contact between wound-sieve tubes and regular phloem. *Protoplasma* **130**, 27–40.
- Smith, L.M., Sabnis, D.D., and Johnson, R.P.C.** (1987). Immunohistochemical localisation of phloem lectin from *Cucurbita maxima* using peroxidase and colloidal-gold labels. *Planta* **170**, 461–470.
- Terry, B.R., and Robards, A.W.** (1987). Hydrodynamic radius alone governs the mobility of molecules through plasmodesmata. *Planta* **171**, 145–157.
- Tiedemann, R., and Carstens-Behrens, U.** (1994). Influence of grafting on the phloem protein patterns in Cucurbitaceae. I. Additional phloem exudate proteins in *Cucumis sativus* grafted on two *Cucurbita* species. *J. Plant Physiol.* **143**, 189–194.
- Towbin, H., Staehelin, T., and Gordon, T.** (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. *Proc. Natl. Acad. Sci. USA* **76**, 4350–4354.
- Van Bel, A.J.E., and Kempers, R.** (1997). The pore/plasmodesm unit: Key element in the interplay between sieve element and companion cell. *Progr. Bot.* **58**, 278–291.
- Wolf, S., Deom, C.M., Beachy, R.N., and Lucas, W.J.** (1989). Movement protein of tobacco mosaic virus modifies plasmodesmal size exclusion limit. *Science* **246**, 377–379.