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Distribution of α-Galactosidase in *Cucurbita pepo* [1]

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BRIAN THOMAS[2] AND JOHN A. WEBB[3]
Department of Biology, Carleton University, Ottawa, Ontario, Canada K1S 5B6

ABSTRACT

The distribution of α-galactosidase (α-D-galactoside galactohydrolase [EC 3.2.1.22]) in *Cucurbita pepo* has been determined in an attempt to assess its involvement in hydrolysis of transport sugars of the raffinose oligosaccharide series ([α-1-6-0-galactopyranosyl]$_n$ sucrose). Extracts prepared from leaves and petioles at different stages of development, roots, flowers, dry and germinating seeds, all contained appreciable levels of α-galactosidase activity. Chromatography of these extracts on DEAE-Sephadex resolved the enzyme into three active isozymic forms. These isozymes were present in all regions of the plant analyzed but their relative proportions varied between tissues and changed within leaf and petiole tissues during development and in seeds during their germination. The level of total α-galactosidase activity in the leaf blade measured on a fresh weight or total protein basis remained constant at all developmental stages analyzed. The occurrence of these isozymes in mature exporting leaves indicates an effective intracellular compartmentation between their location and the sites of galactosyl oligosaccharide biosynthesis, accumulation and movement in the tissue. We have used these results to comment on the transport pathway of galactosyl oligosaccharides between the phloem and surrounding tissues in this plant.

Members of the raffinose family of oligosaccharides, (α-1-6-0-galactopyranosyl)$_n$ sucrose, are widely distributed in the higher plant kingdom with the lowest mol wt member of the series, raffinose, occurring most frequently (7). They commonly appear among the soluble storage products of seeds (1, 16) and roots (15, 16) and have been reported in leaves (15). In many plant species their occurrence appears restricted to the seed where they are synthesized during maturation of the tissue and rapidly disappear during the initial stage of germination (1). During the past 2 decades, however, investigations of phloem translocation in a number of widely divergent species have established that these oligosaccharides may also accompany sucrose as major transport sugars in a number of plant families (23, 25). Studies of their metabolism have been mostly confined to seed tissue, where it is generally acknowledged that their enzymic hydrolysis is affected by α-galactosidase (α-D-galactoside galactohydrolase [EC.3.2.1.22]) and considerable evidence for the occurrence and properties of this enzyme in germinating seed extracts has been accumulated (3). It would seem reasonable, therefore, to expect those plants that transport these oligosaccharides in their vascular tissue to possess similar α-galactosidase activities in their immature and developing organs, but the nature and function of α-galactosidase activity in plant tissues other than the seed have received scant attention. The enzyme has been briefly reported to be in leaves (3, 8) where an involvement in galactolipid metabolism of organelle membranes was suggested, and it has also been reported in walls of epicotyl tissue (12) and in plant cell and tissue cultures (10, 13). Our interest in the distribution of α-galactosidase in *Cucurbita pepo* resulted from our discovery that immature importing leaves possess an extremely active ability to hydrolyze raffinose, stachyose, and verbascose entering their tissue from the phloem and are incapable of any detectable synthesis or accumulation of these sugars. In contrast, mature exporting leaves demonstrate both a synthesis and a negligible *in vivo* hydrolysis of these oligosaccharides (20, 22, 23). We hypothesized that an extremely active α-galactosidase system is operating in immature leaf tissue, while in mature leaves its activity drastically declines due either to loss or inactivation or, alternatively, through an effective compartmentation becoming established during leaf development. We reject the concept of loss because of our subsequent discovery that extracts from mature leaves of *C. pepo* contain at least three active isozymic forms of α-galactosidase (18). Preliminary experiments indicated that the bulk of the α-galactosidase activity in mature leaf tissue is indeed compartmentalized in the cell wall (unpublished data) and this conforms with increasing evidence for restriction of glycosidase enzymes to cell walls (13). Before extending our studies of the intracellular location of α-galactosidase activity we considered it of interest to discover whether the three isozymic forms are distributed in other tissues of *C. pepo* and to attempt to relate their presence to the utilization of the galactosyl oligosaccharides in these tissues. These results are reported here.

MATERIALS AND METHODS

Plant Material. Plants of the Early Prolific Straight-Neck Squash, *Cucurbita pepo* L. var. *melopepo* f. *torticollis* Bailey (W. A. Burpee, Philadelphia) were used in all experiments. Leaf blades, petioles, flowers, and roots were obtained from plants that had been germinated in Vermiculite and grown in Perlite in controlled environment cabinets (20). Blades and petioles were harvested when the plants were between 2 and 5 weeks old. The morphological age of the blades was determined by a leaf plastochron index (5), based on a petiole length of 30 mm (19). Between LPI[4] 0 and 0.6 the blades had expanded their surface areas from about 8 to 20% of their final size and during this period they demonstrated maximal rates of import. The transition from a totally importing to a totally exporting blade occurred during the interval between LPI 0.5 and 1.5 (50% expanded). At LPI 0.9 only the basal half of the blade was importing. At LPI 2.5 (80% expanded) export from the blade approached its maximal rate. The blade was 100% expanded at LPI 4.0 (19). Flowers were obtained as fully expanded buds prior to opening from 6- to 7-week-old plants and roots were obtained by excising the entire rooting system from 1-week-old seedlings. Seed extracts were prepared from whole seeds imbibed where appropriate on filter

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[2] Present address: Department of Botany, Plant Science Laboratories, University of Reading, Whiteknights, Reading, England RG6 2AS.

[3] To whom correspondence should be addressed.

[4] Abbreviation: LPI: leaf plastochron index; LI, LII, LIII: isozymic forms of α-galactosidase.

paper in glass Petri dishes at 27 C in darkness.

Enzyme Preparations. For determination of total α-galactosidase activity source material was rapidly weighed and homogenized in a pestle and mortar using 2 volumes of 100 mM sodium phosphate buffer (pH 7.0) in the presence of acid-washed silver sand. The extract was transferred quantitatively to a centrifuge tube with a further 2 volumes of buffer solution and centrifuged at 25,000*g* for 30 min at 2 C. The supernatant was assayed for α-galactosidase activity. During centrifugation of seed extracts a lipid layer collected on the surface of the supernatant and a Pasteur pipette was used to remove the clear supernatant from below.

For preparation of the isozymes the crude buffer extracts obtained from source material as described above were treated with solid ammonium sulfate and the protein fraction precipitating in the 30 to 60% range was collected by centrifugation (18). The precipitate was redissolved in 2 ml of extraction buffer and after centrifuging at 10,000*g* for 10 min at 2 C, the supernatant was passed through a column of Sephadex G-100 (85 × 1.5 cm) and eluted with 100 mM sodium phosphate buffer (pH 7.0), flow rate 0.25 ml/min at 2 C. Fractions of 5 ml were collected and each was assayed for α-galactosidase activity. For all tissues examined activity was confined to a single peak which emerged from the column at a constant elution volume. The active fractions were pooled for each tissue and reduced in volume to 1.5 ml by ultrafiltration using an Amicon PM-10 membrane. Further purification of the α-galactosidase fraction was effected on a DEAE-Sephadex A-50 column (30 × 1.5 cm) which had been equilibrated with 0.01 M NaCl in 100 mM sodium phosphate buffer (pH 7.0) at 2 C. The enzyme sample was run onto the column followed by 5 ml of the equilibration buffer. The column was then eluted at a flow rate of 0.2 ml/min with a linear gradient of 0.01 to 0.5 M NaCl in a total volume of 150 ml sodium phosphate buffer (pH 7.0) at 2 C. Fractions of 3 ml were collected and each fraction assayed for α-galactosidase activity.

Enzyme Assays. α-Galactosidase activity was measured using *p*-nitrophenyl-α-D-galactopyranoside as the substrate (18). Assay mixtures contained 200 μl of 3 mg/ml substrate in 200 mM sodium acetate buffer (pH 5.0) and 200 μl of suitably diluted enzyme preparation in 100 mM sodium phosphate buffer (pH 7.0). After incubation at 35 C for 15 min the reaction was stopped by adding 2 ml of 5% Na_2CO_3 and the *p*-nitrophenol released was measured at 400 nm. Blanks were prepared by adding enzyme after Na_2CO_3. The unit of enzyme activity (U) was defined as the quantity that hydrolyzes 1 μmol of substrate/min under the conditions outlined above. Total protein was measured using crystalline BSA as standard (11).

RESULTS AND DISCUSSION

α-Galactosidase Activity in Leaves. Our earlier results had shown that young leaves (LPI <0.3) rapidly hydrolyzed imported raffinose, stachyose, and verbascose, and they appeared incapable of synthesizing or accumulating these oligosaccharides (20, 23). Older leaves (LPI >1.5) by contrast were able to synthesize and accumulate these sugars (23) and if translocation from the blade was curtailed (*e.g.* by a petiole cold block) they appeared to demonstrate little metabolic turnover within this mature tissue (22). If the level of α-galactosidase activity is a controlling factor in this system our results may be explained through a depletion of total enzyme activity during leaf maturation or through an appreciable proportional shift among the three isozymic forms of which one or more is inactive or compartmentalized elsewhere in the cell.

In the first series of experiments we assayed total α-galactosidase activity in the fifth leaf of *C. pepo* (19) at progressive developmental stages over a period of LPI 0 to LPI 2.5. Several leaves were taken at each LPI interval and the units of α-galactosidase activity measured per leaf were plotted againts the average fresh weight and the average total protein content per leaf (Fig. 1). Despite the age variability of the source material it is clear that a straight line relationship is obtained in both cases indicating that the level of α-galactosidase activity remains remarkably constant throughout leaf development to maturity. The proportion of the protein component of the leaf's fresh weight soluble in the phosphate buffer does not alter over this period.

In addition to measuring total α-galactosidase activity for whole leaves we also measured the distribution of activity between the proximal and distal regions of the fifth leaf at developmental stages between LPI 0.5 to 2.5. It is known that the physiological development of the leaf proceeds basipetally (19). Leaves were cut at right angles to the midrib to give approximately two equal halves. The halves were assayed for α-galactosidase activity separately and the specific activity values obtained from the two areas of the leaf compared (Table I). When assayed statistically by a paired *t* test procedure (21) the pairs of values were found to be not significantly different at the 5% probability level. This again supports the above evidence that the level of α-galactosidase activity, at least as measured in leaf extracts, does not alter during development of the blade from the immature to the mature stage.

We also assayed the levels of total α-galactosidase activity in successive leaves on the same plant. The results in Table II were obtained from three plants where the 7th, 6th, and 5th leaves were at the importing, transitional, and exporting stages of physiological development, respectively (19). Again there is no significant evidence of a loss of total α-galactosidase activity from extracts of leaves during their maturation. The results also show that there is much less variability between leaves on the same plant than between equivalent leaves on different plants grown under identical conditions.

The above values for total α-galactosidase activity presumably represented the combined *in vitro* activity of at least three isozymic forms of the enzyme (18). The physical form in which the enzyme exists may determine its activity *in vivo* and a distinction between

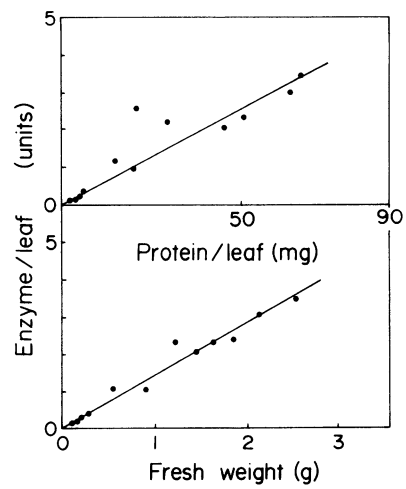

FIG. 1. Relationship between α-galactosidase activity and phosphate buffer-soluble protein or leaf fresh weight in the fifth leaf of *C. pepo* at progressive developmental stages over the range LPI 0 to 2.5.

Table I. Distribution of α-galactosidase between proximal and distal portions of the 5th leaf at progressive stages of development.

Leaf Age (LPI)	Enzyme Activity[1]	
	Proximal	Distal
0.56	59	37
0.81	80	73
0.88	49	41
1.39	48	46
1.47	51	42
1.84	105	110
2.41	44	43

[1] milliunits/mg protein

a depletion of an active form and an increase in levels of an inactive form of the enzyme could be masked when measured *in vitro*. We determined the distribution of α-galactosidase activity among the isozymic forms separated in the eluates obtained from DEAE-Sephadex columns. Although the over-all recovery of total α-galactosidase activity usually lies between 50 to 60% of that found in crude homogenates it is clearly not possible from this method to determine the absolute recovery for each isozyme. Although they show some variability in their stability (18) we have assumed here that the recoveries of the isozymes from a particular tissue were constant relative to each other. The pattern of relative distribution in an extract from a tissue at a given stage of development has been consistently reproducible. We therefore persevered with this approach to identify and compare the relative isozyme composition of a particular tissue during its developmental stages and also between dissimilar tissues.

The elution profiles showing relative enzyme activity of the three isozymic forms as a function of O.D. at 400 nm for importing (LPI <0.3), transitional (LPI 0.5–1.0) and exporting (LPI 1.5–2.5) leaves of *C. pepo* are shown in Figure 2. LI forms a constant proportion (about 15%) of the total recovered α-galactosidase activity. Of the other two isozymes the activity of LII is considerably reduced in the exporting leaves compared to those that are importing or those that are at the transitional stage. The reduction of LII activity is accompanied by an increase in the activity of LIII. It is not possible at this stage to say whether this is due to isozyme interconversion, although bearing in mind the constancy of the total activity it is tempting to predict that this may be so.

Table II. α-Galactosidase levels in consecutive leaves of three plants.

Leaf No.	Plant A		Plant B		Plant C	
	LPI	Enzyme Activity[1]	LPI	Enzyme Activity[1]	LPI	Enzyme Activity[1]
7	0.19	57	-0.19	59	0.39	45
6	1.19	67	0.81	70	1.39	48
5	2.19	61	1.81	68	2.39	49

[1]Enzyme activity, milliunits/mg protein

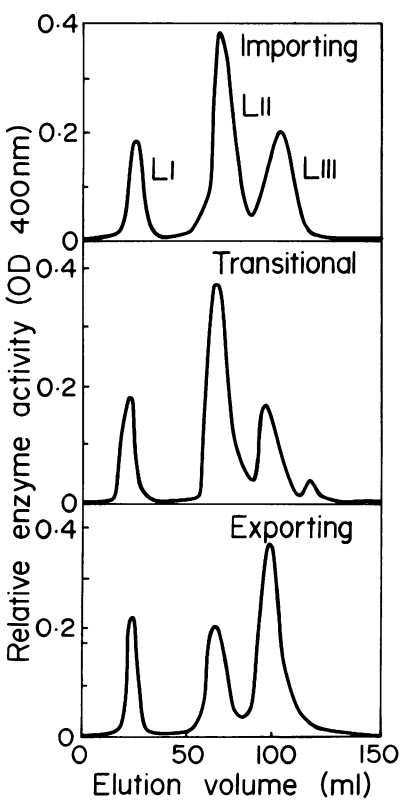

FIG. 2. DEAE-Sephadex chromatography of α-galactosidases from importing (LPI < 0.3), transitional (LPI 0.5–1.0), and exporting (LPI 1.5–2.5) leaves of *C. pepo*.

These results produce no direct evidence to explain the differences between immature and mature leaf tissues toward hydrolysis of the galactosyl oligosaccharides. If both LI and LIII are the inactive forms *in vivo*, there is still an appreciable level of LII in the exporting leaf. These differences can be resolved if we make an assumption that the α-galactosidases are confined to the apoplast, we could then logically conclude that phloem unloading in immature *Cucurbita* tissue involves a direct discharge into the apopolast while in more mature blades, at the stage when excess carbohydrate begins to be produced and the synthesis and accumulation of the galactosyl oligosaccharides occur, loading into the phloem proceeds via the symplast. There is increasing evidence for glycosidases in the cell walls of a wide range of species, as mentioned above, but the pathways of phloem loading and unloading among higher plant species have been little studied. Reports from the laboratories of both Fondy and Geiger (6) and Giaquinta (9) have indicated that in *Beta vulgaris* sucrose loading takes place from the apoplast.

α-Galactosidase Activity in Petioles. Young, rapidly expanding petioles are not only very active in photosynthetic carbon assimilation, but they are also active "sink" regions analogous to young leaves insofar as they rapidly withdraw sucrose and the translocated galactosyl oligosaccharides from the phloem (24). Data in Table III indicate comparatively high levels of total α-galactosidase activity in this young tissue. The distribution of activity among the isozymic forms is shown in Figure 3. While LII activity is prominent and higher than LIII, the situation deviates appreciably from that described above for immature leaves in that LI demonstrates the highest level of activity. Mature petioles also show a striking contrast to mature blades as seen in Table III and Figure 3. There is here a distinct change in the pattern of α-galactosidase activity, total activity is much lower than in the immature tissue, and LII and LIII are greatly reduced. However LI remains the dominant form. Exposure of mature petioles to $^{14}CO_2$ under conditions for photosynthesis labels sucrose heavily but fails to label the galactosyl oligosaccharides and they do not contribute their photoassimilates to the translocation stream (unpublished data). Removal of sugars from the phloem in mature petioles is also extremely slow (23, 24). The need for α-galactosidase activity in mature petioles is much reduced and indeed the data in this report suggest that there is some measure of correlation.

α-Galactosidase Activity in Flower Buds and Roots. In addition to their presence in leaves and petioles, α-galactosidases were found in appreciable quantities in expanding flower buds and the roots of 1-week-old seedlings (Table III). If the results are expressed on a fresh weight basis expanding flower buds appear to be a particularly rich source of the enzyme whereas roots have relatively low amounts. The difference is far less pronounced when the results are expressed on a protein basis. Extracts from both flowers and roots contain three isozymes of α-galactosidase (Fig. 4), which appear to correspond to those found in leaves and petioles. The elution pattern, in terms of quantities of the individual isozymes, are quite distinctive for the organs concerned. In flower extracts LI and LIII are present in approximately equal amounts and are the predominant forms of the enzyme whereas in the roots LIII is the major isozyme. Although this is probably not an exact representation of the *in vivo* situation, it seems likely

Table III. α-Galactosidase levels in different parts of plant.

	Enzyme Activity	
	mU/g fresh wt.	mU/mg protein
Immature leaves	585	54
Mature leaves	505	59
Immature petioles	498	181
Mature petioles	186	113
Roots	143	31
Flowers	984	51
Seeds (dry)	114	11
Seeds, imbibed 24 hours	140[1]	16
Seeds, imbibed 48 hours	179[1]	13

[1]Fresh weight of seeds prior to imbibition

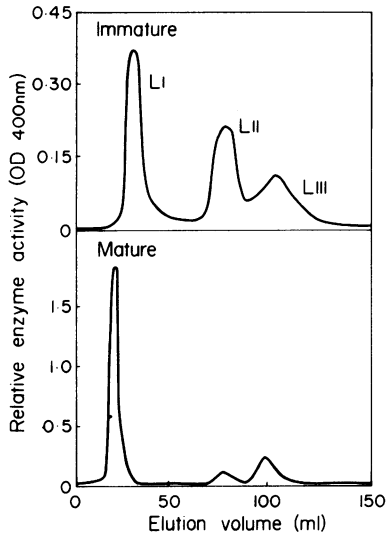

FIG. 3. DEAE-Sephadex chromatography of α-galactosidases from immature and mature petioles of *C. pepo*.

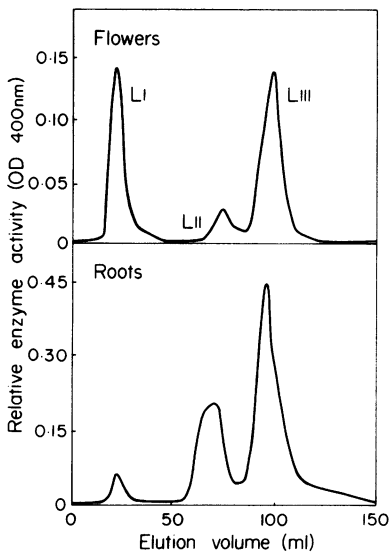

FIG. 4. DEAE-Sephadex chromatography of α-galactosidases from expanding flower buds and the roots of 1-week-old seedlings from *C. pepo*.

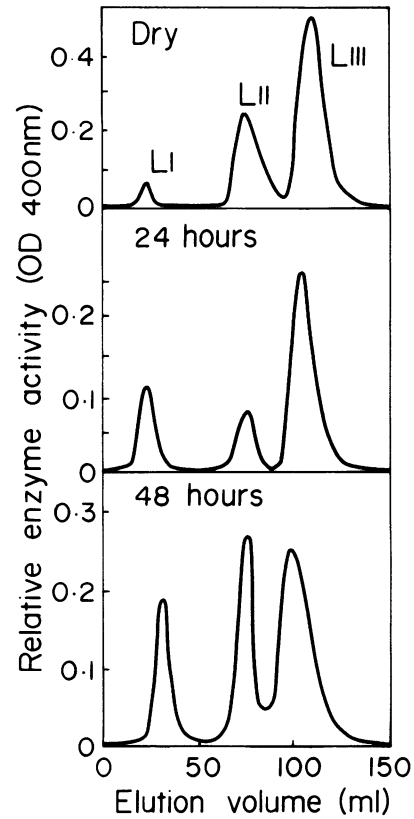

FIG. 5. DEAE-Sephadex chromatography of *C. pepo* α-galactosidases from dry seeds, seeds imbibed for 24 hr, and seeds imbibed for 48 hr.

that such large differences between organs could not be accounted for by selective loss or interconversion of isozymes during preparation of the extracts. Both the young roots and flowers are metabolic sinks and as such are regions in which we would expect the transported oligosaccharides to be rapidly hydrolyzed. We can assume therefore that although the α-galactosidases of these two regions differ both quantitatively and qualitatively they are appropriate to the needs of the organ concerned.

α-Galactosidases in Seeds. Storage oligosaccharides are subject to similar constraints as transport sugars in that they must be degraded prior to their utilization. In the *Cucurbitaceae*, galactosyl sucrose oligosaccharides function as storage sugars in seeds in addition to their role as transport sugars in the phloem. When extracts of *C. pepo* seeds were run on DEAE-Sephadex we recovered three α-galactosidase isozymes similar to those identified above (Fig. 5). Barham *et al.* (2) have shown the presence of two α-galactosidases, differing in their mol wt, in the seeds of several species. In *Vicia faba* the relative amounts of the two forms alter during germination, this shift being apparently correlated with the initiation of stachyose and raffinose hydrolysis (3). Analysis of extracts from seeds of *C. pepo* reveals comparable changes in enzymic form during imbibition. In dry seeds LIII was the predominant form of the enzyme but upon imbibition and germination the levels of LI and LII increased (Fig. 5) and after 48 hr the pattern resembled that from an immature leaf (Fig. 2). This shift from LIII to LI and LII was accompanied by an increase in total α-galactosidase activity (Table III). An analysis of the carbohydrate content of these seeds (unpublished data) has shown that there is a rapid depletion of ajugose, verbascose, stachyose, and raffinose during the first 24 hr of germination. The presence of these compounds and α-galactosidase in the dry seeds indicates that they are either synthesized or accumulated during seed maturation. Dey and Pridham (3) have suggested that α-galactosidases and the galactosyl sucrose oligosaccharides are spatially separated in maturing seeds of *V. faba* and we assume that the situation is apparently similar in *C. pepo*.

The results presented here show that α-galactosidase activity is distributed throughout the *Cucurbita* plant. Analysis of the enzyme shows that in all tissues a similar set of three isozymes are present in at least trace amounts. The significance of the three isozymes and variations in their levels is difficult to assess at present. If the isozymes are produced during extraction by dissociation of one large enzyme made up of active subunits we would have expected the elution profiles from the DEAE-Sephadex columns to have shown a consistent pattern irrespective of the enzyme source. We have previously discovered that each isozyme has a distinctive set of properties (18), the most striking difference being in their response to changes in pH. When *o*-nitrophenyl-α-galactoside is used as the substrate LI and LII are active over a limited pH range with well defined optima at pH 5.5 and 4.5, respectively. LIII on the other hand is active over a pH range of 2.5 to 7.0 with a poorly defined optimum at pH 5.5 (18). Combination of appropriate isozymes would thus enable the enzyme to operate in a range of locations with different local pH values. In addition the presence

of the LIII form would enable the enzyme to function in regions where the pH is poorly controlled and hence variable.

The presence of comparatively high levels of α-galactosidase activity in mature leaves, where we suspect that very little if any hydrolysis of the galactosyl oligosaccharides occurs, suggests that α-galactosidases may have an alternative role in this tissue other than the degradation of transport sugars. This is supported by the presence of these enzymes in spinach leaves (8) in which the presence of stachyose and raffinose as transport sugars has not been reported. Some workers (3, 8) have suggested that α-galactosidases are involved with the metabolism of galactolipids in membranes of cell organelles which are particularly abundant in the photosynthetic lamellae of chloroplasts. Physiological evidence indicates that there is little detectable turnover of these compounds in mature leaves of *C. pepo* (14) and significant hydrolysis of these compounds occurs only during senescence (17). Acid hydrolases, including glycosidases, have been designated by some authors as lysosomal enzymes (4, 12). Under autolytic conditions, therefore, α-galactosidases may be concerned with both membrane and wall degradation. If there is a specific location which can be called the lysosome in plant cells, localization of α-galactosidases within it might explain their apparent lack of activity in mature leaves. While we favor this location to be in the cell wall region, further experimental work is underway in our laboratory to confirm the intracellular location of these isozymes.

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