EMBRYO-LETHAL MUTANTS OF ARABIDOPSIS THALIANA: ULTRASRTUCTURAL ANALYSIS OF EMBRYOS FROM NORMAL AND ABORTED SEEDS

By

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Bachelor of Science

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Stillwater, Oklahoma

1984

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
December, 1986

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ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to all of the people who assisted me in this project. In particular, I am especially indebted to my adviser, Dr. David W. Meinke, for providing guidance, support, inspiration, and valuable time in reviewing draft copies of this document.

I would also like to thank the other members of my committee, Drs. Charlotte Ownby, Ulrich Melcher, and Paul Richardson, for their advisement during the course of this work.

Other members of the lab, Linda Franzmann and Chris Monnot, were particularly helpful. Linda did most of the planting and screening of the various mutants; Chris watered the plants and helped in screening mutants.

Special thanks go to Janice Green-Pennington and Denise Rex at the Oklahoma State University Electron Microscope Center for cutting and staining thin sections for the electron microscope.

Lise Weavers deserves my deepest appreciation for friendship, encouragement, and general organization of the figures in this thesis.

I extend a special thanks to my parents for providing financial and moral support during my graduate work at

Oklahoma State University.

This research was funded in part by the College of Arts and Science at Oklahoma State University and NSF Grant #PCM 82-15667 awarded to David W. Meinke.

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°C	degrees centigrade
g/gal	grams per gallon
hr(s)	hour(s)
M	molar concentration
ml(s)	milliliter(s)
n=5	haploid chromosome number equals five chromosomes
NAA	napthleneacetic acid
um	micrometer

CHAPTER I

MODEL SYSTEMS AND APPROACHES TO THE ANALYSIS OF DEVELOPMENTAL MUTANTS

Review of Developmental Genetics and Mutant Analysis

The process of development has been of interest to biologists for many years. The first investigations in the area of developmental biology were approached primarily from a descriptive point of view, although the German anatomist Wilhelm Roux recognized as early as 1888 the need for more detailed analytical studies. Since that time, developmental biology has grown into one of the most exciting fields of scientific research. Modern developmental biologists use a variety of descriptive, molecular, and genetic methods in their research. One of the most popular methods of current research is the analysis of mutants. Many interesting developmental mutants have been described in Drosophila (Sang 1984), Caenorhabditis (Sternberg and Horvitz 1984), Dictyostelium (Godfrey and Sussman 1982) and the mouse (Bennet 1980). Of these classical systems, perhaps the most thoroughly studied is Drosophila. Mutants of Drosophila are currently being used to describe how groups of cells in the young embryo are programmed to specific developmental fates. One

of the most interesting patterns of abnormal development is found in the homoeotic mutants, where one body part is replaced by another (Sang 1984).

Plant Developmental Genetics

The field of plant developmental genetics is relatively new when compared to the extensive studies that have been performed with Drosophila. Plant developmental biologists consider mutant analysis an important part of their arsenal of techniques. Recent reviews can be found on developmental mutants in maize (Scandalios 1982), Arabidopsis (Meyerowitz and Pruitt 1985; Estelle and Somerville 1986), and seed plants in general (Marx 1983).

Recessive lethals are one of the most common classes of mutants found in mutation research, and as a result have been widely used in the analysis of animal development (Hadorn 1961; Wright 1970). Embryo development in higher plants has also been studied through the use of lethal mutants. The most comprehensive studies have dealt with the defective kernel mutants of maize (Neuffer and Sheridan 1980; Sheridan and Neuffer 1980, 1981; Sheridan et al. 1984), variant cell lines of carrot unable to complete somatic embryogenesis in vitro (Breton and Sung 1982; Guiliano et al. 1984), and of particular interest to this study, embryo-lethal mutants of Arabidopsis thaliana (Meinke 1985; Meinke 1986).

Characteristics of Arabidopsis

Arabidopsis thaliana (L.) Heynh. is a small weed-like member of the Brassicaceae (Cruciferae) that can be found in many parts of the temperate world. Arabidopsis has a compact basal rosette, an erect stem, and an indeterminant inflorescence with many fruits or siliques (fig.1). Arabidopsis has been described as a model system for studies in plant development (Meinke and Sussex 1979a) and plant molecular genetics (Meyerowitz and Pruitt 1985). Some of the useful characteristics of Arabidopsis for genetic, developmental and biochemical studies include the small plant size; simplicity of growth conditions; short life cycle; large number of seeds produced; low chromosome number (n=5); reduced levels of repetitive DNA (Meyerowitz and Pruitt 1985); availability of an Agrobacterium transformation system (Lloyd et al. 1986); well characterized Capsella pattern of embryo development (fig.2); and the presence of a linkage map (Koorneef et al. 1983).

Embryo-Lethal Mutants of Arabidopsis

Embryo-lethal mutants of Arabidopsis have been reported by several groups interested in different aspects of mutagenesis and embryogenesis in higher plants (Müller 1963, Rédei 1970; Meinke 1985). Perhaps the most extensive study was that of Müller (1963), where approximately 3,000 probable embryonic lethals were identified. More recently,

Figure 1. Drawing of Arabidopsis thaliana (L.) Heynh. Mature plant showing basal rosette, main stem with terminal inflorescence and lateral branches (A); trichomes on the surface of leaves (B); flower at the time of pollination (C); petal (D); stamens (E,F); compound pistil (G); mature silique splitting along both sides of the central septum to reveal two rows of seeds (H); and mature seed (I). Drawing from Ross-Craig (1948).

Figure 1

Figure 2. Stages of embryo development in Capsella bursa-pastoris. The embryo is composed of two major parts: the embryo proper and the suspensor. The embryo proper (stippled) develops into the mature embryo, whereas the suspensor degenerates during later stages of development. Embryos from Arabidopsis thaliana pass through a similar series of developmental stages. (Adapted from Raghavan 1976).

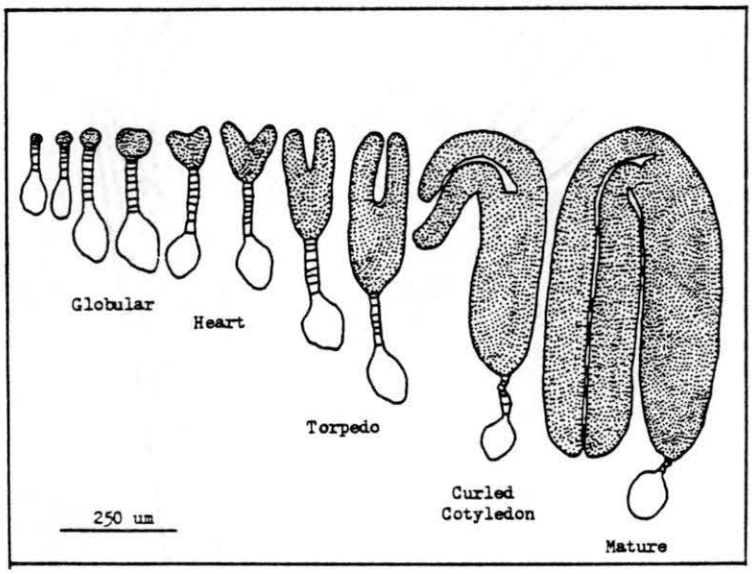

Figure 2

Meinke (1985) described over 30 new recessive embryo-lethal mutants that have been isolated and analyzed in a series of continuing experiments. Several different approaches have been taken in the analysis of these mutants. Meinke (1982) and Baus (1985) reported evidence of gametophytic gene expression in mutants showing a non-random distribution of aborted seeds along the length of heterozygous siliques. In another study, Marsden and Meinke (1985) described abnormal development of the suspensor in an embryo-lethal mutant of Arabidopsis. Heath et al. (1986) described the seed storage proteins found in normal and aborted seeds of Arabidopsis, and Baus et al. (1986) examined the response of mutant and wild-type embryos on a variety of tissue culture media.

The purpose of this project was to describe at an ultrastructural level the timing and formation of protein bodies and lipid bodies in the maturing cells of mutant and wild-type embryos. The timing and formation of these storage organelles in normal cells was crucial because this information was then used to characterize the developmental age of embryonic cells from selected embryo-lethal mutants.

Protein Bodies as Developmental Markers

Storage proteins of plant seeds are deposited in membrane-bound organelles called protein bodies. These protein bodies are restricted to specialized tissues of

the seed such as the starchy endosperm and aleurone layer of monocots, and the mesophyll of cotyledons and occasionally the hypocotyls of dicot embryos (Weber and Neumann 1980). Seed storage proteins by definition are synthesized in developing seeds, accumulate during seed maturation, are broken down and used as a nitrogen source during germination, and are not found in other tissues of the mature plant. Seed storage proteins and protein bodies can be used as developmental markers of cellular differentiation because they are present only during a very short phase of the life cycle.

The formation, contents, and distribution of protein bodies have been characterized in many dicotyledonous plants (for recent review see Weber and Neumann 1980). Many of the descriptive studies have dealt with members of the legume family such as Pisum (Varner and Schidlovsky 1963; Smith and Flinn 1967; Swift and Buttrose 1973); Phaseolus (Miege and Mascherpa 1976; Öpik 1966; Harris et al. 1975); Vicia faba (Baily et al. 1980; Neumann and Weber 1978); Lupinus (Survorov and Sobolev 1972; Davey and Van Staden 1978) and Glycine max (Tombs 1967; Lott and Buttrose 1978a). One reason for the interest in this family may be the wealth of information published on their well-characterized seed storage proteins. Cruciferous species analyzed for protein body development include Sinapis alba (Rest and Vaughn 1972; Werker and Vaughn 1974; Kirk and Pylotis 1976; Schopfer 1979); Brassica napus

(Hofsten 1970,1974); B. campestris (Hofsten 1974) and Crambe abyssinica (Hofsten 1973; Smith 1974). From these studies, it has been concluded that the protein bodies of cruciferous species have much in common with the protein bodies from other plant families. Protein bodies in these crucifers range from 1-10 μm in diameter and are found in both the hypocotyl and the cotyledons of mature embryos. Also, small dense inclusions called globoids are commonly found in the protein bodies of Brassica, Sinapis, and Crambe (Weber and Neumann 1980). The globoids of Crambe were found to contain sulfur, magnesium, and calcium (Hofsten 1973). It is believed that these globoids represent an accumulation of the phosphorus containing compound phytin (Weber and Neumann 1980).

This project was initiated after arrested embryos from several different embryo-lethal mutants of Arabidopsis were found to have unusual shapes that did not correspond to a given stage of normal development. The mutants in question contained arrested embryos with a variety of phenotypes ranging from large green embryos without a defined hypocotyl or cotyledons to a mutant characterized by reduced cotyledons and an enlarged hypocotyl. The primary goal of this project was to use the presence of protein bodies as a developmental marker to identify mutants that had continued cellular differentiation after the embryo had become developmentally arrested at the morphological level.

In order to determine whether certain mutants had abnormal patterns of development at the cellular level, the normal pattern of development had to be characterized first. Therefore, one of the objectives of this project was to establish the site and time of protein body development in wild-type embryos. These results are presented in Chapter 2. The results of descriptive studies on the formation of protein bodies in arrested embryos from four embryo-lethal mutants (115D-4A, 112A-2A, 130B-A, and 114D-1A) are presented in Chapter 3. This information was then used to complement biochemical studies on the relative levels of seed storage proteins in wild-type and mutant embryos. Combining these two techniques has provided valuable information on the synthesis and acumulation of seed storage proteins in wild-type and mutant embryos.

Results from both descriptive and electrophoretic studies have shown that seed storage proteins of Arabidopsis thaliana are synthesized at low levels during the linear cotyledon stage of development. These proteins accumulate in the protein bodies of the hypocotyl and cotyledons only during the curled and mature cotyledon stages of development. Arrested embryos from mutant 115D-4A lack mature protein bodies and accumulate at most very little if any seed storage protein. On the other hand, mutant 112A-2A embryos contain relatively normal amounts of seed storage proteins and have mature protein bodies in both the hypocotyl and cotyledon. Mutant 130B-A embryos contain

significant amounts of storage proteins, but mature protein bodies are found primarily in cells of the hypocotyl. Embryos from mutant 114D-1A contain reduced levels of seed storage proteins and protein bodies at various stages of development in both the hypocotyl and cotyledons. Arrested embryos from lethal mutants of Arabidopsis thaliana therefore differ not only in their pattern of abnormal development, but also in their extent of cellular differentation as reflected by different patterns of protein body formation and storage-protein accumulation.

CHAPTER II

ARABIDOPSIS THALIANA: ANATOMICAL AND ULTRASTRUCTURAL ANALYSIS OF WILD-TYPE EMBRYOS

INTRODUCTION

Normal embryo development involves a complex set of synchronized events. As described in the first chapter, one approach to the study of embryo development is through analysis of mutants that are blocked at specific stages. By comparing these mutant embryos to wild-type embryos, it may be possible to learn more about the processes that take place during normal embryo development. Before mutant embryos can be analyzyed, the events that take place during normal embryo development must first be documented. The purpose of this chapter is to briefly describe the formation of protein bodies during the maturation phase of normal embryo development in Arabidopsis thaliana. Emphasis is placed on wild-type embryos from the torpedo to the mature cotyledon stages of development because it is during these stages that large quantities of seed storage proteins are packaged into protein bodies.

MATERIALS AND METHODS

Growth of Plants

Mature wild-type seeds of Arabidopsis thaliana strain "Columbia" were suspended in water and then transferred to 3 inch pots filled with a 12:3:1 mixture of coarse vermiculite, potting soil and sand. The soil surface was misted periodically to prevent the seeds from drying out. After the seeds had germinated (usually 3-5 days), the soil around the plants was top-watered with either distilled water or a dilute fertilizer solution. After two pairs of true leaves were formed, the plants were watered daily with a solution containing 4 g/gal of 7-6-19 All Purpose Hyponex and Ø.25 g/gal of 15-16-17 Peat Lite Special. The plants were grown at 24 C° ± 2 C° on light benches with 16 hr/8 hr light/dark cycles. After approximately 3 weeks, the plants began to flower and produce seeds. At this time, siliques (fruits) were harvested for tissue processing.

Preparation of Tissue for Microscopy

Siliques were split open along the central septum under a Wild M-7 dissecting microscope. The stage of embryo development was determined by dissecting several representative seeds from each silique with fine forceps. Seeds containing embryos at either the linear to early-curled cotyledon or mature cotyledon stages were placed on filter paper moistened with 4% glutaraldehyde in

a Ø.2 M sodium phosphate buffer at pH 6.8 during the dissection process. If intact seeds were to be fixed, the seed coat was punctured to allow maximal passage of fixing fluids.

If isolated embryos were to be fixed the seed coat could easily be dissected away under a dissecting microscope with fine-tipped forceps. The tissue was then placed immediately in a small glass vial containing 4% buffered glutaraldehyde on ice for 6-8 hours. The fixative was drawn off with a pasteur pipet and the tissue was rinsed 3 times with cold phosphate buffer. Post-fixation was done overnight (8-1Ø hours) in 1% osmium tetroxide in the same phosphate buffer on ice. The tissue was then rinsed 2 times in cold buffer, transferred to a clean glass vial, and then rinsed 3 more times in cold buffer to ensure removal of any residual osmium. At this point the tissue could be stored for several days at 4° C in phosphate buffer. It should be noted that solutions containing osmium tetroxide were handled very carefully with gloves under the fume hood. The waste osmium was placed in a large bottle under the fume hood and tagged for appropriate disposal.

As soon after fixation as possible, the tissue was dehydrated in a graded ethanol series. The seeds and embryos were drawn up in a pasteur pipet and transferred to a new vial along with a few drops of phosphate buffer. Residual buffer was then removed before fresh liquid was

added. The ethanol dehydration was therefore performed in a single vial. The tissue was passed through a 10%-20%-40%-60%-80%-95% ethanol series for 10 minutes each, then through 3 changes of absolute ethanol for 15 minutes each. After dehyrdration, the tissue was infiltrated with 1:3, 1:1, and 3:1 dilutions of Spurr's resin:absolute ethanol for 2-3 days in each solution. The tissue was then infiltrated with a pure solution of Spurr's resin for 2-3 more days. This infiltration scheme was needed in order to get maximal penetration of the resin into the dense embryonic tissue. Each piece of tissue was then individually embedded in a Beem capsule containing approximately 1ml of fresh Spurr's resin. Sealed Beem capsules were then placed in an oven at 68° C for 72 hours to polymerize the resin. Samples were stored in labeled capsules until sectioning.

Light Microscopy

Polymerized blocks were trimmed to a pyramidal shape at the tip surrounding the tissue with a razor blade and then thick-sectioned at approximately 1 µm with glass knives fitted with LKB truffs on a Sorvall "Porter Blum" model MT-2 ultra-microtome. Sections were lifted out of the water in the truff with a fine-hair loop and transferred to one of the two etched rings on a fluorescent antibody microscope slide. Using these slides with etched rings made it easier to keep track of sections during staining and

microscopic examination. Slides with drops of water containing sections were then placed on a slide-warming tray at 70° C until the water evaporated. The sections would then adhere to the slide during staining and rinsing. The sections were stained with a solution of 1% toluidine blue in 1% sodium borate for 1 minute on a 70° C hot plate and rinsed with a fine stream of distilled water. Initial micrscopic examinations were made without a coverslip in order to take immediate preliminary notes on the sections. Temporary slides were prepared by adding one drop of immersion oil to the ring on the slide and placing a coverslip over the sections. For permanent slides, a drop of Permount mounting media was used instead of immersion oil. These permanent slides were left on the slide-warming tray for a least 24 hours before viewing, while the temporary mounts could be examined under the microscope immediately. Sections were examined with an Olympus OM-10 compound light microscope with a photo tube and 35mm camera attached for photomicroscopy.

Electron Microscopy

Blocks were prepared in the same way as in sectioning for light microscopy. Thick sections were were made through part of the tissue, and then the blocks were taken to the Oklahoma State Electron Microscope facility for thin sectioning. Silver-grey thin sections were made using an Sorvall ultramicrotome model MT-6000 fitted with a Diatome

diamond knife. The sections were mounted on 200 mesh copper grids and stained with 5% aqueous uranyl acetate and 0.3% aqueous lead citrate. Sections were viewed with a JEOL 100-CX II transmission electron microscope. All of the thin sections for electron microscopy were prepared by Janice Green-Pennington and Denise Rex at the Oklahoma State University EM facility.

RESULTS

Light Microscopic Examinations

Embryos at the heart stage and earlier were found to contain very little in the way of storage material. At the light microscopic level, numerous empty vacuoles and nuclei with large nucleoli were present, but no protein bodies were seen. Cells in these young embryos were small (5-15 μm) and stained very pale with toluidine blue.

Serial sections through 11 embryos at the linear to early-curled cotyledon stages were examined. These embryos filled approximately 1/2 to 2/3 of the seed and were found to have differentiated at the cellular level into these distinct types of tissue: the epidermis, the mesophyll of the developing cotyledons and hypocotyl, the vascular tissue, the shoot apical meristem, and the root apical meristem. Many of these tissue types could easily be distinguished after examination at 200x (fig. 3). No significant differences could be seen in the amount or intensity of stain in the hypocotyl or cotyledons. The only

Figure 3. Light micrograph of wild-type early-curled cotyledon embryo. Scale bar = 40 μm.

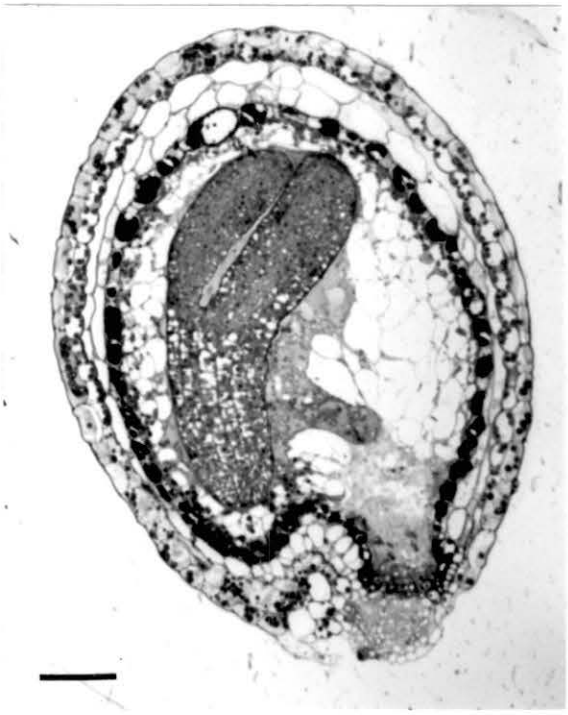

Figure 3

organelles clearly visible at the light microscope level were nuclei, nucleoli, and large transparent vacuoles. The vacuoles did not appear to contain storage protein or any other materials.

Sections through 4 embryos at the mature cotyledon stage of development revealed that the embryo completely filled the seed. These embryos were also found to contain the same distinct types of tissues found in the linear to early curled-cotyledon embryos. At this stage of development there were no significant differences seen in the staining of the hypococtyl (fig. 4) and the cotyledons (fig. 5). Organelles that could be seen in the cells of these embryos included nuclei, nucleoli, small lipid droplets and fairly large (5-10 µm) dark-blue staining protein bodies (fig. 6). The protein bodies in these embryos appeared to fall into at least two phenotypic classes. The hypocotyl and cotyledon cells appeared quite similar with respect to protein and lipid bodies. Under the light microscope, individual lipid bodies were difficult to distinguish but lipid-rich areas of the cells were readily apparent by their pale color. The cells of the mature embryo ranged in size from 8-22 µm in diameter. These cells had various shapes and sizes in different parts of the embryo, but not much could be determined about their ultrastructure at the light microscope level. The light microscope did prove useful when screening through individual embryos to determine the stages of development

Figure 4. Light micrograph of hypocotyl from mature wild-type embryo. Scale bar = 25 µm.

Figure 5. Light micrograph of cotyledon form mature wild-type embryo. Scale bar = 25 µm.

Figure 6. Light micrograph of cells from mature wild-type hypocotyl. (pV) = provascular tissue, (L) = lipid-rich area, and (Pb) = protein body. Scale bar = 1Ø µm.

Figure 4

Figure 5

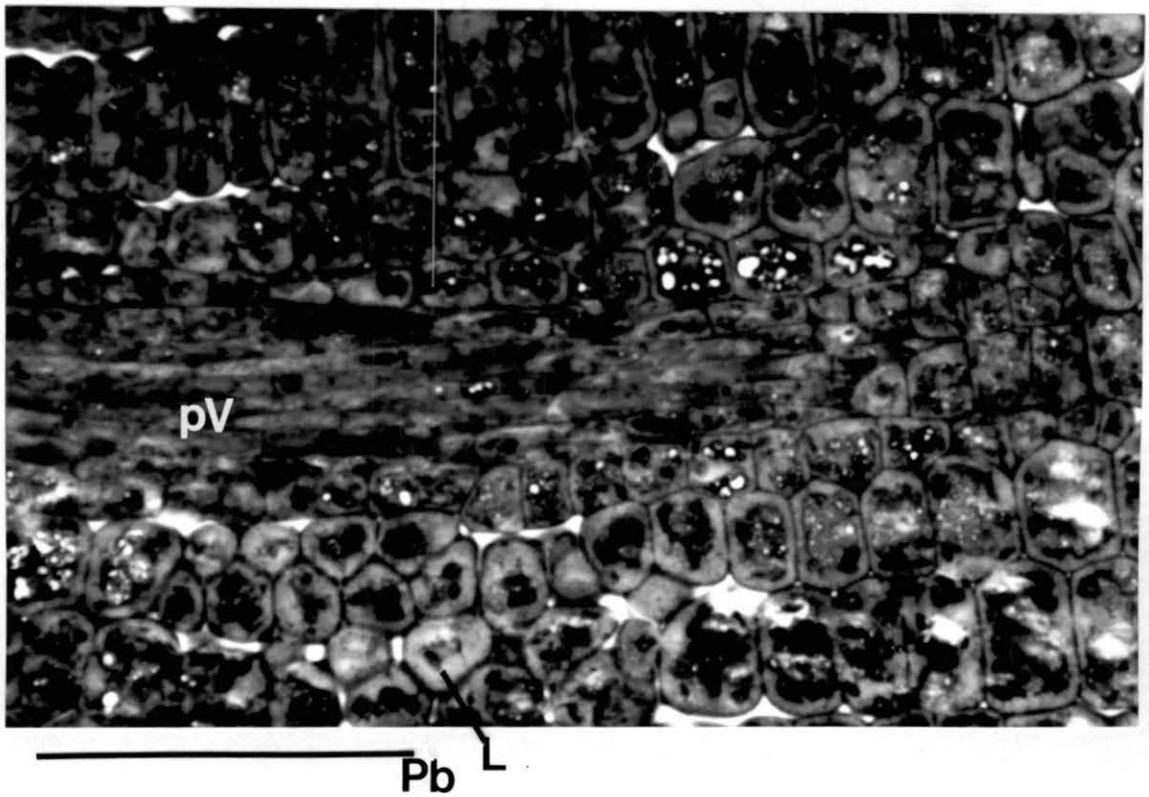

Figure 6

when protein bodies were beginning to fill.

Electron Microscopic Examinations

Results from electron microscpy were much more conclusive than from light microscopy. The organelles that could be seen under the electron microscope include: nuclei, nucleoli, plastids, mitochondria, endoplamic reticulum (ER), lipid bodies and developing protein bodies. Embryos at the linear to early-curled cotyledon stages were found to contain significant amounts of rough ER in both the hypocotyl (fig.7) and cotyledons (fig. 8), but individual embryos contained variable amounts of protein in their vacuoles. Even embryos taken from adjacent seeds in the same silique could be found to contain different levels of storage protein in their protein bodies. The linear to early-curled cotyledon embryos contained cells with many small lipid bodies (figs. 7,8). The developing protein bodies in these immature embryos had already developed many small inclusions (see fig. 8). The lipid bodies appeared to be closely associated with each other and were distributed primarily near the periphery of the cells.

Organelles that could be seen in sections of both the hypocotyl (fig. 9) and cotyledon (fig. 1Ø) from mature embryos included mature plastids, nuclei, nucleoli, numerous lipid bodies and a few (3-1Ø) large protein bodies. There was very little ER seen in these cells, probably due to the reduced amount of protein synthesis in the

Figure 7. Electron micrograph of hypocotyl from wild-type embryo at early-curled cotyledon stage of development. Numerous immature protein bodies (Pb) and lipid bodies (Lb) are visible. (Nu) = nucleolus and (ER) = endoplasmic reticulum. Scale bar = 2 μm.

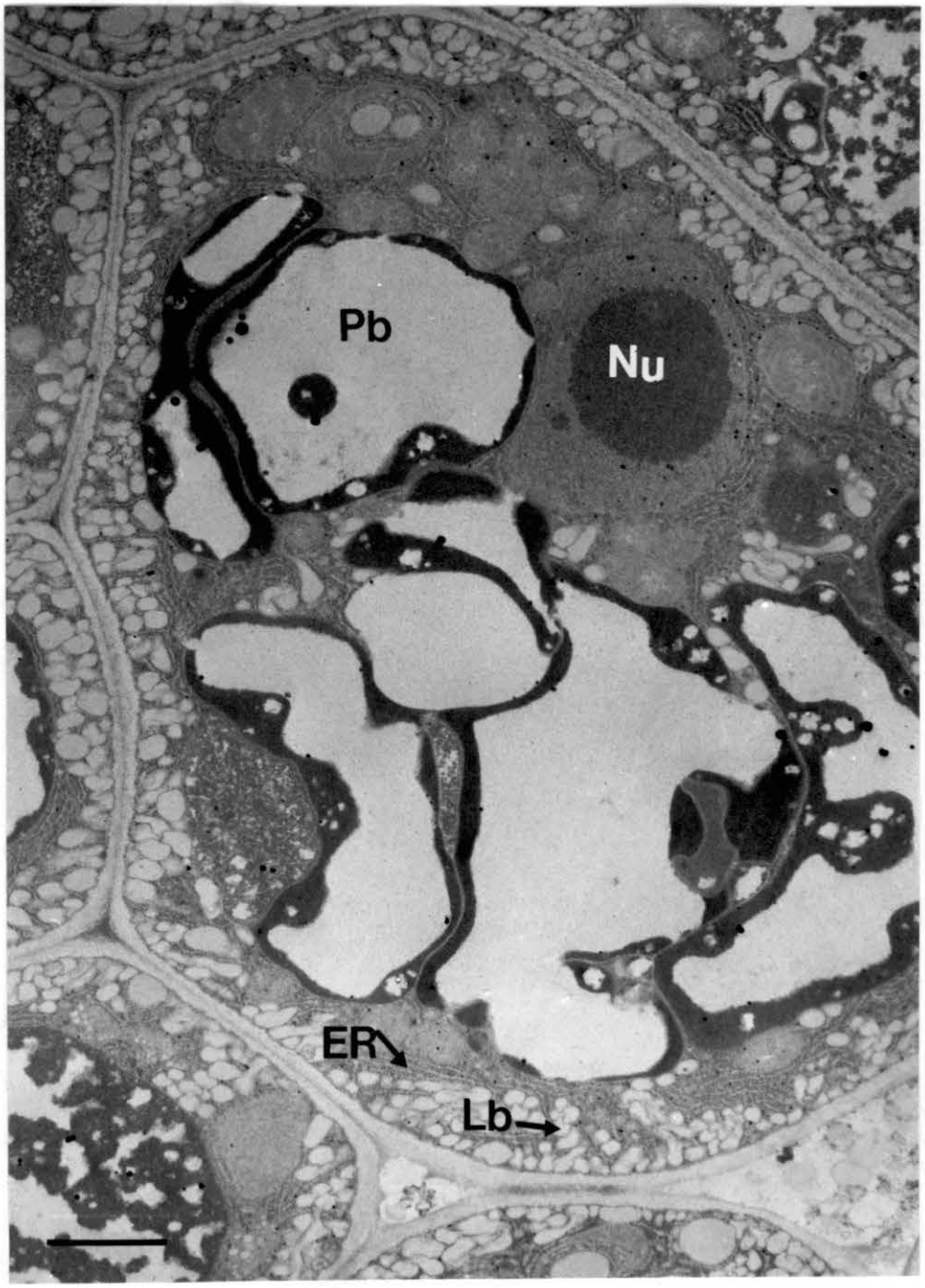

Figure 7

Figure 8. Electron micrograph of cotyledon from wild-type embryo at early-curled cotyledon stage of development. (Pb) = immature protein body, (i) = inclusion in protein body, and (Lb) = lipid body. Scale bar = 2μm.

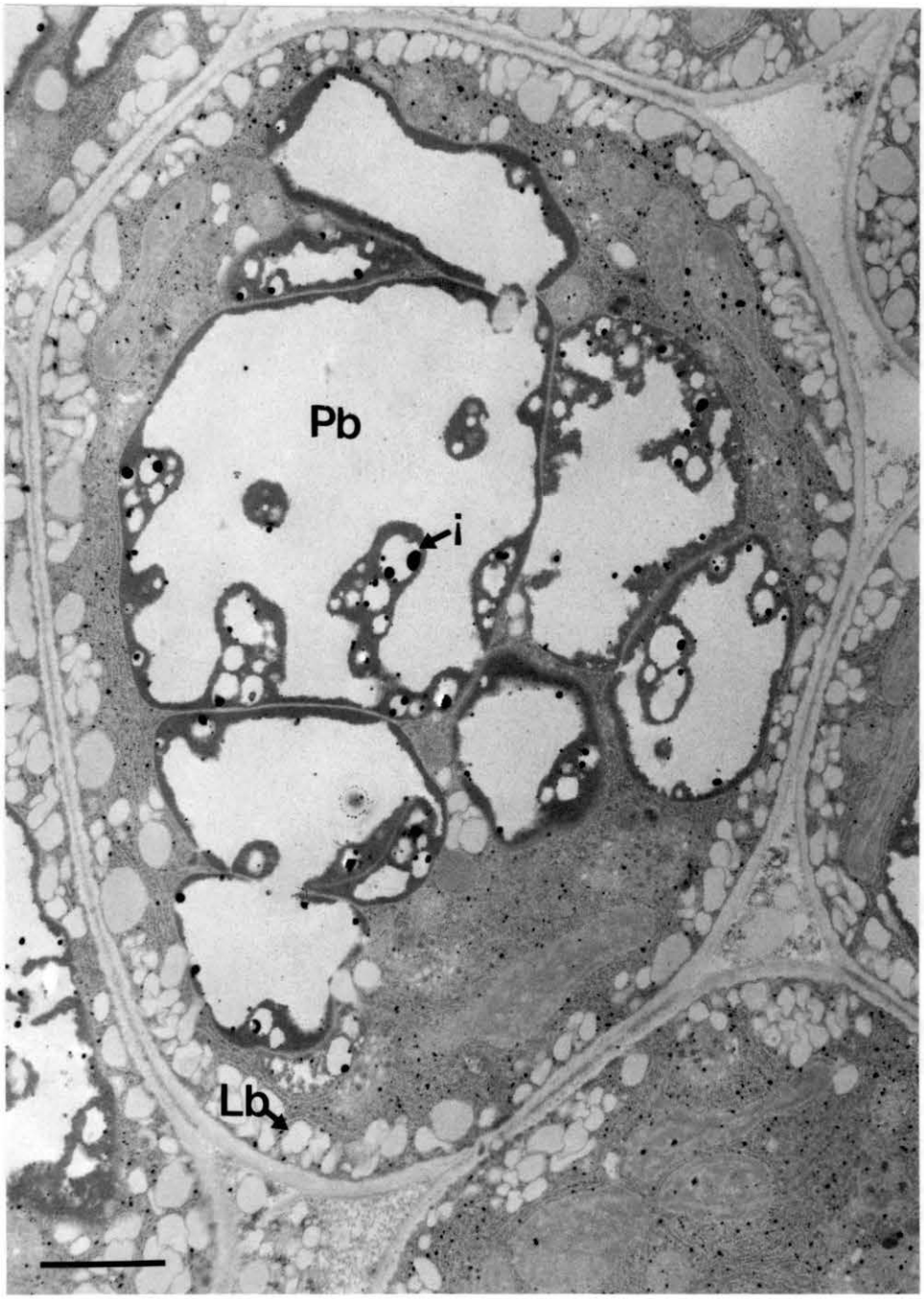

Figure 8

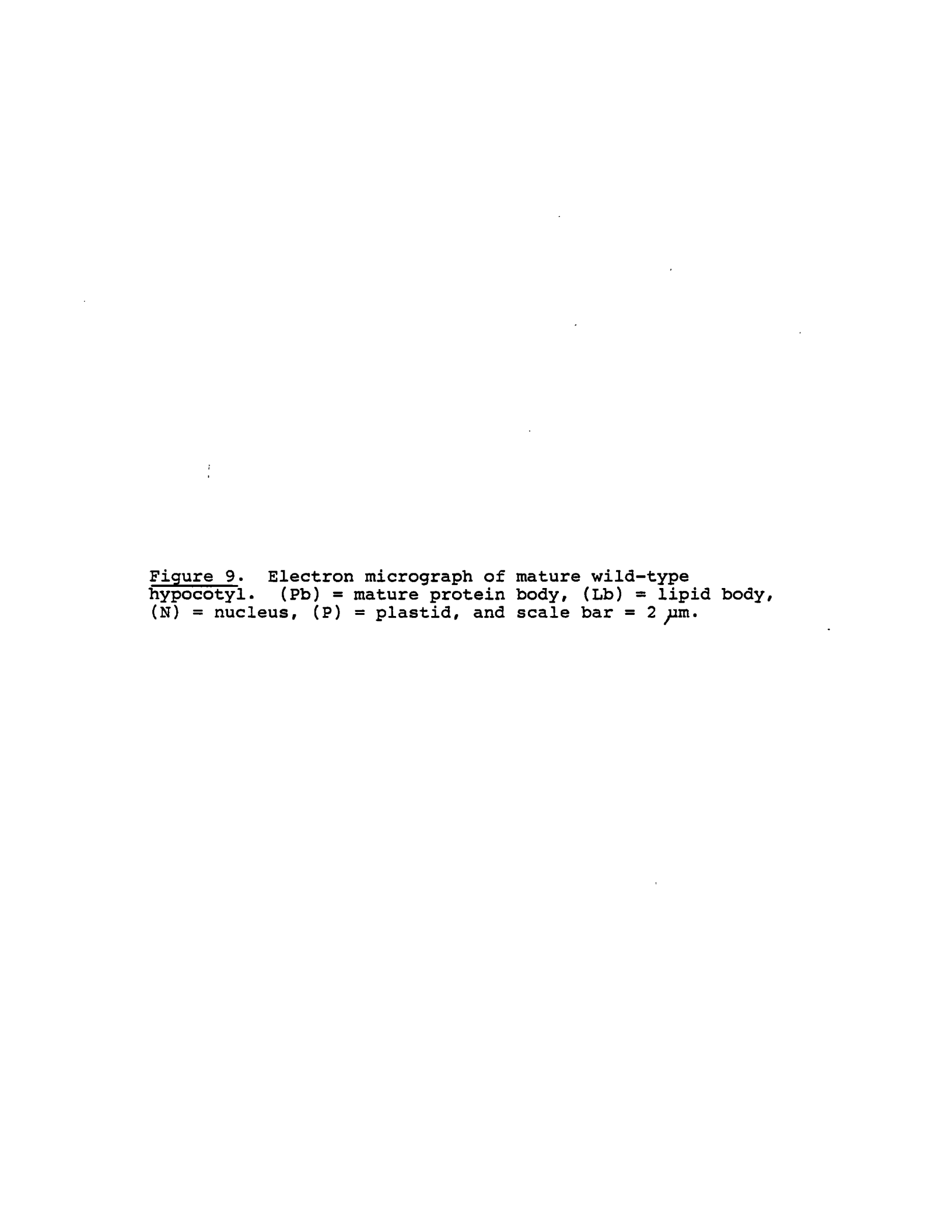

Figure 9. Electron micrograph of mature wild-type hypocotyl. (Pb) = mature protein body, (Lb) = lipid body, (N) = nucleus, (P) = plastid, and scale bar = 2 μm.

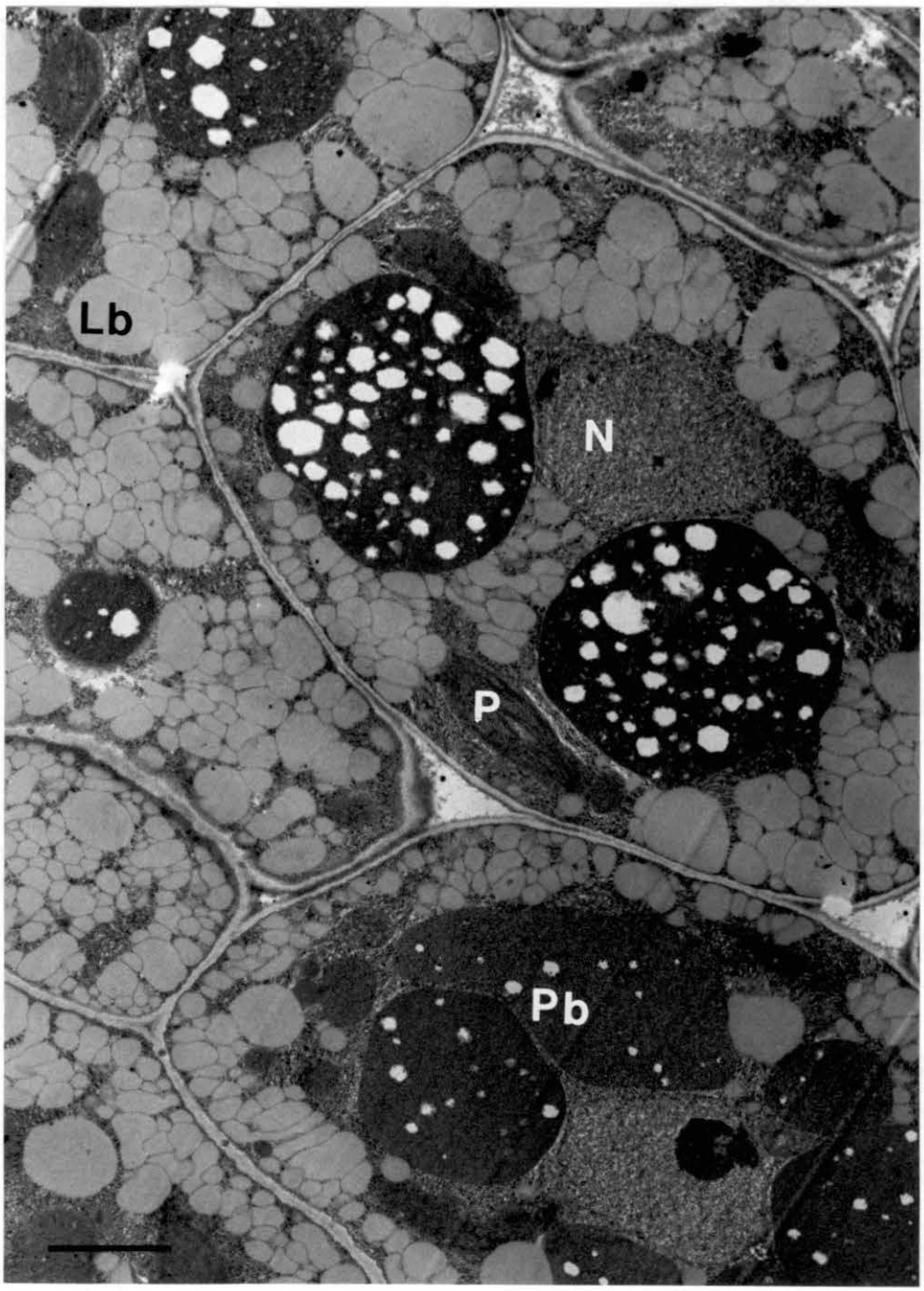

Figure 9

Figure 1Ø. Electron micrograph of mature wild-type cotyledon. (Pb) = mature protein body, (Lb) = lipid body, and (P) = plastid. Scale bar = 2 µm.

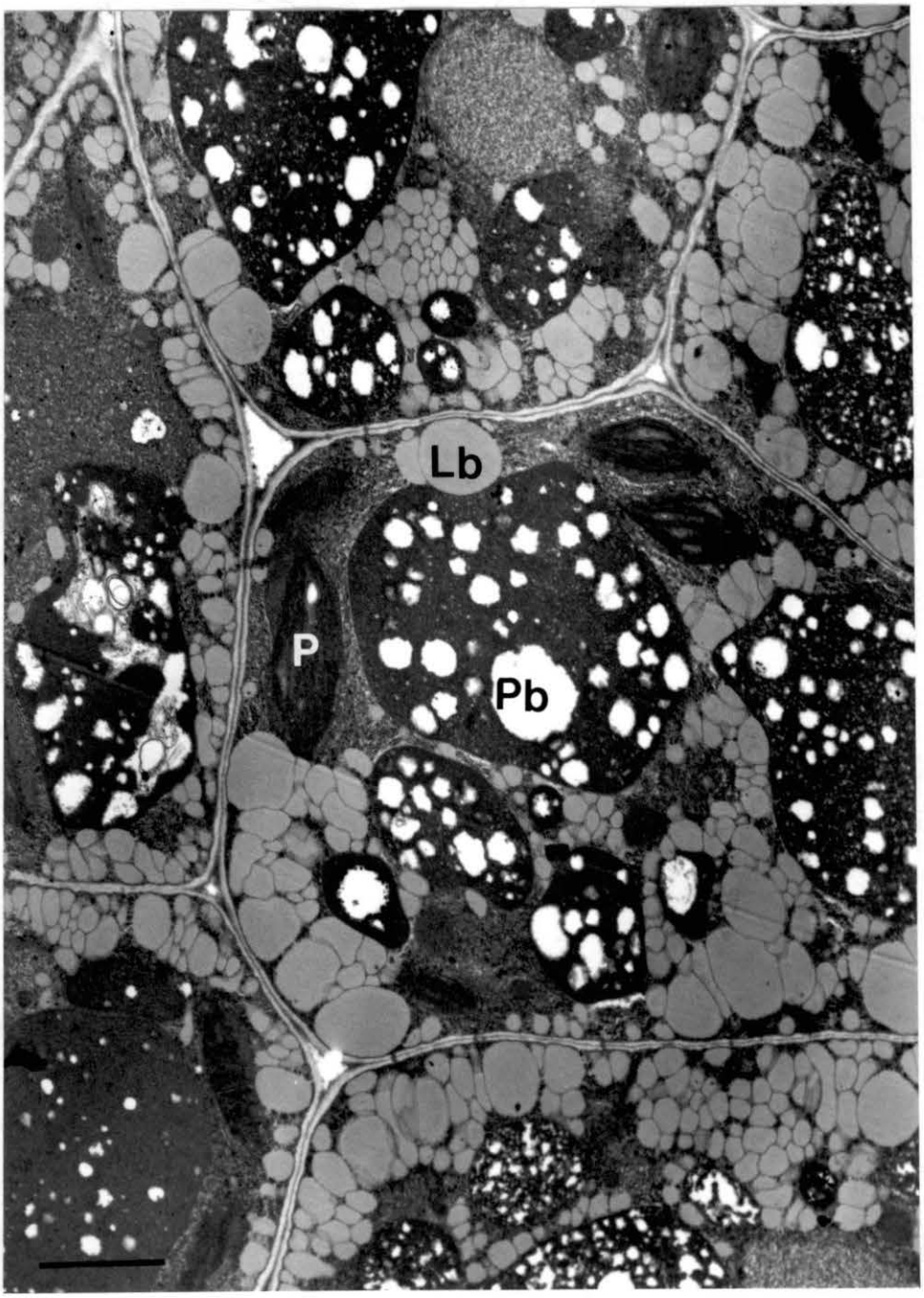

Figure 10

embryo as it prepares for desiccation. Mature protein bodies seemed to have a wide range morphological classes. The ranges went from those with a smooth-appearing homogenous matrix with few inclusions to those with a more granular appearance and contained many inclusions. The full range of protein bodies were seen in cells of the hypocotyl and the cotyledons (fig. 9). Occasionally one cell would contain protein bodies at each end of this morphological range. Lipid bodies in mature embryos were much larger than those found in the linear to early-curled cotyledon embryos and were distributed randomly throughout the cells. It therefore appears that lipid bodies coalesce during cellular maturation, forming fewer but larger lipid bodies in the mature embryo (figs. 8,10).

DISCUSSION

Results from the microscopy of wild-type embryos were consistent with other ultrastructural studies on members of the Brassicaceae. Hofsten (1974) included electron micrographs of cells with mature and immature protein bodies of Brassica species. Her results were very similar to those obtained in this study. She found that protein bodies varied in size from 1-10 µm and were found in both the hypocotyl and cotyledons. The protein bodies of Brassica also contained electron-dense crystalline bodies or globoids. X-ray analysis of these globoids in Crambe revealed that they contained sulfur, magnesium, and calcium

(Hofsten 1973). Rest and Vaughan (1972) described two types of protein-containing bodies in embryos of Sinapis, aleurone grains and myrosin grains. The aleurone grains were predominant in cells throughout the embryo, while the myrosin grains were found in specialized cells in both the hypocotyl and cotyledons. The myrosin grains were found to contain significant quantities of myrosin, a thioglucosidase.

In Arabidopsis, there seemed to be no specificity for the localization of protein bodies; cells in both the internal and external regions of the hypocotyl and cotyledons were rich with these storage organelles. Protein bodies are limited to the tissues of late stage embryos as none were seen in any embryo earlier than a curled cotyledon embryo. Early-curled cotyledon embryos from the same silique were found to contain differing amounts of protein in their protein bodies. It therefore appears that the precise timing of protein deposition in these embryos is somewhat variable.

Embryos at the linear to early-curled cotyledon stages contained many small lipid bodies while in the same embryo very little protein could be found in the protein bodies. This suggests that lipid body formation precedes deposition of seed storage proteins. In all cases where mature protein bodies were found, the cells also contained large quantities of lipid. In embryos that contained large amounts of ER and partially filled protein bodies, there was

already an abundance of lipid. The protein bodies in mature Arabidopsis embryos resembled the aleurone grains described in Sinapis (Rest and Vaughan 1972) and were predominant throughout the embryo. In mature embryonic cells, no specialized (myrosin) cells were seen that contained only smooth protein bodies with no inclusions.

CHAPTER III

ANATOMICAL AND ULTRASTRUCTURAL ANALYSIS OF MUTANT EMBRYOS

INTRODUCTION

Four embryo-lethal mutants of A. thaliana with interesting phenotypes were chosen for ultrastructural analysis. These mutants were each given a number when the original seeds were planted following ethyl methane-sulfonate (EMS) seed mutagenesis. The mutants chosen for this study were 115D-4A, 112A-2A, 13ØB-A, and 114D-1A. All four of these mutants segregated with a Mendelian pattern of inheritance when grown at both low and high temperatures (Meinke 1985).

Mutant 115D-4A was characterized by the presence of aborted seeds containing large green embryos that lacked any defined hypocotyl or cotyledons (see fig. 11). This mutant is known as the "green blimp" because of the abnormal shape of the arrested embryo. The green blimp embryo had the ability to form normal roots in culture, but has formed only abnormal shoots on a variety of shoot-inducing media. The embryos of mutant 112A-2A had

Figures 11-14. Embryos from mutants 115D-4A (11), 112A-2A (12), 130B-A (13), and 114D-1A (14) as seen through the dissecting microscope. Scale bar = 25 μm.

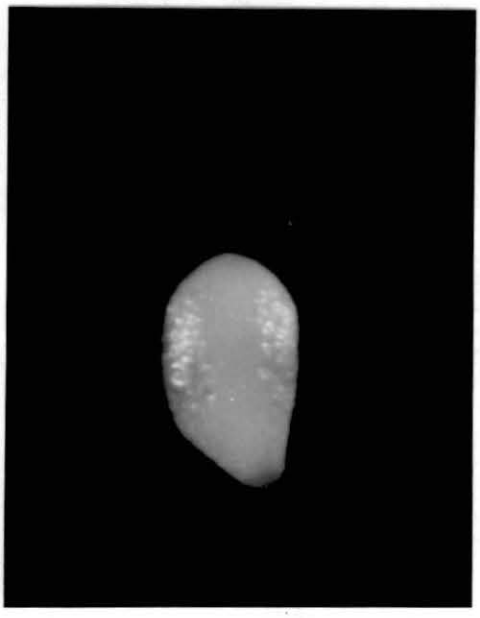

Figure 11

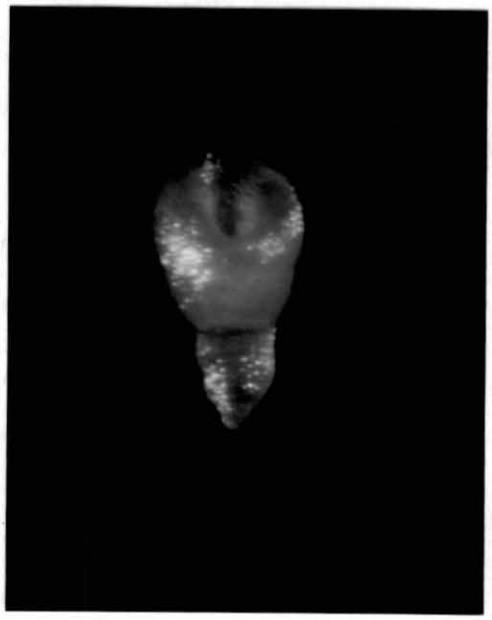

Figure 12

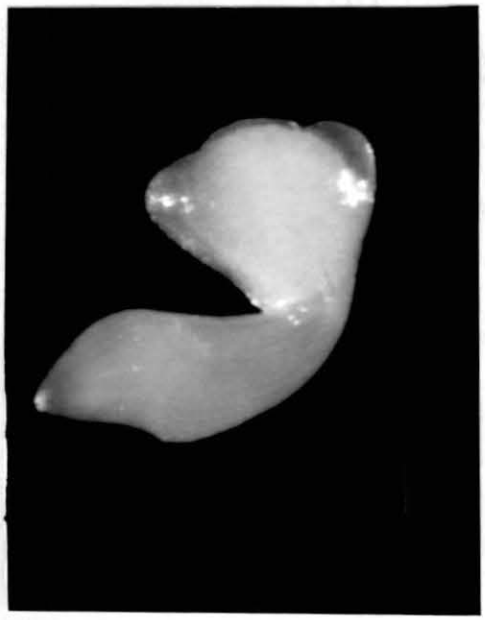

Figure 13

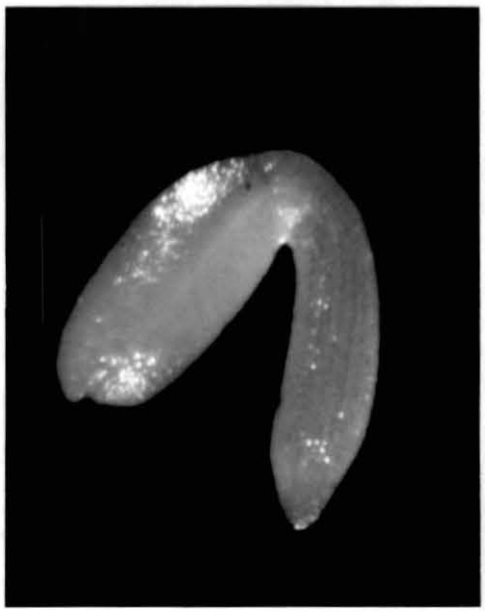

Figure 14

partially or fully fused cotyledons and a reduced hypocotyl (see fig. 12). These embryos formed into plants that failed to produce roots when rescued in tissue culture. Mutant 112A-2A has been named the "rootless, fused-cotyledon" mutant because all tissue derived from 112A-2A embryos lacked the ability to form roots in tissue culture. Mutant 130B-A had a very interesting phenotype; the embryos were composed of a fairly normal hypocotyl and two reduced cotyledons (see fig. 13). In tissue culture this mutant has formed fairly normal but slow-growing plants that set seed. Because of the slow growth habit, 130B-A has also been called the "Pokey" mutant. Homozygous mutant plants produced some phenotypically normal seeds in addition to many aborted seeds; therefore 130B-A is likely to be a leaky mutant. Mutant 114D-1A was characterized as an albino mature mutant, meaning that this mutant embryo actually completed normal embryogenesis but failed to turn green (see fig. 14). Homozygous mutant seeds from this line were able to germinate on soil but formed albino seedlings that soon died. This mutant should actually be characterized as a seedling-lethal rather than an embryonic-lethal.

The mutants described above were chosen for more detailed studies because they exhibited a wide range of abnormal development and clearly differed from normal embryos. The phenotypes of these mutants were interesting, but more detailed ultrastructural analysis has shed light

on some of the events that take place during and after embryo abortion. After studying the phenotypes of these mutants, several conclusions were drawn about normal embryo development. First, the hypocotyl and cotyledons probably develop independently of each other because in mutants 112A-2A and 130B-A one of these structures can form while the other is greatly reduced. Second, the embryo can complete embryogenesis and germinate without the accumulation of chlorophyll as in mutant 114D-1A. Finally, from 115D-4A it appears that embryonic cells can continue to divide and form a fairly large embryo without forming any of the characteristic structures found in normal embryo development. The primary purpose of this chapter is to describe the ultrastructure of these embryos with respect to protein and lipid bodies, then to use this information to determine the stage of developmental arrest at the cellular level.

MATERIALS AND METHODS

Wild-type seeds of Arabidopsis thaliana strain "Columbia" were treated with the mutagen EMS, then planted and screened for embryonic lethals according to Meinke (1979). Over 30 recessive embryo-lethal mutants with a wide range of lethal phases were isolated (Meinke 1985). The four mutants chosen for this study had been maintained as heterozygotes for several generations before this study was initiated. Phenotypically normal seeds from

Figure 15. Light micrograph of 115D-4A mutant embryo in seed. Scale bar = 10 μm.

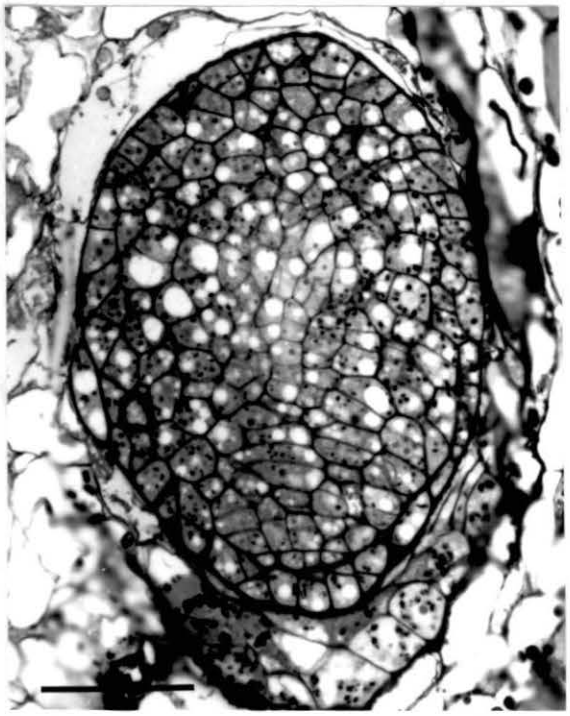

Figure 15

heterozygous plants were saved and planted as described in Chapter 1. Two-thirds of the resulting plants were expected to segregate for the mutant phenotype, while one-third were expected to be wild-type. Mutant embryos were dissected out of aborted seeds from siliques that also contained phenotypically normal seeds at the mature green stage. The mutant tissue was processed for microscopy in the same manner as detailed for wild-type tissue in Chapter 2. Thick sectioning for light microscopy was performed on glass knives. Thin sectioning for electron microscopy was performed by the staff at the Oklahoma State University E.M. facility using diamond knives.

RESULTS

After examination of serial sections through 13 different 115D-4A mutant embryos under the light microscope, it appeared that the mutant embryos ranged from 80 to 200 um in length. Some mutant embryos nearly filled the entire seed and still showed no differentiated hypocotyl or cotyledons. Most of the 115D-4A embryos were fairly small in size at approximately 100 um in length (fig. 15). Cells in the "blimp" embryo ranged in size from 8 to 20 um in length. In all of these embryos, large empty vacoules could be seen in many cells at the light microscope level (see fig. 15). Many small dark-staining structures that resembled starch granules could also be seen in the cells of the smaller embryos but were not as

Figure 16. Electron micrograph of 115D-4A mutant embryo. (V) = vacuole, (P) = plastid, (Lb) = lipid body, (N) = nucleus, and (Nu) = nucleolus. Scale bar = 2 μm.

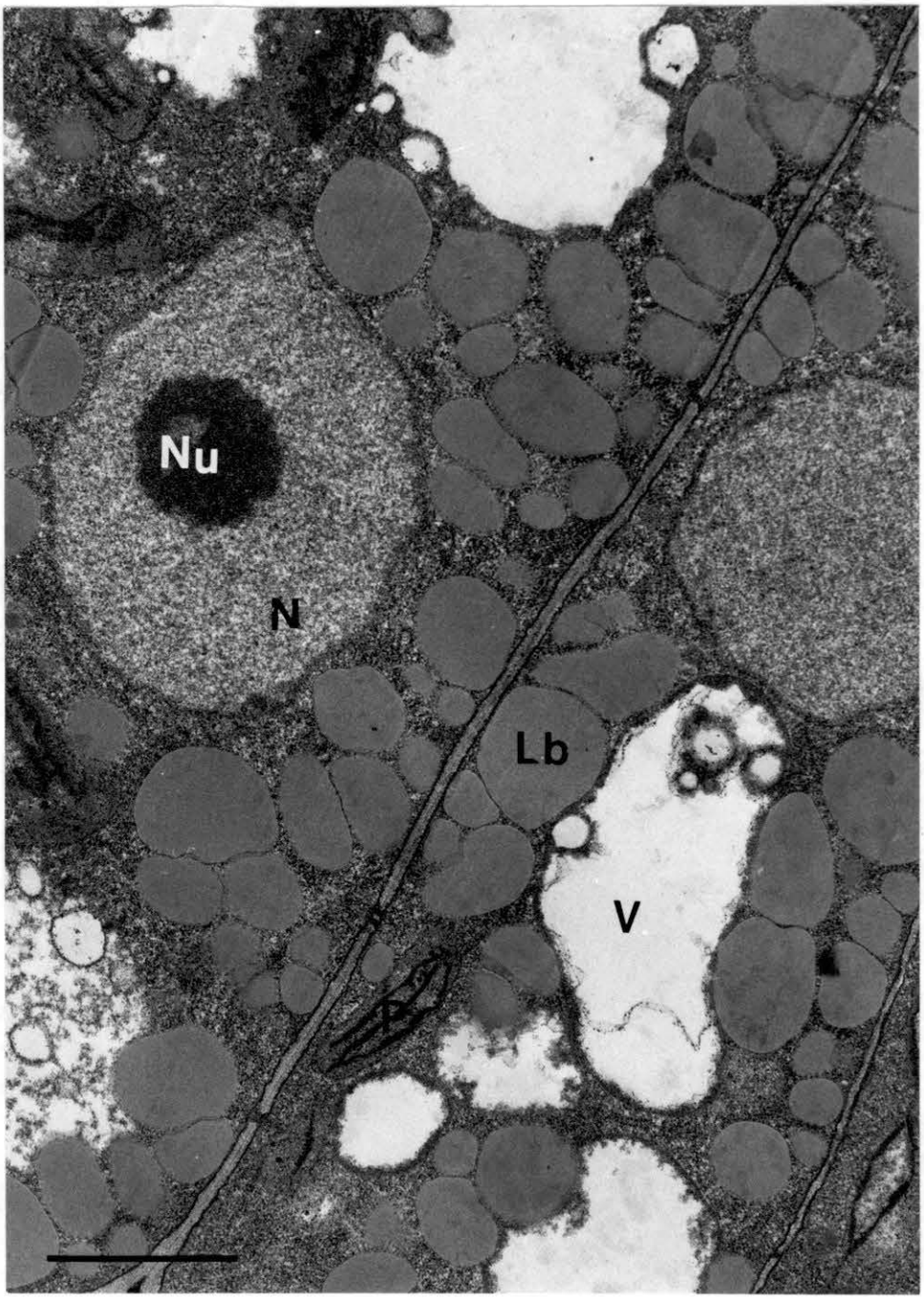

Figure 16

Figure 17. Electron micrograph of 115D-4A mutant embryo. (V) = vacuole, (Lb) = lipid body, (P) = plastid, (N) = nucleus, and (Nu) = nucleolus. Scale bar = 2 μm.

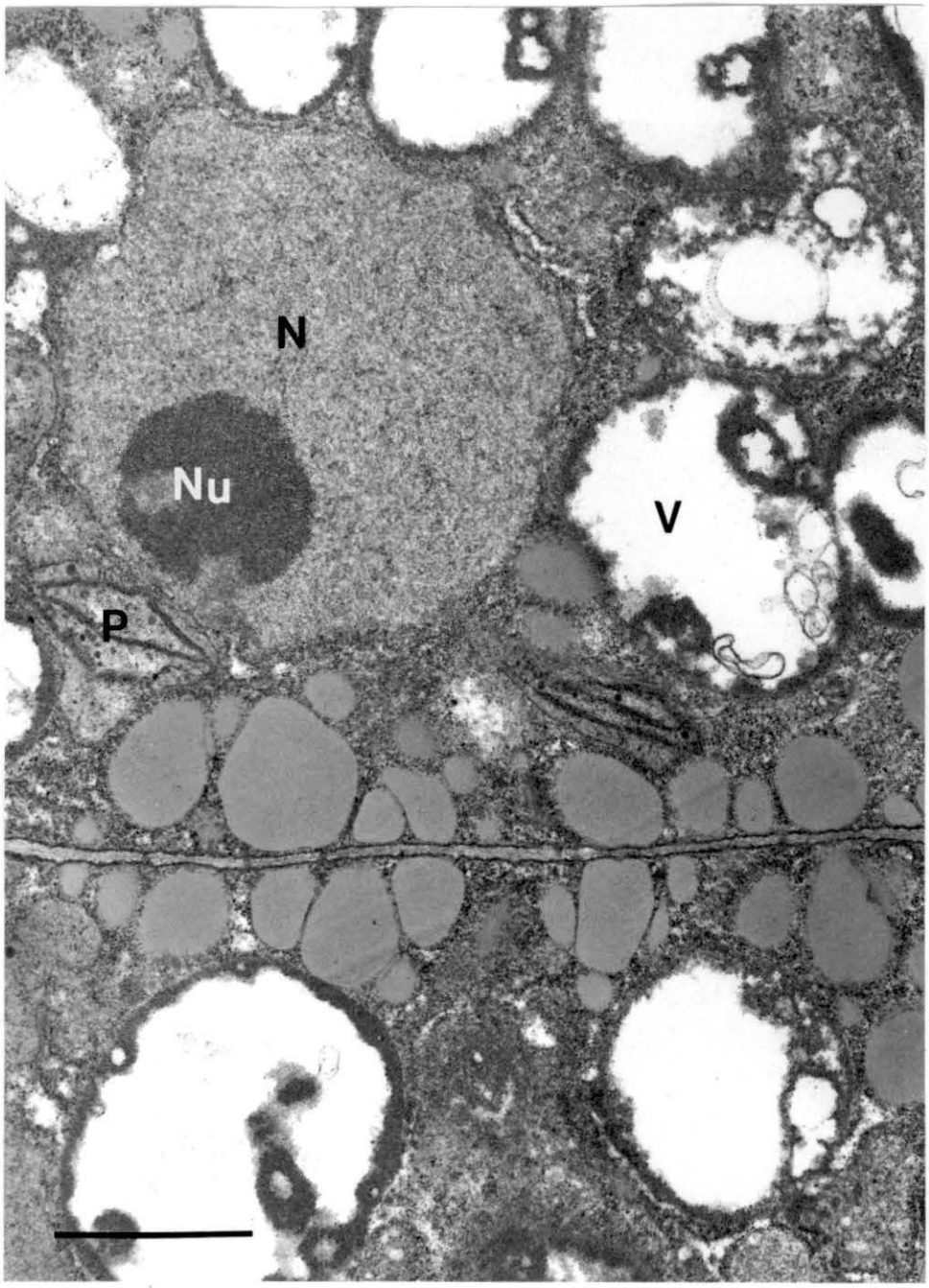

Figure 17

apparent in the larger embryos. Several of the larger embryos that were examined had extensive vascular differentiation in the internal cells, while the smaller embryos seemed to have more disorganized internal cells. All embryos that were examined were a pale-green or green color prior to fixation. The results from electron microscopic examinations of 115D-4A were in agreement with results from light microscopy. The cells of 115D-4A embryos did contain large vacuoles (see fig. 16). Some embryos did contain a small amount of electron-dense material around the edges of the vacuoles but in these embryos, there was no visible ER (see fig. 17). None of the 115D-4A embryos examined contained mature protein bodies. This suggests that there was a reduced level of storage-protein synthesis in the 115D-4A cells when compared to wild-type cells at the cotyledon stages of development. The vacuoles in cells of these embryos did not contain any of the crystalline or globoid structures that were seen in mature protein bodies (see figs. 16,17). The cells of 115D-4A embryos also contained numerous large lipid bodies (see fig. 16). These lipid bodies were much like the ones found in mature wild-type embryos while the large vacuoles resembled those found in early-curled cotyledon embryos.

112A-2A, the rootless, fused-cotyledon mutant was green in color and nearly filled the seed in all cases. Eight different mutant embryos were exmined under the light

Figure 18. Light micrograph of 112A-2A mutant embryo. Hypocotyl damaged guring dissection. Scale bar = 25 μm.

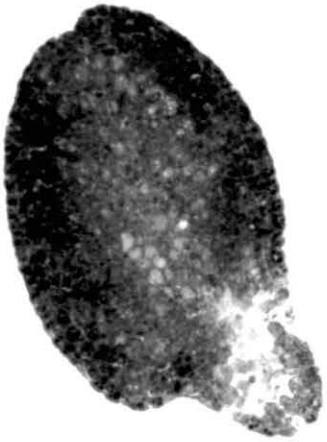

Figure 18

Figure 19. Electron micrograph of 112A-2A hypocotyl. Two morphological types of protein bodies are present (Pb_1 and Pb_2) along with numerous lipid bodies (Lb) and plastids (P) Scale bar = 2 μm.

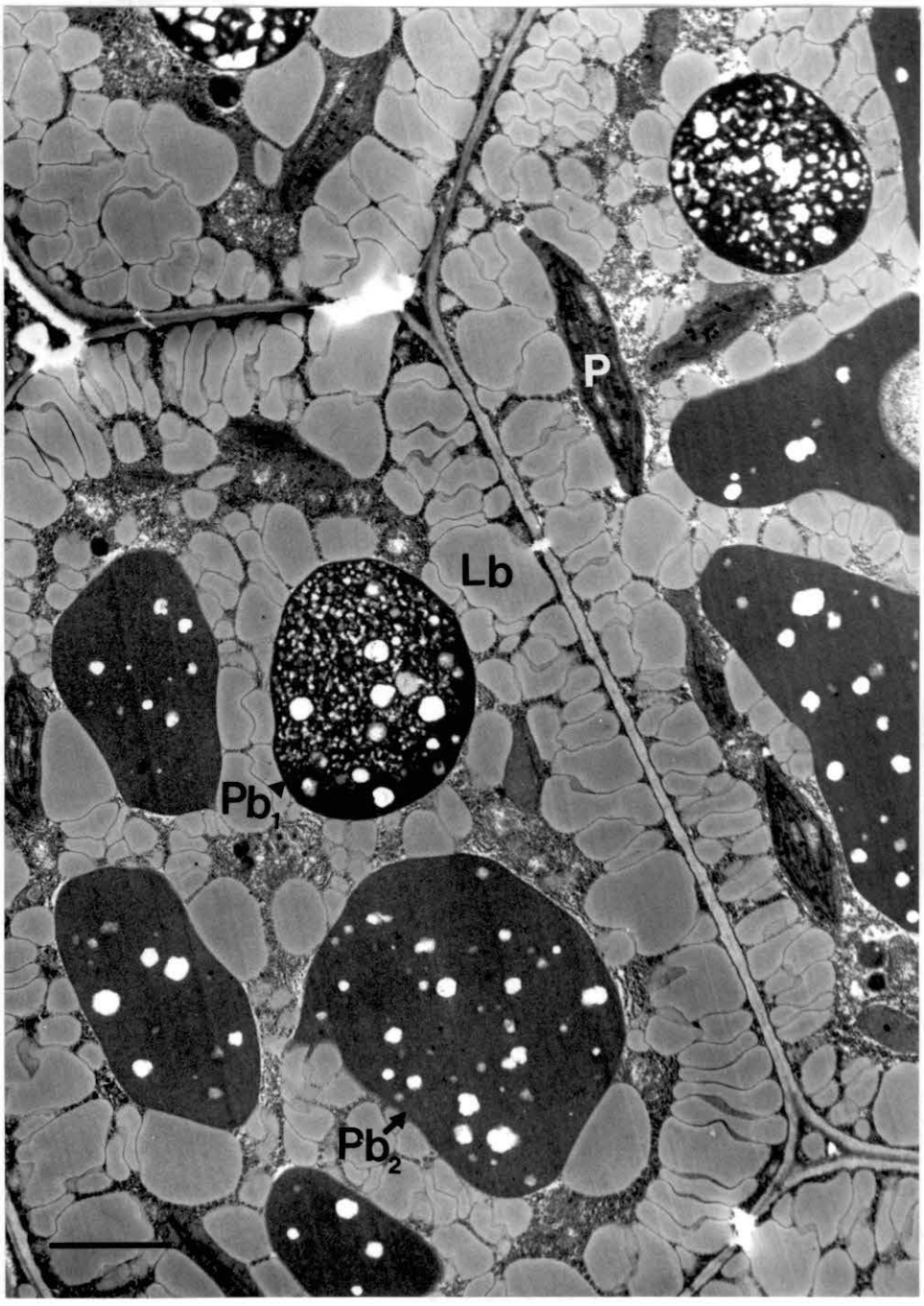

Figure 19

Figure 2Ø. Electron micrograph of 112A-2A cotyledon. Numerous protein bodies (Pb), lipid bodies (Lb), and plastids (P) are visible, along with a distinct nucleus (N) and nucleolus (Nu). Scale bar = 2μm.

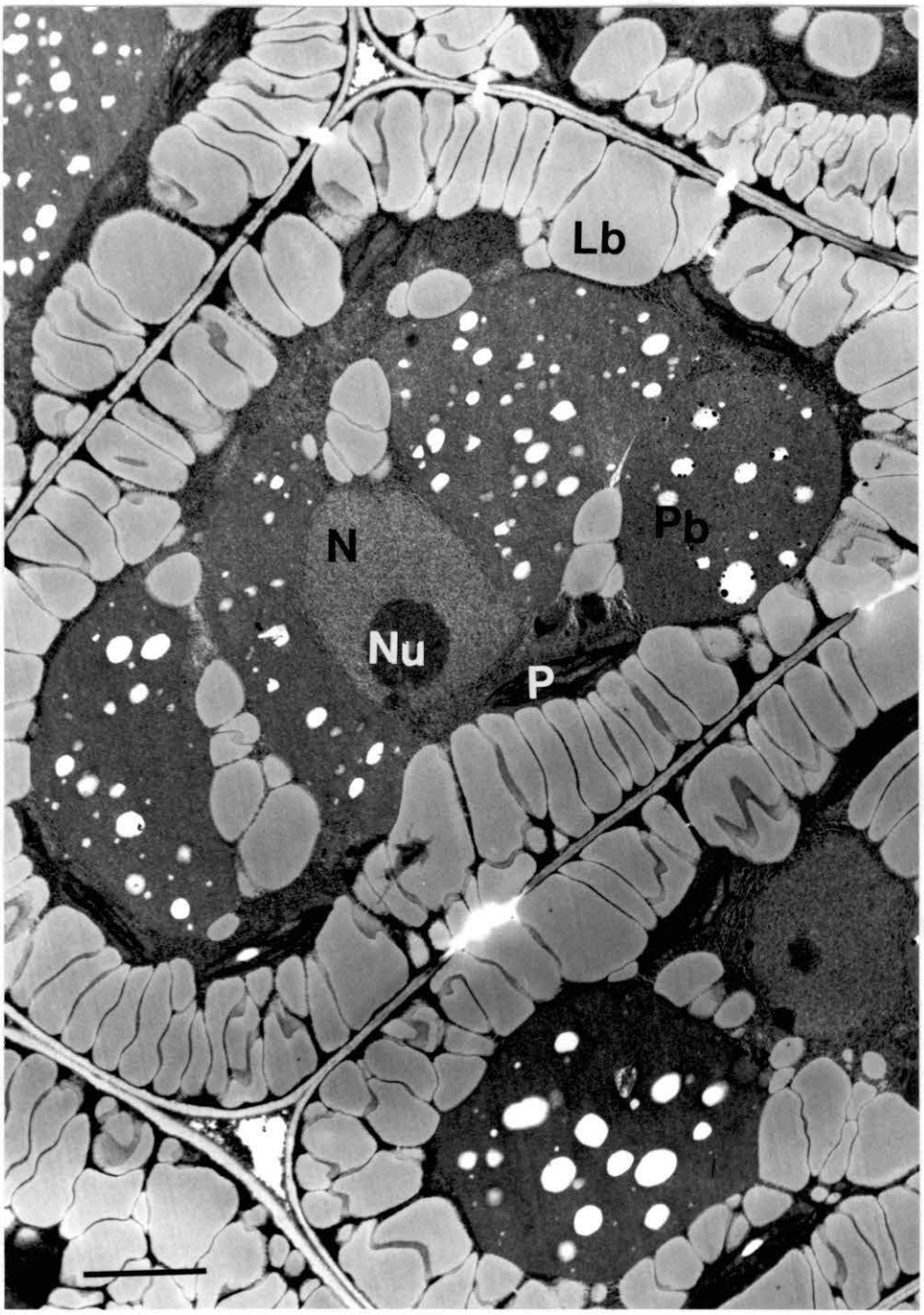

Figure 20

microscope and all were found to contain epidermal, mesophyll, and vascular tissues. Each embryo contained cells with mature protein bodies in both the hypocotyl and cotyledon regions (see fig. 18). These protein bodies ranged in size from 6-1Ø um in diameter. The hypocotyl was greatly reduced in all cases and no root apical meristem could be seen. The cotyledons of the 112A-2A embryo were fused along one or both edges forming a cup-like structure (see fig. 14). The internal region of the 112A-2A embryo did contain clusters of small cells that were not organized into any distinguishable structure.

Examination with the electron microscope revealed that the cells of mutant 112A-2A resembled the cells of a mature wild-type embryo. These mutant embryos had protein bodies and lipid bodies in both the hypocotyl (fig. 19) and cotyledon region (fig. 2Ø). There a wide range in the morphological types of protein bodies found in the cells of mutant 112A-2A embryos. These ranged from protein bodies with a relatively smooth appearance and few inclusions to those with a fairly granular appearance and many small inclusions or globoids (see fig. 19). The cells of the 112A-2A embryo appeared to be packed with large lipid bodies, perhaps even more than the amount found the cells of wild-type embryos. Nine different embryos from 13ØB-A (the "pokey" mutant) were sectioned and examined under the light microscope. The mutant embryo filled the entire seed and was composed of a fairly normal, green hypocotyl and

Figure 21. Light micrograph of 130B-A mutant embryo. This embryo is composed of a large hypocotyl and cotyledons. Scale bar = 25 µm.

Figure 21

Figure 22. Electron micrograph of 130B-A hypocotyl. Numerous protein bodies (Pb) and lipid bodies (Lb) are present. Same embryo as in Figure 23. Scale bar = 2 μm.

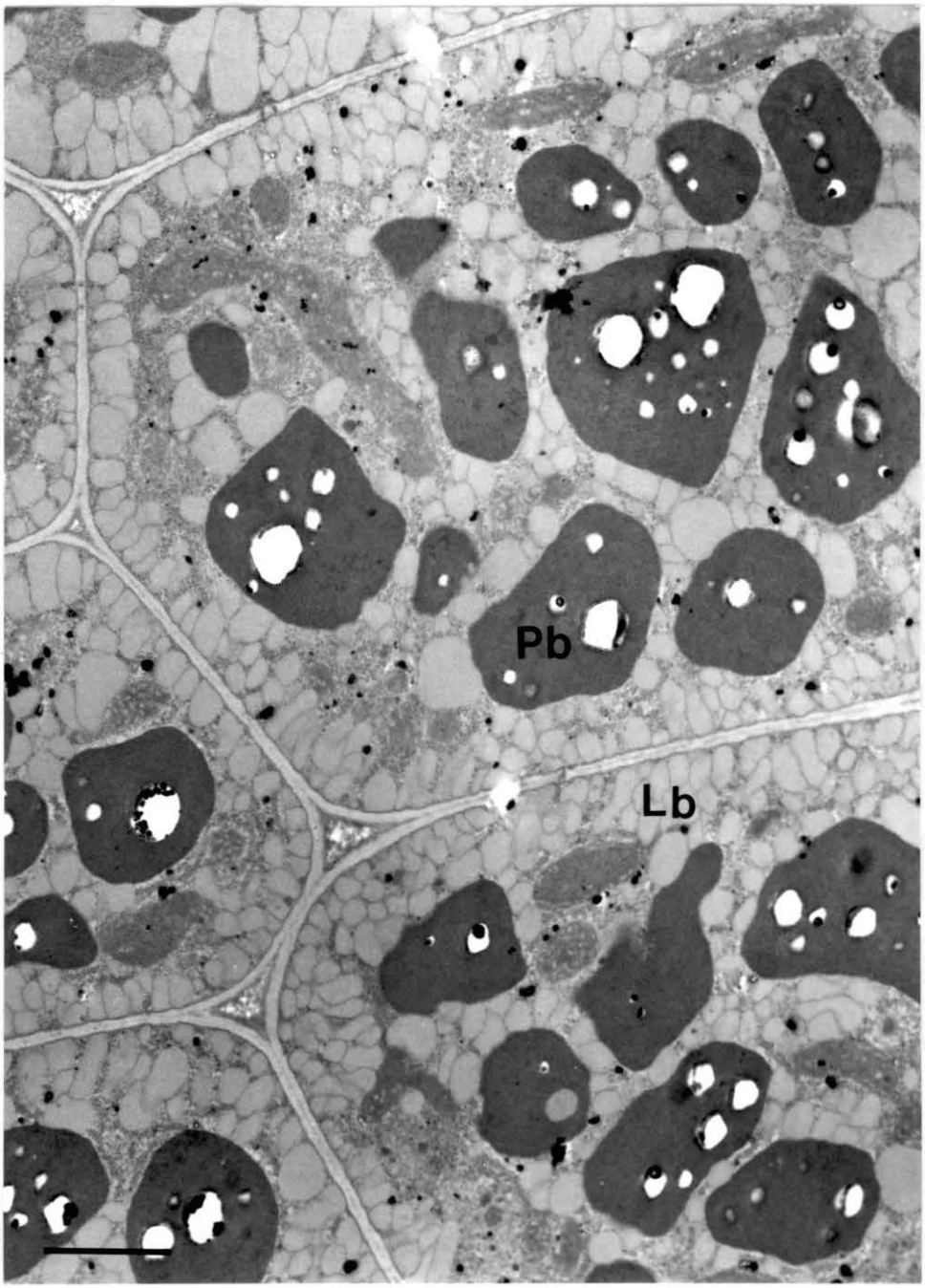

Figure 22

Figure 23. Electron micrograph of 13ØB-A cotyledon. Immature protein bodies (Pb), endoplasmic reticulum (ER), and lipid bodies (Lb) are visible. Same embryo as in Figure 22. Scale bar = 2 µm.

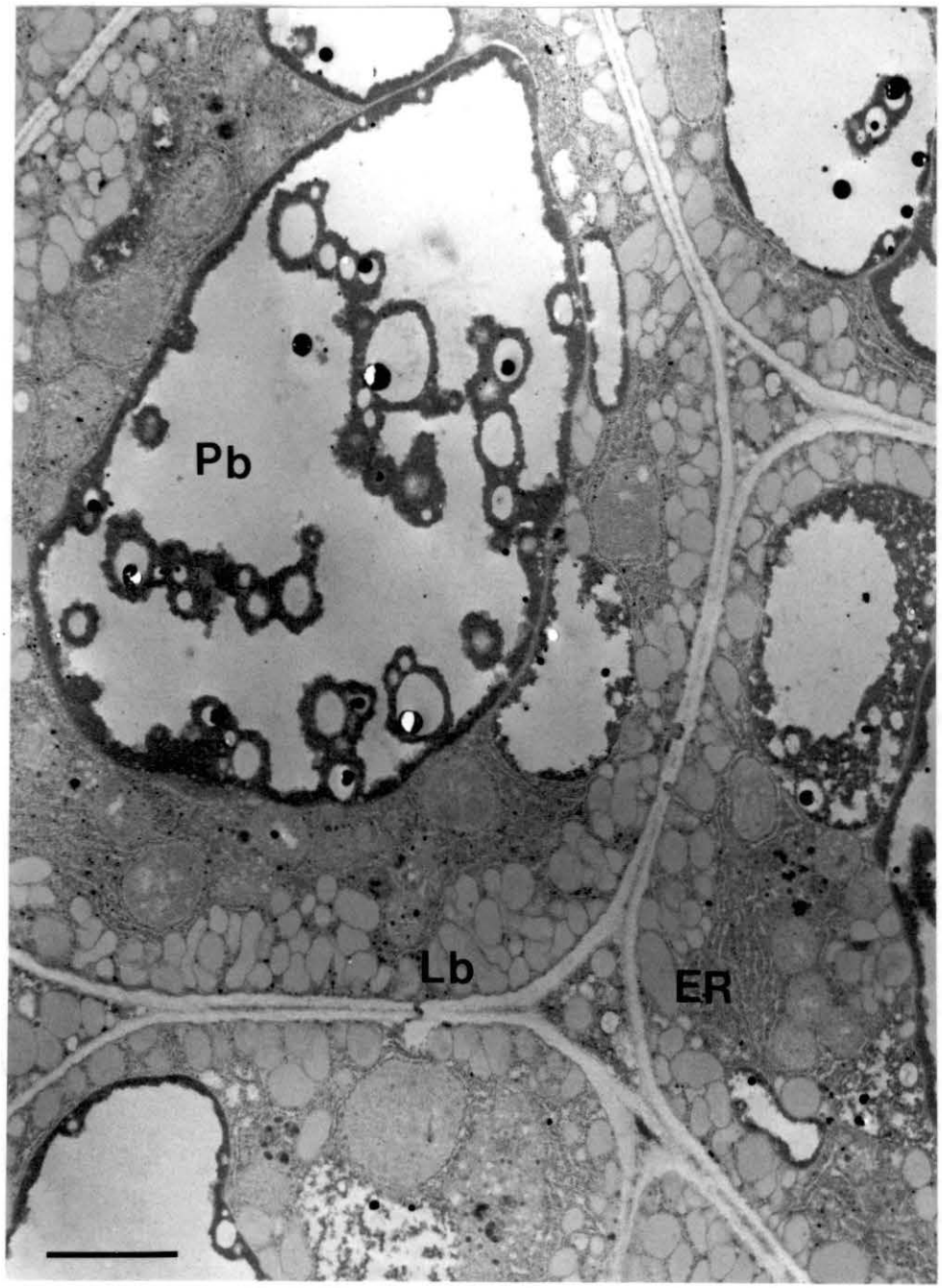

Figure 23

slightly pale cotyledons. These features made it difficult to distinguish the aborted seeds from the phenotypically normal seeds. For this mutant it was necessary to dissect the embryo from each seed in order to exclude stray normal embryos. The 13ØB-A embryo had fully differentiated internal tissues such as a root apical meristem and vascular tissue. These mutant embryos had a slightly enlarged hypocotyl but the cotyledons varied in size from severely reduced (fig. 13) to fairly large distinct structures (fig. 21). Under the light microscope the hypocotyl stained quite well for protein bodies. The cotyledons stained pale when compared to the hypocotyl and appeared to contain large empty vacuoles rather than mature protein bodies (fig. 21). These observations were confirmed with the electron microscope. Cells of the mutant hypocotyl contained mature protein bodies and many lipid bodies (see fig 22). At the ultrastructural level the 13ØB-A hypocotyl cells looked much like the cells of a wild-type mature embryo. The protein bodies in the 13ØB-A hypocotyl appeared have a very narrow range of morphological types with all protein bodies having a smooth appearance and relatively few inclusions. Figure 23 shows cells from the cotyledon region of the same mutant embryo that was just described in the hypocotyl region. There was a clear difference in the amount of storage material found in the protein bodies of the hypocotyl and cotyledons. The cotyledon cells had immature protein bodies that contained

Figure 24. Light micrograph of 114D-1A mutant embryo. This embryo has a normal hypocotyl and cotyledons. The root apical meristem was damaged during dissection. Scale bar = 25 µm.

Figure 24

Figure 25. Electron micrograph of 114D-1A hypocotyl. Several large immature protein bodies (Pb) and lipid bodies (Lb) can be seen along with a nucleus (N) and nucleolus (Nu). Scale bar = 2 µm.

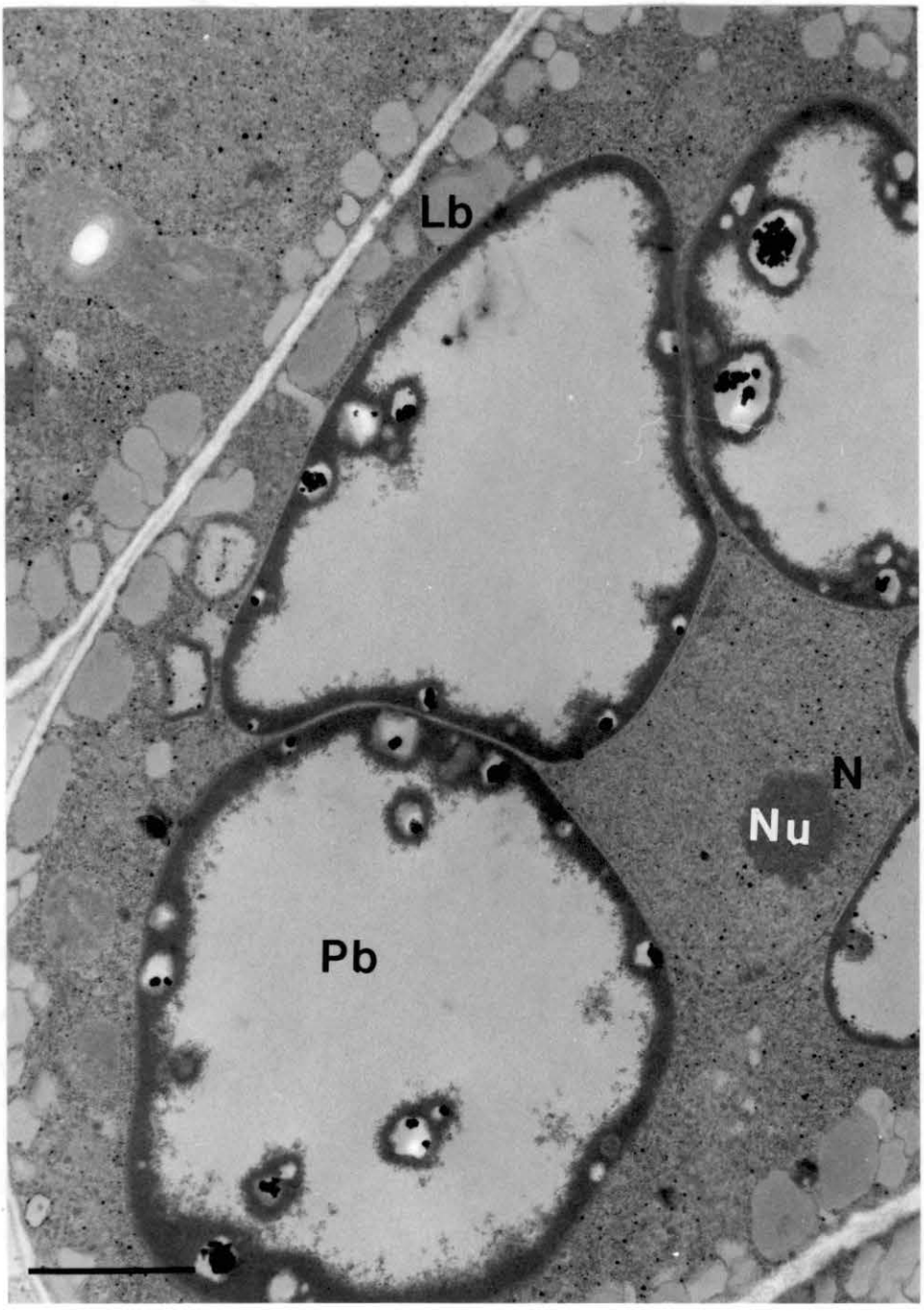

Figure 25

Figure 26. Electron micrograph of 114D-1A cotyledon. Large protein bodies (Pb) and lipid bodies (Lb) can be seen along wth a nucleus (N). Scale bar = 2 µm.

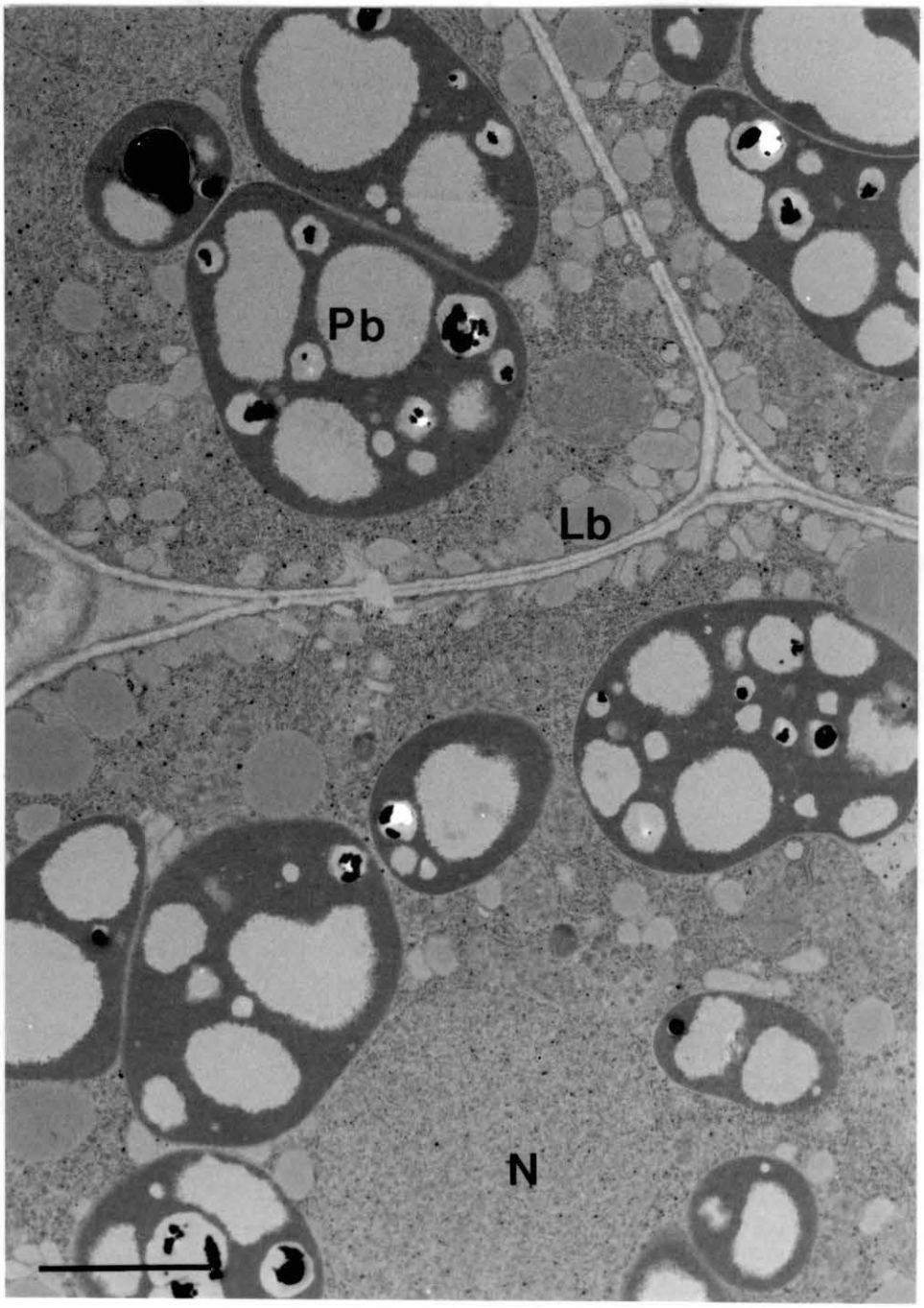

Figure 26

very little seed storage proteins. The storage proteins present in the 13ØB-A cotyledons appeareed to be distributed mainly around the edges of the developing protein body (fig. 23) much like cells of wild-type embryos during the late stages of development (fig. 8). The cotyledon cells also contained large amounts of ER (see fig. 23), indicating that high levels of protein synthesis may be occuring in these cells. It appears that the cotyledon cells are delayed in their development and resemble wild-type cells at the early-curled cotyledon stage of development.

Serial sections were taken through six different 114D-1A mutant embryos and examined with the light microscope. Except for their white color, these mutant embryos were morphologically identical to mature wild-type embryos (fig. 24). These albino embryos had the expected types of differentiated tissue and morphology (fig. 24). In some 114D-1A embryos, mature protein bodies could be seen in the cells of the hypocotyl, while the cotyledon cells had more immature protein bodies. In other 114D-1A embryos, just the reverse was seen with the cotyledons having mature protein bodies and the hypocotyl having more immature protein bodies. This became more apparent after examintion under the electron microscope, where it became clear that hypocotyl and cotyledon cells of the same mutant embryo could contain variable amounts of storage protein (see figs. 25,26). It appeared that the timing of protein

deposition in the cells of the hypocotyl and the cotyledons was variable. The cells of both the hypocotyl and cotyledons also appeared to contain a reduced level of storage lipid bodies when compared to wild-type embryos at the same stage of development. The lipid bodies of 114D-1A embryos were relatively small and mainly distributed around the edges of the cells. The more mature protein bodies in this mutant appeared to have a narrow morphological range, with most having a smooth appearance and several large inclusions (fig. 26). Even the cells with more immature protein bodies had several small inclusions (fig. 25). Very little ER was visible in the cells that contained relatively small amounts of storage proteins. From this, it appears that protein synthesis or accumulation in 114D-1A embryos may actually be altered in the presence of the mutant gene.

DISCUSSION

Microscopic analysis has revealed some interesting facts about each of these mutants. Mutant 115D-4A or "the green blimp" was the original mutant of interest because it had characterstics of both immature and mature embryos. The morphological "globular" shape resembles that of an embryo at a very early stage of development, but the size and color are that of a much later stage embryo. The initial question concerning this mutant was whether cells

of the aborted embryo more clearly resemble the cells of an immature or a mature embryo. At the macroscopic level this mutant has what appears to be a relatively normal level of chlorophyll, which would indicate that this mutant has the ability to mature with respect to chlorophyll synthesis and accumulation. But at the ultrastructural level, the cells of this mutant appear to be devlopmentally immature because of the accumulation of a reduced number of lipid bodies and the absence of mature protein bodies. It is surprising that an embryo could have this high level of chlorophyll accumulation and still have relatively immature protein bodies. This makes it difficult to say exactly what developmental stage the cells of this mutant represent, but it is clear that mutant 115D-4A is not simply a mature embryo that lacks defined hypocotyl and cotyledons.

In the case of mutant 112A-2A, cells of the arrested embryo appear to have matured at the ultrastructural level with the accmulation of numerous lipid bodies and mature protein bodies even though morphogenesis has clearly been altered. The absence of a root apex and the inablity to form roots in culture is a relatively rare phenotype. One other example of rootless plants has been described in tobacco plants regenerated from mutagenized protoplasts that were resistant to high concentrations of NAA (Muller et al. 1985). The fused cotyledon phenotype is also quite interesting but is not as rare; this phenotype has been reported in somatic embryos by several groups (Crouch 1982;

Jelaska 1977; Lippmann and Lippmann 1984). Even though the hypocotyl is greatly reduced in size the cells that make up this structure still have the ability to form normal storage protein bodies and lipid bodies. Future studies will be performed on this mutant in order to determine whether the mutant phenotype is caused by abnormal levels of plant hormones or perhaps faulty hormone receptors. Two general conclusions were drawn after ultrastructural analysis of mutant 112A-2A; first, it is possible to have an embryonic lethal that is not blocked in storage protein accumulation, and second, cells that make up a reduced structure at the macroscopic level (hypocotyl of 112A-2A) can still accumulate normal levels of storage proteins in their protein bodies.

Perhaps the most interesting results from this study were obtained from mutant 13ØB-A, the "pokey" mutant. This mutant has an altered pattern of morphology and cellular maturation in the cotyledons but not in the hypocotyl. At the macroscopic level the cotyledons are often reduced in size. At the ultrastructural level the cotyledon cells resemble those of a wild-type embryo during the early cotyledon stage of development because of the abundance of ER around the immature protein bodies. At the same time the hypocotyl develops into a relatively normal mature structure at both the macroscopic and ultrastructural level. From this, it appears that the timing of development can be delayed in one part of the embryo while it is not

affected in other parts. Because this mutant embryo can be rescued in tissue culture and the plant grows slowly, it appears that the mutant gene not only affects morphogenesis but also affects growth at the gross morphological level. One possible way to explain the presence of immature protein bodies in the cotyledons might be to conclude that the cells of a reduced structure cannot further differentiate, but the cells of the reduced hypocotyl in mutant 112A-2A do contain mature protein bodies. Therefore mutant 13ØB-A represents another mutant that forms an abnormally reduced structure, but in this mutant the reduced phenotype also coincides with a delayed pattern of protein body formation.

Mutant 114D-1A also has an altered timing of cellular maturation within different tissues of the same embryo. In this mutant though, there is variabilty as to which portion of the embryo devlopes slower than the other. This mutant also has an albino phenotype which is accompanied by the absence of mature plastids in the cells of both the hypocotyl and cotyledons (see figs. 25,26). Because Arabidopsis embryos are normally green at the mature stage and 114D-1A embryos reach a mature cotyledon shape but stay white, it appears again that morphological maturation and chlorophyll synthesis occur independently of each other. It also appears that events at the cellular level are very sensative to changes at the biochemical level. The results from mutant 114D-1A seem to be the reverse of those from

mutant 112A-2A; in mutant 112A-2A there was abnormal morphology but normal cellular differentiation, and in mutant 114D-1A there was normal morphology but altered cellular differentiation.

In this study, several mutants with different phenotypes were examined for their patterns of morphological development and formation of protein and lipid bodies. Abnormal morphology was seen in mutants 115D-4A, 112A-2A, and 130B-A. Abnormal patterns of protein body formation were seen in mutants 130B-A and 114D-1A. In the cotyledons of mutant 130B-A there were only immature protein bodies, while in the hypocotyl there were mature protein bodies. These mature protein bodies in mutant 130B-A were found to have a narrow range of morphological types. Mutant 114D-1A also appeared to have protein bodies with a narrow morphological range. Both mutants had protein bodies with a relatively smooth matrix and few inclusions. In these 114D-1A embryos both mature and immature protein bodies were found. The cells of wild-type and 112A-2A embryos had protein bodies with a wide range of morphological types, while 115D-4A embryos did not contain any mature protein bodies. It appears that a variety of differences can be found in the protein bodies of these mutant embryos. Mutations that affect embryo development at the morphological level may also alter the timing and formation of storage protein and lipid bodies.

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