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VACUOLE FORMATION IN WHEAT STARCHY ENDOSPERM

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Abstract

The formation of vacuoles in wheat (Triticum aestivum cv. Highbury) starchy endosperm cells was studied using electron microscopy. Some vacuoles were always present, even in the coenocytic cytoplasm. The first formed endosperm cells were highly vacuolated, but became filled with cytoplasm as they grew older. Various-sized pieces of cytoplasm were found in vacuoles of developing endosperm cells, probably as a result of autophagic sequestration. The membranes of the autophagic vacuoles appeared to originate from the rough endoplasmic reticulum and from extensions of already-formed vacuoles. Autophagic activity was confirmed by localizing the hydrolytic enzyme acid phosphatase within the vacuoles. The rough endoplasmic reticulum (RER) also stained positive for this enzyme.

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Introduction

Despite numerous studies, the precise mode of deposition of storage proteins in the developing starchy endosperm of wheat remains unclear. While this process has been studied by numerous investigators, a great deal of controversy exists concerning the process. One aspect is the route by which the storage proteins enter the vacuoles or whether they are deposited into vacuoles at all. An early hypothesis suggested that protein bodies were independent organelles capable of protein synthesis and deposition. This theory was supported by Morton et al. (1964) who reported that protein bodies contained DNA and ribosomes (with lower sedimentation values than those of the cytoplasm) within a distinctive membrane, and were capable of in vitro protein synthesis. Similarly the suggestion that vacuoles are the site of protein synthesis is based on the presence of ribosomes associated with protein bodies (Barlow et al., 1974). These results are not consistent with more recent studies, which have led to the proposal of two different mechanisms. The first is that the protein is synthesized on the rough endoplasmic reticulum (RER) and transported possibly via the Golgi apparatus into vacuoles (Greene, 1981; Donovan et al., 1982). Alternatively, the protein may be deposited directly within the RER lumen to form a body surrounded by a non-intact membrane of RER origin (Miflin et al., 1981; 1983).

One problem in determining the precise mechanism is obtaining reproducible and artifact-free specimens. For example, some workers have reported direct connections between RER and vacuoles (Campbell et al., 1981; Parker, 1982) while others have found none (Bechtel et al., 1982a, b; Bechtel and Barnett, 1986a, b). Furthermore, workers differ in their views as to what constitutes "good fixation" of specimens (Miflin et al., 1981; 1983).

In the present paper we report electron microscopic studies which show that storage proteins are present within vacuoles during the initial stages of protein deposition in developing wheat endosperm. We also show that the enlargement of these vacuoles during the early stages of development occurs by an autophagic process involving the RER and at least one hydrolytic enzyme (acid phosphatase) may be present within the vacuoles when protein deposition is occurring. The results differ from studies conducted on root tips

(Marty, 1978; Marty et al., 1980) in that vacuoles are always present in developing endosperm, even at the earliest stages of differentiation. Our results may explain some of the problems experienced by early workers in interpreting the relationships between RER, vacuoles, and protein bodies.

Materials and Methods

Wheat (Triticum aestivum cv Highbury) used for this study was grown either in field plots or in glasshouses at Rothamsted Experimental Station, Harpenden, England during the summer of 1987. Individual heads were tagged at anthesis and harvested at various times during development, typically; day of flowering, 2, 4, 7, 9, 11, 14, 18, 21, 28, 35, 42, and 50 days after flowering (DAF). The middle florets were used for microscopy. Fixation for microscopy was conducted as previously described (Bechtel et al., 1982a) using 2% glutaraldehyde (v/v) and 2% paraformaldehyde (w/v).

Acid phosphatase activity was localized at the ultrastructural level using a modified Gomori method (Gomori, 1952). Wheat endosperm tissue was fixed 1 h in 2% glutaraldehyde-2% paraformaldehyde in 0.05 M cacodylate buffer at pH 6.8 and 19 C. Tissue was washed 3 times for 10 min each with buffer and once overnight to remove fixative. Localization media consisted of 8.0 mM βglycerol phosphate or 2.7 mM para-nitrophenyl phosphate in 0.05 M sodium acetate buffer (pH 5.5) and 2.4 mM lead nitrate. Incubation of wheat endosperm was at 37 C for 1 h followed by 4 washes in acetate buffer. Lead contamination was checked for by adding a drop of ammonium sulfide to the used wash buffer. Contamination was detected by the presence of a dark precipitate. No lead was detected following the second buffer rinse. Controls used for acid phosphatase localization were incubation medium minus the substrate and medium minus substrate and lead nitrate. Samples were then prepared for light and electron microscopy as previously described (Bechtel et al., 1982a).

Results

A light microscopic study confirmed previous results concerning the presence of vacuoles early in endosperm formation (Morrison and O'Brien, 1976; Mares et al., 1977). In order to study the formation of new vacuoles and enlargement of existing vacuoles, an examination of developing tissues was conducted by electron microscopy. The time frame for observation was from 14-17 DAF when cell division in the outer endosperm tissue had ceased and these cells possessed little storage protein. Examination was limited to the subaleurone region, the first two cell layers beneath the aleurone.

A feature that was frequently observed was the presence of areas of cytoplasm surrounded by membranes (Figs. 1-3). The amount of cytoplasm within the membranes was extremely variable in size and the cytoplasm was composed only of groundplasm, ribosomes, and occasional membranes (Figs. 1-3). Organelles such as plastids, mitochondria, Golgi bodies, lipid droplets, and RER, that were typically present within endosperm cells, were never observed in these sequestered regions. Some regions were less than a micrometer in diameter while others were several micrometers across (Figs. 1 and 2). Present in the cell during the sequestration of cytoplasm within membranes was RER which had lost some or all of its ribosomes and had become distended (Figs. 3 and 4). Other membranes seemed more directly involved in sequestering the cytoplasm and were connected to pre-existing vacuoles (Fig. 5) or surrounded portions of cytoplasm (Fig. 6). Another phenomenon observed was vesicles and RER surrounding portions of cytoplasm which were devoid of most organelles (Fig.7). These partially surrounded regions matched closely to those found within membranes (compare Figs. 1-3, 6 to 7). By 17 DAF possible remnants of cytoplasm were observed within vacuoles along with protein bodies (Fig. 8).

Cytochemical analysis for acid phosphatase was conducted to determine if this general hydrolase, which is typically associated with lysosomal systems, was localized within any of the structures associated with sequestered cytoplasm. A modified Gomori technique was used to localize acid phosphatase activity in developing endosperm cells. Para-nitrophenyl phosphate gave more consistent staining, than did βglycerol phosphate without large, extremely dense deposits that obscured cell ultrastructure. The buffer-only control lacked any staining of the endosperm (Fig. 9). Controls using incubation media minus the substrate all showed a small amount of background staining consisting of small, dense dispersed deposits in RER, mitochondria, cell walls, and nuclei (Figs. 10 and 11). In contrast, the samples with substrate present showed selective staining of the RER, small vesicles, vacuoles, portions of some protein bodies, and cytoplasm present within vacuoles.Vacuoles containing cytoplasm showed reaction product on, in, and around the sequestered cytoplasm (Figs. 12-14). The periphery of some protein bodies and portions of the protein granules

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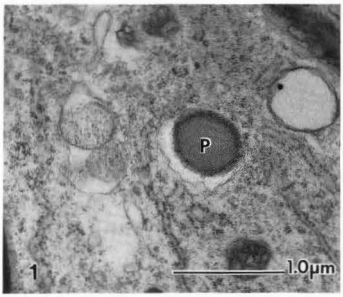
Figs. 1-8. Electron micrographs of autophagic vacuoles. 1. Regions of cytoplasm of 14 DAF endosperm enclosed within vacuole membranes are characteristic of autophagic vacuoles (A). P=protein body. 2. Massive region of cytoplasm of 14 DAF endosperm in various stages of digestion (A) surrounded by intact membrane (arrows). 3. RER of 14 DAF cell showing dilations (arrows) and an autophagic vacuole (A) containing cytoplasm (C) and protein granules (P). 4. RER in 14 DAF endosperm cell exhibiting dilation into vesicles as well as vesicles in various stages of losing ribosomes (*). 5. Long membranous extension (arrow) continuous with autophagic vacuole (A) and closely associated with RER of 14 DAF cell. 6. Small portion of 14 DAF cytoplasm (C) surrounded by ribosome-less cisternal membrane (arrows). 7. Vesicles (*) lining up and surrounding a portion of 14 DAF cytoplasm destined to become autophagocytized. 8. Remnants of cytoplasm (C) and membrane (arrow) in autophagic vacuole (A) from 17 DAf subaleurone cell.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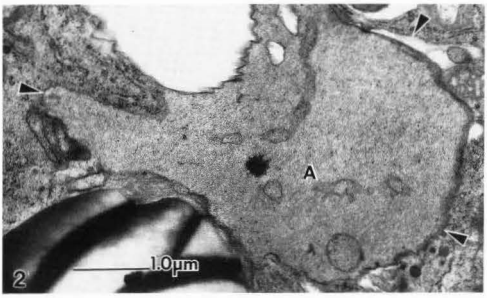
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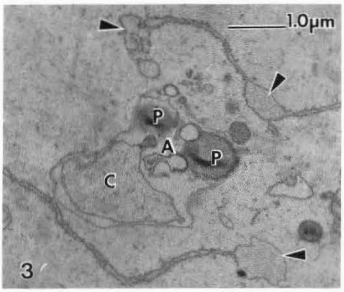
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A
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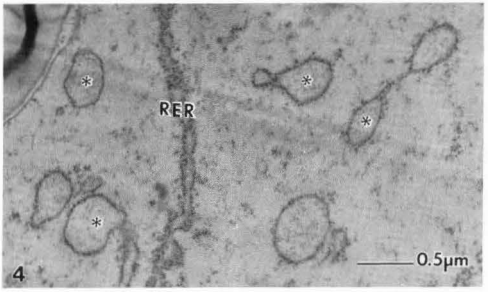
C
3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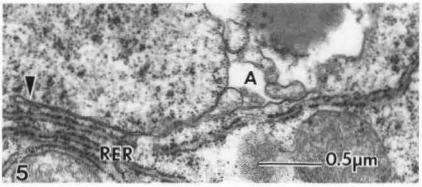
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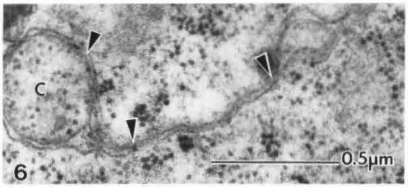
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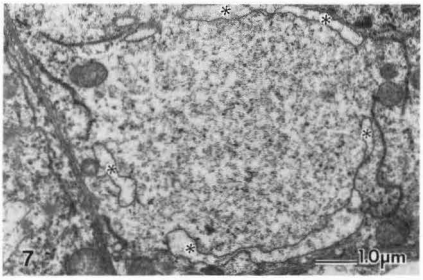
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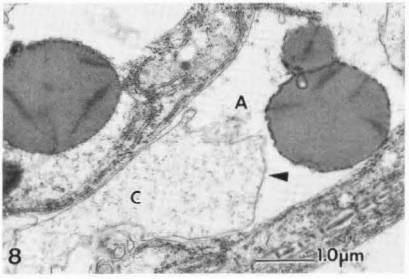

A
C
1.0μm
8

also stained positively (Figs. 15-17). Staining of the RER was not uniform. RER associated with vacuoles stained positive while that further away did not stain (Fig. 18). In many cases positively-stained ER was found to be continuous with the tonoplast, with expanded regions of ribosome-less ER, or connecting vacuoles with expanded ER (Figs. 19-22). These ER-vacuole connections were apparently limited to the period of vacuole development because none were observed past this period. The thin filament-like extensions of vacuoles also stained positive (Fig. 23). Golgi bodies were not usually stained, but the few that were had reaction product in the trans cisternae and in the trans Golgi network (Fig. 24). Vesicles of various sizes were also stained, some of which were located near Golgi bodies and protein bodies (Figs. 24 and 25).

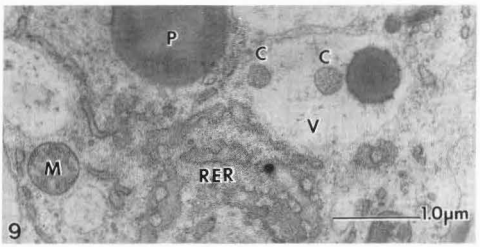
Discussion

The initiation of vacuoles from the ER and from the process of autophagy has been documented for meristematic root tissue (see Marty et al., 1980 for a review). Our results suggest that a similar system may function in the initial production of some of the wheat endosperm vacuoles. The finding of regions of cytoplasm within intact "vacuole" membranes could possibly result from a particular plane of sectioning through the endosperm cell. We feel this explanation is highly unlikely because of several observations. First, the observed regions of cytoplasm never possessed any organelles other than free ribosomes. Since the membranes surrounding the cytoplasm always lacked ribosomes, and smooth ER in wheat endosperm was limited to tubular elements that interconnected with RER (Bechtel and Barnett, 1986a, b), it would not be possible to obtain planes of section that would resemble what we have observed. Furthermore many of our micrographs are very similar to those from root meristems for which corresponding high voltage electron microscopy has confirmed the three-dimensional structure of the autophagic system (Marty et al., 1980). Fixation artifacts could also account for various membranous configurations. We have previously investigated our fixation scheme using freeze-fracture, freeze-etch techniques on fresh, glycerol treated and chemically treated wheat endosperm tissue and found that while, our routine fixation could result in breakage of the tonoplast (Bechtel and Barnett, 1986a), we did not observe this phenomenon with the present samples. Probably the most convincing evidence for the operation of autophagy in wheat endosperm is the acid phosphatase localization within the sequestered cytoplasm.

Acid phosphatase activity was localized within ER, vesicles, vacuoles, and protein bodies and activity was highest during meristematic activity and vacuole growth. The nonuniform localization of the enzyme may be caused by poor penetration of the reagents (Moore et al., 1987) or by uneven enzyme distribution. The presence of hydrolytic enzymes in vacuoles of plant tissues is a common phenomenon (Thayer and Huffaker, 1984; Henry and Steer, 1985; Wittenbach et al., 1982) and associated with autophagy (Marty et al., 1980). The working hypothesis for autophagy and vacuole formation in root meristems is that tubular provacuoles (= the trans Golgi network of Griffiths and Simons, 1986; = GERL of Novikoff, 1976) wrap themselves around portions of cytoplasm to eventually form a double membrane bounded structure. The enclosed piece of cytoplasm then becomes digested (Marty, 1978; Marty et al., 1980). We have observed a very similar situation in wheat endosperm except that the "provacuole" membranes are derived from RER and pre-existing vacuoles rather than from the trans Golgi network. As the entire endomembrane system tends to be interrelated and interconnected, this difference may not be of fundamental significance. In fact, the Golgi apparatus is preparing to package storage proteins at this time (Bechtel et al., 1982a; Bechtel and Barnett, 1986a, b) and may not be able to handle the extra burden of participating in autophagy. This is supported by the fact that very few Golgi bodies stain positive for acid phosphatase whereas the RER stains heavily. Other plant systems also show varied acid phosphatase localization (Henry and Steer, 1985; Gartner and Nagl, 1980; Pyliotis et al., 1979) indicating that a single mechanism of autophagy in plants probably does not exist.

The observations of vesicularization of RER and of vacuoles continuous with ER may be related to vacuole initiation. Previously we had been unable to identify these continuities using U.S. wheats grown under field conditions conducive to fast growth (35 days from flowering to combine harvest; Bechtel et al., 1982a; Bechtel and Barnett, 1986a,b). As these continuities are limited to the period of vacuolation prior to storage protein deposition, they probably contribute little to the direct flow of storage proteins into the vacuoles as previously reported (Simmonds, 1978; Campbell et al., 1981; Parker, 1982). Furthermore, the localization of acid phosphatase within the RER lumen lends credence to the view that ER functions in vacuole production.

Figs. 9-17. Acid phosphatase cytochemistry of 14 DAF wheat endosperm. 9. Buffer only control showing lack of staining of mitochondrion (M), RER, protein body (P), and cytoplasm (C) in vacuole (V). 10. Incubation medium without substrate shows minimal staining of cell wall (CW), mitochondrion (M), and RER (*). 11. Minus substrate control exhibits a small amount of staining of mitochondrion (M) and nucleus (Nu). 12. Early stage of autophagy with cytoplasm in vacuole (V) surrounded by thin membrane (arrows) and with acid phosphatase localized in vacuole (*). 13. Autophagic vacuole in 12 DAF wheat endosperm showing acid phosphatase localization (*). 14. Two autophagic vacuoles (A and B) and a vesicle (*) staining positive for acid phosphatase in 12 DAF endosperm; A in a more advanced stage of digestion than B. 15. Acid phosphatase localization (*) around protein granule (P) and in vesicle (arrow). Note dense inclusion (D) is not positive for localization. 16. Positive localization of acid phosphatase (*) in protein body (P) and RER. 17. Protein body (P) with positively-stained regions (*).


P
C
C
V
M
RER
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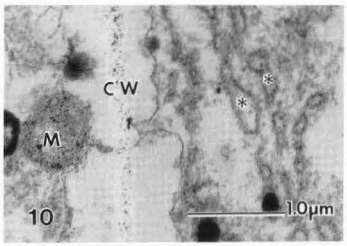
9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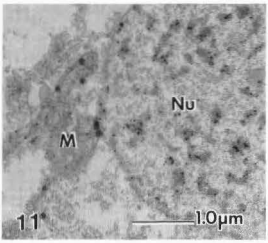
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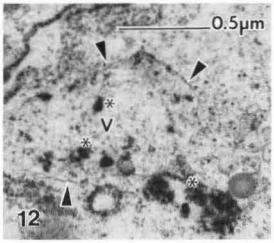
11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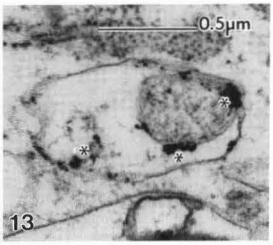
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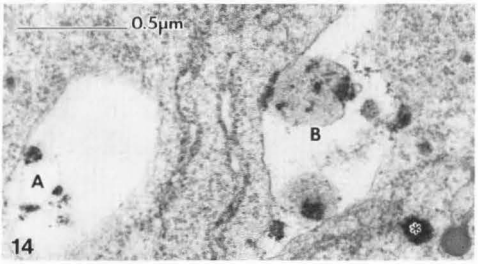
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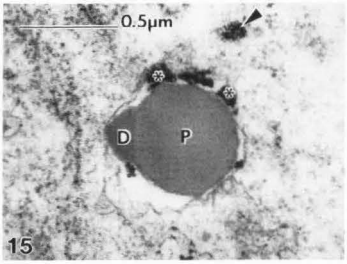
14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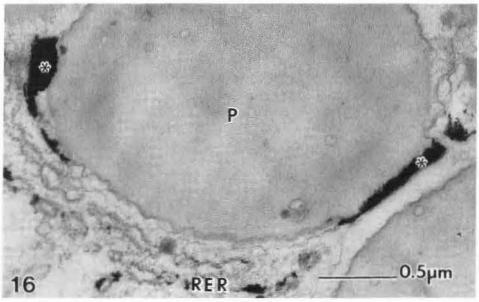
D
P
15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RER
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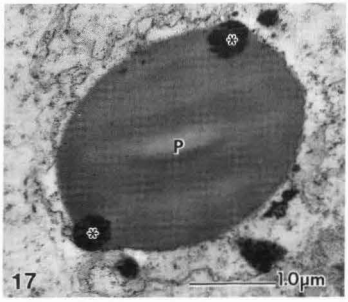
P
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17

Localization of acid phosphatase within protein bodies presents an interesting dilemma. The function of protein bodies as autophagic organelles during seed germination had been hypothesized (Matile, 1968) and since proven (Van der Wilden et al., 1980); the presence of hydrolytic enzymes is necessary for the mobilization of storage reserves (Van der Wilden et al., 1980). Most enzymes present during germination, however, are synthesized de novo and are different from the ones present during seed maturation. It appears that autophagy is active only in endosperm cells for a short period of time and probably does not contribute much membrane to the overall tonoplast system as observed in more mature cells; the bulk of the vacuole enlargement results from fusion of various vesicles during grain filling (Bechtel et al., 1982b). The process is important because autophagy is a normal cellular process that assists in ridding cells of obsolete metabolic machinery and aids in recycling components. Although the vacuoles are produced by endosperm cells for the deposition of storage proteins, it is surprising to find that protein reserves are being deposited into newly formed vacuoles which also contain the hydrolytic enzyme acid phosphatase. Little is known about the substrate specificity of hydrolytic enzymes that are present in developing cereal endosperms, and other hydrolases are probably also present within the vacuoles. This is supported by the similar activity profiles given by the bulk protease and acid phosphatase assays (data not shown). What effects these enzymes have on the protein storage reserves is unknown. Separation of the hydrolases and protein granules in subcompartments of the vacuole could prevent mixing of the enzymes and storage proteins.

A possible function for the phosphatases is to break down phytic acid, a major component of the aleurone cell, the outermost endosperm tissue. We have frequently observed subaleurone cells that appear to be intermediate in position and character between aleurone and starchy endosperm cells in young developing endosperms. Since the mature wheat grain has a single layer of aleurone cells, the intermediate cells must have become typical starchy endosperm cells. Hydrolytic enzymes including acid phosphatase could have a role in regulating the differentiation of such cells by digesting aleurone-specific cytoplasmic components.

Acknowledgment

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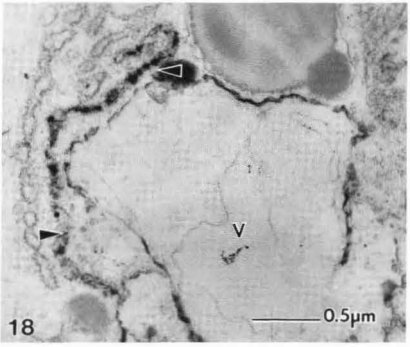
Figs. 18-25. Acid phosphatase localization in 14 DAF wheat endosperm. 18. Section through wheat endosperm showing only the RER (arrows) next to vacuole (V) staining positive for acid phosphatase while RER further away does not stain. 19. Positively-stained RER continuous with tono- plast (arrow) of vacuole (V). 20. RER in process of vesicle formation exhibiting positive staining for the enzyme. 21. Acid phosphatase positive RER and vesicle (*) continuous with a vacuole (V). 22. Two vacuoles (V) connected by positively-stained RER (arrow). 23. Positive localization in long, thin extension (arrows) of vacuole (V). 24. Golgi bodies in wheat endosperm with acid phosphatase localized in the trans Golgi network and budding vesicles (arrows). 25. Vesicles (*) near Golgi body (G) and protein body (P) that have stained positive for enzyme.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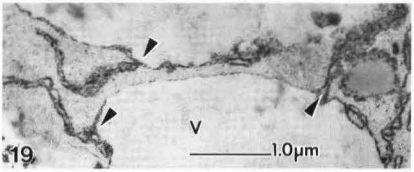
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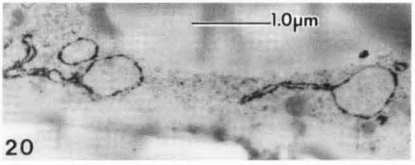
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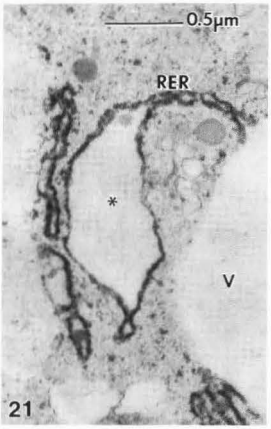
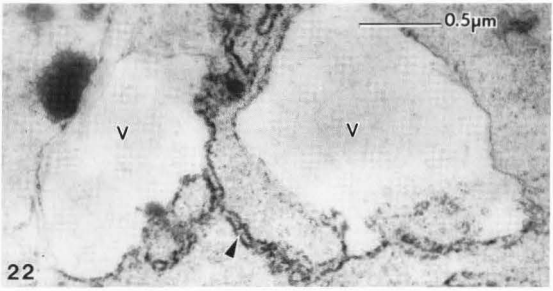

0.5μm
RER
*
V
21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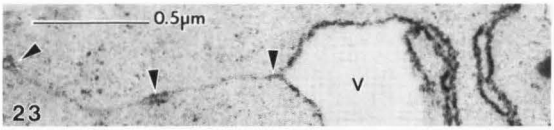
V
V
22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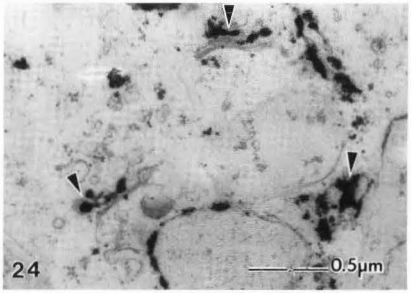
V
23


24
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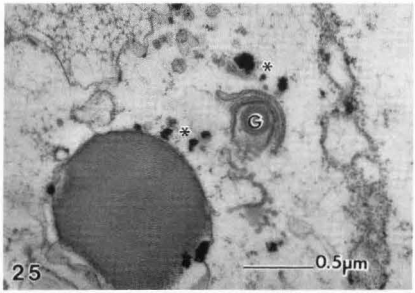
*
*
G
25
0.5μm

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Discussion with Reviewers

Reviewer I: Couldn't the phenomenon you describe be compared to the extensive membrane complexes described by Mares et al., 1976, Plant Sci. Lett. 7:305-311?

Authors: The process you refer to was observed in modified aleurone cells very early in development and involves the proliferation of RER. Our system occurs much later in development, is for the starchy endosperm cells, and the RER may possibly function in autophagy. The two systems are not comparable.

M.G. Smart: Was any attempt made to reconstruct membranes "surrounding" portions of cytoplasm (or high voltage TEM of thick sections attempted)?

Authors: We have a limited number of serial section reconstructions, but they have not been of much help in visualizing the three dimensional aspects of this part of the cell. Our freeze-fracture work has been very useful, however, and shows close relationships between RER and vacuoles.

B.A. Stone: In assessing the acid phosphatase cytology can it be certain that the ER has, or does not have, associated ribosomes?

Authors: RER is RER when ribosomes are attached and proteins are secreted into its lumen. When ribosomes fall off and are not replaced the ER becomes smooth ER by definition. It no longer is capable of participating in protein synthesis.

Reviewer IV: What is the frequency of this proposed mechanism of vacuole formation?

Authors: We do not have any numbers for its occurrence, but feel it is prominent during the starchy endosperm cell growth stage when these cells undergo a large expansion in cell volume.