Plant Physiol. (1990) 93, 617–622
0032-0889/90/93/0617/06/$01.00/0

Received for publication December 1, 1989
and in revised form February 3, 1990

Carbohydrate Metabolism in Photosynthetic and Nonphotosynthetic Tissues of Variegated Leaves of *Coleus blumei* Benth.[1]

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ABSTRACT

Mature, variegated leaves of *Coleus blumei* Benth. contained stachyose and other raffinose series sugars in both green, photosynthetic and white, nonphotosynthetic tissues. However, unlike the green tissues, white tissues had no detectable level of galactinol synthase activity and a low level of sucrose phosphate synthase indicating that stachyose and possibly sucrose present in white tissues may have originated in green tissues. Uptake of exogenously supplied [^{14}C]stachyose or [^{14}C]sucrose into either tissue type showed conventional kinetic profiles indicating combined operation of linear first-order and saturable systems. Autoradiographs of white discs showed no detectable minor vein labelling with [^{14}C]stachyose, but some degree of vein labeling with [^{14}C]sucrose. Autoradiographs of green discs showed substantial vein loading with either sugar. In both tissues, *p*-chloromercuribenzenesulfonic acid had no effect on the linear component of sucrose or stachyose uptake but inhibited the saturable component. Both tissues contained high levels of invertase, sucrose synthase and α-galactosidase and extensively metabolized exogenously supplied ^{14}C-sugars. In green tissues, label from exogenous sugars was recovered as raffinose-series sugars. In white tissues, exogenous sugars were hydrolysed and converted to amino acids and organic acids. The results indicate that variegated *Coleus* leaves may be useful for studies on both phloem loading and phloem unloading processes in stachyose-transporting species.

The sink-to-source transition in leaves is characterized by an increased synthesis of characteristic transport carbohydrates such as sucrose (8), sorbitol (12), mannitol (6), and raffinose family oligosaccharides (18, 24) as well as a concomitant reduction in enzymatic activities associated with the degradation of these compounds (7, 8, 12, 18). Additionally, it has been suggested that there is some sort of restriction of the phloem unloading pathway, possibly the occlusion of plasmodesmata, which allows net export of transport assimilates (see review, ref. 22). However, the processes which determine whether a leaf tissue will display loading or unloading of phloem tissue are not as yet known (22).

Recently, a rather elegant system for the study of phloem loading and unloading has been recognized, this being the variegated leaf of *Coleus blumei* Benth. (5, 27). The nonchlorophyllous areas of the mature variegated leaf behave from all appearances as true 'sinks' for photoassimilates produced by the green photosynthetically active areas (4, 5, 27), and therefore provide an ideal simplified system for the study both of phloem loading (in the green regions) and phloem unloading (in the white regions) in the same leaf. To date, however, there has been no complete examination of carbohydrate metabolism in these variegated leaves. *Coleus*, a member of the Lamiaceae, contains substantial levels of stachyose and high activity of galactinol synthase in its leaves (9). It is probable, therefore, that like other members of this family (*e.g. Lamium, Elscholtzia, Marrubium, Origonum* [11]; *Salvia* [13]), *Coleus* also translocates stachyose in the phloem. The leaf of variegated *Coleus* therefore provides an unusual opportunity to study the synthesis, phloem loading, phloem unloading and catabolism of the raffinose-family series in the same leaf. The aim of the present study was therefore to characterize the metabolism of carbohydrates in both green and white tissues of variegated *Coleus* leaves, focusing on the raffinose family of oligosaccharides.

MATERIALS AND METHODS

Chemicals

All buffers, reagent chemicals and enzymes were purchased from Sigma Chemical Co., St. Louis, MO. [U-^{14}C]Sucrose (SA[2] = 350 mCi mmol^{-1}) was purchased from ICN Biochemicals, Irvine, CA. [^{14}C]Stachyose (SA = 1.6 mCi mmol^{-1}), [^{14}C]galactinol (SA = 1.2 mCi mmol^{-1}), and [^{14}C]raffinose (SA = 1.3 mCi mmol^{-1}) were extracted from mature pumpkin (*Cucurbita maxima* cv Big Max) leaves after photosynthetic labeling with $^{14}CO_2$ and purified by ion exchange and paper chromatography as described previously (16).

Plant Material

Coleus plants (*Coleus blumei* Benth.) were propagated from cuttings obtained from plants purchased at a local nursery. Rooted cuttings were planted in a mixture of soil:sand:peat mix (1:1:1, by volume) and kept in a greenhouse under natural lighting and approximately 30 °C/20 °C day/night temperatures. Plants were watered daily with half-strength Hoagland

[1] Supported by a grant from the National Science Foundation (DCB 8901785).

[2] Abbreviation: SA, SPS, sucrose phosphate synthase; SS, sucrose synthase; GS, galactinol synthase; PCMBS, *p*-chloromercuribenzenesulfonic acid.

solution. Approximately 8 weeks after planting, plants were transferred to a growth chamber set for a 16-h 30 °C day/8-h 20 °C night cycle, and allowed to equilibrate to the new growth conditions for 1 week prior to use in experiments.

Diurnal Carbohydrate Analysis

Ten leaf discs (6 mm diameter) were excised from the interveinal areas of green and white tissues of mature leaves at 4-h intervals throughout the day/night cycle. Soluble carbohydrates were extracted and analysed by HPLC as described previously (15). Starch remaining in the extracted discs was digested with amyloglucosidase (20) and released glucose quantitated spectrophotometrically using a commercially available glucose detection kit (Sigma HK 10) following the manufacturer's recommendations.

Enzyme Extraction

Leaf tissue (1 g) from green or white areas of mature leaves was ground on ice using a mortar and pestle in 3 mL of grind buffer (SS, SPS: 50 mM Mops-NaOH, 10 mM $MgCl_2$, 5 mM DTT, 0.25% [w/v] BSA [pH 7.5]; invertase, GS, α-galactosidase: 50 mM Mops-NaOH, 20 mM 2-mercaptoethanol [pH 7.5]). The extracts were filtered through cheesecloth, transferred to microfuge tubes and centrifuged for 4 min at 10,000*g* in a microcentrifuge. Aliquots (0.5 ml) of supernatant were desalted on 2 mL Sephadex G25 columns equilibrated with grind buffer. Protein contents of the desalted extracts were determined by the Bradford method (1).

Enzyme Assays

SS and SPS were assayed using 70 μL desalted enzyme as described by Robbins and Pharr (20).

GS was assayed as described by Madore *et al.* (16) except that the galactinol was quantitated by HPLC as described in an assay for stachyose synthase by Pharr *et al.* (17).

Invertase activity was assayed over the pH range of 4.0 to 7.6 using 50 μL desalted enzyme in 150 μL 50 mM McIlvaine buffer at the appropriate pH and a sucrose concentration of 125 mM. Assays were run at 30 °C for 30 min and stopped by boiling for 1 min. The glucose content of 10 μL aliquots of the assay mixture was then determined spectrophotometrically as described above for starch analysis.

α-Galactosidase activity was also determined over the pH range 4.0 to 7.6 using 50 μL enzyme and 50 μL McIlvaine buffer containing 0.3% (w/v) *p*-nitrophenyl-α-galactoside. The assays were run at 30 °C for 30 min and stopped by the addition of 2.9 mL 5% (w/v) sodium carbonate. The amount of p-nitrophenol released was determined spectrophotometrically (7).

Sugar Uptake Studies

Kinetic studies of [^{14}C]sucrose and [^{14}C]stachyose uptake were performed on green and white leaf discs as described previously (14). Leaf tissues were abraded with carborundum prior to excision of leaf discs (26). Autoradiography of labelled discs was performed as described previously (14).

Metabolism of fed ^{14}C-sugars (stachyose, galactinol, raffinose, sucrose) was determined by extracting discs after a 30 min uptake period in 5 mM sugar using the same uptake protocol as for the kinetic studies. Neutral, basic, acidic, and ethanol-insoluble fractions were obtained and the neutral fractions further characterized by thin layer chromatography (14).

RESULTS

Both green and white tissues from mature *Coleus* leaves contained substantial levels of soluble sugars of the raffinose series (Fig. 1). Only the green tissues contained appreciable levels of starch (Fig. 1). Soluble sugars in general were higher in green than in white tissues, although the levels of stachyose, raffinose and galactinol in white tissues often approached those in green tissues at periodic intervals in the diurnal cycle. In the green tissues, starch and sugar levels showed distinct diurnal patterns (Fig. 1). Levels of sucrose and the raffinose oligosaccharides declined throughout the night and then showed two peaks at 4 and 12 h into the day. These oscilla-

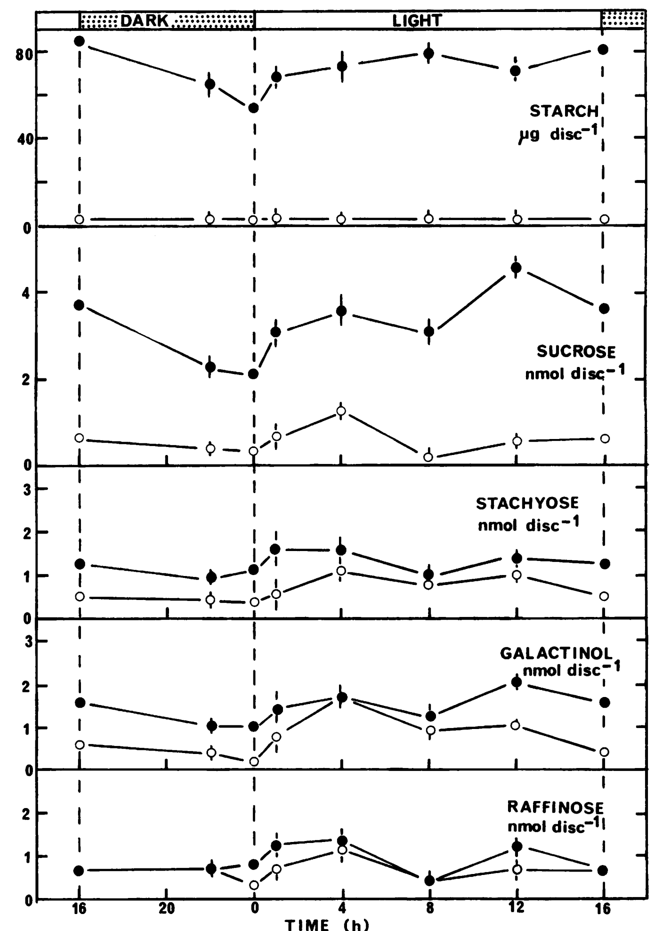

Figure 1. Diurnal carbohydrate levels in variegated leaf tissues of *C. blumei* Benth. Green tissues (●), white tissues (○). Data represent the means of two experiments (± SE).

Table I. *Enzyme Activities in Green and White Tissues of Variegated Coleus Leaves*

Data represent the means of triplicate experiments (± = SE).

	Enzyme Activity	
	White	Green
	μmol mg⁻¹	*protein h⁻¹*
Sucrose synthase	0.41 ± 0.01	0.60 ± 0.10
Sucrose phosphate synthase	0.41 ± 0.01	1.06 ± 0.02
Galactinol synthase	ND[a]	26.30 ± 2.70

[a] Not detected.

tions in soluble sugar levels were also seen in the white tissues but were somewhat dampened.

The presence of raffinose-family oligosaccharides in the white tissues did not appear to be a result of *de novo* synthesis in these tissues, as the white tissues contained no detectable GS activity (Table I). Similarly, white tissues contained substantially lower activity of SPS, indicating that some of the sucrose found in white tissues may also not have been synthesized there (Table I). Activities of SS, normally associated with sucrose catabolism, were similar in both tissue types.

White tissues had greatly elevated levels of invertase (Fig. 2a) and α-galactosidase (Fig. 2b) activities compared to green tissues. Although both tissues showed appreciable activity of both enzymes across a broad pH range, only the white tissues showed distinct peaks of activity in the alkaline range (pH 7.2 for invertase, Fig. 2a; pH 7.0 for α-galactosidase, Fig. 2b) indicating the possibility of distinct alkaline forms of these enzymes in the white tissues.

Both green and white tissues were able to take up exogenously supplied [^{14}C]sucrose and [^{14}C]stachyose and did so at comparable rates and with similar kinetic profiles typical of combined linear first-order and saturable components (Fig. 3a,b,c,d). Autoradiography revealed that green tissues showed extensive vein labeling following uptake of either [^{14}C]sucrose (Fig. 4a) [^{14}C]stachyose (Fig. 4b). Minor vein labeling was apparent in white tissues fed [^{14}C]sucrose (Fig. 4c), but not [^{14}C]stachyose (Fig. 4d). The linear components of uptake of either sugar in either tissue type were insensitive to the inhibitor PCMBS (Fig. 3a,b,c,d). The saturable component of sucrose uptake was inhibited by PCMBS by approximately 30% in both green (Fig. 3a) and white (Fig. 3b) tissues while the saturable component of stachyose uptake was inhibited by 80% and 50% in green (Fig. 3c) and white (Fig. 3d) tissues, respectively.

Both green and white tissues were able to rapidly metabolize exogenously fed ^{14}C-sugars into a neutral, basic, acidic and insoluble compounds (Table II). Sucrose was the most effectively metabolized sugar, with only 10 to 15% of the label remaining as sucrose following a 30 min labeling of either green or white tissues. In green tissues, label from sucrose was utilized for the synthesis of raffinose-family oligosaccharides; in white tissues the label appeared predominantly as glucose, fructose, and basic compounds. Exogenously fed raffinose-family sugars (stachyose, raffinose, galactinol) were also appreciably metabolized, particularly in the white tissues. Again, only the green tissues incorporated label from these sugars into other members of the raffinose series; in the white tissues the label was recovered again primarily in hydrolysis products (*e.g.* raffinose in the case of exogenously fed stachyose), hexoses and basic and acidic components (Table II).

DISCUSSION

The presence of stachyose and raffinose in white tissues would appear to be a result of phloem transport and not *de novo* synthesis, based on the lack of detectable levels of galactinol synthase in these tissues (Table I) and the lack of synthesis of stachyose or raffinose from exogenously supplied sugars, particularly galactinol (Table II). The presence of galactinol itself in the white tissues, however, may not reflect phloem-derived sugar exclusively. Although galactinol is quite commonly detected in petiole and stem extracts (11, 16), it is not normally considered to be a principal phloem sugar. Since the white tissues are apparently capable of limited synthesis

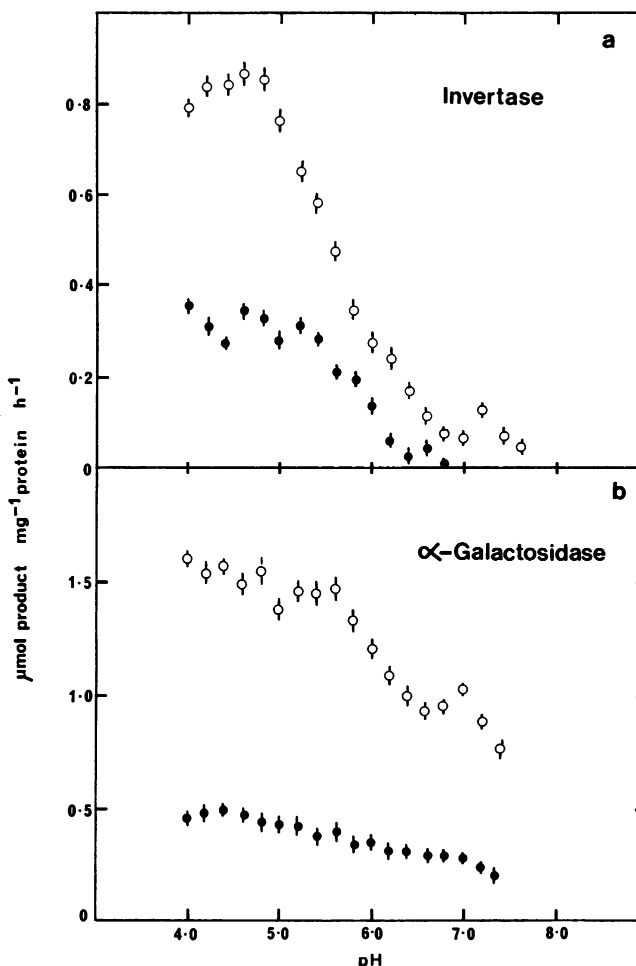

Figure 2. Effects of pH on hydrolytic enzyme activities in crude extracts from *Coleus* leaf tissues. a, Invertase activity; b, α-galactosidase activity. Green tissues (●), white tissues (○). Data represent the means of two experiments (± SE).

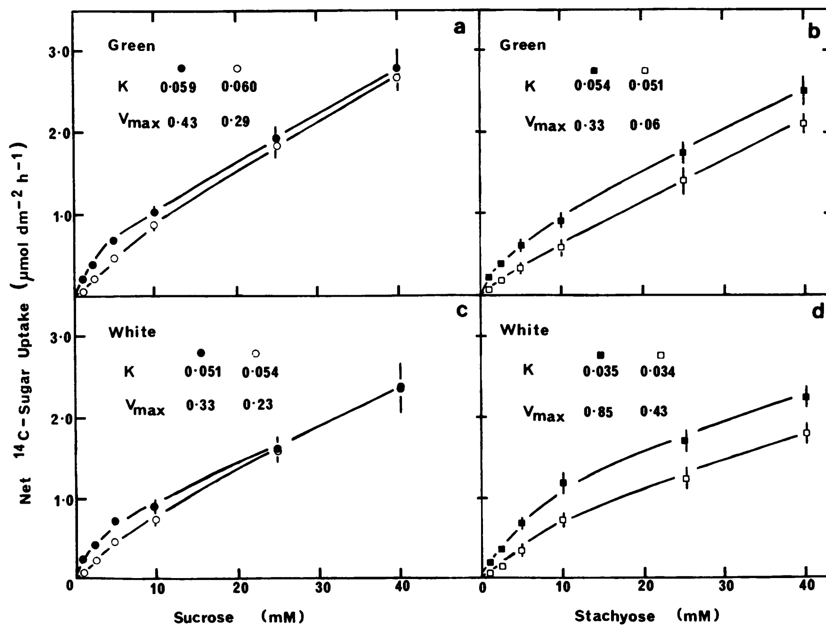

Figure 3. Kinetic profiles of ^{14}C-sucrose or ^{14}C-stachyose uptake into variegated *Coleus* leaf tissues. a, [^{14}C]Sucrose uptake into green tissues; (●), control; (○), tissues pretreated with 15 min with 2 mM PCMBS. b, [^{14}C]Stachyose uptake into green tissues; (■), control; (□), tissues pretreated 15 min with 2 mM PCMBS. c, [^{14}C]Sucrose uptake into white tissues; (●), control; (○), tissues pretreated 15 min with 2 mM PCMBS. d, [^{14}C]Stachyose uptake into white tissues; (■), control; (□), tissues pretreated 15 min with 2 mM PCMBS. Data represent the means of three experiments (± SE).

of galactinol from exogenous sugars (Table II), it is probable that they do contain galactinol synthase but the use of crude extracts, which we know contain high levels of hydrolytic and other enzymes, interfered in some manner with the assay of galactionol synthase preventing the detection of the product, galactinol. Further studies using purified enzyme preparations should resolve this question.

The data presented here do clearly show that variegated *Coleus* leaves contain raffinose-series oligosaccharides and the enzymes for their synthesis and degradation. Green leaf tissues are able to synthesize stachyose from exogenously supplied radiolabeled sugars, and white tissues are able to metabolize exogenously fed sugars to amino acids and organic acids, presumably through respiratory activity. What is particularly interesting in this study is the observation that there is rapid metabolism of an exogenously supplied sugar in both the green and the white tissues. It would appear from the data presented here that although vein loading occurs in the green tissues following an exogenous sugar feeding, some of the fed sugar is not directly loaded, but instead is hydrolysed and resynthesized into phloem-mobile or storage metabolites. This is in agreement with previously reported findings for cucurbit species (10, 16, 19, 21) in which exogenously fed sugars have been shown to undergo interconversions in addition to being transported.

The extensive interconversions of exogenous sugars may in part explain the observations of Turgeon and Wimmers (26) who reported a significant 'time lag' in the delivery of exogenous sugars to the minor veins of *Coleus*. These researchers suggested that phloem loading in *Coleus* occurred via an indirect route through the mesophyll, but they did not specifically examine the possibility of metabolism of exogenous

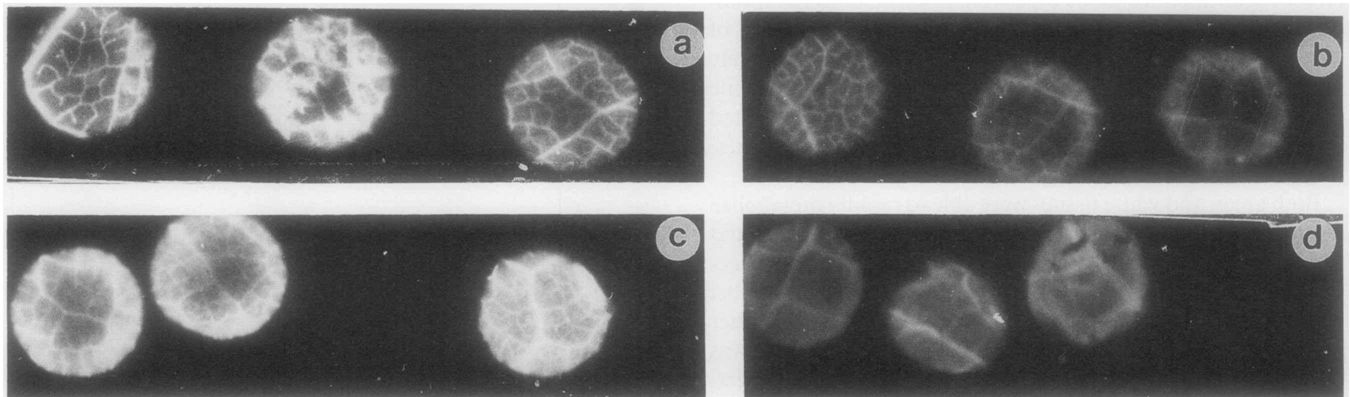

Figure 4. Autoradiographs of *Coleus* leaf discs floated on 5 mM [^{14}C]sucrose or [^{14}C]stachyose for 30 min. a, Green leaf tissue, [^{14}C]sucrose; b, green leaf tissue, [^{14}C]stachyose; c, white leaf tissue, [^{14}C]sucrose; d, white leaf tissue [^{14}C]stachyose. Autoradiographic and photographic exposure times were the same in all cases.

Table II. *Metabolism of Exogenously Supplied ^{14}C-Labeled Sugars by Leaf Discs from Green and White Tissues of Variegated Coleus Leaves*
Data represent the means of three experiments (± SE).

Compound Recovered	^{14}C Sugar Fed							
	Stachyose		Raffinose		Galactinol		Sucrose	
	Green (1076 ± 25)[a]	White (1036 ± 76)	Green (672 ± 22)	White (671 ± 13)	Green (854 ± 44)	White (799 ± 32)	Green (1866 ± 106)	White (1830 ± 110)
	% ^{14}C recovered							
Neutral	86.1	55.6	72.3	49.1	77.3	56.3	46.2	44.8
Verbascose	5.1				4.6		1.2	
Stachyose	42.9	28.4	10.6		23.3		7.8	
Galactinol	10.4	0.9	9.0	0.7	17.8	35.8	4.3	0.9
Raffinose	6.3	8.5	20.5	24.2	12.8	0.2	1.1	0.7
Maltose	5.4	3.7	5.6	9.8	5.5	5.8	1.4	1.6
Sucrose	7.5	2.6	9.3	7.5	4.6	3.5	9.3	14.5
Galactose	3.3	3.2	5.1		3.8	4.6		
Glucose	2.2	2.7	6.2		2.7	3.4	9.1	11.7
Fructose	3.1	6.0	5.7	6.7	2.1	2.8	12.0	15.1
Basic	3.3	15.9	7.0	20.8	7.3	19.2	22.3	32.9
Acidic	4.0	13.8	8.0	15.8	3.9	13.6	29.1	19.1
Insoluble	6.6	14.7	12.6	14.4	12.5	10.8	2.6	3.2

[a] Total ^{14}C per disc (dpm).

sucrose in the mesophyll as one reason for the delay in delivery of exogenous sugar to the phloem. As seen in the present study, metabolism of exogenous sugars, and of sucrose in particular, is very extensive in *Coleus* and it is possible that the delay in phloem loading observed in previous studies was due to the need to resynthesize hydrolyzed sucrose into a phloem-mobile form. The data in the present study show clearly that most of the sucrose taken up in a 30 min labeling period is metabolized to nonsucrose metabolites, and that only in green tissues do stachyose and other members of the raffinose-sugar biochemical pathway account for a significant portion of the label. However, this possibility in no way detracts from the hypothesis put forward by Turgeon and Wimmers (26) that movement of assimilates in the *Coleus* leaf may follow a symplasmic pathway.

In contrast to another previous report (27), our data point to a substantial effect of PCMBS on sugar uptake in *Coleus*. The reason for this difference may be explained by the kinetic data presented here which show that PCMBS is only inhibitory to the saturable component of either sucrose or stachyose uptake and has no effect on the linear component. The previous study reported uptake only at a concentration of 25 mM, which is well out of the range of the PCMBS-sensitive saturable component. However, we did not observe the previously reported (27) stimulation of sucrose uptake in white tissues in response to PCMBS at any sucrose concentration; the reasons for this difference are not clear. Additionally, we did find that, although white tissues rapidly metabolized exogenously fed sugars, they did show vein labeling with exogenous sucrose, in contrast with previous findings (27). We are hesitant to suggest that the appearance of label in the vein areas necessarily represents label in the sieve elements, but more probably represents label taken up into vein parenchyma and surrounding tissues. However, only microautoradiography will resolve the location of this label.

Enzymatic data suggest that the white tissues may also contain alkaline forms of the principal hydrolytic enzymes invertase and α-galactosidase, not present in the green tissues, which may aid in degradation of imported sugars (7). A previous analysis (5) of import of photosynthetically-derived ^{14}C-labeled assimilates from green tissues into white tissue regions also showed extensive metabolism of phloem-derived solutes into compounds similar to those described here for exogeonous sugars. It is interesting to note that in this previous study these researchers also detected the presence of 'unknown' compounds (*cf.* ref. 5, U1 U2 and U3, Fig. 10) whose chromatographic behavior are very much like those of stachyose, raffinose and galactose run in the same solvent systems (M Madore, personal observation). This suggests that raffinose sugars may indeed have been transported to white sink tissues but were so rapidly metabolized that their presence in the photoassimilate supply was easily overlooked in these experiments which dealt only with carbon metabolism in sink tissues. Unfortunately, however, these results may have left the incorrect impression that *Coleus* does not transport any sugar except sucrose (*cf.* ref. 26).

In summary, the data from the present study confirm that the variegated *Coleus* leaf provides a useful system for the study of vein loading and unloading in a stachyose-synthesizing species. Indeed, the presence of raffinose-family oligosaccharides in *Coleus* leaves further aligns this species with members of the Cucurbitaceae in terms of their phloem transport physiology. It has been well documented that the phloem of both *Coleus* (2) and cucurbits (21, 26) is unusual in that two types of sieve-tube-associated cells can be distinguished, one (intermediary cells) abundantly connected by plasmodesmata to the associated sieve elements and also to

adjacent bundle sheath cells and another (companion cells) symplasmically isolated from vascular parenchyma cells.

Recent evidence has indicated that the plasmodesmata between the bundle sheath and intermediary cells of *Cucurbita pepo* are fully functional (23), suggesting a role for these plasmodesmata in the loading of the abaxial phloem. In *Coleus,* however, the functioning of these plasmodesmata could not be demonstrated, leaving the question of the role of intermediary cells in phloem loading still in question (3). It is intriguing, though, that the presence of intermediary cells has been found to be commonly associated with the stachyose biosynthetic pathway (see 22 for review). The present data confirm that this is true also for *Coleus*. It will be interesting to now see if this correlation holds in white tissues, which neither make stachyose nor show phloem loading of this compound, and so may not possess intermediary cells.

ACKNOWLEDGMENTS

The author acknowledges the excellent technical assistance provided by Carol M. Boyd and Michelle V. Gadus in carrying out this research.

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