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Phloem Loading in *Coleus blumei* in the Absence of Carrier-Mediated Uptake of Export Sugar from the Apoplast[1]

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ABSTRACT

Phloem loading in *Coleus blumei* Benth. leaves cannot be explained by carrier-mediated transport of export sugar from the apoplast into the sieve element-companion cell complex, the mechanism by which sucrose is thought to load in other species that have been studied in detail. Uptake profiles of the export sugars sucrose, raffinose, and stachyose into leaf discs were composed of two components, one saturable and the other not. Saturable (carrier-mediated) uptake of all three sugars was almost completely eliminated by the inhibitor *p*-chloromercuribenzenesulfonic acid (PCMBS). However, when PCMBS was introduced by transpiration into mature leaves it did not prevent accumulation of ^{14}C-photosynthate in minor veins or translocation of labeled photosynthate from green to nonchlorophyllous regions of the leaf following exposure to $^{14}CO_2$. The efficacy of introducing inhibitor solutions in the transpiration stream was proven by observing saffranin O and calcofluor white movement in the minor veins and leaf apoplast. PCMBS introduced by transpiration completely inhibited phloem loading in tobacco leaves. Phloem loading in *C. blumei* was also studied in plasmolysis experiments. The carbohydrate content of leaves was lowered by keeping plants in the dark and then increased by exposing them to light. The solute level of intermediary cells increased in the light (phloem loading) in both PCMBS-treated and control tissues. A mechanism of symplastic phloem loading is proposed for species that translocate the raffinose series of oligosaccharides.

Sucrose-proton cotransport is considered by most investigators to be the driving force for phloem loading in those species that have been analyzed in detail (3, 4, 9). According to this hypothesis, sucrose passes from mesophyll cell to mesophyll cell through plasmodesmata, enters the extracellular space (apoplast) in the vicinity of the phloem, and is cotransported with protons into the SE-CCC[2] by a carrier located in the plasma membrane. An attractive feature of this model is that it accounts for two fundamental characteristics of translocation in the phloem: selectivity for export sugar and the very high concentration of sugar in the SE-CCC.

The suggestion has also been made that loading might occur by a symplastic route, *i.e.* through plasmodesmata from mesophyll cells to the SE-CCC (16, 29). There is little direct, and no compelling, evidence for such a pathway. However, the presence of large numbers of plasmodesmata at the interface between the companion cells and bundle sheath in the minor veins of some species has stimulated considerable interest in the symplastic phloem loading concept. In a large and taxonomically diverse group of plants, some of the companion cells of the minor vein phloem are specialized to the extent that they are structurally distinct from 'ordinary' companion cells or companion cells specialized as transfer cells and are called 'intermediary cells' (for discussion see ref. 25). Intermediary cells, found in members of the Cucurbitaceae (21, 27), in *Coleus blumei* (5), and in a variety of woody species (6, 7), are large, densely cytoplasmic, and are always connected to each other and to bundle sheath cells by numerous fields of plasmodesmata (27).

One of the most convincing lines of evidence for the apoplastic phloem loading pathway is the inhibition of both sucrose-proton cotransport and phloem loading by the sulfhydryl-modifying compound PCMBS. This inhibitor is relatively impermeant and therefore must block uptake from the apoplast (8, 9). We previously noted that uptake of exogenous sucrose into the minor veins of *C. blumei* leaves is insensitive to PCMBS (30), calling into question the relevance of sucrose-proton cotransport in the process of phloem loading in this species. Madore and Lucas (15) also demonstrated that, although PCMBS inhibits vein loading of [^{14}C]sucrose, it does not block transport of $^{14}CO_2$-derived assimilate from mesophyll cells to the minor veins of *Ipomoea tricolor*. However, results of this type must be viewed with caution since the site of accumulation of label is unknown. It is not clear that transport to the vein, either from [^{14}C]sucrose or ^{14}C-photoassimilate, involves loading of sugar into the SE-CCC; the label could accumulate instead in the bundle sheath or phloem parenchyma cells. To show that true phloem loading in *C. blumei* occurs in the absence of sugar-proton cotransport from the apoplast it is necessary to demonstrate that: (a) PCMBS inhibits carrier-mediated uptake of export sugars in this plant; (b) PCMBS can be delivered effectively to the apoplast surrounding the SE-CCC; (c) photosynthetically derived, as well as exogenous, sugar accumulates in the minor veins in the presence of PCMBS; (d) this photosynthate enters the SE-CCC of the veins in the presence of PCMBS; and (e) translocation of photosynthate occurs in the presence of PCMBS. These issues are addressed here in turn.

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[2] Abbreviations: SE-CCC, sieve element-companion cell complex; PCMBS, *p*-chloromercuribenzenesulfonic acid.

MATERIALS AND METHODS

Plant Material

Coleus blumei Benth. cv Candidum plants were grown in a greenhouse in artificial soil as described (30). Seeds, originally from G. W. Park Seed Co. (Greenwood, SC), are no longer available from that source; they may be obtained from the authors.

Sugar Uptake Studies

Leaf discs (5.6 mm in diameter) were cut, under the surface of Mes-NaOH buffer (pH 5.5, containing 2 mM $CaCl_2$) from the green portion of mature leaves abraded with carborundum. Incubations in buffered ^{14}C-labeled sugar solutions (5–40 kBq·mL^{-1}; 2 mL volume) were carried out in 3.5-cm plastic Petri dishes for 1 h. The label in buffer-washed (30 min; 4°C) leaf discs was measured by scintillation counting (24). Details of the procedures have been described (30).

^{14}C-Labeled Sugars

[U-^{14}C]Sucrose was obtained from Amersham Corp. (Arlington Heights, IL). Labeled raffinose and stachyose were prepared from mature leaves of *Cucurbita pepo* L. plants supplied with $^{14}CO_2$ for 15 min followed by a 15 min chase in room atmosphere. Labeled leaves were flash frozen in liquid N_2 and extracted in 80% ethanol (v/v) saturated with $BaCO_3$ at 60°C. Extracts were passed through ion-exchange columns (Bio-Rad AG2-X8 [formate] anion, and AG50W-X8 [hydrogen] cation resins). Radioactive compounds were purified by TLC on tapered silica gel G plates (Uniplates; Analtech, Newark, DE) with chloroform/acetic acid/water (6/7/1; two developments). This solvent system resolved all compounds of interest except raffinose and galactinol which were separated on tapered plates with isopropanol/water (5/1). For two-dimensional identification of sugars in translocation experiments, analytical silica gel GHL plates (Analtech) were used with the two solvent systems described above. Labeled sugars were localized with x-ray films.

Transpiration

To follow the path of transpired solutions, the cut end of the petiole of an excised leaf was immersed in 0.1% (w/v) saffranin O for 30 min in the light (300 μmol photons·m^{-2}·s^{-1}). Movement of solutions in the leaf apoplast was monitored by allowing the leaf to transpire a 0.1% calcofluor white (Sigma) solution for 30 min. Leaf tissue was fixed in ethanol/acetic acid/chloroform (6/1/3) for 6 h, dehydrated in ethanol, and embedded in Spurr medium (23). Thin (1 μm) sections were viewed under epifluorescent UV light (Olympus BH-2 microscope, New Hyde Park, NY).

Labeling with $^{14}CO_2$

In translocation experiments, detached leaves were enclosed in a Plexiglas chamber and $^{14}CO_2$ (0.5 MBq) was generated inside the chamber by addition of excess 80% lactic acid to $Na_2{}^{14}CO_3$ ($6.6 \cdot 10^5$ MBq·mmol^{-1}) (30). During the labeling and subsequent transport periods, the cut end of the petiole was kept in water (control) or PCMBS solution.

Autoradiography

Tissue pieces or whole leaves were frozen in powdered dry ice, lyophilized, pressed flat between polished steel plates in a large vise, and pressed against x-ray film (hyperfilm-βmax, Amersham) as described (30).

Plasmolysis

Plants were kept in low light (15–20 μmol photons·m^{-2}·s^{-1}; 14 h day, 10 hr night) for 5 d and then in complete darkness for 2 d to reduce the solute content of the phloem. Leaves from darkened plants were excised and allowed to transpire PCMBS (1 mM) or water (control) for 60 min. To facilitate transpiration without allowing significant photosynthesis to occur, the leaves were exposed to dim light (15–25 μmol photons·m^{-2}·s^{-1}) and low RH (30%), and air was circulated over them with a fan. Observations of transpired saffranin movement in such leaves demonstrated the efficacy of the procedure. The upper surfaces of the leaves were abraded with carborundum and small (1 mm·2.5 mm) pieces of tissue were excised and placed, abraded side down, on filter paper saturated with either PCMBS (1 mM) or Mes buffer (controls) for 30 min in the dark to allow further exposure to the inhibitor. The leaf pieces were then either illuminated (500 μmol photons·m^{-2}·s^{-1}) or kept in the dark for 90 min on either PCMBS (1 mM) or Mes buffer (controls). In some experiments, tissue pieces were incubated in either sucrose or stachyose (20 mM). To test for plasmolysis the tissue was submerged in sorbitol solution for 30 min in the dark and fixed at room temperature in the dark in 2% glutaraldehyde (25 mM Hepes-KOH buffer [pH 6.8]) with sufficient added sorbitol to achieve the same total osmolality as the previous solution. The percentage of plasmolyzed intermediary cells was determined in plastic-embedded sections with a ×100 oil-immersion objective (Leitz Ortholux microscope) (26). For each osmoticum treatment, 15 replicates (tissue pieces) were analyzed, each with 8 to 16 intermediary cells. Sectioning and microscopic evaluations were conducted on coded samples to avoid investigator bias. Statistical significance was evaluated by the one-tailed Mann-Whitney test (2).

RESULTS

PCMBS-Sensitivity of Sugar Uptake

We first tested the effectiveness of PCMBS in inhibiting the uptake of export sugars into cells. *Coleus blumei* translocates the oligosaccharides raffinose and stachyose, as well as sucrose (14). Uptake curves of all three sugars were similar to those reported for sucrose in other species: a saturable component of uptake, which has been studied extensively and which is due, at least in the case of sucrose, to carrier-mediated cotransport with protons (12, 19), occurs in conjunction with a first-order (linear) mechanism (Fig. 1). When PCMBS (1 mM) was included in the incubation medium, the saturable component of sugar uptake was inhibited by 85 to 94% (Fig. 1). These

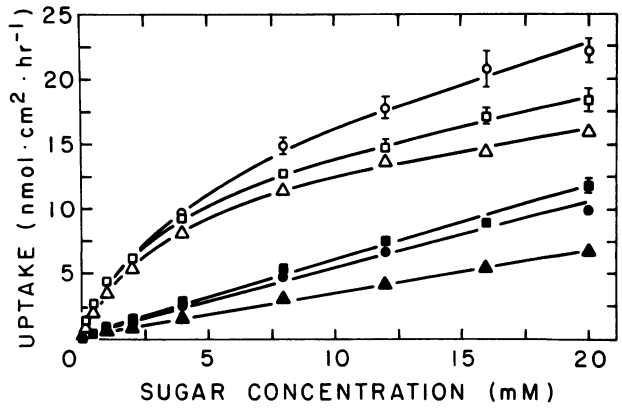

Figure 1. Net influx of ^{14}C-labeled sucrose (○, ●), raffinose (□, ■), and stachyose (△, ▲) into leaf discs at increasing sugar concentrations either with 1 mM PCMBS (closed symbols), or without PCMBS (open symbols). Experiments on the different sugars were not run concurrently.

results demonstrate that PCMBS is an effective inhibitor of carrier-mediated sucrose, raffinose, and stachyose uptake in *C. blumei*.

Penetration of PCMBS

To ensure that PCMBS was able to penetrate into the apoplast of the minor veins it was introduced via the transpiration stream in detached leaves. Microscopic observations of whole leaves indicated that saffranin O dye introduced in the same way (40 min transpiration period) readily penetrated to the farthest extremities of the minor vein network. In other experiments, calcofluor white was introduced by transpiration and plastic-embedded sections of tissue were examined by epifluorescence microscopy. The dye stained the cell walls of the vascular bundle, including all cells of the phloem, intensely, indicating that there is no barrier to diffusion of material from the xylem to the phloem in minor veins. The concentration of PCMBS in the apoplast was calculated from the concentration of the uptake solution (1 mM), the amount of solution transpired (obtained by difference in weight of solution before and after the experiment), the water content of the leaf (91% of fresh weight), and the assumption that apoplast water constitutes 15% of total leaf water content (13). The mean apoplast concentration of PCMBS delivered by transpiration ranged between 0.5 and 1.8 mM in the experiments reported here. This is a very conservative estimate of local PCMBS concentration around the phloem cells of the minor veins since it is an average value for the lamina while the highest concentration of inhibitor was in the veins.

Following transpiration of 0.5 mM PCMBS, vein loading of exogenous [^{14}C]sucrose was completely inhibited in tobacco leaf tissue (not shown). Transpiration of PCMBS also fully inhibited vein loading in *Vicia faba* leaves (1).

Vein Loading in PCMBS-Treated Leaves

For studies of photosynthate transport to veins, leaves that had been treated with PCMBS as described above were exposed to $^{14}CO_2$ for 5 min. Leaves were flash frozen, lyophilized, and autoradiographed after a further 15 min period in room atmosphere. Accumulation of ^{14}C-labeled photosynthate in the minor veins was apparent in both PCMBS-treated and control leaves (Fig. 2).

Entry of Photosynthate into the SE-CCC

We used the plasmolysis technique to determine if solute is transported from the mesophyll into intermediary cells in the presence of PCMBS. The carbohydrate content of the leaf was first lowered by darkening the plants, and PCMBS was introduced by a combination of transpiration and incubation of small tissue pieces on PCMBS solution. Plasmolysis was then used to determine whether the solute content of intermediary cells would increase as a result of exposure to exogenous sugar or to light.

In preliminary experiments, control leaf tissue (no PCMBS) was plasmolyzed in graded steps of osmoticum. The greatest discrimination between leaves kept in the light and dark was obtained at 800 mOsm/Kg sorbitol; at this osmolality many of the intermediary cells in dark-treated, but few of those in light-treated, tissue pieces were plasmolyzed. Therefore, in subsequent experiments the response of intermediary cells to this single concentration of osmoticum was used as an index of solute content. While this method does not determine the point of incipient plasmolysis, and thus does not measure absolute solute levels, it is a reliable test for differences in solute potential and it has the advantage that many replicates can be run and differences in response to experimental treatments can be evaluated statistically.

In samples from control leaves, the solute content of intermediary cells increased significantly (fewer cells plasmolyzed) when tissue was placed in the light (Fig. 3). This increase must have been due to uptake of solute since intermediary cells are not photosynthetically active; plastids are found infrequently, are diminutive, and contain very few internal membranes and no grana (5). The solute content of intermediary cells also increased when the leaf pieces were incubated, in the dark, on sucrose or stachyose (Fig. 3).

In PCMBS-treated tissue, the solute content of intermediary

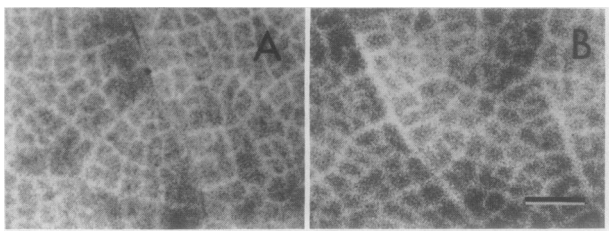

Figure 2. ^{14}C-Labeled photosynthate in minor veins of PCMBS-treated (A), and water-treated (B) control leaves after 5 min of exposure to $^{14}CO_2$ and 15 min of transport. PCMBS was continuously administered by transpiration throughout the labeling and chase periods. No accumulation of label was seen in minor veins when tissue was frozen immediately after the labeling period. X-ray films were used as photographic negatives; therefore, white regions indicate the presence of ^{14}C. Bar = 1 mm. Tissue reduced approximately 20% in area by lyophilization.

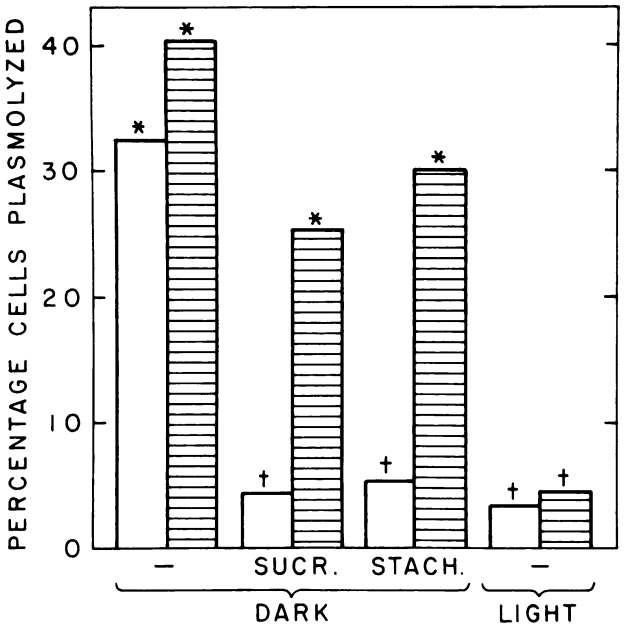

Figure 3. Percentage of minor vein intermediary cells from darkened leaves plasmolyzed by an 800 mOsm/Kg sorbitol solution. Leaves were treated with PCMBS (shaded bars), or water (open bars). After treatment, pieces of tissue were either placed in the light or kept in the dark for 90 min on solutions with or without sucrose or stachyose. The tissue was then plasmolyzed. Different symbols indicate that the values are statistically different at the 95% confidence level.

cells increased significantly in the light, demonstrating that the inhibitor does not prevent loading of photoassimilate into the SE-CCC (Fig. 3). Sucrose and stachyose had little or no effect on solute levels in PCMBS-treated tissue (Fig. 3).

Translocation

Translocation experiments were conducted on leaves that had been excised and treated with PCMBS (1 mM) by transpiration. Label in the leaf was visualized by autoradiography following exposure to $^{14}CO_2$ for 5 min, and a subsequent 1 h translocation period. Translocation from the green to the nonchlorophyllous regions of the lamina was clearly evident in PCMBS-treated leaves although it was reduced in comparison to controls (Fig. 4). Export sugars were analyzed in transport experiments of 15 min duration following exposure of leaves to a 5 min pulse of $^{14}CO_2$ (4): labeled sucrose, raffinose, and stachyose were present in approximately the same proportions in nonchlorophyllous tissue of both PCMBS-treated and control leaves (Table I).

DISCUSSION

The data reported here indicate that carrier-mediated uptake of export sugar from the apoplast is not a sufficient explanation for phloem loading in the leaves of *Coleus blumei*. PCMBS is an effective inhibitor of carrier-mediated sucrose, raffinose, and stachyose uptake from the apoplast yet it does not prevent loading or translocation of photoassimilate. Our results differ from those of Madore (14) in which sugar uptake

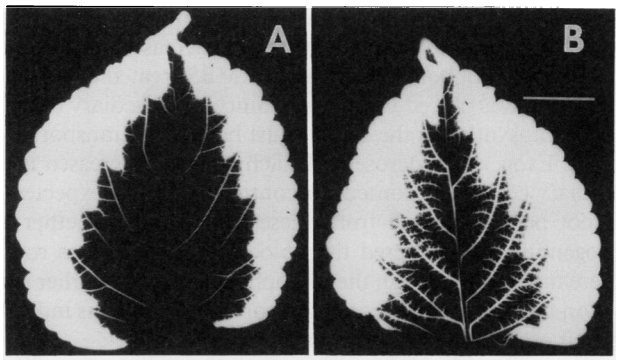

Figure 4. Translocation of ^{14}C-labeled photosynthate from distal green to central nonchlorophyllous regions of PCMBS-treated (A) and water-treated control (B) leaves. Transpiration of PCMBS continued throughout the labeling and transport periods. There was no label in the central nonchlorophyllous region if the tissue was frozen immediately after the $^{14}CO_2$ exposure period. Bar = 2 cm. Leaves reduced approximately 20% in area by lyophilization.

was inhibited by PCMBS to a much lesser extent. The reason for this discrepancy is unknown. Her results with the sucrose carrier, indicating only 30% inhibition, are especially surprising since carrier-mediated sucrose uptake is severely inhibited by this compound in other species (9).

Kinetic profiles of raffinose and stachyose uptake are similar to those that have often been reported for sucrose uptake in that there are both saturable and nonsaturable components. Although we have no direct evidence that the saturable component represents a proton cotransport mechanism, it is presumably carrier-mediated and it is virtually abolished by PCMBS.

A major objective of this study was to determine if photosynthate is able to enter the SE-CCC in the presence of PCMBS. In our previous experiments exogenous sucrose accumulated in the minor veins in PCMBS-treated tissue (30), and in the studies reported here labeled photosynthate did the same. $^{14}CO_2$-Derived assimilate was also transported to the minor veins of PCMBS-treated leaf discs of *Ipomoea tricolor* (15). However, minor veins are complex; the presence of label in veins does not necessarily indicate that it enters the long-distance conducting cells. Two approaches were used to determine if it does so in *C. blumei*. In the first, an increase in the solute content of intermediary cells in carbohydrate-

Table I. *Translocated Radioactive Compounds in Nonchlorophyllous Tissue*

Experiments were performed with and without PCMBS.

Compound	+PCMBS	–PCMBS
	% radioactivity in extract	
Sucrose	7.2	6.2
Raffinose	11.9	15.5
Stachyose	48.3	60.2
Glucose	0.3	0.1
Fructose	1.9	1.5
Galactose	1.8	0.6
Galactinol	3.6	1.9

depleted leaves was monitored by plasmolysis tests. These experiments proved that solute levels increased in the intermediary cells of both control and PCMBS-treated tissue when the tissue was placed in the light. Since intermediary cells are not photosynthetic, the solute must have been transported to them. Exogenous sucrose and stachyose also increased intermediary cell solute content in control tissues, as expected. It is not possible to tell from these experiments whether the exogenous sugar entered the phloem directly or was routed there indirectly through the mesophyll. However, earlier studies on the timing of sucrose transport to minor veins indicates that it enters the mesophyll first (28).

Madore (14) has suggested that the interconversion of sugars in the mesophyll of the *C. blumei* leaf could help explain our previous findings (28) that there is a time delay between application of [^{14}C]sucrose to the leaf and its appearance in the minor veins. We doubt that this is the case. The conclusion drawn from our experiments is that exogenous sucrose, which is translocated in this species, does not enter the minor veins directly from the free space as expected, given the hypothesis of apoplastic loading; it enters the mesophyll first. The fate of the sucrose after it enters the mesophyll is not relevant to this point. It is true that some of this sucrose could be used in the mesophyll to produce other transport compounds and that this process would influence the time course of loading. On the other hand, it is not clear that the site of synthesis of the raffinose series of sugars is exclusively in the mesophyll; these sugars could also be made from exogenous sucrose after it arrives in the SE-CCC.

Exogenous sugars had a limited effect on the solute content of intermediary cells in PCMBS-treated tissue; while there may have been a moderate elevation in solute level, the effect was not statistically significant at the 0.05 level of confidence. In these experiments PCMBS reduced uptake by approximately half at 20 mM sugar concentration and this may be the reason that solute levels did not go up. On the other hand, the results might also reflect a difference in site of uptake, metabolism, or compartmentalization of exogenous sugar in PCMBS-treated tissue.

Of course, plasmolysis tests do not identify the nature of solute in cells. It could be argued that exposure to light resulted in the transport of solute other than export sugars into the intermediary cells. However, this explanation could not account for the results of translocation experiments, the second approach used to demonstrate entry of photosynthate into the SE-CCC. Labeled sucrose, raffinose, and stachyose were transported from green to nonchlorophyllous regions of PCMBS-treated detached leaves after exposure to $^{14}CO_2$.

The amount of photosynthate exported was reduced by PCMBS in comparison to controls (Fig. 4). It is not clear why this was so. Perhaps the PCMBS that entered the leaves by transpiration was toxic to a limited extent; inhibitor introduced this way is concentrated by transpirational loss of water. Indeed, we noticed in some experiments that the edges of leaf discs cut from PCMBS-treated leaves became discolored, as if the cells that had been traumatized by cutting were damaged by the inhibitor. This effect was not seen in discs from control leaves placed on 1 mM PCMBS. We noted also that intermediary cells of darkened leaves plasmolyzed at a slightly higher frequency than controls when treated with PCMBS (two columns on the left in Fig. 3). This effect is not statistically significant in the figure shown but in other experiments it was more obvious and was clearly significant.

Another possible explanation for the inhibitory effect of PCMBS on translocation is that it has a selective effect on phloem loading. There are two types of companion cell in the minor veins of *C. blumei*: intermediary cells and 'ordinary' companion cells (5). The latter cell type does not have the extensive symplastic connections to bundle sheath or other surrounding cells that characterizes intermediary cells. It has been suggested that these companion cells might load from the apoplast while intermediary cells load symplastically (5). Perhaps the reduction of translocation by PCMBS is due to the inhibition of sugar-proton cotransport from the apoplast into the 'ordinary' companion cells of the minor veins. These cells are considerably smaller than intermediary cells and we could not tell by light microscopy whether their solute content increased in the plasmolysis experiments. Resolution of this question will require further study.

What is the mechanism responsible for loading photosynthate into intermediary cells? One possibility that needs to be explored is that hexoses are loaded from the apoplast with subsequent synthesis of oligosaccharides in the phloem. Carrier-mediated glucose uptake is PCMBS-insensitive (8) and we have determined that this is also true of galactose and fructose uptake in *C. blumei* (our unpublished results). Therefore, hexose-proton cotransport into the SE-CCC from the apoplast cannot be ruled out by the data reported here.

There are two other obvious routes by which export sugars could be loaded into the SE-CCC. First, the linear component of sugar uptake from the apoplast, although not usually regarded as a specific phloem loading process, must be considered. It is not clear what this part of the uptake profile represents in a mechanistic sense. Second, loading could be symplastic; the presence of large numbers of plasmodesmata at the interface of the bundle sheath and intermediary cells is tantalizing, though indirect, evidence in favor of transport from mesophyll to phloem without entry of photosynthate into the extracellular space. PCMBS does not inhibit symplastic transport (18).

The difficulty with symplastic loading is that a feasible mechanism for it has not been proposed. It has been suggested that it could operate by an endoplasmic reticulum-mediated transport process that would presumably allow solute to pass through the desmotubule of the plasmodesma (reviewed in Gunning [10]). However, this idea seems at odds with the current view of plasmodesmata structure (20).

We would like to propose another mechanism that could operate in those species, such as *C. blumei*, that translocate the raffinose series of oligosaccharides. According to this model, the intermediary cell acts as a molecular size-discrimination trap. Compounds with low molecular mass, perhaps sucrose and galactinol (21), pass into the intermediary cell from the bundle sheath through the plasmodesmata along a diffusion gradient and are used to synthesize the larger sugars of the raffinose series in this cell. These sugars are trapped in the phloem due to their size. This would require that the molecular size exclusion limit of the plasmodesmata between the bundle sheath and intermediary cell be smaller than normal. Synthesis of the larger oligosaccharides would main-

tain the diffusion gradient for the incoming compounds and the trapping mechanism would cause the buildup of turgor pressure that drives long-distance transport and carries the newly synthesized sugars away. Raffinose and stachyose synthesis has been shown to occur in the mesophyll cells of cucurbits (11, 17), but this does not preclude the possibility that they are made in the phloem as well (22).

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