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Endoplasmic Reticulum Formation during Germination of Wheat Seeds[1]

A QUANTITATIVE ELECTRON MICROSCOPE STUDY

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ABSTRACT

This study demonstrates germination-induced ultrastructural changes in wheat (*Triticum aestivum* L. cv Arthur) aleurone cells. Seeds imbibed for 4 hours in water contained endoplasmic reticulum (ER) or ER-like membranes as vesicles or as short segments of membrane associated with the spherosomes on the periphery of aleurone grains. Aleurone cells incubated between 8 and 10 hours contained abundant ER membranes mainly associated with the nuclear envelope and, to a lesser extent, with the spherosomes surrounding the aleurone grain. The membranes located on the periphery of the nucleus occurred as regions of stacked cisternae. When aleurone cells were analyzed by morphometry, the increase in ER during incubation was found to be greater than 2-fold. During the same incubation period, other organelles did not change significantly. The early increase in ER was not affected by gibberellin incubation. Thus, the rapid proliferation of ER observed during the early stages of germination in aleurone cells of wheat is not likely to be controlled directly by gibberellin.

The aleurone layer, a thin band of cells that surrounds the endosperm of cereal grains, functions largely in synthesis and secretion of α-amylase and other hydrolytic enzymes. The synthesis of α-amylase in barley is thought to occur on membrane-bound polyribosomes (5, 17), is enhanced by the GA, and is inhibited by ABA (10). The GA-induced increase in α-amylase synthesis follows a 3- to 4-h lag, indicating transcriptional control by GA (10).

GA also has been implicated in the stimulation of ER synthesis in aleurone cells (2, 15, 21, 23, 25). Numerous electron microscope studies have indicated a proliferation of ER membranes following GA treatment. Thus, aleurone cells that initially contained a sparse population of ER cisternae and vesicles in the dry seed, contained extensive regions of stacked ER membranes following GA treatment. Although the role that GA plays in the control of membrane synthesis is a subject of controversy (7, 11, 24), a clear stimulation of ER formation in aleurone cells occurs during incubation. From this standpoint, the aleurone cells provide an excellent system in which to study *de novo* membrane synthesis. We report here quantitative measurements of ER development in aleurone cells of wheat and the effect of GA on this membrane proliferation.

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MATERIALS AND METHODS

Preparation of Material. Grains of wheat (*Triticum aestivum* L., cv Arthur) from Illinois Seed Supply Service (Peoria, IL) were degermed by dissecting approximately one-fourth of the embryonic end of the grain with a razor blade. The degermed seeds were then surface-sterilized in a 10% solution of commercial bleach (5.25% NaOCl) for 15 min and thoroughly rinsed in deionized H_2O. These seeds were then imbibed in 20 mM succinate buffer (pH 5.6) plus 10 mM $CaCl_2$ in a ratio of 25 ml media per 10 seeds (6). The seeds were imbibed for 4 h at 20°C, and then 1 μM GA_3 was added to the incubation media ($t = 0$). Samples were taken at 2, 4, 6, 8, and 10 h following addition of GA_3.

Tissue Fixation. Because of the difficulty in fixing seed tissue, a protocol for aleurone cell fixation employing a combination of aldehyde and permanganate fixatives adapted from the protocol of Mollenhauer (19) for investigation of seed tissue was used. Incubated seeds were placed in fixative containing 3% glutaraldehyde, 1.5% *p*-formaldehyde, and 1.5% acrolein in 50 mM collidine buffer (pH 7.2). Aleurone layers were removed using a spatula and forceps under a dissecting microscope. Excess starch was removed, and the layers were transferred to fresh fixative. Aleurone layers were then minced with a razor blade into pieces approximately 1 mm^2. Selected pieces were transferred into fresh fixative and fixed for 15 min at room temperature, then overnight at 4°C. The tissue was rinsed six times for a total of 1 h in 50 mM collidine buffer. Following aldehyde fixation, the tissue was washed in deionized H_2O three times for a total of 30 min and postfixed in 2% aqueous $KMnO_4$ for 40 min on ice. The tissue was thoroughly rinsed in deionized H_2O and soaked in 2% aqueous uranyl acetate overnight at 4°C. Samples were dehydrated through a graded acetone series and embedded in epoxy resin (22). Silver sections were cut using a diamond knife and stained in alkaline lead citrate (20 min) prior to viewing.

For morphometric analysis, electron micrographs of whole cells or portions of whole cells at a final magnification of 22,500 were examined under transparent overlays containing dots spaced 1 cm apart (20). Organelle composition of the cells on a volume basis was determined by counting the number of dots that were superimposed over specific cell components. The analysis was confined to the cytoplasmic area of the cell (*i.e.*, nonnuclear). The average relative error comparing duplicate analyses of individual micrographs was less than 5%. A minimum of nine cells was analyzed from three different aleurone preparations from each time and treatment. ER, mitochondria, microbodies, Golgi apparatus, spherosomes, and leucoplasts (plastids) were identified by their characteristic morphologies. ER, however, could not be separated into its SER or RER components, since the tissue was postfixed in $KMnO_4$, a procedure that destroys ribosome morphology (19).

RESULTS

Ultrastructural Changes during Germination. To understand better the synthesis of ER in higher plants, an investigation into the ultrastructure of wheat aleurone cells was conducted. This tissue may provide a unique opportunity to study *de novo* ER synthesis, since a large and relatively rapid proliferation of ER has been reported in both barley and wheat aleurone cells. The ultrastructure of the cytoplasm of the wheat aleurone cell was dominated by the aleurone grain, which was surrounded by spherosomes forming an aleurone grain-spherosome complex (Figs. 1 and 2). Located between these complexes were mitochondria, leucoplasts, microbodies, ER, and occasional Golgi apparatus. An extensive study of the ultrastructure of aleurone cells from barley has been reported (14, 15). Because of the similarity between barley and wheat aleurone cells, a similar analysis of wheat would be redundant.

The ER in wheat aleurone cells imbibed for 4 h in buffer was typically found in two regions of the cytoplasm. In the first, ER was located on the periphery of the aleurone grain-spherosome complex. Here, the membranes were in relatively short segments, 0.25 to 0.65 μm long, and were often closely appressed to the spherosomes surrounding the aleurone grain (Fig. 1). In the second, ER or ER-like vesicles with a diameter of approximately 0.15 μm were located free in the cytoplasm.

Aleurone cells imbibed for 4 h and incubated with 1 μM GA_3 for up to 4 h were very similar to the cells after only a 4-h imbibition. However, cells incubated with GA_3 for 6 to 10 h showed a significant increase in ER membranes as compared to 4-h-imbibed cells (Fig. 2). In addition, the ER was now located as regions of stacked membranes frequently, although not exclusively, associated with the nucleus. Significant membrane development was also observed on the periphery of the aleurone grain-spherosome complex. Here, the ER membranes often surrounded large portions of the complex, and the location of the membranes was reminiscent of the location of the short segments of ER found in 4-h-imbibed cells. Thus, the content of ER increased over the incubation period, and the ER morphology was altered as well.

Morphometric Analyses of Aleurone Cells. To evaluate quantitatively the morphological changes that occurred during this early phase of germination, electron micrographs of aleurone cells were analyzed by the dot overlay method of Ovtracht *et al.* (20). The major organelle found in the cytoplasm of the aleurone cells was the aleurone grain (Fig. 3). Aleurone grains accounted for between 50 and 60% of the total organelle volume. The fractional composition of aleurone grains did not significantly change during incubation (analysis was conducted at the 5% level). Spherosomes occupied approximately 30% of the total organelle volume. Statistically significant changes did not occur during the incubation period, although the fractional composition appeared to decrease slightly at 8 and 10 h of GA_3 incubation (Fig. 3). Mitochondria composed approximately 5% of the total organelle volume, and the composition was not altered with incubation (Fig. 3).

ER occupied a relatively small percentage of the fractional volume of the cytoplasm, but its composition was most affected (Fig. 4). The ER composition in the aleurone cell was relatively constant between 0 and 4 h incubation, but it increased significantly (analysis also at the 5% level) between 4 and 6 h. The total volume occupied by the ER increased more than 2-fold, with the major portion of this increase occurring between 4 and 6 h of incubation.

Leucoplasts and microbodies occurred at a low frequency in the aleurone cell. Their fractional composition was 1.5 and 0.5%, respectively, and it remained constant over the incubation period; because of their low frequency, however, deviations were high (data not shown). Golgi apparatus occurred infrequently between 0 and 6 h of incubation but occurred with increasing frequency between 6 and 10 h.

GA_3 Effect on Organelle Development. Although GA_3 has a dramatic effect on the synthesis of α-amylase, the direct effect of GA_3 on ER development is a subject of some controversy (7, 9, 15, 16, 24). To determine if the early stimulation of ER synthesis was, in fact, due to a direct effect of GA_3, a series of incubations was performed in the absence of GA_3. Interestingly, aleurone cell ER development was the same whether GA_3 was present or not (Fig. 4). GA_3 also had no effect on the development of aleurone grains, spherosomes, or mitochondria during this early phase of incubation (data not shown). Thus, these data demonstrate that the proliferation of ER membrane in aleurone cells observed here could not be attributed to a direct GA_3 effect.

DISCUSSION

Fixation of Aleurone Tissue. A factor limiting the investigation of the early ultrastructural changes during germination of aleurone cells has been the inability to properly fix tissue for electron microscopy (2, 11, 14, 21, 24). Although adequate fixation of tissue was obtained with a mixture of aldehyde fixatives (glutaraldehyde, *p*-formaldehyde, and acrolein) followed by postfixation with OsO_4, because of the dense cytoplasmic matrix, organelles (except mitochondria) were not clearly delineated. Fixation with $KMnO_4$ alone delineated intracellular membranes but usually left organelles, especially spherosomes, in a distorted state and left the cytoplasm with a granular appearance (2, 11, 14). Here, organelle preservation and membrane intensification were obtained by combining aldehyde fixation with postfixation in permanganate. Thus, this fixation protocol allowed for adequate preservation of tissue for morphometric analysis.

Ultrastructure of Aleurone Cells during Germination. We have undertaken this study to evaluate the ultrastructural changes that occur in aleurone cells during germination. Most significantly, we have quantitated the increase of ER during this period. A significant amount of ER was present in cells imbibed for 4 h. Although rapid cellular changes occurred in cells during imbibition (3, 4), the ER present at 4 h of imbibition was likely present in the dry seed. This conclusion was supported by the fact that the 4-h-imbibed aleurone cells appeared morphologically similar to cells of dry aleurone layers of both barley or wheat (compare, for example, Fig. 1 to Fig. 4 of Jones [14] or Plate 1A of Colborne *et al.* [7]).

The increase of ER in wheat cells has been observed in the first day of germination in wheat aleurone layers by Colborne *et al.* (7). With the data presented here, this ER increase occurs between 4 and 6 h of incubation. In agreement with their observation, a significant quantity of ER membrane is located as stacked cisternae on the periphery of the nucleus.

Other morphological changes in aleurone cells were less pronounced. Jones (15, 16) reported an expansion of the aleurone grain during early incubation. A similar expansion of aleurone grains was not observed here. This can be explained in two ways. First, because of the variability of the data, changes in volume of aleurone grains of less than 10% would be difficult to detect by morphometry. Secondly, Jones (15, 16) reported that the volume increase was less pronounced when cells were fixed with glutaraldehyde than with permanganate fixation. Since aleurone layers were fixed in aldehyde fixatives and postfixed in permanganate, the volume increase of aleurone grains likely would be small.

GA_3 Control of ER Development. Finally, an effect of GA_3 on ER development has been reported (16); however, the nature of this effect is somewhat unclear. GA_3 stimulates [^{14}C]choline incorporation into ER-containing fractions (8) and increases the activities of phosphorylcholine cytidyl and glyceride transferases (1; unpublished results). However, the inability to clearly demonstrate an increase in [^{3}H]glycerol (9) or [^{14}C]acetate (18) incorporation into lipid molecules suggests either that the sites of membrane precursor synthesis in the aleurone cell are not avail-

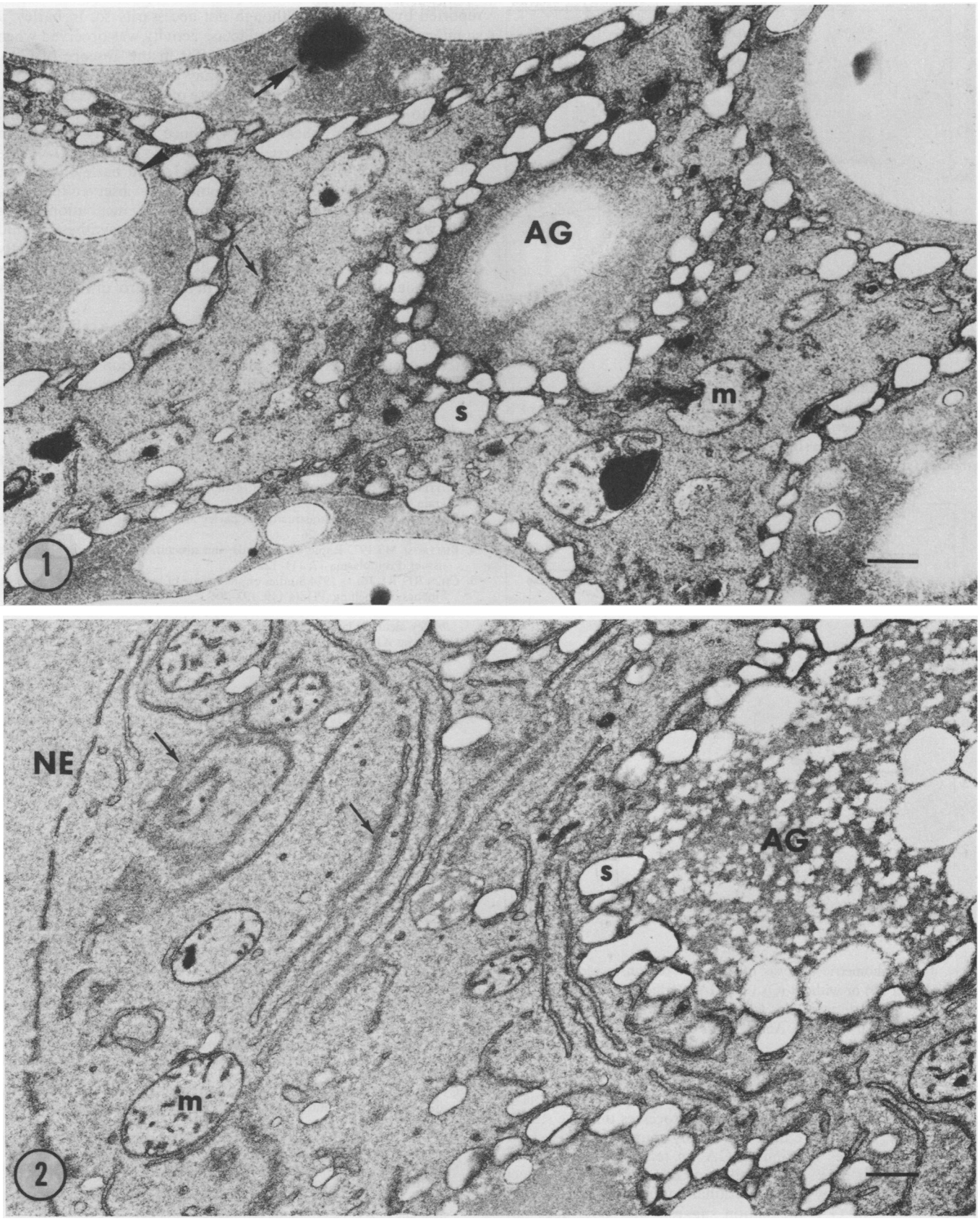

FIG. 1. Aleurone cells imbibed 4 h in water (t = 0). Large aleurone grains (AG) contained both electron-dense and -transparent inclusions (large arrow and arrowhead, respectively). ER or ER-like vesicles are scattered throughout the cytoplasm (small arrows), but stacked ER lamellae are absent. m, Mitochondrion; s, spherosome; bar = 0.5 μm.

FIG. 2. Aleurone cells imbibed 4 h and incubated 8 h plus GA_3. Stacked ER cisternae are now abundant (arrows), frequently in proximity to the nuclear envelope (NE). Bar = 0.5 μm.

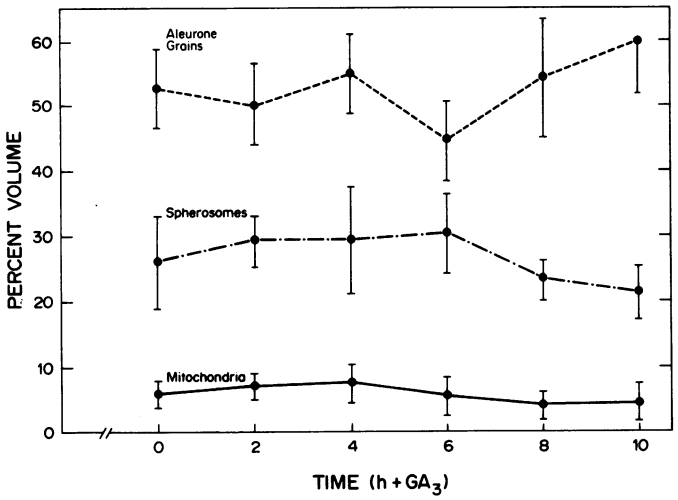

FIG. 3. Morphometric analysis of protein body, spherosome, and mitochondria composition in aleurone cells. Values are an average of a minimum of nine determinations from three separate aleurone cell preparations, and the bars are ±SD. Analysis was conducted by the dot overlay method of Ovtracht *et al.* (20).

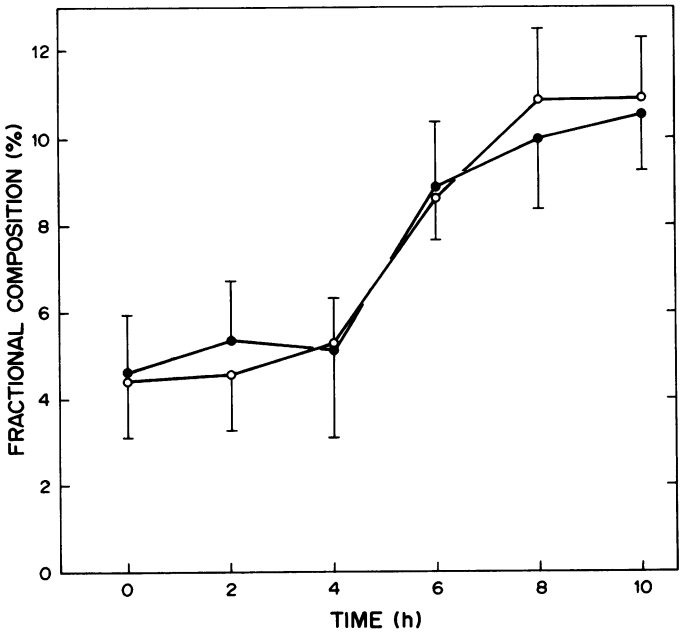

FIG. 4. Morphometric analysis of ER composition in aleurone cells incubated with (●) or without (○) 1 μM GA_3. Values are an average of a minimum of nine determinations from three separate aleurone cell preparations, and the bars are ±SD. Analysis was conducted by the dot overlay method of Ovtracht *et al.* (20).

able for free exchange with externally added glycerol and acetate or that the proliferation of ER following GA_3 treatment is a secondary effect related to the secretory processes that are stimulated by GA_3 (24, 25). We have previously shown that the wheat aleurone system does respond to GA_3 in terms of increased α-amylase secretion (12). The inability of morphometry to detect any significant changes in the fractional volume occupied by ER with up to 6 h of GA_3 incubation suggests that GA_3 does not directly control the early ER synthesis in wheat aleurone cells. This quantitative result is in agreement with qualitative ultrastructural observations made by Colborne *et al.* (7).

These results may be interpreted as contrasting to those recently reported by Jones (16), although not necessarily so. In barley, a significant increase in Cyt *c* reductase activity was observed when aleurone layers were imbibed in buffer in the absence of GA_3. This increase in enzyme activity may be, at least in part, an increase in ER membrane. The increase of Cyt *c* reductase activity was 2- to 3-fold, similar to the 2-fold increase in ER fractional volume observed in wheat (Fig. 4). Since this early increase in membrane in barley occurred in the absence of added GA_3, it is not ER synthesis that is controlled by GA_3. In barley, however, an additional increase in ER development was observed following 18 h of GA_3 incubation. Since the longest GA_3 incubation in this study was 10 h, this stimulation by GA_3 of ER synthesis would not have been observed. Thus, GA_3 appears not to control directly the early synthesis of ER in wheat cells, although a more complex involvement of the embryo and GA_3 in the control of ER synthesis is likely in later stages (16, 24).

In summary, we have demonstrated that aleurone cells of wheat underwent a rapid increase in ER membrane content, that this increase occurred between 4 and 6 h of incubation, and that this early ER synthesis was not influenced by GA_3 treatment.

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