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LIPID PHASE SEPARATIONS AND INTRAMEMBRANOUS PARTICLE MOVEMENTS IN THE YEAST TONOPLAST

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Summary

The tonoplast of *Saccharomyces cerevisiae* contains regions depleted of intramembranous particles as the cells enter stationary phase. Freeze-fracture studies on intact cells from this growth stage show that a dispersed particle distribution predominates if the cell temperature is raised to 40°C but that particle-depleted areas prevail at or below the cell growth temperature of 30°C. Tonoplasts of isolated vacuoles also contain particle-depleted regions. Differential thermal analyses of lipids extracted from isolated vacuoles show an endothermic transition which encompasses the cell growth temperature. These results suggest that the tonoplast at this stage contains patches of gel-phase lipid and that these patches correspond to the intramembranous particle-depleted areas of the freeze-fractured tonoplast.

Introduction

Previous work has shown that during the transition from exponential growth to stationary phase the vacuolar membrane or tonoplast of the yeast *Saccharomyces cerevisiae* contains areas depleted of intramembranous particles surrounded by particle-rich regions [1]. These patterns apparently are caused by the movement of the intramembranous particles laterally in the plane of the membrane in accordance with the fluid-mosaic membrane model [2]. Although a number of factors such as pH, ions, microtubules, etc. may play a role in particle movements (for reviews see Refs. 3—5), the morphology and development of the tonoplast patterns resembles those formed by lipid phase separations that have been reported in a variety of biomembranes (for reviews see

Refs. 6, 7). In this paper we present evidence that during the transition from exponential to stationary phase, the yeast tonoplast contains both gel and fluid state lipids which we believe correspond to the particle-poor and -rich regions, respectively, of the freeze-fractured tonoplast.

Materials and Methods

Culture conditions. Dr. L.H. Hartwell of the University of Washington kindly provided the haploid strain of *S. cerevisiae* A364A (*a ade1 ura1 gal1 tyr1 his1 lys2 trp1*) used in these studies [8]. The cells were grown in a yeast extract, peptone and dextrose medium (medium 1) at 30°C [9] and were harvested during the transition from exponential to stationary phase when a high percentage of the cells contained tonoplasts with the geometric particle pattern [1].

Electron microscopy. Whole cells or subcellular fractions were freeze-fractured by the method of Moor and Mühlethaler [10] with a Balzers freeze-etching unit (BAF 301). The replicas were cleaned with chromic acid, washed with distilled water, picked up on Formvar-coated grids, and examined with a Philips 300 or 400 electron microscope. The shadow direction for the micrographs shown in this paper is toward the top of the page, and the nomenclature of Branton et al. [11] has been used to describe the fracture faces. The statistical tests used are described by Sokal and Rohlf [12]. The procedures for the temperature studies are presented in the appropriate table legends (Tables III and IV).

Vacuole isolation. 1 ml of an early stationary phase culture was added to 1 l of medium 1 in a 2.8 l Ehrlenmeyer flask to start the culture, and the culture was incubated at 30°C on a shaker for 38.5 h. The cells were then washed twice with distilled water, resuspended in 25 ml of a solution of 5 mM Na · EDTA/50 mM dithiothreitol in 0.1 M Tris (pH 8.9) with 1 mg/ml Pronase (Calbiochem, grade B) [13], and incubated for 30 min at 30°C on a shaker. After this pretreatment, the cells were washed twice with 0.6 M KCl, resuspended in 50 ml of a 1 : 10 (v : v) dilution of Glusulase (Endo Laboratories, Garden City, NY) in 0.6 M KCl, and incubated for 2 h at 30°C on a shaker. The protoplasts were sedimented through a sucrose layer to remove cell debris [14]. A Sorvall HB-4 swinging bucket rotor and Sorvall RC-2B centrifuge were used in this and all subsequent centrifugations. The protoplasts were lysed by resuspending them in 10% Ficoll in 0.1 M sorbitol/10 mM sodium citrate (pH 6.8) (for a review see Ref. 14). Five strokes by hand with a Ten Broeck tissue grinder was used to help break the protoplasts. The resulting mixture (gradient 1) was centrifuged for 30 min at 21 500 × *g*. The top layer was removed, diluted 1 : 1 (v : v) with 10% Ficoll in 0.1 M sorbitol/10 mM sodium citrate (pH 6.8), and homogenized by hand as described above. This mixture was placed at the bottom of a centrifuge tube (6 ml/tube), overlaid with 2 ml 7% Ficoll in 0.1 M sorbitol/10 mM sodium citrate (pH 6.8), and this in turn was covered with 2 ml 0.1 M sorbitol/10 mM sodium citrate (pH 6.8). This discontinuous gradient (gradient 2) was centrifuged for 20 min at 21 500 × *g*. The material at the 7% Ficoll/0.1 M sorbitol interface was removed, diluted with 3 parts 0.6 M sorbitol/10 mM sodium citrate (pH 6.8) [14], and centri-

TABLE I

RECOVERY AND SPECIFIC ACTIVITIES OF BIOCHEMICAL MARKERS IN FRACTIONS

Aliquots for the assays were removed from the protoplast lysate, gradient 1 (after the floating material was removed), gradient 2 (after the material at the 7% Ficoll/0.1 M sorbitol interface was removed), gradient 3 (material which remained after lipid-body- and vacuole-enriched fractions were removed), lipid body, and vacuole-enriched fraction. Protein and DNA units are in mg. Enzyme units (Units) are given in $\mu mol \cdot min^{-1}$, and specific activities are given in $\mu mol \cdot min^{-1} \cdot mg^{-1}$ except for the α-mannosidase assay in which the units are given as the change in absorbance at 400 nm $\cdot min^{-1}$ and the specific activity as the change in absorbance at 400 nm $\cdot min^{-1} \cdot mg^{-1}$. The percent recovered is expressed as the percentage of the protoplast lysate activity recovered in the other fractions. n.d., not detected.

Fraction	Protein	DNA	NADPH-cytochrome *c* reductase		Cytochrome *c* oxidase		Alcohol dehydrogenase		α-Mannosidase	
			Units	Spec. act.	Units	Spec. act.	Units	Spec. act.	Units	Spec. act.
Protoplast lysate	970	10	43	0.04	350	0.36	12 000	13	36	0.04
Gradient 1	820	7.7	32	0.04	270	0.33	10 000	13	25	0.03
Gradient 2	160	0.94	4.8	0.03	41	0.26	2 600	17	4.7	0.03
Gradient 3	5.0	n.d.	1.4	0.27	0.79	0.16	19	3.7	n.d.	
Lipid body	3.2	n.d.	0.25	0.08	n.d.		n.d.		n.d.	
Vacuole pellet	10	0.11	0.26	0.02	5.9	0.57	7.9	0.76	4.4	0.42
% recovered	103	84	90		92		105		95	

fuged for 10 min at 10 400 × *g* and then for 20 min at 21 500 × *g*. The material which floated on the top of the gradient was removed as the lipid-body-enriched fraction, and the material which pelleted was used as the vacuole-enriched fraction.

Vacuoles used for the biochemical, enzyme and lipid analyses were isolated by the osmotic lysis procedure described above. For some of the ultrastructural studies, vacuoles were also isolated isotonically from protoplasts by the DEAE-dextran method [15]. After the polybase treatment, the lysate was centrifuged at 500 × *g* for 10 min, and the supernatant was removed and spun at 3000 × *g* for 20 min. This gave a pellet which contained a large number of vacuoles.

Enzyme assays. Sample aliquots (Table I) were assayed for: protein [16]; DNA [17]; NADPH-cytochrome *c* reductase [18]; cytochrome *c* oxidase [18]; alcohol dehydrogenase [19]; and α-mannosidase [20]. All enzyme assays were done at room temperature in 50 mM potassium phosphate buffer (pH 7.2) by a continuous spectrophotometric assay. Herring sperm DNA (Sigma) was used as a standard in the DNA assay, and alcohol dehydrogenase activity was measured in the reverse direction using 1.5 mM NADH and 0.36 mM acetaldehyde as substrates.

Lipid analyses. Lipids from the lipid-body- and vacuole-enriched fractions were extracted by the method of Bligh and Dyer [21]. Lipid fractions were stored as $CHCl_3$ solutions under nitrogen at −4°C until used. Neutral lipids were separated by one-dimensional thin-layer chromatography [22] and phospholipids by two-dimensional thin-layer chromatography [23,24]. Phospholipid phosphorus was determined by the method of Ames [25] and sterol by the *o*-phthalaldehyde method [26]. Ergosterol (Sigma) was used as a standard for the sterol assay, and the absorption was measured at 470 nm. Total lipid weight was determined by adding aliquots of vacuole lipids in chloroform to aluminum pans, removing the chloroform under reduced pressure, and then weighing the pans with a Mettler electronic microbalance ME-22.

Differential thermal analysis. Aliquots of vacuole lipids in chloroform were added to sample pans, the chloroform removed under reduced pressure, and the pans weighed with a Mettler electronic microbalance ME-22. 20 μl of a 50% (v/v) glycerol/water mixture was then added to the sample pan and to an empty reference pan. The pans were sealed and their thermal behavior analyzed with a Mettler TA 2000 thermal analysis system. The samples were cycled between −50 and +60°C at least twice before the heating curves were traced. A heating rate of 5 K/min was used for the recordings. Indium was used as a standard to calibrate the endothermic transitions.

Results

A vacuole-enriched fraction can be isolated from cells during the shift from exponential growth to stationary phase. Although this fraction is more contaminated than vacuole fractions isolated from exponential phase yeast (see Ref. 27 and references therein), about 12% of the α-mannosidase activity, the tonoplast marker, was recovered in the vacuole-enriched fraction, and the specific activity of α-mannosidase was increased approximately 11-fold over that of the protoplast lysate (Table I). This contrasts with the low recoveries

and the same or reduced specific activities in the vacuole-enriched fraction for markers of the nucleus (DNA), microsomes (NADPH-cytochrome *c* reductase), mitochondria (cytochrome *c* oxidase), and cytoplasm (alcohol dehydrogenase). Although an enzyme marker for the yeast plasma membrane was not used, its freeze-fracture ultrastructure is substantially different from that of the tonoplast [10]. Freeze-fractured aliquots from the vacuole-enriched fraction revealed little contamination by the plasma membrane. In addition, the plasma membrane contains a substantial amount of phosphatidic acid and sterol when compared to the tonoplast [28,29]. Our vacuole-enriched fraction (Table II) contained little or no phosphatidic acid and amounts of sterol comparable to that found for tonoplasts isolated from exponential phase cells [28,29]. Lipid bodies are often intimately associated with the vacuole during this stage of cell development [30]. Although it was not possible to completely eliminate the contaminating lipid bodies, Table II shows that our vacuole-enriched fraction differed substantially from the lipid-body-enriched fraction and agrees with the results for tonoplasts from exponential phase cells [28,29]. These analyses plus the freeze-fracture data justified the use of the vacuole-enriched fraction in the subsequent analyses.

Differential thermal analysis of lipids extracted from the vacuole-enriched fraction showed a broad endothermic transition (Fig. 1). It was not possible to determine precisely the onset or end of the transition because of the gradualness of the transition. Although no transition was seen for the cooling curve (Fig. 1D), the endothermic transition was clearly reversible because it was always detected in the heating curves even after several heating and cooling cycles (Fig. 1). The exothermic transition may not be seen in the cooling curves if it occurs over a very wide temperature range. At 30°C, the cell growth temperature, the lipids are in the midst of the endothermic transition, indicating that both gel- and fluid-phase lipid are present in the tonoplast at physiological temperatures.

When isolated vacuoles were freeze-fractured, intramembranous particle-depleted regions were found in the tonoplast (Fig. 2). The DEAE-lysis procedure for isolating vacuoles preserved the in vivo arrangement of particles in the tonoplast better than the osmotic lysis procedure. The geometric particle pattern (particle-rich rows surrounding polygonal particle-depleted areas, Fig. 2C; see Ref. 1 for a description of the particle patterns) was only found in

TABLE II

LIPID AND PROTEIN CONTENTS OF THE VACUOLE- AND LIPID-BODY-ENRICHED FRACTIONS

The ratios are based on weights. No correction was made for the esterified fatty acids in the weight calculations for the sterol ester. Phospholipid weights were calculated by multiplying the lipid phosphorus content by 25.

	Lipid body	Vacuole
Protein/total lipid	0.13	0.92
Phospholipid/total lipid	0.01	0.41
Sterol + sterol ester/phospholipid	17	0.35
Sterol/total lipid		0.07

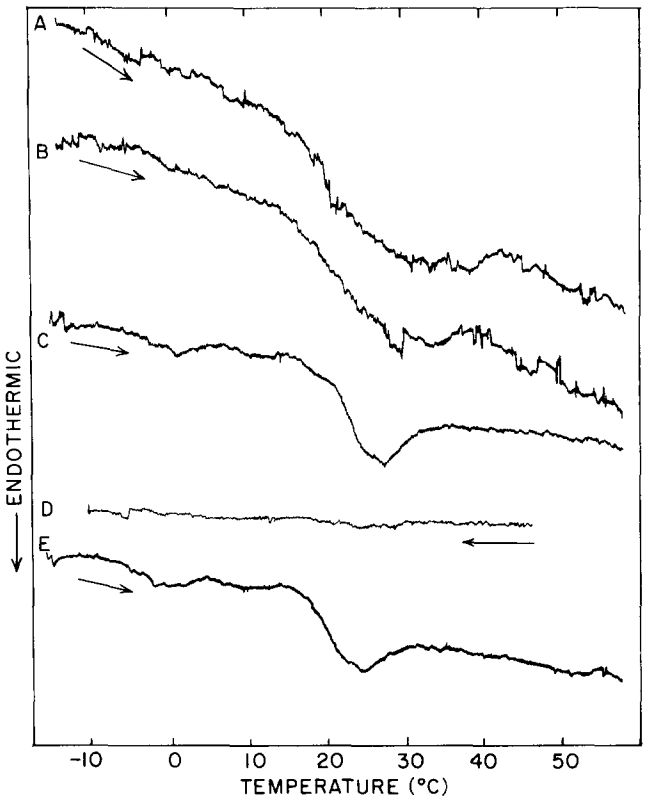

Fig. 1. Differential thermal analysis scans of vacuole lipids. Scans A and B are consecutive heating scans of the same vacuole lipid sample (2.4 mg lipid). Scans C through E are consecutive scans of a vacuole lipid sample (3.1 mg lipid) from a separate experiment.

vacuoles isolated by the DEAE-lysis procedure. However, vacuoles isolated by the osmotic lysis procedure clearly showed large areas depleted of particles (Fig. 2A, B). Neither isolation procedure preserved the scalloped pattern of particle-rich ridges and indented particle-poor areas sometimes seen in vivo [1].

TABLE III

EFFECTS OF TEMPERATURE ON THE INTRAMEMBRANOUS PARTICLE PATTERN

Cells were grown at 30°C. Aliquots (2.5 ml) were removed, equilibrated to 0, 13, 30 or 40°C for 5 min, and then fixed with 0.25 ml of 25% glutaraldehyde also equilibrated to the appropriate temperature. The cells were fixed for 2 h and then processed for freeze-fracture. At least 60 tonoplast fracture faces were counted for each temperature.

Temperature treatment (°C)	Dispersed	Percent aggregated	Geometric
0	19	12	69
13	22	7	71
30	22	14	64
40	65	34	2

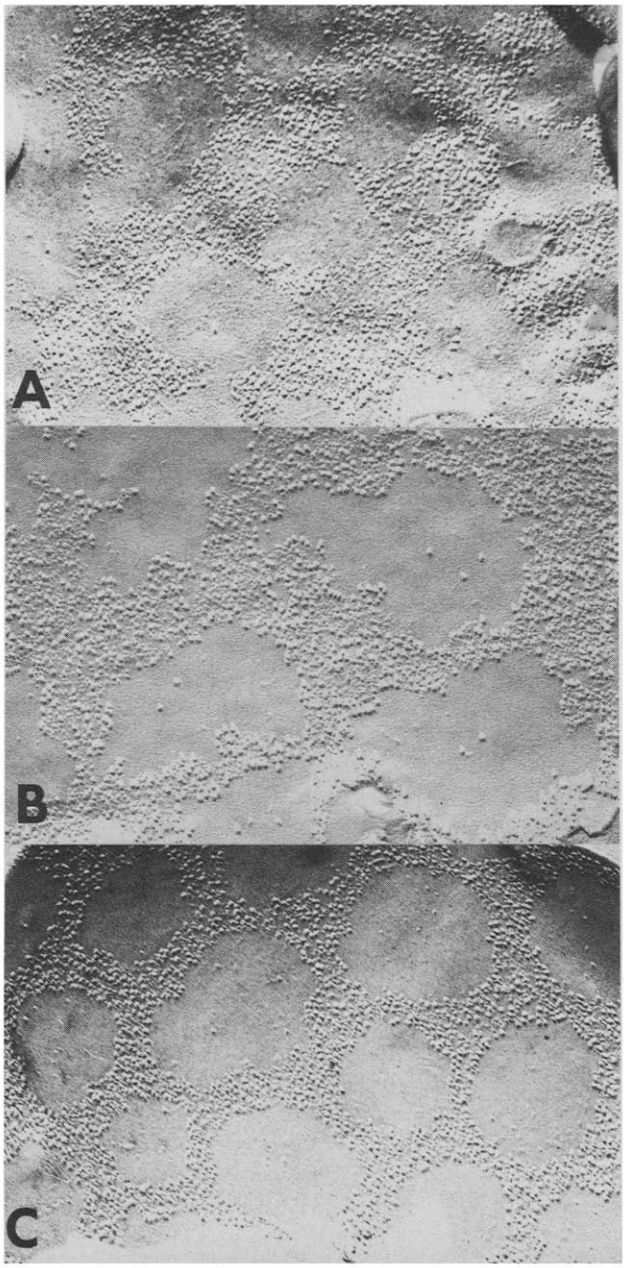

Fig. 2. Protoplasmic fracture faces of the tonoplast of isolated vacuoles. The vacuoles shown in A and B were isolated by the osmotic lysis procedure and show the aggregated particle pattern. The vacuole shown in C was isolated by the DEAE-lysis technique and shows the geometric particle pattern. A, B, C: 59 000×.

Temperature studies on intact cells showed that the intramembranous particle patterns were temperature-sensitive (Table III). At or below 30°C the geometric particle pattern was stable but was converted to a dispersed or aggregated pattern at 40°C. We tried to raise the temperature to 45°C in an attempt to

TABLE IV

EFFECTS OF TEMPERATURE REVERSAL ON THE INTRAMEMBRANOUS PARTICLE PATTERN

Cells were grown at 30°C. Three aliquots of 1.8 ml each were removed from the culture. Two of these were equilibrated to 40°C and one to 30°C. After 5 min one tube at 40°C and one at 30°C were fixed with 0.2 ml of 25% glutaraldehyde (equilibrated to the appropriate temperature). One 40°C aliquot (40 → 30) was returned to 30°C, equilibrated, and after 5 min fixed as described above. The cells were fixed for 2 h and then freeze-fractured. At least 27 tonoplast fracture faces were counted for each temperature regime.

Temperature treatment (°C)	Dispersed	Percent aggregated	Geometric
30	22	44	33
40	50	47	3
40 → 30	14	83	3

disperse more of the particles, but this reduced the cell viability and also caused particle-depleted blisters in the tonoplast similar to the artefacts described by Hasty and Hay [31]. The results presented in Table III show statistically significant differences. A 3×3 test of independence with the G test for temperatures 0, 13, and 30°C showed that the particle pattern frequencies for these temperatures were not significantly different ($G = 2.79$, $P = 0.59$). If the 40°C data were included, the resulting 4×3 test showed a significant difference ($G = 113$, $P < 0.01$). These results confirmed that lowering the temperature had no effect on the particle patterns while raising it had a dramatic effect. These temperature effects were partially reversible (Table IV). Although lowering the temperature from 40 to 30°C did not reconstitute the geometric pattern, an aggregated pattern with particle-depleted regions was formed.

Discussion

During the shift from exponential growth to stationary phase, areas depleted of intramembranous particles are formed in the yeast tonoplast [1]. In this paper, we present evidence that these particle-depleted regions correspond to areas enriched in gel-phase lipid. First, isolated vacuoles clearly show areas depleted of intramembranous particles (Fig. 2). This indicates that the pattern is not due to an interaction of the tonoplast with the cytoplasm via microtubules, microfilaments, metabolic energy, etc., but is intrinsic to the vacuole and most probably to the tonoplast. This observation is consistent with the presence of gel-phase lipid in the tonoplast. Second, the particle patterns are temperature-sensitive (Table III). As the temperature is raised (40°C), the dispersed particle pattern predominates while at or below the cell growth temperature (30°C) the majority of the tonoplasts contain particle-depleted areas. In addition, these pattern changes are at least partially reversible when the temperature is lowered from 40 to 30°C (Table IV). These results are compatible with the idea that as the temperature is raised the lipid becomes more fluid and the particles are free to diffuse to form a more dispersed pattern. At lower temperatures, a phase separation occurs, and the gel-phase lipid areas exclude

intramembranous particles to form particle-poor regions. This interpretation has been used to explain particle-depleted areas caused by low temperature in a variety of biomembranes (for reviews see Refs. 6, 7) including the yeast tonoplast [32]. Direct support for the correspondence of gel-phase lipid patches and particle-poor regions in freeze-fractured membranes has come from studies with bacteria in which the particle-poor regions were isolated and shown to contain less protein and less fluid lipid than the bulk membrane [33,34]. Lastly, differential thermal analysis of isolated vacuole lipids showed a broad endothermic transition which included 30°C, the cell growth temperature. This indicates that both gel-phase and fluid phase lipids are present at the cell growth temperature. This could lead to a lateral phase separation of the tonoplast lipids which in turn would influence the distribution of the intramembranous particles.

Although the presence of gel-phase lipid is probably sufficient to form particle-depleted regions in the tonoplast, the formation of the geometric particle pattern may involve other forces in addition to the gel-phase lipid. The geometric particle pattern is not obtained when the vacuoles are isolated by the osmotic lysis procedure (Fig. 2A, B) or after the temperature is lowered back to 30°C after a short period at 40°C (Table IV). Both of these procedures probably transiently increase the fluidity of the membrane because osmotic swelling of membranes can increase the fluidity of the membrane lipids [35]. When the osmotic conditions or temperature returns to normal, particle-depleted regions (gel-phase lipid areas) are reformed but the geometric pattern is lost. This implies that other forces may be needed to form the geometric pattern. Niedermeyer et al. [36] showed that glycerol could induce artefactual particle-depleted areas in the tonoplast. Maul [37] was able to produce linear polygonal patterns of particles in mitochondria by adding glycerol to cultured cells below their phase-transition temperature. A similar combination of forces along with a lipid phase separation in the yeast tonoplast might be needed to form the geometric pattern.

The presence of gel-phase lipid in the yeast tonoplast at physiological temperatures is unusual in that most biomembranes are fluid at physiological temperatures. By experimental manipulation of the cellular membrane lipids, prokaryotes can be shown to survive with large amounts of gel-phase lipid in their membranes at physiological temperatures (for reviews see Refs. 6, 7). James et al. [38] used fatty acid desaturase mutants in yeast to alter the fatty acid compositions and phase transitions of yeast membranes. Cells enriched in stearolate showed aggregated particle patterns in the tonoplast when grown below or slightly above the phase-transition temperature of the cell lipids. There is evidence that gel-phase lipid may exist at physiological temperatures for animal membranes [39—42], although in some of these studies the sterols must be removed [41] before a clear transition is detected. In a few studies on plants, phase transitions at physiological temperatures in leaves [43], cotyledons [44,45], and algal cultures [46] have been detected. These changes are believed to accompany senescence and loss of membrane function [43—46]. The changes in the yeast tonoplast occur without manipulation at physiological temperatures, only at a certain state in development, and are not involved in the loss of membrane function [1].

The presence of gel phase lipid in membranes can dramatically alter the function of those membranes (for reviews see Refs. 6, 7). The change in yeast is of particular interest because it occurs at a stage in which changes in vacuole function are believed to be important in the development of stationary phase in yeast [47]. At present these changes in vacuole activity can only be correlated with the changes in the tonoplast. However, we have studied the effect of the tonoplast changes on lipid body uptake into the vacuole and have shown that the process is substantially different from what happens in exponential phase cells [30]. Lipid body uptake is restricted to particle-depleted regions of the tonoplast at this stage and resembles phagocytosis. In this paper, we have presented evidence that these particle-depleted areas corresponded to gel-phase lipid regions. This lipid phase separation has a dramatic effect on the uptake of lipid bodies and probably on other vacuole functions as well. It now appears that with the onset of stationary phase, the yeast tonoplast contains a number of different structural domains which may have different functional roles.

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