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Cytodifferentiation in developing leaves of a leafy liverwort, *Plagiochila arctica* Bryhn & Kaal. (Hepaticae)

Basile, Margaret Ruth, Ph.D.

City University of New York, 1991

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A

Cytodifferentiation in developing leaves of a leafy liverwort, Plagiochila arctica Bryhn & Kaal. (Hepaticae)

by

Margaret R. Basile

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1991

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Margaret R. Basile
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This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Abstract

Cytodifferentiation in developing leaves of a leafy liverwort,
Plagiochila arctica Bryhn & Kaal. (Hepaticae)

by

Margaret R. Basile

Advisor: Professor Thomas E. Jensen

A light and electron microscopic investigation of cytodifferentiation in lateral and ventral leaf primordia of Plagiochila arctica Bryhn & Kaal. (Hepaticae) revealed qualitative and quantitative changes in the structure and organization of the differentiating cells as development progressed from primorida to maturity. The most pronounced changes in those structures already present at the start were observed in the plastids, cell walls, vacuoles and mitochondria. These organelles manifested the greatest changes, both qualitative and quantitative during the course of development. In addition, a peculiar feature of cytodifferentiation in leafy hepatics such as Plagiochila arctica was observed, the *de novo* development of membrane-bound structures called "oil bodies". The changes observed in the individual organelles including the walls were similar to changes that occur during cytodifferentiation in leaf cells in flowering plants. What was surprising was the fact that each/all of the liverwort leaf cells at maturity were more highly differentiated than individual cells of flowering plants, manifesting features that help characterize three different tissue/cell types in flowering plants: epidermal, mesophyll, and collenchyma.

Acknowledgements

I wish to thank my advisor, Dr. Thomas E. Jensen, for his support, encouragement and advice throughout the course of this research. I was first introduced to cell biology and electron microscopy through his course. He then suggested I do a tutorial with him based on a paper I wrote for the course. With time, he became my advisor in the graduate program.

Thanks also go to Dr. Jack Valdovinos, Dr. Dwight Kincaid, Dr. Lawrence Crockett, Dr. Myron Ledbetter, and Dr. Bill Buck for serving on my committee and for their interest, suggestions and guidance.

Special thanks go to my family, my husband, Dominick, and my children, Margaret, Christopher and Donald. Without their support and encouragement throughout the years, it would not have been possible to finish this thesis.

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Introduction

All plants go through a developmental sequence as the cells differentiate into a variety of cell types. The details of how this process occurs, although widely investigated, has not been completely elucidated. By using a plant with a simple developmental and differential pattern it may be possible to discern certain aspects of this process which may be less evident in complex plants. The leaves of leafy liverworts, which have only one cell type, seem ideal for this study. Specifically, the leafy liverwort, Plagiochila arctica Bryhn & Kaal., was used to investigate this phenomenon. By way of background to the work done in the past, the following introduction and literature review is presented.

The liverworts or Hepaticae constitute an ancient group of land plants with a fossil record dating from the Devonian. There is some recent evidence to indicate that fossil hepatics bearing strong resemblance to rhyniophytes were present in the Early Devonian (Remy and Remy, 1980; Remy, 1982). Fossils of plants which are unequivocally liverworts and bearing striking resemblances to extant taxa have been collected from Upper Devonian strata (Hueber, 1961). These fossils have been assigned to the Metzgeriales, a modern order that contains both leafy and thallose forms. Presumably, therefore, the capacity for leaf development evolved within the Hepaticae sometime during the Upper Devonian. The first fossils clearly assignable to the order of liverworts consisting entirely of leafy forms, the Jungermanniales, have not yet been

reported earlier than the Jurasic (Halle, 1913-Wiss. Ergebn. Sched . cited by Schuster,1966). The Jungermanniales is presently the largest order in the Hepaticae, containing approximately 85% of the known species. Of the extant classes of land plants, the Hepaticae appear to be amongst the earliest to emerge as a distinct group. In spite of the fact that they have left a record of their existence and evolutionary divergence over a period of approximately 350,000,000 years, their evolutionary position with respect to both the fossil and extant phyla of photoautotrophs continues to be debated (Crandall-Stotler, 1980; Miller, 1974; Mishler and Churchill, 1984; Robinson, 1985; Schuster, 1979; Stoneburner, 1990; among others). It seems highly unlikely that any consensus will be reached until a great deal more salient information about the structure and biology of hepatics is obtained.

Cytological data gathered over the past 20 years are becoming increasingly useful in interpreting the evolutionary relationships of hepatics to other bryophytes as well as to the algae on one hand and other phyla of land plants on the other. These data resulted from comparative studies primarily concerned with ultrastructural changes or differences that are related to cytokinesis, spermatogenesis, and sporogenesis.

The literature pertaining to cytokinesis in bryophytes has been reviewed recently (Brown and Lemmon,1990a). This literature reveals that cytokinesis in all the liverworts investigated conforms to the Phragmoplast-Type, the type that is characteristic of all

photosynthetic land plants (Embryophyta) (Pickett-Heaps, 1972) and a small, heterogeneous assemblage of algae (Mattox and Stewart, 1984). Furthermore, the site of phragmoplast formation may be predicted in bryophytes, as in all other land plants, by the formation of preprophase bands (e.g., Brown and Lemmon, 1988b; Diers, 1965; Fowke and Pickett-Heaps, 1978; Horner et al., 1966; Lehmann and Schulz, 1969; Schnepf, 1973). Preprophase bands described by Pickett-Heaps and Northcote (1966b), have not been discovered in any alga, including those that form phragmoplasts (Brown and Lemmon, 1990a).

Comparative studies on spermatogenesis in bryophytes conducted by Carothers and coworkers over a period of two decades was recently reviewed by Carothers and Rushing (1988). These investigations revealed five ultrastructural features useful for comparative purposes:

1. The shape of the anterior portion of the spline (= a band of microtubules that in the mature spermatozoids extends the length of the gamete).
2. The number of spline microtubules.
3. The spline aperture (i.e., whether open, closed, or inaperturate).
4. The morphology of the lamellar strip (= structure composed of many, closely parallel, diagonally oriented lamellae and located appressed beneath the spline at the anterior end).
5. The structure and relative position of the basal bodies (= the

ànterior portion of the flagella derived from centrioles).

A comparison of these ultrastructural features in twenty-two species of bryophytes indicated that with respect to their differentiation as well as their mature form and structure, the sperm of liverworts is quite variable. Nevertheless, sperm and spermatogenesis in liverworts are, thus far, distinctly different from any alga. On the other hand, they overlap to differing degrees in these features with other bryophytes. Carothers and Rushing (1988) point out that the available data are still too limited to arrive at strong conclusions. Nevertheless, the presently available data support those classifications that treat the Hepaticae, Musci, and Athocerotae as three classes within the Bryophyta (Chopra and Kumra, 1988; Crum and Anderson, 1981; Puri, 1973; Schofield, 1985; Watson, 1971) in contrast to those that place the three groups in separate phyla (Crandall-Stotler, 1986; Mishler and Churchill, 1984).

Comparative studies on sporogenesis conducted over the past ten years and reviewed by Brown and Lemmon (1988a) reveal several features important to the study of bryophyte phylogeny. For example, they found that there are four phenomena that occur in various combinations in the different taxa of bryophytes:

1. Precocious marking during meiotic prophase of the eventual cleavage planes of the spore tetrad, either by lobing of the cytoplasm or discrete infurrowing of the sporocyte wall.

2. Two divisions of the single plastid (in mosses and

hornworts) and migration during meiotic prophase of the resultant four plastids into the cytoplasmic domains that will eventually be cleaved into spores.

3. Development of structural wall precursors during meiotic prophase I that predict the pattern of the spore exine.

4. Development of microtubular systems associated with the initiation of the spore wall and the development of an aperture, both relating to polarity established during meiotic prophase I.

Although no single bryophyte manifests all four of the above listed phenomena, it is evident that a precocious establishment of division polarity and the precocious establishment of spore wall patterning in meiotic prophase I are characters common to Anthocerotae, Musci, and Hepaticae: the Bryophyta.

Another feature found to be common to all the bryophytes studied was the development of two principal wall layers in the spore, an inner intine (endospore) and outer exine (exospore). These two layers are interpreted by a number of investigators cited by Brown and Lemmon (1988a) to be basically similar to the intine and exine of the spores of seed plants. None of the bryophyte spores, however, show the typical complex tectate pattern of sculpturing that characterizes the outer layers of exine in the walls of angiosperm pollen (Brown and Lemmon 1988a). In addition to the features of sporogenesis that are common to all bryophytes studied, there were found several features that correlate well with the several classes and subclasses. For examples, three distinct types

of wall correlate with the three subclasses of mosses: the Andreaeidae, Sphagnidae, and Bryidae. Similarly, in the liverworts, spore wall ontogeny is distinct in the two subclasses, the Marchantiidae and the Jungermanniidae.

In marked contrast to the amount of modern research being conducted on the differentiation of reproductive cells and tissues is the near absence of contemporary studies on the differentiation of the cell and tissue types that compose the vegetative parts of liverworts. Studies directed toward the understanding of specific cell and /or tissue types in the Hepaticae are relatively few and widely scattered in time of publication. The great majority of these studies have been conducted at the light microscope level. The most extensive information obtained by means of light microscopical studies pertains to changes or differences in the shape and segmentation patterns of apical cells and their immediate derivatives (merophytes). Present understanding of these segmentation patterns in relation to stem, leaf, and branch development began with the exemplary studies of Leitgeb (1875). Light microscopical work extending Leitgeb's original observations with respect to branching were conducted by Basile & Basile (1980, 1984), Crandall-Stotler (1972), Douin (1930), Evans (1912), Thiers (1982). These studies have resulted in the identification and characterization of thirteen distinct branching patterns based largely on whether the origin of branch initials is exogenous or endogenous , terminal or lateral, from lateral or ventral

merophytes, as well as where they are initiated with respect to developing leaves. The thirteen types are as follows:

1. Dichotomous-type
2. Frullania-type
3. Microlepidozia-type
4. Acromastigium-type
5. Radula-type
6. Fontinalis-type
7. Bryopteris-type
8. Lejeunea-type
9. Adventive Radula-type
10. Bazzania-type
11. Plagiochila-type
12. Anomoclada-type
13. Aplanolejeunea-type

This is possibly the largest number of branching patterns in any class of plants and includes the most primitive as well as the most derived modes of branch initiation. Branching types 1-6 are exogenous, being initiated by cells in a superficial position. Dichotomous branching, type 1, is the most primitive mode of branching in land plants and apparently the only type exhibited by fossil plants for the first several million years of their fossil record (Chaloner, 1970). Dichotomous branching is fairly common in the Metzgeriales but only rarely observed in the Jungermanniales (leafy liverworts) (Basile & Basile, 1980, 1984; Douin, 1930).

Types 2-4 are monopodial in origin and develop from transformed leaf initials. Types 5-6, also monopdial, originate laterally from superficial cells of the stem. Exogenous, monopodial branching is considered by comparative morphologists to be the most derived mode of branching and is characteristic of shoot/stem branching of most seed plants. The remaining seven branch types are initiated endogenously. Endogenous initiation of branches is more characteristic of root branching in the taxa more recently evolved than bryophytes (e.g., seed plants). Nevertheless, in more ancient phyla such as Bryophyta and Lycopodiophyta an endogenous origin of leafy shoots may be characteristic of a number of included taxa (Schuster, 1966).

While branch initiation has been fairly well characterized, the cells and tissues comprising the branching stems of leafy liverworts have not. The outer one or two layers of cells of the stems are referred to as cortical cells and the remaining inner cells are referred to as medullary cells. The number of layers of cells comprising the medullary tissue of a stem varies from one to several, depending on the species. Except for position, there appears to be no set of cellular or subcellular features that are used to characterize the cell types that make up the cortical and medullary tissues of liverwort stems. Consequently, there is presently very little data upon which to compare stem anatomy of hepatics with that of other plants.

With regard to leaf initiation and development, the classic

observations on the Jungermanniales by Leitgeb (1875) were extended to include the Calobryales by Campbell (1920), and to include the Metzgeriales by Buch (1930). More recent studies on leaf development in the Jungermanniales were conducted by Grill (1958), and Bopp and Feger (1961). It had been determined by Leitgeb (1875) that leaves in the Jungermanniales are initiated by a two-celled primordium that differentiates at the anterior portion of each merophyte. A merophyte is the cell (and its derivatives) that is formed following each segmentation division of a shoot apical cell. In the Jungermanniales each merophyte divides to form five cells - two comprise the two-celled leaf primordium, the other three contribute to the cortex and medullary tissue. Each of the paired leaf primordia function initially as apical cells. Campbell (1920) and Buch (1930) determined that leaves in the Calobryales and Metzgeriales, respectively, are initiated from a single cell primordium that functions initially as an apical cell. Grill (1958) conducted a detailled study of the pattern of cell divisions responsible for generating the multilobed, highly dissected leaves of Trichocolea tomentella (Ehrh.) Dum. Bopp and Feger (1961) analysed the patterns of cell divisions responsible for generating the remarkable variety of leaf forms that develop in a wide variety of species of the Jungermanniales. They concluded from their analysis that the initial stage of leaf development is dominated by apical growth, but the apical growth period is usually of short duration. Following the initial apical growth period, there occurs a

second stage in which the non-apical cells become equally meristematic. They divide synchronously with a regular alternation of transverse and longitudinal divisions. The final form of the leaf is determined by which one or more of five basic patterns of cell division function during the third and final stage of leaf development. The five basic patterns distinguished by Bopp and Feger (1961) are as follows: "equal growth" in which there is a regular alternation of transverse and longitudinal divisions (i.e., a continuation of the pattern established during the second stage of leaf development); "group growth" in which synchronous growth is confined to discrete groups or populations of cells; "serial growth" in which growth in certain populations of cells is restricted to transverse divisions; "fanning or 'fan' growth" in which serial growth is accompanied by broadening at the basal or distal ends due to localized longitudinal cell divisions; and, "irregular growth" in which the planes and location of divisions is irregular. During the course of development of a particular type of leaf morphology, a particular sequence of any combination of these five modes may occur. In the development of some leaf forms, one or more of the five modes may be completely suppressed.

All the research reviewed relevant to leaf initiation and development indicates that, in general, leaf ontogeny in leafy liverworts corresponds to leaf ontogeny in the great majority of land plants. That is, leaf primordia are initiated from superficial cells in a regular phyllotactic sequence at the flanks of the shoot

apex. Initial development is by means of apical growth and final development is accomplished by means of intercalary growth. There is one major difference between the leaves of most leafy liverworts and most other groups of land plants. Although there are several species of liverworts with multistratose leaves, the leaves of most species of the Jungermanniales are one cell layer thick. The only other major groups of plants to have unistratose leaves are the Musci in the Bryophyta and the Hymenophyllaceae in the Polypodiophyta. Many of the features of the cells making up the unistratose leaves of liverworts, those that can be discerned with a hand lens or light microscope, are considered to be important taxonomic characters. Consequently, these features (e.g., their size, their shape, the presence or absence of corner wall thickenings, and the size, shape, and number of "oil bodies") are well documented in the taxonomic literature on this group (e.g., Schuster 1966, 1979). Many features not discernable with a hand lens or light microscope have gone uninvestigated. At the outset of the research reported here, there had been no electron microscopical study of either the structure or the development of the cells composing the leaves of leafy liverworts. Consequently, there has been a lack of sufficient data for characterizing the cells that compose the leaves of leafy liverworts to the same extent that the cells composing the leaves of other plants have been characterized. It follows that there has also been insufficient bases for comparing the cell types that compose the leaves of leafy liverworts with any other cell type,

although it should be pointed out that more than one hepaticologist (e.g., Schuster, 1966; Smith, 1990) has referred to the leaf cells of the Jungermanniales as collenchymatous.

The purposes of this thesis research was to obtain information that is presently lacking about the subcellular structure and organization of the cells comprising the leaves of a representative species of the leafy liverworts and to use this information both to better characterize the cells as well as to compare them to cell types that compose the leaves of other land plants, especially flowering plants. An important part of this study was to follow the developmental changes in the cell organelles correlated with the development of leaves from primordium to maturity.

The liverwort, Plagiochila arctica Bryhn & Kaal. (Plagiochilaceae, Jungermanniales), was chosen for this study. It is representative of a large, cosmopolitan genus that contains over five hundred species worldwide. Moreover, in its general morphological features, P. arctica appears to be representative of the majority of species of succubous leafy liverworts. None of the features of the leaves revealed by light microscopy and reported in the taxonomic treatment of P. arctica (e.g., Schuster, 1980) suggests that the leaf cells of this species will prove to be unique or unusual when compared to those of other leafy liverworts. An additional reason for choosing this plant is that in the past twelve years, it has been used in a number of studies on the chemical regulation of leaf and branch initiation (e.g. Basile, 1979; Basile and Basile 1984,

1987, 1990,1991; Basile et al., 1987). It seemed desirable to continue to develop this plant as a model system for studying leafy liverwort development and differentiation. Preliminary studies appeared to confirm this opinion (Basile and Jensen, 1986, 1987, 1989).

Methods & Materials

Plant Material.

The plants used in this research were derived from specimens of Plagiochila arctica Bryhn & Kaal. (Plagiochilaceae) collected in Alaska and identified by Dr. William C. Steere (W.C. Steere #74-80). Plagiochila arctica is one of the over 500 species composing the large cosmopolitan genus, Plagiochila Dumort. As the name implies, P. arctica is an arctic plant and is circumpolar in its distribution. In nature it shows a wide tolerance of environmental conditions, which may explain why it is easily maintained in axenic culture, in vitro. The original stock cultures were started from pieces of gametophyte tissue using a "washing machine" technique (Basile, 1972). The plants used in this investigation were regenerated from tissue fragments prepared from stock cultures. To prepare the fragments, the contents of a mature stock culture were ground in a sterile stainless steel microblender accessory on a Waring Blender connected to a voltage regulator and run for 20 secs at 60 V. The resulting suspension of gametophyte fragments was taken up in a B-D Cornwall 10ml pipetting syringe fitted with a 15 gauge needle and inoculated into culture vessels containing fresh nutrient medium (Basile and Basile, 1988).

Culture Media.

The nutrient medium on which the plants used in this investigation were cultured consisted of a combination of macronutrients, micronutrients, a supplementary carbon source, and

a buffering agent (See Appendix I). The macronutrients included $(NH_4)SO4$, $Ca(NO_3)_2.4H_2O$, $MgSO_4.7H_2O$, KH2PO4. The micronutrients included $Fe(NH_4)_2SO_4.6H_2O$, $MnSO_4.H_2O$, $ZnSO_4.7H_2O$, $CuSO_4.5H_20$, $(NH_4)_6Mo_70_{24}.4H_2O$, $CoSO_4.7H_2O$, H_3BO_3, $Na_3VO_4.16H_2O$. The inorganic nutrients were supplemented with 1% w/v glucose in order to increase the rate of growth and development of the inoculum. A buffering agent, 2-(N-morpholino)-ethanesulphonic acid (MES buffer) was added at 0.05 M to combat rapid pH shifts common in ammonium salt-containing media.

The pH of the medium was adjusted to 4.8 with NaOH . It was then cold sterilized through 0.20 µm Nucleopore membrane filters.

Culture Conditions.

Plants used in this study were cultured in 30 cc Wheaton Type serum bottles and closed with Wheaton type stoppers vented by means of a cotton-plugged, 18 gauge hypodermic needle (Basile, 1964). Each bottle contained 10ml of nutrient medium at the time they were inoculated. The cultures were incubated on lighted shelves in an air conditioned room thermostatically controlled to maintain a temperature of 19 +/- 2 C. They received continuous illumination of between 700 and 1000 lux from cool white fluorescent bulbs. Incubation periods were from nine to twelve weeks.

Microtechnique.

Techniques used for the preparation of specimens for electron microscopy for the most part followed standard established techniques as described in the literature (i.e., Hayat, 1989;

Mollenhauer, 1964). Minor modifications made are described below and in Appendices. For both transmission electron microscopy (TEM) and light microscopy (LM) observations, plants were harvested at 9-12 weeks. Shoots that had grown relatively straight and achieved a length of about 5-10 mm were selected. Most were then fixed according to the following schedule (Appendix II, III): -

1. Fixed by adding glutaraldehyde to the culture medium to give a working concentration of 3% v/v and letting stand at room temperature for 1 hr. (To some cultures 2% tannic acid was added to the fixation solution to possibly help stabilize microtubules.)
2. Rinsed 3 x in 0.1 M cacodylate buffer (pH 6.8).
3. postfixed for 1 hr in 2% osmium tetraoxide (OsO_4) in 0.1 M cacodylate buffer (pH 6.8).
4. Rinsed 2x in cacodylate buffer.
5. Dehydrated through a graded ethanol series (50%, 70%, 95%, 3x 100%).
6. Rinsed through 3 changes of 100% propylene oxide.
7. Embedded either in Spurr's (Spurr, 1969) or EPON 12 (Luft, 1961) embedding medium using standard embedding techniques.
8. Polymerized either at 70 F. overnight when in Spurr's, or at 35 F overnight, 50 F overnight, and for 3 days at 65 F when in EPON 12.

Flat embediment molds were used throughout because it was found that it was easier to maintain the proper orientation of the

shoot apices in these molds than in Beem capsules.

For scanning electron microscopy (SEM), plants were harvested at 9-12 weeks. Suitable leafy shoots 5-10 mm in length were selected. They were then prepared for the SEM according to the following schedule (Appendix IV).

1. Fixed by adding glutaraldehyde to make a working concentration of 3% v/v culture medium at room temperature for 1 hr.
2. Rinsed 3x in 0.1 M cacodylate buffer (pH 6.8).
3. Postfixed in 2% osmium tetraoxide (OsO_4) in 0.1 M cacodylate buffer (pH 6.8) for 1 hr at room temperature.
4. Rinsed 2x in cacodylate buffer.
5. Dehydrated through an alcohol series - 10%, 30%, 50% 70%, 90%, 3x 100% - for ten minutes for each solution.
6. Critical point dried (CPD) using a SAMDRI-790 critical point dryer.
7. Mounted on copper stubs using double stick tape.
8. Gold coated in a Technics Hummer II for observation in the SEM (JEOL U-3 & Amray 1830).

<u>Microscopical Analysis.</u>

For TEM and LM observation, blocks were initially trimmed to a pyramid of 0.5 mm and then thick sectioned (2-4 μm) on an LKB 8800 Ultramicrotome III using a glass knife. Sections were cut and regularly examined with a light microscope until the area just above the apical region could be located and the block properly oriented . Once the desired area and proper orientation were established, the

block was retrimmed to a pyramid of .25 mm (and 1 mm in depth) and sectioned using a diamond knife (Dupont Instruments). Both thick and thin sections were cut using different regions of the knife. A standard procedure was followed at this point. First a thick section was cut and mounted on a glass slide to identify the area of the plant being sectioned. Then 20-30 thin sections (70-90 nm) were cut and mounted on thin bar grids (SPI 200 mesh HH &/or HS). Six to nine sections were mounted on each grid. Thick sections (1-2 µm) were stained with 0.5% Toluidine Blue (Trump et al., 1961). Thin sections were first post stained with uranyl acetate /methanol stain (Stempak and Ward, 1964) and then with lead citrate (Reynolds,1963) according to the authors' published procedures. The thin sections were then observed and photographed in the transmission electron microscope (TEM) (Hitachi HS 9 and H 7000) using Kodak 4869 film. For each area of the grid observed and photographed, the area was first photographed at low magnification, i.e., 1000x, to correlate the TEM with the LM sections and to aid in identifying the areas in higher magnification micrographs. The areas of interest were then photographed at magnifications of 3000-20000x to obtain micrographs to be used for both qualitative and quantitative analysis. The negatives of all TEM photomicrographs were printed at 2.5 their original magnification. A replica of a defraction grating was periodically photographed at the various magnifications used to ascertain that correct magnifications were being recorded.

Photography

Thick sections were photographed using an Olympus Model BHS

Laboratory Microscope with a fully automatic 35mm camera module, model PM-1035ASP and Kodak Panatomic-X film. Negatives were developed in Microdol-X, 1:3 and printed on Kodak RC paper. The prints were then developed in Kodak Dektol solution and fixed in Kodak hypofixer solution.

Thin sections were photographed using the photosystems of Hitachi Model HS 9 or Model H 7000 transmission electron microscopes and Kodak 4869 film. Negatives were developd in Kodak D-19 and printed on Kodak RC paper using a Durst enlarger. The prints were then developed in Kodak Dektol solution and fixed in Kodak hypofixer solution.

Scanning electron microscope preparations were photographed using the photosystems of either a Joel U-3 or Amray 1830 scanning electron microscope and Polaroid type P/N 55 film. Negatives were processed with hyposulfite and printed on Kodak RC paper using the same methods as the negatives taken with the TEM .

Reproductions were made using a Polaroid MP-3 enlarging system. Polaroid type P/N 55 film was used. Negatives were processed with hyposulfite and printed on Kodak RC paper using the methods described above.

Morphometric Analysis.

There are quite a few texts that describe morphometrics and how to do it. Weibel has written a number of these (e.g., 1973, 1980). Another one of note is that of Aherne and Dunhill (1987). Others who have contributed many articles to the field describing "how-to" methods include, for example, Bertram and Bolender (1990),

Loud et al (1965), van Diest et al. (1989), Weibel and Bolender (1973), Weibel & Gomez (1962), and Weibel et al. (1969) . Many others have used morphometrics in their research. A few examples of these include those of plants - de Soyza et al. (1990), Kincaid and Schneider (1983), Khatijah and Briarty (1990); algae - Sicko-Goad (1982); cyanobacteria - Jensen and Rachlin (1984); animals - Bertram and Bolender (1986), Loud et al (1965.

In this study, electron micrographs were first analyzed qualitatively to investigate changes in the cells and the organelles within the cells during leaf development. The same micrographs were then used to do a quantitative study. Based on the various methods described in the literature, it was decided that point counting (Weibel, 1979) would be the best method to estimate relative volume of the various organelles within the cells during leaf development. Point count lattices were constructed by applying "dots" to sheets of transparent plastic paper laid over standard sheets of graph paper. Distances of either 1 cm or 2 cm apart were marked off for the respective grid lattices. A lattice was thereby constructed of points one cm apart, and another of points 2 cm apart. The lattices were randomly placed over the micrographs and secured with clips so that they did not slide while measurements were being taken.

For counting whole cells, micrographs were taken at 3,000x and enlarged to 7,500x. This magnification was chosen because (1) it enabled one to see entire cells in a single micrograph, and (2) it was sufficiently large enough that adequate counts could be made of the

major organelles, i.e., nucleus, chloroplasts, vacuoles, mitochondria, oil bodies and cell walls. The 1 cm grid point lattice was used for these counts.

For analysis of individual organelles, (e.g., chloroplasts) micrographs were taken at 10000-20000x and enlarged to 25,000-50,000x. A 1or 2 cm grid lattice was then used .

Morphometric analysis was performed employing a Macintosh SE. An analysis of the major organelles found within the first 9 leaves starting with the two-celled leaf primordium was done (Tables 1-3). Statistical analysis of the morphometric data was carried out using a Statview 512 program. The relative percentages of the leaf volume taken up by major organelles was determined (Table 1). The increasing dimensions of the leaves as they matured was recorded (Table 2). Third, the changes in plastid development from the apical cell to the mature leaf were observed (Table 3).

Results

Leafy Shoots and the Origin of Leaves at the Shoot Apex

The leafy shoots of Plagiochila arctica have typical dorsiventral symmetry with their leaves arranged on the stems according to a 1/2 phyllotaxy (Fig. 1-2). The leaves are initiated from segments produced by the regular divisions of a tetrahedral apical cell with three cutting faces. Each of the segments, termed a merophyte, becomes separated from the apical cell by a wall formed parallel to each of the three cutting faces. Therefore a full cycle of divisions of an apical cell results in the formation of a series of three merophytes (Fig. 3). Each of the merophytes undergoes further divisions. The precise sequence of divisions of each merophyte following its separation from the apical cell was not a part of this investigation. According to Leitgeb (1875), followed by Evans (1912), the first series of divisions of a single merophyte results in five cells with predictable developmental fates . The three deeper lying cells contribute to the stem tissues (cortex and medullary). Two cells in a superficial position in each merophyte serve as leaf primordia . These first two leaf primordia cells are therefore smaller than the apical cell from which they originated. The development of the leaves from these two leaf primordia was the purpose of this study. Although a full cycle of divisions of the apical cell cutting off merophytes always results in the formation of three sets of five cells - the two-celled leaf primordia and their three subtending cells contributing to the stem tissue, only two of the three two-celled leaf primordia continue to develop into leaves

in P. arctica. The third primordium either fails to develop any further or develops into a single slime papilla or two slime papillae (Fig. 3-4).

Transverse sections taken at the anterior portion of the apical cell show a centrally located apical cell surrounded by three ranks of merophytes (Fig. 5-6). The two ranks of merophytes giving rise to leaves are normally those furthest away from the substratum (when the plants are growing on a solid surface). They are referred to as lateral merophytes. Those in which any further development of the two-cell primordium is either completely suppressed, or give rise to slime papillae are normally closest to the substratum and are referred to as ventral merophytes.

The least developed lateral leaf primordium was arbitrarily designated as leaf number one. Each successive, further developed leaf was assigned a sucessively higher number. There were typically eight or nine leaves at different developmental stages visible in transverse sections through the anterior region of a shoot apex of P. arctica (Fig. 5). Some of the more salient, qualitative features of subcellular organization observed through the combined use of LM and TEM, beginning with the apical cell and progressing through the cells composing the nine sucessively older leaves follow. The various organelles of the leaf were both qualitatively and quantitatively (Table 1) analyzed. The increase in cell size was measured (Table 2). Since the plastids were a prominent feature of each leaf cell which changed with the growth and age of the leaf, the changes in development from plastid to chloroplast was also

recorded (Table 3).

The Apical Cell

The apical cell of P. arctica, like that of almost all leafy liverworts, is roughly triangular in transverse section (Fig. 6-7). It is 17-19 μm along each cutting face (Basile, 1979). As it is triangular in longitudinal section proceeding down into the stem, the sections become increasingly narrower in diameter. In a cross-section taken approximately through the center of the cell where the nucleus usually can be seen, the cell is 12-15 μm in diameter (Table 2). Except for its distinctive shape, the general appearance and subcellular organization of the apical cell viewed at low magnification are typical of meristematic cells. The nucleus is usually found to be centrally located and surrounded by a dense cytoplasm (Fig. 6). In some cases it can be seen slightly off center (Fig. 7), closer to the cutting face where the last merophyte appears to have been cut off. The merophyte that is still in the one-celled stage was the last one formed (Fig. 5-7). Plastids appear to be numerous and more or less uniformly distributed throughout the cell (Fig. 6-7). They are small, their length averaging in size to be 1.5 μm, their width, 1.1 μm (Table 3). They lack well developed thylakoids (Fig. 8-9). The interior of the plastid is mainly composed of stroma which occupies 67-86% of the inner organelle space. The weakly developed thylakoids occupy the remaining13-33% of the space. The grana are few in number, typically 1-4 per plastid containing 2-3 thylakoids per grana stack. An occasional elongated plastid may be seen, presumably prior to dividing (Fig. 10).

Mitochondria, ranging in size from 0.44 x 0.40 - 0.84 x 0.44 μm, are recognizable scattered through the cytoplasm even though their cristae are not well developed (Fig. 9-10). Vacuoles are numerous and relatively small, 0.72 - 1.04 μm in diameter. They are not uniformly distributed (Fig. 6-7). Larger numbers are sometimes found aggregated between the nucleus and one of the cutting faces/walls (Fig. 8). There is some evidence to indicate that these aggregations of vacuoles predict where the next segmentation division of the apical cell will occur (Hebant et al., 1978). Vacuoles may appear relatively electron transparent or empty, but often contain granular material or short pieces of membrane-like material (Fig. 10). Endoplasmic reticulum, rough and smooth can also be seen (Fig. 9-11). Golgi appeared few in number, in that rarely more than one per section through the apical cell was observed (Fig.12). They are typical in appearance, being composed of 3-4 flattened sacs with small vesicles nearby. Numerous plasmodesmata can be seen traversing the cell walls between the apical cell and the surrounding ventral and lateral merophytes (Fig. 9-11,14). Occasionally a branched plasmodesmata was observed (Fig. 12). They may be as numerous as 30 per 4 μm line section when cut longitudinally through the cell wall or spaced on the average 133.3 nm apart. Microtubules were not abundantly evident in the apical cell in most micrographs but cortical microtubules, 25 nm in diameter, were observed both in the apical cell and in the adjoining merophytes. They were sometimes seen in groups of three (Fig. 13). In addition to lomasome areas, groups of linear membranes

were sometimes seen between the cell membrane and the cell wall (Fig. 12).

Leaf One - TheTwo-Celled Leaf Primordium

Both cells making up this primordial leaf have a typical meristematic appearance (Fig. 15). That is, the cytoplasm is dense, the nucleus occupies a major portion (24%) of the cell and occupies a central position (Fig. 15). The two cells at this stage vary in size, typically between 11.7 x 7.0 - 12.6 x 7.7 µm. The plastids, are still numerous, small (1.28 x 1.2 - 1.76 x 1.52 µm), and distributed around the nucleus (Fig. 5,15). They continue to contain a few weakly developed thylakoids, showing little differentiation from the plastids of the apical cell, containing 1-4 grana per plastid wth 2-3 thylakoids per grana stack. However, an occasional elongated plastid is visible, ranging from 2.2 x .76 - 2.52 x .92 µm. Dumbell shaped plastids are present, apparently ready to divide (Fig. 16). Mitochondria are present, averaging 0.8 x 0.76 µm, interspersed among the plastids and vacuoles. They show little internal structure at this stage, with no or very weakly developed cristae. The vacuoles are still numerous, mostly small and scattered (Fig. 15-16.). They range in size from 0.60 x 0.48 - 1.24 x 0.84 µm. A few larger vacuoles can be seen, which are approximately 1.68 x 1.6 - 1.76 x 0.88 µm. These are apparently products of smaller fusing vacuoles. They still appear to contain granular contents and membraneous inclusions. Endoplasmic reticulum continues to be present. Golgi are present but because they are not numerous, they are not readily seen in any given section. Numerous plasmodesmata

connect the leaf cells via their adaxial walls with the apical cell (Fig. 14, 16), up to 7.5 per µm line segment. They are typical of plasmodesmata transversing the wall from cell to cell as seen in other plants. Their size falls within the range (20-100 nm) of that found in other plants, as they are 40 nm in diameter. The adaxial walls at this stage are still attached to the apical cell and are thinner, 0.08 -0.16 µm in diameter, than the outer free walls, 0.28-0.32 µm in diameter. In general, the most recently cut off merophyte has a thinner inner wall than the inner walls of the other merophytes in direct contact wth the walls of the apical cell. The outer walls are already cutinized at this very early stage of leaf development (Fig. 15). "Lomasome-like" structures are present which appear to be adding material to the cell walls (Fig. 16). Cortical microtubules are present seen in cross section at right angles to the wall (Fig. 13).

Leaves Two to Four

The leaves rapidly develop from the two-celled stage. Development proceeds by transverse and longitudinal anticlinal divisions and by cell enlargement. Because of the strictly anticlinal divisions, a leaf is formed that is comprised of a single cell layer of cells (Fig. 18).

As the cells increase in number by cell division, their size varies, typically between 11.06 x 9.19 - 13.19 x 6.6 µm. There are some changes in some of the organelles that can be correlated with leaf cell maturation. The nucleus does not noticeably change. It remains prominent and centrally located in cells unless they have

just divided, in which case they lie closer to the newly forming wall (Fig. 18). New wall formation was observed in this and other stages of leaf development. Wherever it was observed during this investigation, it corresponded to the phragmoplast-type (e.g., Pickett-Heaps and Northcote, 1966a) as expected. The phragmoplast first develops in the mid area of the leaf cell and continues to develop outwards to the existing walls of the original cell (Figs. 18-22). Numerous microtubules can be seen in longitudinal section at right angles to the newly forming wall (Figs.19-20). Many small vesicles appear to be coalescing to form the primary wall (Figs. 21-22). Microtubules can be seen beyond where the vesicles are already forming the new wall on either side toward the position of the existing side walls apparently directing the formation of the newly forming wall (Figs. 21-22). In addition to the microtubules that are seen apparently directing new wall formation in dividing cells, microtubules can be seen in cross section parallel to existing cell walls. They are often seen in groups of three or four along the cell wall (Fig. 23A). Groups can sometimes be seen along the cells walls opposite each other perhaps indicating the forerunner of a preprophase band. However, preprophase bands have yet to be clearly demonstrated in P. arctica. Microtubules can also be seen in longitudinal section underlying the cell wall when the plasmodesmata are cut in cross section (Fig 23B).The vacuoles are for the most part still small and numerous, ranging in size from 0.80 x 0.72 - 2.2 x 1.92 μm, but some begin to coalesce to form larger vacuoles. Some small, vacuole-like structures are visible

approximately 0.40 x 0.32 µm which contain lomasome-like contents (Fig. 24). Lattice or waffle-like inclusions are sometimes seen in the vacuoles (Fig. 24). Beginning in leaf two and continuing through leaf four the plastids show increased numbers of thylakoids making them more recognizable as chloroplasts. Although small plastids (1.28 x 1.0 µm) are still visible, most fall within the range of 1.6 x 1.4 - 2.08 x 1.64 µm. A few elongated plastids 2.56 x 1.28 - 2.6 x 1.48 µm are occasionally present. The number of grana range from 2-5 per section through the plastids. The thylakoids that make up each grana stack usually range from 2-5. Occasionally as many as seven are seen. The interior of the plastid is still mainly composed of stroma which occupies 66-77% of the space. The developing thylakoids occupy 23-34% of the interior of the plastid. Chloroplasts are seen which appear to be dividing. Mitochondria are present and increasing in size. Most range in size from .56 x .48 - .88 x .60 µm. Occasional larger ones are seen, 1.04 x .48 - 1.24 x .60 µm. They begin to show signs of increased internal structural complexity. By leaf four, recognizable cristae are evident (Fig. 24). There are no obvious changes in either the endoplasmic reticulum or the Golgi (Fig. 24). Golgi of up to five flattened discs are visible (Fig 24). Plasmodesmata are abundantly distributed along all the walls between adjacent leaf cells. (Fig. 23B). When cut in cross section through a wall, they appear to be randomly distributed with some pairs or groups of three being spaced as closely as 0.08-0.12 µm apart. In longitudinal section they appeared as close as 0.06-0.16 µm apart. The corners of the cell walls show increased

thickening, 0.4-0.6 μm between cells at the outer wall (Fig. 18). This appears to be the result of either an increased or a more localized "lomasome-like" activity at the juncture of two or more cells (Fig. 25). Outer walls continue to be thicker, 0.16-000.20 μm, in width than the inner walls, 0.04-0.12 μm. A cuticle appears to be present (Fig. 26).

Leaves Five and Six

Cell division continues in leaves five and six, as does increase in leaf size. The cells continue to expand in the adaxial-abaxial direction causing an increased thickness of the leaf. The leaf cells have enlarged to approximately 9.3x14.7 μm. The centrally located nucleus is still the most prominent structure (Fig. 29). Although numerous, small vacuoles are still present, most have coalesed to form fewer, larger vacuoles (Figs. 28A-D). In some, globular inclusions can be seen which may be contributing to the "lomasome like" material or may be early developing oil bodies (Fig. 28C). Thylakoid and grana differentiation continue to progress within the plastids and they clearly may now be referred to as chloroplasts (Figs. 27-28). Stacks of grana appear to be grouping together to form larger stacks and are connected. An occasional larger plastid can be seen. In this case the position of the grana stacks appears to be shifted, as though the chloroplast is preparing to divide (Fig. 27,30). Mitochondria with more developed cristae are scattered throughout the cytoplasm (Fig. 28A). The endoplasmic reticulum and Golgi are without obvious change. The plasmodesmata are becoming more separated correlated with cell expansion. In a typical thin

section as few as two-six can be seen in longitudinal section per 4 µm line section of wall (Fig. 30). They can be seen also in cross section where they appear to be randomly dispersed (Fig. 28B&D). Deposition of cell wall material by the "lomasome-like" structures continues, especially at the corners where cells meet (Fig. 29). A section through three cells of a leaf shows the nucleus is still usually centrally located surrounded by the remaining organelles (Fig. 29). Cell plate formation continues to be seen as the phragmoplast type (Fig. 31).

Leaf Seven

By leaf seven, the central nucleus has become somewhat less dominant in appearance in many of the cells, especially in the cells in which vacuolar coalescence has progressed to the point of forming a few prominent vacuoles with only very few smaller vacuoles remaining (Figs. 32,33). Cell size has increased to 15.4x20.3 µm (Table 2). Vacuoles still contain some granular material but appear to be more electron transparent than in earlier stages. Functioning chloroplasts are clearly evident by their more fully developed thylakoids and the beginning of the appearance of starch grains (Fig. 32). Lipid inclusions are occasionally present. There is an increase in the size of the chloroplasts (Figs. 32-34) and the grana appear more clearly interconnected. The number of thylakoids per grana ranges from two-seven. The mitochondria continue to show increased development of cristae (Figs. 32-33). There is no noticeable change in the appearance or the distribution of the endoplasmic reticulum or Golgi from the preceeding stage.

The numerous plasmodesmata continue to be more spaced wherever the walls increase in their linear dimensions, with as few as 3-4 per 4 μm section. Localized thickening of the cell walls, at least in some cells appears to be continuing as evidenced by the still present "lomasome-like" structures depositing material at the inner surfaces of the walls. The "lomasome-like" material appears as aggregates of small vesicles which in turn appear to be depositing their contents to the cell wall (Figs. 32-34). Occasionally membraneous inclusions can be seen in the area between the plasma membrane and the cell wall, apparently adding material to the cell surface as do the more typical looking lomasome areas (Fig. 35). Microtubules can still be found in small numbers in the cortical cytoplasm close to the walls.

The most noteworthy change in the differentiation of the leaf cells at this stage is the first clear evidence of the formation of structures called "oil bodies" (Figs. 32-35). They seem to appear *de novo.* The first evidence is the formation of small oil droplets in vacuole-like compartments. Oil bodies viewed through the TEM are single-membrane bound sac-like structures that contain spherical droplets of "essential oils", classified as terpenes or sesque-terpenes. Although only one to four oil bodies are seen per section at the EM level, at the light microscope level four to eight can be seen per cell when viewing a whole cell with oil immersion. Oil bodies are widely distributed in the Hepaticae, especially in the Jungermanniales. Although terpenes are produced in a variety of land plants, their accumulation and storage within membrane-bound

structures in the cytoplasm appears to be a unique feature of the hepatics. Differences in the size, shape, and number of oil bodies, and the chemical composition of their contents, have come to be important taxonomic characters in the Hepaticae.

Leaf Eight

By leaf eight, the cells are almost fully enlarged (18.9x25.6 um). The nucleus no longer dominates the view of a sectioned leaf cell in the majority of cells. Most of the originally small, separate vacuoles have coalesced into what appear in the micrographs to be two large vacuoles (Figs. 36,37) for the most part devoid of contents. One appears to be positioned just below the adaxial free surface and the other below the abaxial free surface. What appears to be two, more probably represents optical sections of a single, doughnut- shaped central vacuole, the long axis of which is in the plane of the internal cell walls. The vacuole now takes up a major portion of the cell volume. The bulk of the non-vacuolar portion of the cytoplasm is generally restricted to a central "strand" broad enough to include the nucleus connected with another band along the internal wall surfaces of the cell (Figs. 36,37). Fully differentiated chloroplasts containing starch grains and well developed thylakoids are seen to be more or less evenly distributed in the band of cytoplasm along the internal cell walls (Figs. 36-40) (Table 3). Lipid inclusions are sometimes seen within the chloroplasts (Fig. 40). Mitochondria with well developed cristae are also present (Figs. 38-40). There is still no obvious change in the endoplasmic reticulum or Golgi. The plasmodesmata are more widely spaced

(1-3 per 4 µm line segment), but still form abundant interconnections between adjacent cells (Figs. 38-39). Cortical microtubules are still visible. They appear in groups of two, three or four (Fig. 41). The cell walls are not quite fully expanded. The continued deposition of wall material by "lomasome-like" structures is still evident (Fig. 36). All corners between two or more adjoining cells have increased thickening (Figs. 36-38). Oil bodies have developed further. Several large spherical droplets of oil are usually seen contained within a single membrane limited structure (Figs. 36-37). The droplets are variable in size.

Leaf Nine

By leaf number nine, most if not all of the leaf cells have fully matured and average in size 20.4x30 µm . The nucleus occupies a relatively small portion of the cell (2%). Most of the leaf is occupied by a large central vacuole (Figs. 42A&B,43). The cytoplasmic contents surround the vacuole and occupy less than 1/3 of the cell (see Table 1). Within this band of cytoplasm, fully developed chloroplasts containing as many as 6-15 starch grains and well developed grana containing 2-9 thylakoids per grana stack are dispersed (Figs. 42-48). Well developed mitochondria continue to be seen (Figs. 44-49). Except for the closer packing imposed by vacuolar enlargement, there is no apparent change in the endoplasmic reticulum or Golgi (Fig. 45). The plasmodesmata are at their furthest spacing (0-3 per 4 µm line segment) over the surfaces of all the now mature internal cell walls. Oil bodies continue to be seen, each containing several large oil droplets (Fig. 42A&B). Other

small vacuole-like compartments are seen containing what may be small oil droplets (Fig. 46). These may be newly forming oil bodies. Cortical microtubules continue to be seen (Fig. 49). Corners between adjacent inner and outer walls continue to show increased thickening (Fig 42-43,47). A cuticle is evident (Fig. 42, 46). By this stage the cells have achieved thickened corners appearing collenchymatous as described by various bryologists (e.g., Schuster, 1966) (Figs. 50A-D). Figure 50A is that of Plagiochila arctica. Figures 50B-D are those of other Plagiochila species.

Slime papillae:

Before summarizing some of the more salient results of this investigation of leaf development, it seems worthwhile to mention something about the development of slime papillae (Fig. 51). When slime papillae are formed, they develop from a two-cell primordium at the ventral surface of the plant, the same type of primordium that in the lateral position gives rise to leaves. They arch over the apical cell (Fig. 57) and appear to function as a protective device for the growing apex. They secrete a mucous like substance. They may occur singly or in pairs, one coming from each of the two cells of the ventral primordium. The cells of developing papillae have a different appearance than leaf cells. They are spherical in cross section (Fig. 50) and may appear as a chain of 2-3 cells in exterior view (Fig. 3, 57). They are well developed and appear to contain the same organelles as the leaf cells, i.e., nucleus, vacuoles, plastids, mitochondria, oil bodies, endoplasmic reticulum, and Golgi. The vacuoles remain small. The cell contents continue to appear

meristematic (Figs. 51,52). In longitudinal section often three or more Golgi can be seen (Fig. 52). The mitochondria often contain cristae. The vacuoles may be more or less empty, or may contain inclusions, some are grid-like and some vesicular (Fig. 53). The cell walls are surrounded by a cuticle (Figs. 51,55). The slime papillae usually only grow for a short period of time. As they mature the wall thickening increases (Figs. 54-56). The wall becomes quite thick, layered and "sculptured" (Fig. 56). The internal cytoplasmic contents degenerate (Figs. 54, 55). They may be retained for a while on the growing stem as the plant matures but often wither and are sloughed off, apparently having fulfilled their function.

Summary of Results

By following the changes in subcellular organization in developing leaves of P. arctica, a number of significant features regarding the structure and development of leaves of this representative leafy liverwort were ascertained. The subcellular organization and composition of the leaves at the two-cell primordial stage were characteristic of meristematic cells. Except for their position there was nothing to distinguish them as leaf cells. For example, there was no observed difference between the two-cell primordia in the ventral position in which further development is either totally suppressed or continues to give rise to slime papillae and those in the lateral position that continue to develop into leaves.

The membraneous organelles that exhibited the most

pronounced changes during the course of leaf development were the plastids, the mitochondria, the vacucles, and the oil bodies. High proportions of plastids showing the well developed grana typical of mature chloroplasts and mitochondria with developed cristae were not obvious until leaf five-six. The complete coalescence of small vacuoles to form a single, large central vacuole was not apparent in the majority of leaf cells until leaf nine. The beginning differentiation of membrane-bound oil bodies was not discerned until leaf seven. These developed rapidly, so that they were fully differentiated in leaf nine.

Significant developmental features were also observed in the nine leaf sequence with respect to the cell wall and associated structures. From the start, an abundance of plasmodesmata were seen traversing all the interior walls of the leaf cells. Although the plasmodesmata became more widely spaced as the cells enlarged, there was no apparent change in their structure and number. That is although an occasional branched plasmodesmata was seen, there did not seem to be any additional plasmodesmata formed. Another important feature that showed early in leaf development was the appearance of an electron translucent layer that coated the entire outer, exposed surfaces of the leaves. Subject to further verification, this layer is presumed to represent a cuticle. Changes in the cell walls per se take place more gradually, over the nine leaf sequence. Most cells were not fully expanded before leaf nine. Thickening of the walls was not uniform, The thickest regions were at the internal corners of the walls where three or more cells

abutted. the next thickest were the outer corners where two cells met, then the free, surface-facing walls under the cuticle. The thinnest were the internal walls that help separate adjacent cells and through which plasmodesmata pass.

The fully mature leaf cells of P. arctica can now be characterized as follows: The cells which comprise the leaves are unistratose and polyhedral; the outer, free surface walls are coated with a cuticle; both the corners of the outer walls and the inner walls are unequally thickened at the corners - that is the corners where two or more cells adjoin are always thicker than the rest of the walls. Plasmodesmatal interconnections are abundant. This is most evident in the walls between the first few cells which make up the future leaf. As the leaf cells continue to divide , there is no apparent increase in the number of plasmodesmata. They appear to remain, but are spread out over a greater wall surface. There is a full complement of membranes and membrane-bound organelles typical of plant parenchyma cells. That is, the nucleus, chloroplasts, mitochondria, endoplasmic reticulum, Golgi and vacuoles are all present. The plastids are differentiated containing numerous thylakoids in stacks of grana, and numerous starch grains are present in the chloroplasts. Lipid inclusions are also sometimes seen. Mitochondria develop cristae and are indistinguishable from those of other plants. The vacuoles are sometimes empty in appearance but often contain granular, vesicular or membraneous inclusions. And, in addition to the typical organelles found in other plant cells, perhaps their most distinguishing feature is that they

contain, within each of the mature leaf cells, oil bodies, membrane-bound structures peculiar to the Hepaticae.

Discussion

The main purpose of this thesis research was to obtain original information about the subcellular structure and organization of the cells composing the leaves of a representative leafy liverwort, Plagiochila arctica. This information could then be used to compare and contrast these cells with cells composing the leaves of other land plants, especially flowering plants. Because the approach was developmental, it is possible to discuss some of the changes in subcellular structure and organization that led to the fully differentiated leaf cells.

The organellar components of plant cells that more clearly distinguish them from other eukaryotic cells are the walls, the plastids (or chloroplasts), and the prominent central vacuole. Differences in the structure, composition, and distribution of these same organelles are often critical determinants of the different cell types that occur in land plants. For the foregoing reasons, this discussion will largely focus on the development and differentiation of the walls, plastids, and vacuoles in relation to the development and differentiation of leaf cells. Additional attention will be given to the oil bodies, however, since these structures are unique to the cells of liverworts.

Cell wall formation

There are three aspects of cell wall formation that warrant discussion based on the results of this study. These are the formation of new walls, the "prediction" of the site of new wall

formation, and elaboration or modification of wall surfaces.

As pointed out in the "Results" section, the walls of the two cells comprising the primordium are uniformly thin as is characteristic of meristem and/or parenchyma cells (Fig. 15). During the course of the development of each new leaf, numerous new cell walls are formed in the process of generating the cells that comprise a mature leaf. It could be seen that in a dividing leaf cell of Plagiochila arctica the newly forming cell plate begins its formation in the middle of the cell (Figs. 18-22). Microtubules could be seen at right angles to the forming cell plate (Figs. 19-22). A great number of vesicles could be seen, some of which had apparently recently fused in the forming cell plate (Figs. 21,22). Pieces of endoplasmic reticulum, entrapped in the forming cell plate, were visible. The entrapped pieces of endoplasmic reticulum are thought to be the precursors of the plasmodesmata (e.g. Hepler, 1982; Lloyd, 1987). Extending beyond the phragmoplast, microtubules can be seen which are thought to be directing the future formation of the extending cell wall (Figs. 21,,22). Occasional profiles of Golgi and their derived vesicles are seen (Fig. 24). It can thus be confirmed that the new walls in Plagiochila arctica are laid down by means of a phragmoplast has been described in higher plants (Pickett-Heaps and Northcote, 1966a). Although the involvement of phragmoplasts in new cell wall formation had been established for other bryophytes (e.g., Brown and Lemmon, 1988b; Diers, 1965; Fowke and Pickett-Heaps, 1978; Horner et al., 1966; Lehmann and Schulz, 1969; Schnepf, 1973), it had not been previously established for any leafy

liverwort.

The cell walls of higher plants may range in thickness from 0.1 µm to many micrometers. The cell wall adds rigidity to the cell and allows limited communication between plant cells. The cell wall is composed of cellulose fibers held together in a matrix of protein and polysaccharide. Cortical microtubules have been observed lying parallel to the cell wall which in flowering plants are believed to direct the orientation of cellulose microfibrils (Ledbetter and Porter, 1963; Ledbetter, 1967). The formation of the cell wall begins with the formation of a cell plate (Hepler, 1982; Lloyd, 1982). In P. arctica throughout the development of the leaves cortical microtubules were observed. They were first seen in the apical cell (Fig. 13). They continued to be seen through subsequent stages of leaf development (Figs. 23A&B, 41, 49).

Like the phragmoplast, another aspect of new wall formation that is considered characteristic of land plants is the formation of a preprophase band. Immunofluorescence microscopy has been used to demonstrate the presence of a preprophase band in higher plants which determines the site of subsequent cell division (e.g., Gunning and Sammut, 1990; Wick and Duniec, 1983, 1984; Wick et al., 1981). This is a 1-3 µm band of up to 100 microtubules which encircles the cell just below the plasma membrane prior to prophase. This band disappears before mitosis begins.

However, the presence or absence of a preprophase band does not seem to be consistent in the bryophytes. A preprophase band has been shown to be present in some bryophytes. However depending on

the stage of development of the plant they were studying, other researchers have not found preprophase bands either in the species they looked at, or in the particular stage of development or type of cell under investigation.

A few examples of the research noting the presence or absence of preprophase bands are as follows. Using indirect immunofluorescence, it was shown in the hornwort, Phaeoceros laevis (Brown and Lemmon, 1988b), that an asymmetrical preprophase band was present. The microtubules of the preprophase band are in a tight band over the plastid isthmus but are in a widely spaced band on the other side of the cell over the nucleus. The band encircles the cell in the future plane of division (Brown and Lemmon, 1985, 1988b).

The moss genus, Sphagnum, was shown to have a preprophase band (Schnepf, 1973). Preprophase bands were not found in protonematal stages of the moss, Funaria (Schmiedel et al., 1981) or Physomitrella (Doonan et al., 1987). However, they were found in developing leafy shoots of these species (Schmiedel et al., 1981; Doonan et al., 1987). These developmental studies were done using either mosses or hornworts.

In the hepatics, the thalloid liverwort, Marchantia, was used. Two species, Marchantia polymorpha and M. berteroana, were studied. Distinct preprophase bands were not observed in these species in maturing thallose tissue (Fowke and Pickett-Heaps, 1978). However, in observing developing Marchantia gemmae, groups of microtubules were seen which the authors concluded "may represent

a modified or primitive preprophase band" (Fowke and Pickett-Heaps, 1978). Other authors in studying Marchantia spp. found broad preprophase bands containing a few microtubules in cells dividing to form mucilage papillae (Galatis and Apostolakos, 1978a, 1978b).

In Plagiochila arctica, in a cell with already formed walls containing numerous plasmodesmata, cortical microtubules can be seen lying parallel to the cell wall in a section just below the plasma membrane and cell wall (Fig. 23B). They can also be seen next to the cell wall in a cross section through a cell wall (Figs.13, 23A,41,49). However, the presence of a preprophase band in Plagiochila arctica has not yet been established using standard thin sectioning techniques. In some cells, though, aggregations of cortical microtubles were seen (Fig. 13). These may indicate the future presence of a preprophase band. It will take the use of indirect immunofluorescence methods, such as those used by Brown and Lemmon (1990a, 1990b) to clearly demonstrate whether or not a preprophase band is present in the dividing leaf cells of the leafy liverwort, Plagiochila arctica.

Another feature of the walls of plant cells is the presence of plasmodesmata. In P. arctica, as in higher plants, plasmodesmata are present in the leaf cells. Plasmodesmata were first described by Tangl in 1879 (cited by Robards and Lucas, 1990). "Plasmodesmata are cytoplasmic connections between adjacent plant cells, constituting the structural and functional analogs of gap junctions found between the cells of animals; as such, they create an intercellular continuum - the symplasm." (Robards and Lucas, 1990).

Since then they have been reported at some stage of development in all higher plants examined (for review see Robards and Lucas, 1990) as well as in many lower plants. The frequency of plasmodesmata varies greatly between different cell types. There may be less than 1 to several dozen per square micrometer of cell surface (Holtzman and Novikoff, 1984). In higher plants depending on the species looked at, their frequency has been reported as between 0.1-10.0 per μm^2 (Robards and Lucas, 1990), and between 3-15 per μm^2 (Gunning and Robards, 1976). Their structure has been examined at the electron microscope level. It is now generally accepted that they consist of three parts: a narrow desmotubule derived from pieces of endoplasmic reticulum being caught up in the forming cell plate. This is surrounded by a space, or cytoplasmic sleeve, which in turn is surrounded by the plasmalemma. The desmotubule is described as containing a central dot, 1.0 nm in diameter, surrounded by a pale ring about 12 nm in diameter, covered by an electron opaque layer a few nanometers thick. The width of the cytoplasmic sleeve has been estimated as 5-6 nm (Overall et al., 1982). Depending on the plants or plant parts looked at, the total diameter of the plasmodesmata has been estimated as being from 20 nm (Schnepf and Sych, 1983), to 35-40 nm (Gunning and Robards, 1976), to as high as 50-100 nm (Holtzman and Novikoff, 1984). In P. arctica they fall within this range, being approximately 40 nm in diameter (Fig 23B).

As in other plants, plasmodesmata have been observed in bryophytes. Plasmodesmata were examined in bryophytes at the light microscope level before 1914 (reviewed by Pisnernik, 1914).

Pisnernik examined 29 species and found plasmodesmata in 27 of those looked at (Pisnernik 1914). Five of these were Hepaticae. Wiegand (1954) also examined Hepaticae at the light microscope level and found plasmodesmata in sixteen of twenty-two species examined. Both investigators blamed their lack of finding plasmodesmata in all species examined on the methods used. Whenever young leaf cell walls were examined, the presence of plasmodesmata was observed. More recently, plasmodesmata were examined in a moss, Sphagnum palustre L. (Schnepf and Sych, 1983). They examined cross-sections of inner walls of fixed young buds which would include developing leaves. Their work too was done at the light microscope level. As the young leaves of Sphagnum are uniformly 15 μm in thickness they considered it "valid to compare the number of plasmodesmata in the different walls directly" (Schnepf and Sych, 1983). They estimated there to be 12-22 plasmodesmata per μm^2 in S. palustre. They found the plasmodesmata to be distributed evenly when comparing living cells of the same cell type. However, in a wall between a particular cell that would remain metabolically active (chlorocyte) and a cell that would soon die (hyalocyte), they found a diminished number of plasmodesmata when compared to the number of plasmodesmata between two chlorocytes. As they noted a decrease in the number of plasmodesmata per μm^2 area after equal cell divisions and an increase in cell size, they attributed this to a thinning out of the number of plasmodesmata caused by cell wall expansion. After a later unequal division, the number of plasmodesmata increased in

certain cell walls. However, they reported no evidence for secondary formation of plasmodesmata. They also saw no branched plasmodesmata indicating division of existing plasmodesmata as reported by Krull (1960). In another moss, Polytrichum, the number of plasmodesmata per parenchyma cell cross wall was estimated to be 9-12 per μm^2, whereas in the leptoid end wall there were 16-20 per μm^2 (Eschrich and Steiner, 1968).

Plasmodesmata were observed in Plagiochila arctica also. They were looked at throughout the developmental stages of the leaves. In P. arctica there are a greater number of plasmodesmata seen in a section through a wall between the apical cell and that of a recently cut off merophyte compared to that of a wall between mature leaf cells. When seen in longitudinal section their number can be as many as 7.5 per μm line segment in the merophyte walls (Fig. 9-12) compared to as few as 0-3 per μm line segment in mature leaves (Figs. 38-39). When the section is cut through the wall so that the plasmodesmata are seen in cross section, their number per area of wall can be better estimated. In a single profile if μm^2 sections are compared of the cell wall of a young leaf, their are 5-30 plasmodesmata per μm^2 (Fig 23B). In mature leaves there were from 0-5 per μm^2. This decrease is presumed to be caused by cell wall expansion as noted by Schnepf and Sych (1983). Their distribution therefore would appear to be random, not distributed in pit fields (Fig. 16). This corresponds with that of the distribution of plasmodesmata as seen by Wiegand (1954) in Hepaticae at the light microscope level. Occasionally a branched plasmodesmata was

observed in P. arctica (Fig. 12) as reported by Krull (1960) for another genus. However no direct evidence for an increase in the number of plasmodesmata was observed. As a lesser number of plasmodesmata were observed in older walls, it is assumed that there was no increase over the initial number formed during cell wall formation. This corresponds with the observations of others studying bryophytes (i.e., Schnepf and Sych, 1983).

Another feature of wall development that the leaf cells of P. arctica shares with other land plants, is that of the elaboration or modification of wall surfaces. As the leaves of P. arctica are a single layer thick, each cell contains outer walls that interface with the atmosphere such as epidermal cells do in higher plants. In the apical cell and the leaf primordium which has just been cut off from the apical cell and has not yet expanded, the outer wall is approximately 0.28-0.32 µm in thickness. Then as the cells divide and expand to form the leaves, there is at first a slight thinning and then a gradual thickening of the outer wall as the cells develop to maturity. The outer wall of the young leaf cells, leaf 2-5 range in thickness from 0.16-0.20 µm. In the early stages as the leaves continue to develop they are also dividing and increasing in overall size so there is little change in the thickness of the wall. However, as the leaves reach maturity by leaf 8-9 the outer walls have increased to 0.44-0.66 µm. In addition to obvious thickening of the entire cell wall, a cuticle is present. Additionally the corners between two cells show increased thickening up to 2 µm between adjacent cells (Figs. 29, 35, 36). There is also a thickening between

all the corners of the interior walls of the cells especially where three or more cells meet (e.g., Figs. 6, 12,18, 38, 43, 50A-D). Because of this thickening the leaf cells of the family Plagiochilacae, and even most of the Jungermanniales, have been refered to as being "predominantly collenchymatous (Schuster, 1966). In bryophytes these thickenings are often referred to as trigones (Schuster, 1966; Schofield, 1985). This feature fits the description of angular collenchyma as described by Cutter (1978). Others have defined collenchyma as "plant tissue in which the primary cell walls are thickened, especially at the cell corners." (Lackie and Dow, 1989). Bryologists, e.g., Smith (1990), have described many leafy liverwort leaf cells as being collenchymatous, that is "of cells having thickened corners." Most leaf and stem cells of flowering plants are collenchymatous. Collenchyma cells are "plastic" in nature. Liverworts are not 'higher plants' and therefore, these cells may not be collenchyma in a strict higher plant sense. However, by tradition they have been designated as collenchymatous by bryologists because of their great similarity to higher plant collenchyma (e.g.., Schuster, 1966).

The presence of "lomasome" like areas is often seen. These apparently are the site of deposition of new wall material. They appear to be areas outside the plasma membrane which contain small vesicular-like inclusions apparently adding material to the cell wall. They are not just seen at the corners of the outer walls (Figs. 26,29,32) and where the corners of the inner walls meet (Figs. 18,20,25), but may be dispersed along the cell wall (Fig. 17).

Occasionally layers of membraeous like material are also observed (Figs. 12,35). This has also been observed in higher plants. Occasionally a small vacuole-like compartment can be seen within the cell containing similar appearing inclusions (Figs. 9, 24).

Chloroplast development

Another important feature of higher plants shared by bryophytes is the presence of abundant, lens-shaped chloroplasts in the leaves. The existence of chloroplasts in plant cells has been known for some time. They were first observed by Nehemiah Grew in the 17th century. Ingen-Housz (1779) showed that only the green parts of plants had the ability "to correct bad air" in sunlight. Tschirch (1884) first postulated the presence of a chloroplast membrane. Van Mohl (1873) observed starch grains within the chloroplasts and Von Sachs (1887) demonstrated that they were the product of assimilation of CO_2 and light. Therefore, before the end of the 19th century the existence and essential function of chloroplasts was known.

The photosynthetic cells of plants all contain chloroplasts as it is within this organelle that photosynthesis takes place. Chloroplasts have been described from many plants. In higher plants most of the chloroplasts are found in the palisade and spongy mesophyll of leaves. They are also found in guard cells of the epidermis. In many plant species the chloroplasts of other epidermal cells are small and few in number or the plastids are not developed into chloroplasts. In addition, chloroplasts are found in the outer cells of the stem and any other green part of the plant such as

immature flowers, fruits, pods or seeds. Chloroplasts are generally not found in the meristematic cells of the shoot and root. Higher plant chloroplasts are generally 4-8 μm in diameter, though in many species they can be smaller or larger. There is a wide range in the number of chloroplasts found per cell. For example, in the palisade cells of spinach leaves there are 300-400 chloroplasts per cell. In contrast the leaves of the cocoa tree, Theobroma cacao, have only three chloroplasts per cell.

The basic structure of a mature chloroplast is the same. That is, it is an organelle bounded by a double membrane 100 Å (Mercer, 1960) to 300Å thick (Weier and Thomson, 1962). Between the two membranes, outer and inner, is an intermembrane space. Within the chloroplast is found another membraneous component the thylakoid membrane, so named by Menke (1962). The thylakoid membrane is frequently stacked in grana. There are inter-thylakoid and inter-granal connections creating a single, membrane enclosed cavity distinct from the surrounding stroma (Heslop-Harrison, 1963). The grana are 3000-6000Å in diameter. There are usually 5-20 grana per chloroplast in angiosperms, but there may be as few as two or as many as 60. Each part of the chloroplast is specialized in some way. The outer membrane is highly permeable. The inner membrane is less permeable and contains transport proteins. The stroma contains many soluble enzymes. The thylakoids are the site of the photosynthetic light absorbing system, the electron-transport chain, and an ATP synthetase.

It is generally accepted that the chloroplasts of plants

originally develop from preexisting plastids (e.g., Gunning and Steer, 1975), although it has also been shown that mature chloroplasts can divide and thereby increase the number of existing chloroplasts (Possingham and Saurer, 1969). The plastids are largely undifferentiated. They are double membrane limited and the internal stroma has a granular appearance. No to few thylakoids are seen. In young meristematic tissue, in recently derived cells, the plastids have this appearance. As the cells mature, thylakoids can be seen developing and eventually stacks of grana are visible. In plastids the interdisc space in the grana is 50-60Å. Lipid inclusions begin to appear. These osmiophilic granules or plastoglobuli may be few or many in number. In spinach they are small, 50-220 nm and as many as 800-1500 may be found in each chloroplast (Lichtenthaler, 1969). In older cells, particularly in storage tissue, starch granules are visible. In mesophyll cells there are usually 1-5 starch grains per chloroplast, but more are found in other chloroplasts such as bundle sheath chloroplasts of C_4 plants. The mature chloroplast is usually elongate in shape, contains a few to several stacks of grana, lipid inclusions and starch grains. The stacks in the grana are closer in mature chloroplasts and the spacing may be 20Å or less. Grana are interconnected in the chloroplasts by helical frets and all helices wind in the same direction, usually right handed (Paolillo, 1970). In a study of 910 grana from 7 species of flowering plants, it was found that there was a range of 5-47 grana/plastid (Paolillo, 1970).

Chloroplasts have been studied in bryophytes to a limited degree, mostly describing fully differentiated chloroplasts. The

gametophytic cells of Anthoceros have been examined (Brown and Lemmon, 1988b). The mature gametophyte cell contains one large chloroplast, 40 μm in length. It is surrounded by a double membrane, has similar structures to grana, and contains a pyrenoid as do most algae. The pyrenoid is 4-10 μm long and is surrounded by starch grains. The gametophytes of Phaeoceros also contain only one large chloroplast in which pyrenoid bodies are found (Bold et al., 1987). The mature leaves of mosses, e.g., Polytrichum, Atrichum, contain chloroplasts with and without pyrenoids, and are similar in structure to chloroplasts of higher plants (Paolillo, 1964, 1969). There have been a limited number of studies of plastid development. However, most studies have been done using mosses. Just a few will be reviewed for comparison with those of Plagiochila arctica.

In one study the plastids of the capsule and sporogenous cells of Polytrichum commune were examined using both light and electron microscopy (Paolillo, 1964, 1969). The plastid profiles of the sporocytes were described as flattened lobed structures, containing few or no lamellae and no starch. If they contained lamellated structures, they were "composed of parrallel discs, or compartments" (Paolillo, 1970). The locular space was 50Å, and the interdisc space was equal or greater. In comparison the chloroplasts of the cells of the capsule wall contained many grana with a similar locular space, 50Å, but a much lower interdisc space, 20Å to no space at all (Paolillo, 1970). An area called a plastid center was identified as the possible site of lamellar expansion.
The plastids were not all uniform in appearance in the cells of the

capsule wall. Some contained bands of discs rather than grana as in Euglena (Gibbs, 1960). Most chloroplasts are found in the cells of the inner wall. As the chloroplast develops each has stacks of grana.

Another study was of the development of chloroplasts in six species of mosses during sporogenesis (Jensen and Hulbary, 1978). Development of chloroplasts in sporogenesis is somewhat different than that of gametophytic tissue of leaves. In sporogenesis, there is a reduction in chloroplast number until only one chloroplast is found per sporogenous cell. This reduction may occur by no increase in plastid number during mitotic divisions or by fusion of plastids within the sporogenous cell (Jensen and Hulbary, 1978). Starch grains, lipid inclusions and thylakoids stacked in grana (2-15 thylakoids per grana) connected by a fretwork are first seen during this reduction process. However, the single chloroplast then becomes dedifferentiated, the lamellar system is reduced and only occasional starch grains and lipid inclusions are present. In this spore-mother-cell stage of development the plastid then divides twice to produce four plastids. In these plastids, few to no starch grains and lipid inclusions are seen. The grana-fretwork system becomes reduced and most of the remaining thylakoids appear single (Jensen and Hulbary, 1978). This would compare to the plastids in the early stages of leaf development in Plagiochila arctica. There is then a secondary accumulation of starch in these cells, though the grana-fretwork system still remains reduced. Invaginations of the inner plastid membrane were observed in the sporocytes before meiotic divisions (Jensen and Hulbary, 1978). After meiosis, each

spore contains a single plastid with large starch grains and a weakly developed membrane system. The plastid then forms lobes, which are blebbed off to form proplastids (Jensen and Hulbary, 1978). The inner membrane forms invaginations which appear to be released as vesicles within the plastid stroma. The vesicles may fuse, forming new membranes within the plastid. The grana-fretwork system develops. The proplastids are about 2 µm at this stage. As the plastid matures, it increases in size as well as internal complexity. The mature spore may contain 15 chloroplasts 5 µm in length surrounding a central nucleus (Jensen and Hulbary, 1978).

During development the sporogenous cells are surrounded by an inner and outer tapetal layer. The layer of cells on either side of the sporogenous tissue is secretory. The plastids in these layers differ in appearance from those of the sporogenous tissue. The cells have a dense cytoplasm with numerous cellular organelles. The plastids in this tissue vary in appearance in different species. In some species, e.g., Mnium rostratum, there are large starch grains and the grana-fretwork system is difficult to detect. In others, e.g., Mnium cuspidatum, there are fewer starch grains and a distinct grana-fretwork network can be seen (Jensen and Hulbary, 1978).

A recent publication (Duckett and Renzaglia, 1988) reviewed electron micrographs of bryophyte plastids published over the last 25 years by a number of different authors. The bulk of the literature had to do with mosses. There were differences observed in the appearance of thylakoid systems within the plastids. Some have few thylakoids, whereas others contain many grana and an extensive

fretwork. It was stated that "in most bryophytes the meristems, including apical cells, contain chloroplasts similar to those in mature photosynthetic tissue" (Duckett and Renzaglia, 1988). A few hepatics were mentioned. These authors did not observe plastids in the stem apices of hepatics, other than in Cryptothallus (a thalloid, subterranean, saprophytic hepatic), that understandably lacked a well-defined lamellar system including grana. A few examples of chloroplasts in the Jungermanniales, leafy liverworts, are also mentioned in the review. The similarity of plastids in the stem apex and the mature leaf of Nardia scalaris are pointed out. They are 1.5-2 μm in diameter, contain large lipid globules and 3-5 thylakoid arrays. Each grana is approximately 0.5 μm and contains 3-5 thylakoids. Starch grains are between the groups of thylakoids not against the enclosing membrane. The plastids in mature leaf cells of Trichocolea tomentella have compact grana of 10-20 thylakoids. Young leaf cells and the meristematic region of the stem have grana with fewer thylakoids (Duckett and Renzaglia, 1988).

These examples of leafy liverworts do not include studies of plastid development in the leaves of leafy liverworts from the primordium to the mature leaf.

In Plagiochila arctica there is a distinct pattern of development from undifferentiated plastids in the apical cell to fully mature chloroplasts in mature leaves of this leafy liverwort. In the apical cell and the leaf primordia, the plastids are small and numerous. They range in size from 0.92x.72 to 2.2x1.24 μm. Lamellae are weakly developed or absent (Fig. 6). As the leaf primordia develop to

leaves, so also do the plastids develop and mature. Lamellae begin to aggregate in stacks forming grana. Two to four thylakoids per grana stack are observed. The plastids average 1.28x1.2 to 1.76x1.52 μm in size. As the leaves continue to mature, the internal organelles including the plastids continue to do so also. By leaves 3-4, the grana contain stacks of 2-5 thylakoids. As many as five grana stacks can be seen per chloroplast profile (Fig. 24). The chloroplasts continue to increase in size, being an average of 1.64x1.4 to 2.08x1.64 μm. By leaf 7 starch grains first become visible in the chloroplasts. They are dispersed among the grana (Fig. 32). By leaves 8-9, fully mature chloroplasts can be seen. Their grana contain up to nine thylakoids per grana stack. As many as ten grana stacks can be seen in a single chloroplast section. Their is also an increase in the number of starch grains per chloroplast. As many as 6-15 can be seen per plastid profile (Fig. 43-48). The chloroplasts have increased in size, to an average of 3.76x1.76. Lipid inclusions are occasionally seen in the chloroplasts (Fig. 46). Another interesting feature seen in observing chloroplast development in P. arctica was that of apparent chloroplast division as noted by Jensen and Hulbarry (1978) in mosses and Possingham and Saurer (1969) in spinach. This process was observed in both young leaves (Fig. 16) and older leaves (Fig. 27). The chloroplast appeared to be pinching off in the central portion forming two lobes (Fig. 16). The orientation of the grana stacks appeared rearraged where one stack was more or less at right angles to the rest (Fig. 27).

The chloroplasts of the mature leaves of Plagiochila arctica

are comparable to those observed in other bryophytes and also those of higher plants. Chloroplasts were also observed in the young stem tissue below the apex. These too had developed stacks of grana and the presence of starch grains was observed. The development of the chloroplast from plastid is typical of those as described for other bryophytes (Jensen and Hulbary, 1978). Their size fits within the range of those reported for both bryophytes (Jensen and Hulbary, 1978; Duckett and Renzaglia, 1988) and those of flowering plants (Alberts et al., 1983). Their number of grana/plastid falls with the range of flowering plants (Paolillo, 1970).

Vacuole development

Most living, vegetative cells of plants contain vacuoles. In embryonic or meristematic stages of development of cells, vacuoles are usually small, numerous, and occupy only a small part of the cell. In mature cells, they are large and occupy most of the cell volume. They can comprise as much as 80-95% of the cell. The vacuoles are surrounded by a single membrane, the tonoplast, which separates each vacuole from the cytoplasm. Vacuoles are believed to arise from vesicles derived from the endoplasmic reticulum and the Golgi apparatus. Or, alternatively, vacuoles can arise from preexisting vacuoles. Although they mostly contain water, they store and transport nutrients, metabolites and waste products. Transitory membraneous inclusions are sometimes seen during development. Their most important functions are to help maintain the proper water balance in the protoplasm as well as to maintain or exert turgor pressure and therefore maintain cell size and shape. Other functions

such as storage, autophagy and degradation of materials, have also been attributed to vacuoles in different cell types. In some cells they have been shown to contain hydrolytic enzymes and these are therefore considered to be analagous to lysosomes. Different vacuoles, demonstrating different functions, can be found in the same cell (Alberts et al., 1983).

When the cells of bryophytes are described, vacuoles generally are mentioned as one of the organelles included in the cells as in other plants (e.g., Schofield, 1985). Ultrastructrual studies are usually limited to just mentioning their presence while concentrating on some other aspect of the cell, even when specifically discussing leaves (e.g., Chopra and Kumra, 1988).

As in most vegetative cells of plants the mature leaf cells of leafy liverworts contain much water. This water is mainly contained in large centrally located vacuoles which comprise the major portion of the cell at maturity. As in other plant cells, their major functions appear to be to maintain the proper water balance in the cell and to exhibit turgor pressure and thereby help to maintain cell shape and size.

In Plagiochila arctica, vacuoles are present from the beginning of development of the leaves. They can be seen in the apical cell. The apical cell, except for its position and tetrahedral shape, has the appearance of a typical meristematic cell. In the apical cell the vacuoles are small and numerous and usually surround the central nucleus (Fig. 6). Prior to cell division, i.e., the cutting off of a merophyte, more vacuoles appear to collect toward the side of the

cell where the next merophyte will be cut off. This was observed by Hebant et al. (1978) in Polytrichum commune and Equisetum maximum. This can also be seen in Plagiochila arctica (Fig. 7). In cell division in angiosperms, such as the unequal division of cells in the epidermis of developing leaves of wheat, the displacement of the nucleus to one end of the cell and vacuoles to the other has been observed (Pickett-Heaps and Northcote, 1966a). In the filamentous protonematal apical cells of the moss, Physcomitrium turbinatum, (now P. pyriforme) an uneven distribution of vacuoles was also observed prior to cell division (Jensen and Jensen, 1984).

In Plagiochila arctica as the cell develops and matures the vacuoles become increasingly larger and less numerous. Apparently, they not only increase in size by the addition of more material and water, but also by the fusion of many smaller preexisting vacuoles. Throughout the development of the leaf cells the vacuoles continue to be seen. They vary in size, shape and contents. Some appear very empty and clear (Figs. 34, 43). Others contain a granular matrix (Fig. 37). Some contain membraneous bodies (Fig.39). Others contain a lattice-like appearing structure (Figs. 24, 53). Structures such as these, membranous bodies and lattice-like structures, have been seen in the cells of higher plants and in cyanobacteria (Jensen, 1985). In Plagiochila arctica, none of these features seem to be correlated with a particular leaf, e.g., leaf 2 or leaf 6, or a particular stage of development, but occur sporadically throughout the maturing leaves. At maturity, however, the leaf cell contains a large central vacuole which occupies most of the cell. It appears mostly empty but may

contain some granular material (Fig. 42A&B). The cytoplasmic cell contents containing the nucleus and the other cell organelles can be seen displaced to the periphery of the cell and surround the large central vacuole. Occasionally, a few small vacuoles are still present.

Oil bodies

Oil bodies as found in the Hepaticae are a unique feature unlike anything found in higher plants. They are discrete membrane bound structures containing oil droplets. The early information relating to the discovery and identification of oil bodies has been recorded by many authors. Credit is generally given to the following for the early work. The first to observe and describe oil bodies was Hübener (1834). Mirbel (1835) recognized them as distinct bodies in a thallose liverwort. Pfeffer (1874) named them 'Oelkorper' and Holle (1857) is credited with doing the first detailed study of oil bodies. Goebel (1898) described them as essential oils. Müller (1905, 1939) did a descriptive study of the oil bodies and determined that they contained terpenoids.

Oil bodies are widely distributed in the Hepaticae, especially in the Jungermanniales. Although terpenoids are produced in a variety of land plants, their accumulation and storage within membrane-bound structures in the cytoplasm appears to be a unique feature of the hepatics. Differences in size, shape and number of oil bodies, and the chemical composition of their contents, have come to be an important taxanomic character in the Hepaticae.

Oil bodies vary in size from being very small and many per cell to massive and one per cell. They also differ in being present in all

tissue cells, as in the Jungermanniales, or restricted to specialized cells of the thallus, as in the Marchantiales.

The chemistry of oil bodies was discussed by Müller (1905, 1939). He referred to them as ethereal oils and discussed the optical properties, specific gravity and saponification values of these oils. Specificity of these oils is correlated with diverse types of odors in the Hepaticae. Not all possess a distinctive odor, but in those that do, these aromatic odors are significant in species determination since the odors are not identical between species but distinctly different. Oil bodies are usually colorless, or appear grayish when opaque and segmented or granulate. In a few instances they are brownish. A few Hepaticae have bright blue oil bodies.

The functional significance of these oil bodies is not yet clearly understood. Some believed that they are a repellent to snails (e.g., Stahl 1888). However, others found some species eaten by snails (e.g., Ochi 1960). A recent study found that aphids can get nutrients from bryophytes (Thomas, 1991). Although a few species of liverworts are eaten by insects, many are not. Various authors have thought the oil bodies might be a protection against excessive light (Hieronymus, 1892), desiccation (Gavaudan ,1927; Chalaud, 1931), or against cold temperatures . However, in each case examples of a liverwort species that can grow under these conditions has been found. Schuster (1979) noted that Müller (1939) had suggested that they may have had a physiological function in past ages that is no longer expressed. This idea he says "may be valid but remains wholly speculative." Both Müller and Schuster found oil

bodies present in most of the species they investigated growing in northern areas. Müller collected and examined 115 northern species, while Schuster collected and examined more than 200 (Schuster, 1966). Schuster also noted that the perennial forms of liverworts generally possess them and this may be associated with overwintering, as the annual or biennial forms generally do not contain oil bodies. All this descriptive work of Müller and Schuster was done using the light microscope.

In discussing the cells of Hepaticae using oil bodies as the major criteria they are classified as consisting of two types. The first class consists of those with oil bodies and small, numerous chloroplasts but without pyrenoids. The Jungermanniae are one group that fit into this class. The other class contains no oil bodies, but complex pyrenoids and only one or a few large chloroplasts per cell. The Anthocerotae fit into this class. Schuster (1966) further divided this classification by recognizing two types of oil bodies. Those found in the Jungermanniae which are small to large and may be two to many per cell. They are not usually found in specialized cells but in unmodified cells and the cells usually contain chloroplasts. The second group are large spherical oil bodies, usually one per cell. They fill the cell, and the cells that contain the oil body are usually differentiated in size from neighboring cells. They are few in number and never bear chloroplasts.

In the oil bodies of the Jungermanniae, there is a great deal of variation as to number, size, form and segmentation of the oil bodies. Hence they make a distinctive taxonomic character.

The oil bodies of Plagiochila arctica fall into the description of being "several per cell, usually of relatively fine segments (finely botryoidal), usually moderate in size" "the 'grape cluster' type of Müller" (Schuster, 1966, 1979) (Figs. 32,33,34,36,37).

The origin of oil bodies has been attributed to many different sources. For example some of the light microscopists (e.g., Gauvaudan, 1927; Schuster, 1966) felt they originated in the cytoplasm. Others (e.g., Pfeffer 1874; Pihakaski 1966, 1968) felt they originated in vacuoles. Still others attributed their origin to either the chloroplasts (Kozlowski, 1921) or to mitochondria (Bergdolt, 1926). A few ultrastructural studies have been published on oil bodies in the Hepaticae (e.g., Pihakaski, 1966, 1968; Suire 1970). None of these studies included the leafy liverwort, Plagiochila arctica.

More recently, Galatis et al. (1978a, 1978b) have studied oil bodies at the ultrastructural level specifically studying oil body cell differentiation and the origination of the oil body. They used the thallose liverwort, Marchantia paleacea for their investigations. In Marchantia, the oil bodies occur in specialized cells. Because of this feature, these authors felt they could distinguish their development at an early stage. They occur in meristematic appearing cells with a cytoplasm rich in ribosomes, central nucleus, rough ER, mitochondria, dictyosomes, plastids, vacuoles and undeveloped microbodies. They describe the differentiation of the oil body cell (OBC) as including two distinct processes: 1 "the formation of additional cytoplasm and organelles" and 2 " the reorganization of the cell ... which mirrors the

establishment of polarity." The nucleus is displaced towards one end of the cell and the vacuoles to the other. "... an area is formed clear of large organelles in which the OB will appear later." The presence of microtubules and fusing vesicles is subsequently noted. Their earliest stage of oil body development was recognized as a "rudimentary 'vacuole'". As development proceeds, they recognized the OBC by its larger size, location and association with microtubules, as well as the accumulation of dictyosome vesicles and ER membranes around it. Its membrane is formed by the fusion of vesicles. Lipophilic material is later synthesized in the single oil body. As these are specialized OB cells in the thalloid liverwort, Marchantia, which usually only contain one oil body at maturity, not many in every cell as in the Jungermanniales, similar developmental features have not been described for the leafy liverworts in which development does not appear to be the same. In leafy liverworts where the oil bodies occur in all the cells of the leaves, there is no polarization of the cell before oil body formation, because all the leaves of the cell perform the same function. None are specialized cell types.

Two investigators who did study oil body origin at the electron microscope level in leafy liverworts were Pihakaski (1966, 1968) who observed oil bodies in Bazzania and Lophozia, and Suire (1970) who observed them in Radula. Various methods of oil body development were described. One suggestion was the development of oil bodies is from vacuolar-like structures in which the ER or the Golgi apparatus secrete substances as small droplets. Another

suggestion was that lipid droplets begin to form aggregations which later change to oil globules. The cytoplasm surrounding them differentiates and a membrane delimiting the whole structure is formed. Another suggestion is that oil bodies originate as dilations of ER membranes as vacuoles do, but these structures later develop differentially.

In Plagiochila arctica, the presence of oil bodies is first clearly observed at the seventh leaf stage (Fig. 32). They seem to appear *de novo* . Their is no apparent movement of the nucleus or vacuoles to a polar area of the cell as in the specialized cells of Marchantia. At this stage the appearance of a number of small oil droplets in a sac-like structure delimited by a membrane can be observed (Fig. 32). As the leaves mature the droplets become larger, either by fusion or synthesis of further lipophilic material and the oil body itself increases in size. In a fully mature leaf, several oil bodies can be seen, each containing a few to many densely staining oil globules (Fig. 37). Prior to the clear observation of oil bodies, the earlier cells appear to contain many small vacuolar like bodies, some containing much dense staining material, some more opaque. If the more densely staining vacuolar-like bodies are the precursors of the oil bodies in Plagiochila arctica, it would appear that the oil bodies in P. arctica arise from vacuolar-like structures as suggested by some earlier observers (e.g., Hölzl, 1969; Pfeffer, 1874; Pihakaski, 1968). Because their is no polarity established in P. arctica leaf cells before the formation of oil bodies, a distinct area is not established in the part of the cell where the formation of oil bodies

occurs, as in Marchantia where polarity is established and the single oil body is formed in the central portion of the cell. In P. arctica oil bodies occur throughout the cell. It must again be pointed out that in Marchantia one oil body is contained per specialized cell, almost filling that cell. All the cells of the thallus do not contain oil bodies. In Plagiochila arctica few to several oil bodies are found in all the cells of the mature leaves They do not fill most of the cell. Rather, at maturity the central vacuole is the most prominent feature of the cell. Oil bodies are also present in the stem cells.

Because the oil bodies differ in size, number and shape, as well as in odor or lack of it, they are an important taxonomic character in the identification of the hepatics that do contain them. In many bryophytes, the sporophytic stage between species shows greater variation than the gametophytic stage. Therefore, since the oil bodies occur in the gametophytic stage this makes them very important in the taxa in which they occur.

Conclusions.

This TEM study confirms and further expands what has been previously known about leaf initiation and development in leafy liverworts.

The results are consistent with what is known about subcellular organization in meristematic cells and the changes that occur in them as they differentiate into specialized cell types.

The results of this research also reveal the previously unrecognized fact that all mature cells of leafy liverworts are highly differentiated, manifesting the combined features of a number of

specialized cell and tissue types found in flowering plants:

1. Increased thickening of outer or free walls, the absence of intercellular spaces between the cells and the deposition of cutin,characteristic of epidermal cells and tissues (Fig. 42).

2. The capacity to synthesize and accumulate essential oils (i.e., various types of terpenoids) also characteristic of epidermal cells/tissues of flowering plants, but with the unique feature of enclosing them within specialized membrane bound oil bodies (Fig. 37).

3. Abundant, lenticular, starch synthesizing/storing chloroplasts characteristic of chlorenchyma cells/mesophyll tissue (Fig. 46).

4. Secondarily thickened corners, characteristic of collenchyma cells/tissues (Fig. 35).

5. Numerous plasmodesmata for intercellular transport between adjacent cells (Figs. 6,10,23B).

The differentiation of the separate structural components of liverwort leaf cells while of real interest, is not in itself especially remarkable. What is remarkable is that so many of the components of the individual cells are characteristic of different specialized cells and tissues of higher plants. The single (unistratose) layer of cells that comprise the leaves of liverworts such as Plagiochila arctica have many of the combined features of the epidermal and mesophyll tissues of the leaf lamina as well as the collenchyma tissue of petioles and stems of flowering plants.

Table 1

Relative percentages of leaf volume taken up by organelles during leaf development of Plagiochila arctica Bryhn & Kaal.

	Nucleus	Plastid	Vacuoles	mitochondria	oil bodies
Leaf 1	24 (1.1)	11(0.6)	19(1.2)	7(1.3)	0
Leaf 2-4	22(0.9)	12(0.7)	21(1.1)	7(1.2)	0
Leaf 5-6	18(1.0)	20(0.9)	23(0.9)	7(1.5)	0
Leaf 7	14(1.1)	23(1.3)	37(0.6)	6(1.1)	9 (1.3)
Leaf 8	6(0.8)	15(1.1)	60(0.7)	5(1.0)	10 (1.2)
Leaf 9	2(0.9)	6(1.0)	80(0.5)	2(0.9)	7 (1.0)

Standard errors in parentheses

Table 2

Dimensions in µm through cross-sections of the developing leaves of Plagiochila arctica.

	Leaf number					
	one	2-4	5-6	7	8	9
length	12.0(1.4)	13.2(1.3)	14.7(1.5)	20.3(1.0)	25.6(0.8)	30.0(0.7)
width	7.3(1.5)	8.5(1.2)	9.3(1.4)	15.4(0.9)	18.9(0.8)	20.4(0.8)

Standard errors in parentheses

Table 3

Changes in plastid development related to leaf development in *Plagiochila arctica* (Hepaticae)

"Leaf"	Plastid size	%stroma	%thylakoid	%starch	#grana	#thylakoid/grana	#st grn
Apical	1.5 µm	76	24	0	1-4	2-3	0
1	1.4 µm	76	24	0	1-4	2-3	0
2-4	2.2 µm	71	29	0	2-5	2-5	0
5-6	2.4 µm	63	37	0	3-7	3-5	0
7	2.8 µm	60	38	2	3-8	2-7	1-2
8	3.0 µm	61	34.5	4.5	3-9	3-8	1-4
9	3.76 µm	45	39	16	6-10	2-9	6-15

Plastid size given as average length.
% given as average percent.
# given as ranges found.

Figure 1. Scanning electron micrograph (SEM) of Plagiochila arctica Bryhn et Kaal. SEM of whole plant showing dorsiventral symmetry. Leaves 6-11 are visible. Leaves 1-5 are hidden by the older leaves. 200x.

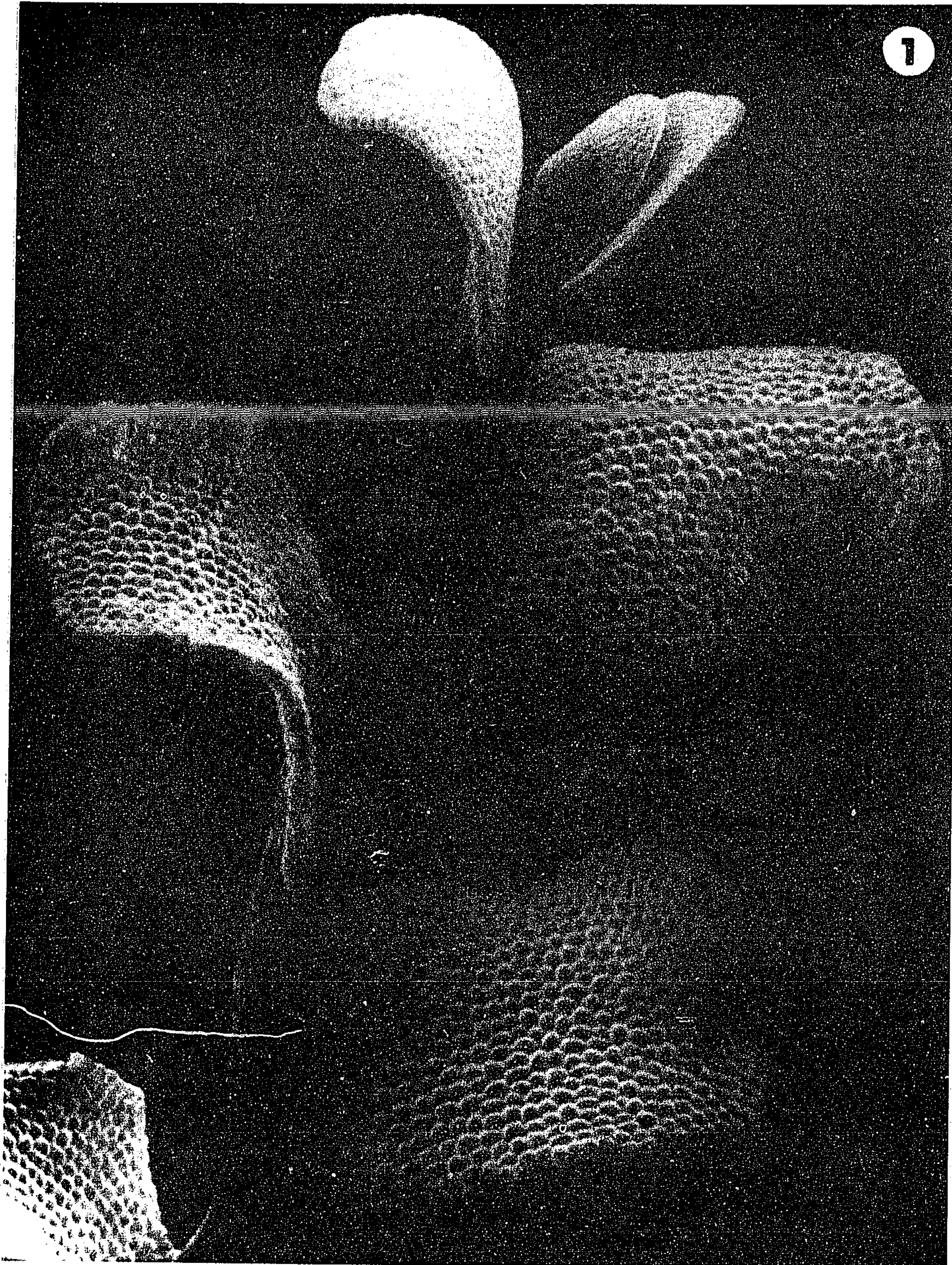

1

Figure 2. Scanning electron micrograph (SEM) of <u>Plagiochila arctica</u> Bryhn et Kaal. SEM of apical portion of plant showing dorsiventral symmetry. Leaves 5 on are visible. Leaves 1-4 are hidden by the older leaves. 300x.

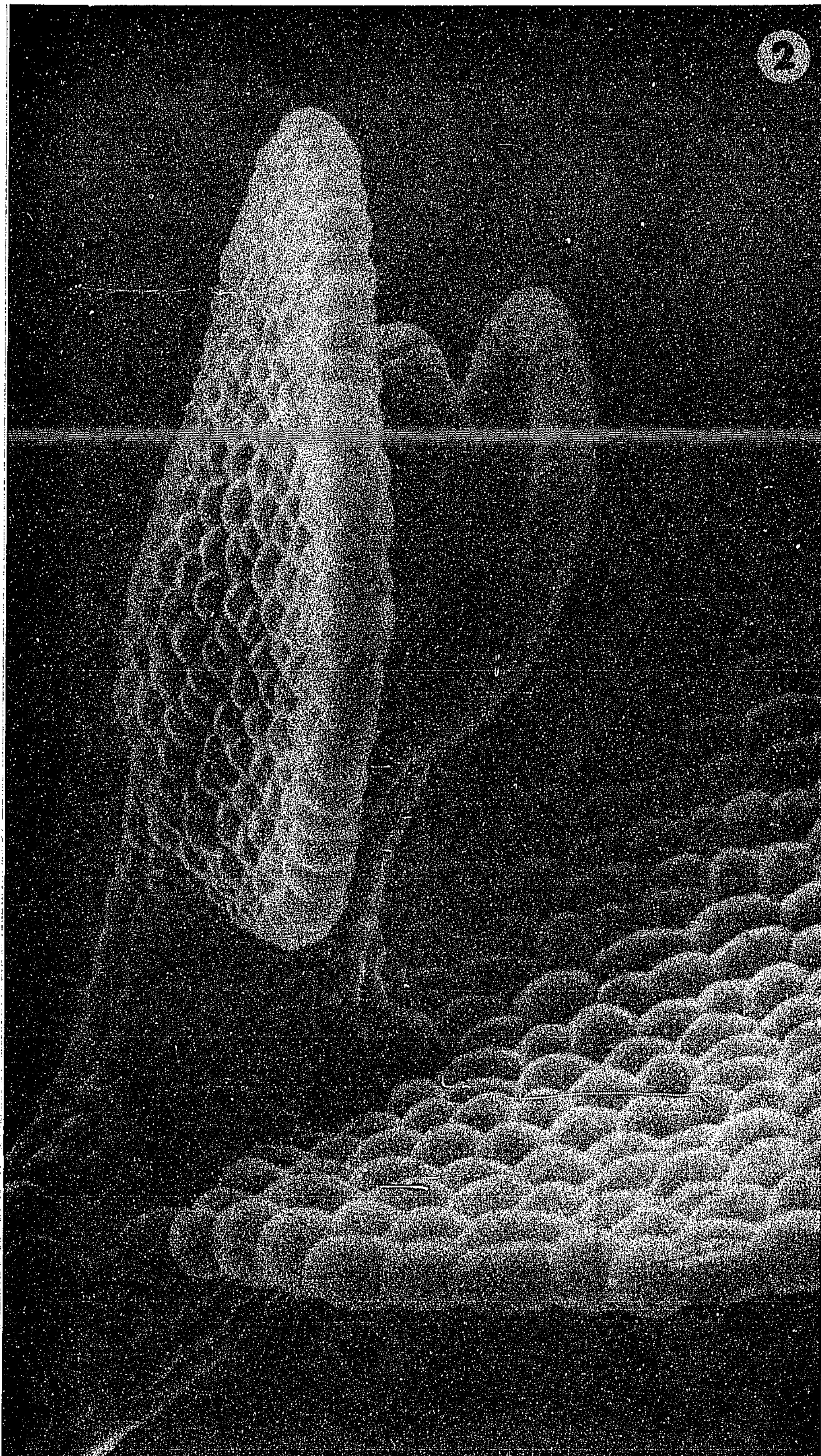

2

Figure 3. Scanning electron micrograph of Plagiochila arctica showing the apical cell (A), the newly cutoff merophytes and young leaves (L1 and L2 are labeled) and a single slime papilla (SP). Scale bar = 100 µm.

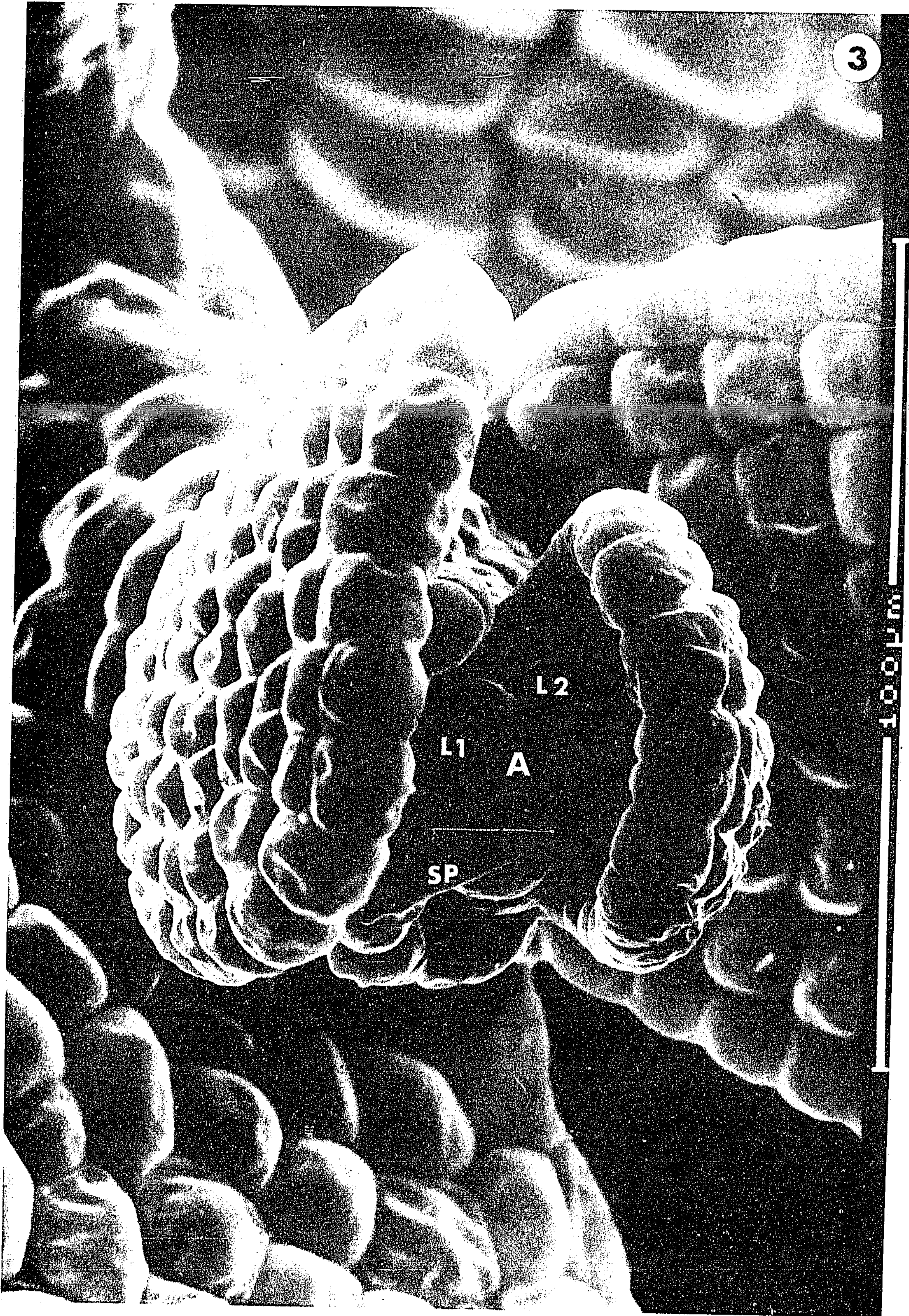

3
L2
L1
A
SP
100µm

Figure 4. Scanning electron micrograph (SEM) of the apical portion of Plagiochila arctica showing the ventral side. The dorsiventral symmetry of the leaves is evident. A single slime papilla is present at the apex, and a pair of slime papillae (SP) are evident several cell rows below. Scale bar = 100μm.

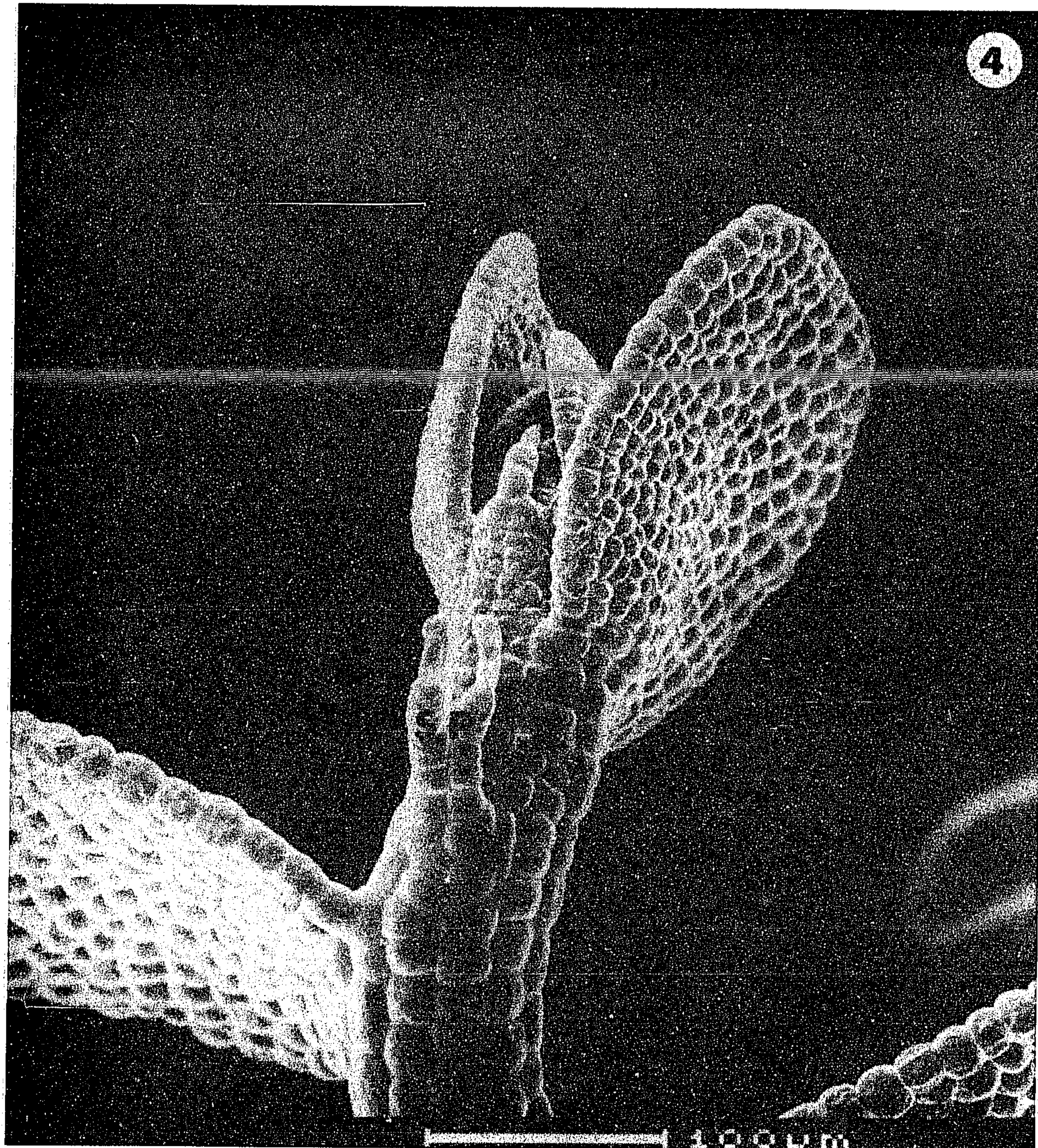

4
100um

Figure 5. Composite of ninety-six transmission electron micrographs of Plagiochila arctica showing a cross section through the apical cell, merophytes, first eight leaves and the slime papillae. 75x.

5

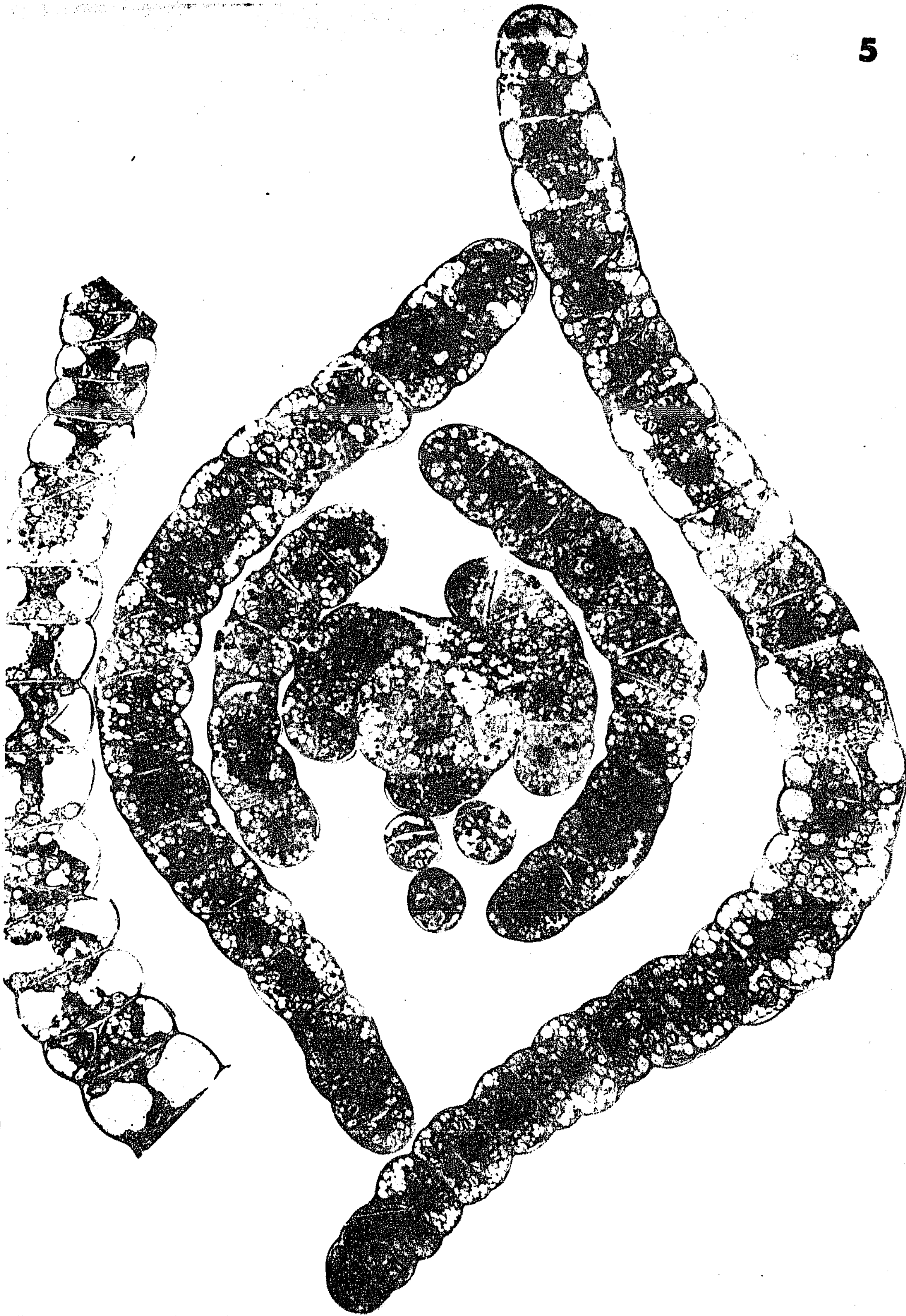


Figure 6. Transmission electron micrograph of a cross section through the apical cell of Plagiochila arctica with a centrally positioned nucleus (Nu). Numerous small plastids (Pl), mitochondria (Mi) and vacuoles (V) are evident. The ventral merophyte is at the lower edge of the micrograph. The left lateral merophyte has divided to form a leaf primordium. The right merophyte is still in the single cell stage. A portion of a young leaf can be seen in the upper right hand corner. 7500x.

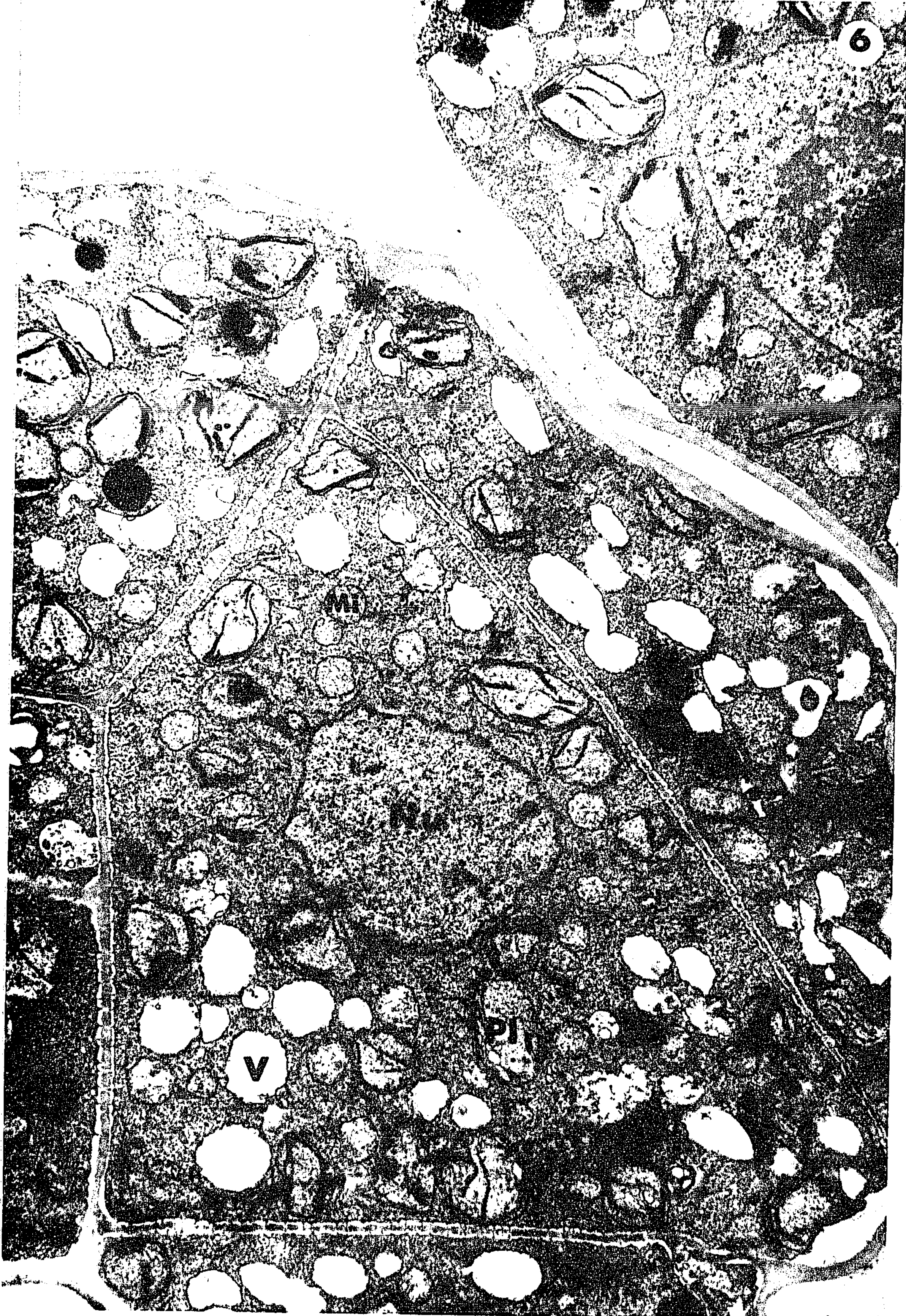

6
Mi
Nu
Pl
V

Figure 7. Transmission electron micrograph of a cross section through the apical cell of Plagiochila arctica with the nucleus (Nu) off center. The right lateral merophyte is the most recently cut off and is still in the single cell stage. Plastids (Pl) , mitochondria (Mi) and vacuoles (V) are evident in the apical cell and the merophytes. 7500x.

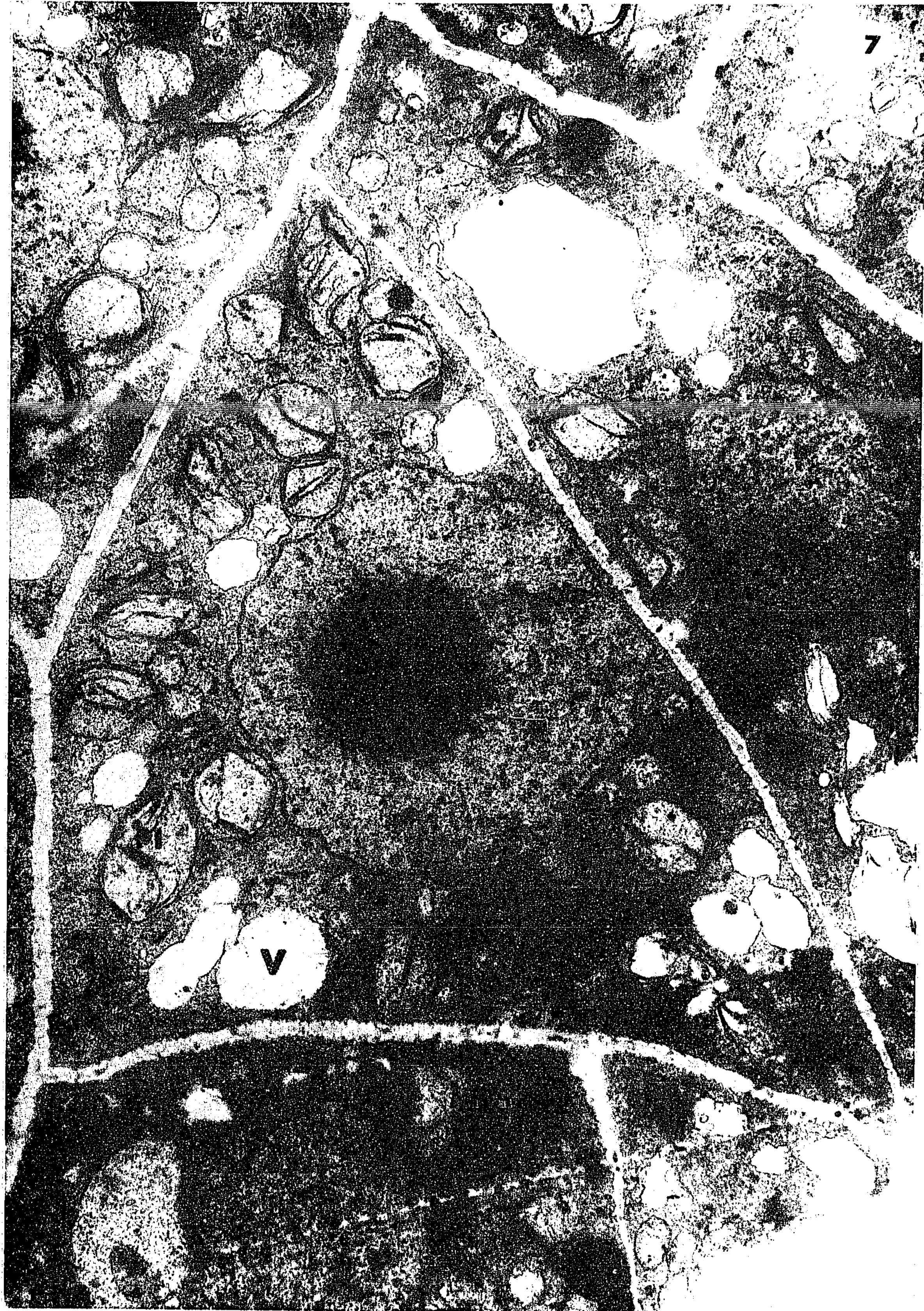

7
V

Figure 8. Transmission electron micrograph of a longitudinal section through the apical cell of *Plagiochila arctica* and the two lateral merophytes. In this section there is a preponderance of vacuoles evident on the side of the apical cell indicating that the next merophyte will be cut off at that side (Hebant et al., 1978). Many plasmodesmatal connections (arrow) are evident between the apical cell and the adjoining merophytes. Corner thickenings are already evident where three cells meet. 7500x.

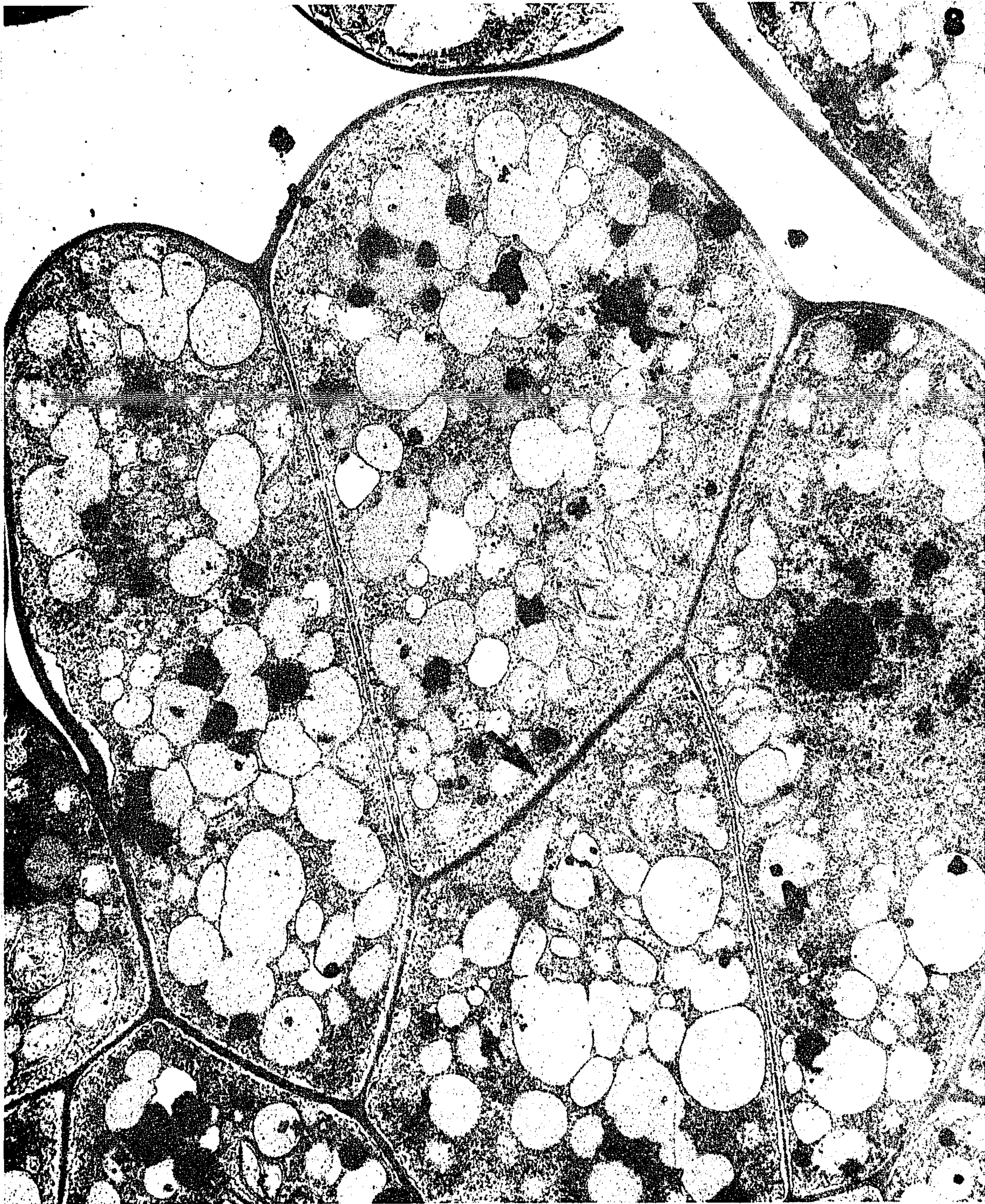

8

Figure 9. TEM of part of the apical cell and adjoining merophyte of Plagiochila arctica. Plastids (Pl) and mitochondria (Mi) are present. A few small vacuoles containing globular inclusions are evident. Endoplasmic reticulum is abundant. A portion of the nucleus (Nu) is shown. Many plasmodesmatal connections (Arrow) between the apical cell and its adjoining merophytes are seen. 9300x.

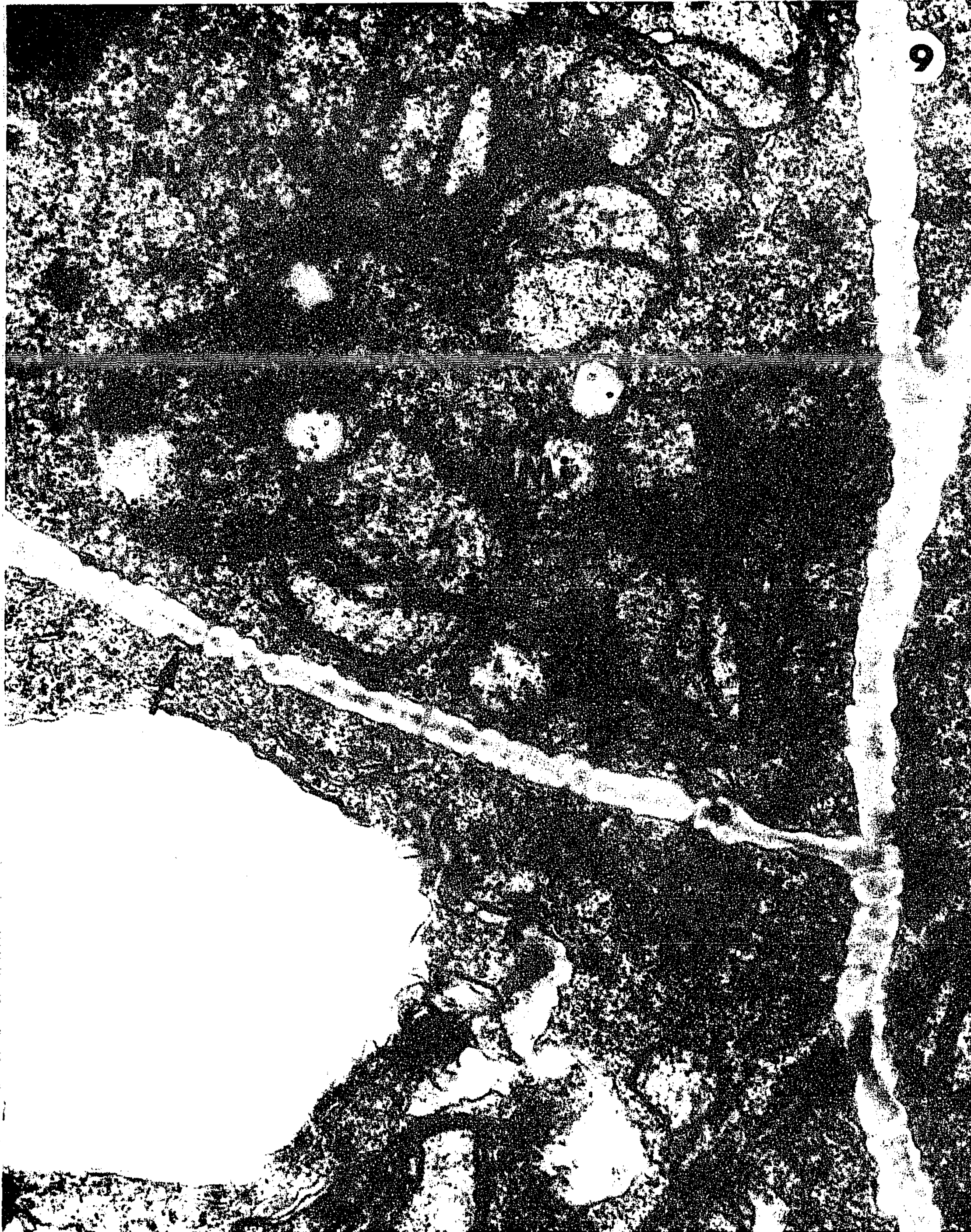

9
M

Figure 10. TEM of a portion of an apical cell of Plagiochila arctica and its adjoining merophytes. Plastids (Pl) and mitochondria (Mi) are evident. Vacuoles(V) mostly empty in appearance are present as is one that contains a membranous like material. Endoplasmic reticulum is seen in all three cells. A portion of the apical nucleus (Nu) is seen. Many plasmodesmatal connections are present. 9300x.

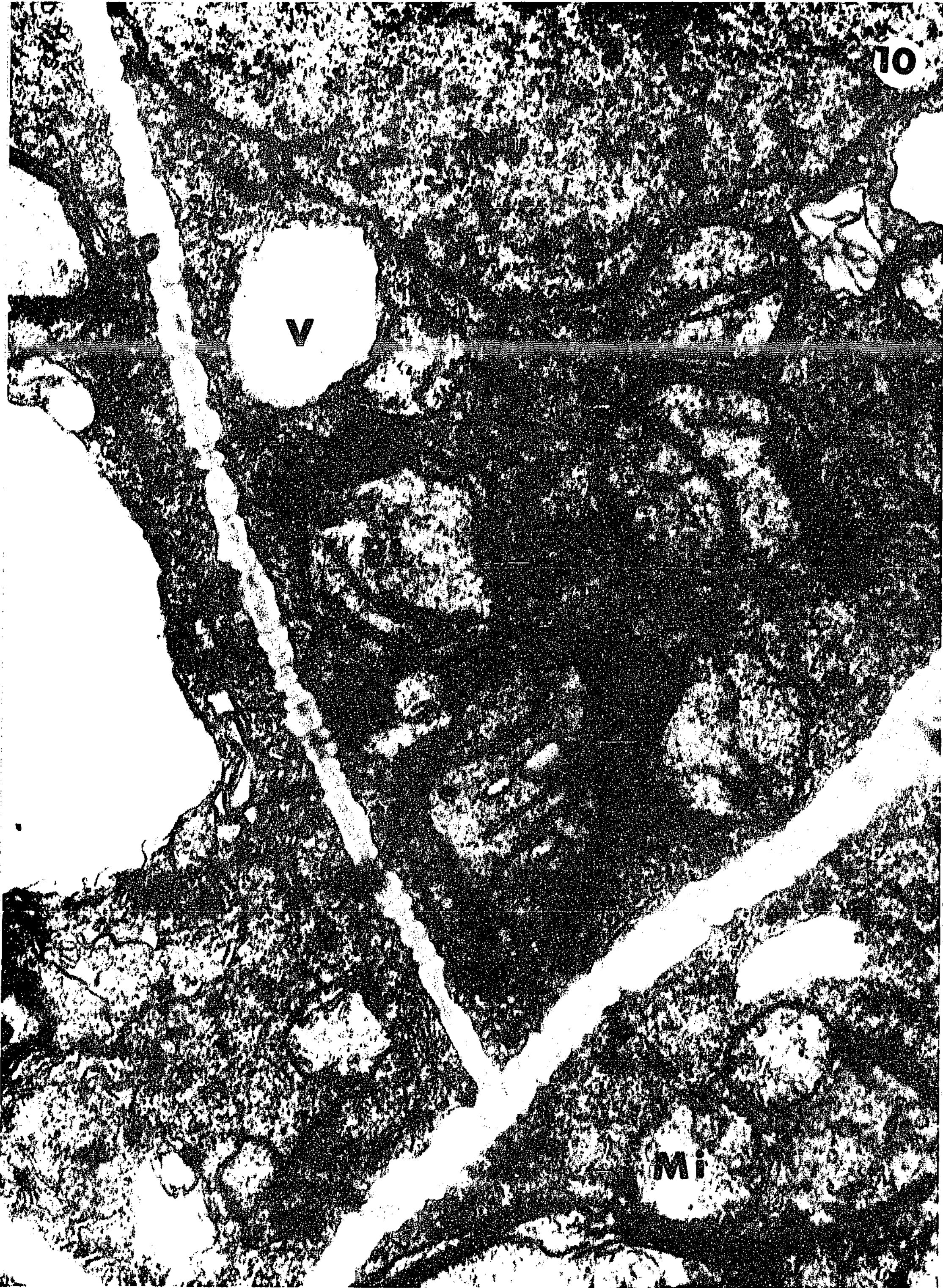

10
V
Mi

Figure 11. TEM of a portion of an apical cell of Plagiochila arctica with adjoining ventral and lateral merophytes. An elongated plastid (Pl) is seen. It contains only a few thylakoids. Vacuolar-like areas are contained within the plastid. Presumably it has elongated prior to division. Mitochondria (Mi) are present. A number of vacuoles (V) are seen, mostly appearing empty. Two in the ventral merophyte contain globular like inclusions. Plasmodesmatal connections are numerous between the apical cell and the merophytes. A portion of the apical nucleus (Nu) with its nuclear membrane (NM) is visible. 9300x.

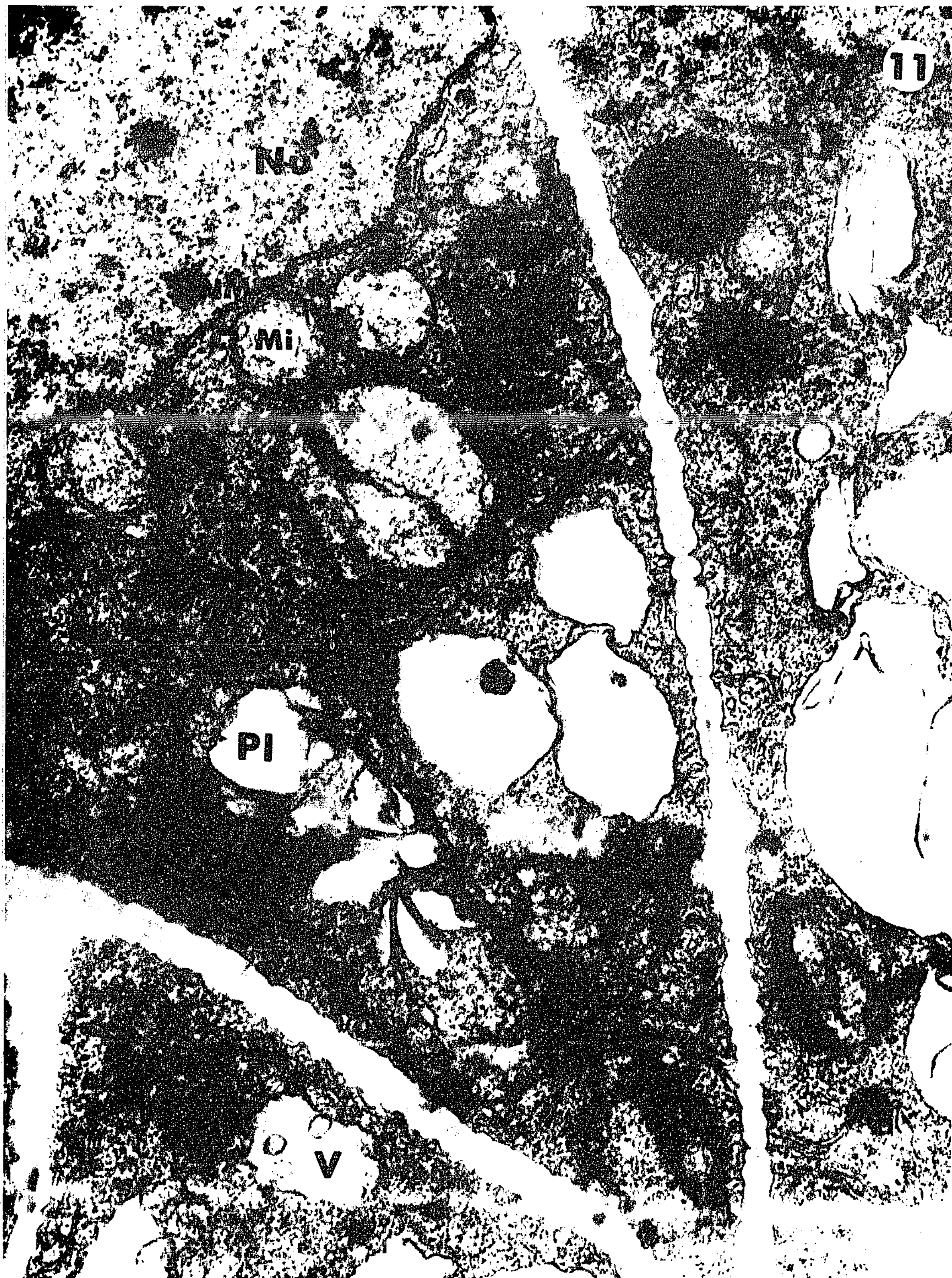

11
No
Mi
Pl
V

Figure 12. TEM through a section of Plagiochila arctica. In the two celled leaf primordium a typical Golgi complex (G) of five layered compartments and vesicles which have budded off is evident. A plastid (Pl) and mitochondria (Mi) are also seen, as are vacuoles. In the adjoining apical cell a membranous addition (Single arrow) to the wall is evident. Plasmodesmatal connections are seen. A branched plasmodesma (double arrows) is visible connecting the leaf primordium and the apical cell. 31000x.

12
G
L
Mi

Figure 13. TEM through a section of Plagiochila arctica showing cortical microtubules (Arrows) in the apical cell and adjoining merophyte. 50000x.

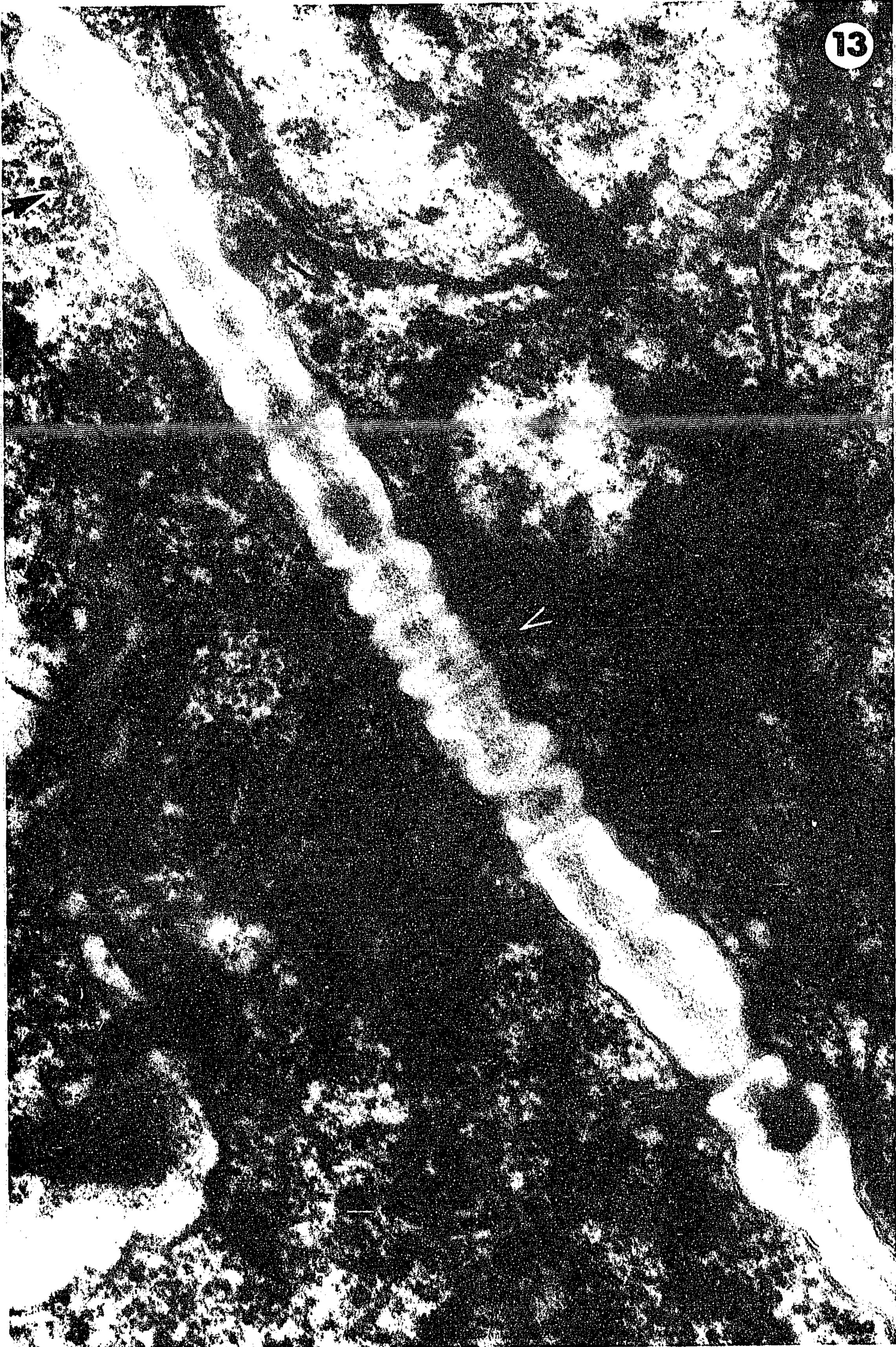

13

Figure 14. TEM of a section through the base of the apical cell and adjoining cells of Plagiochila arctica. Note the numerous plasmodesmatal connections (Arrows) between the apical cell and adjoining cells. 7750x.

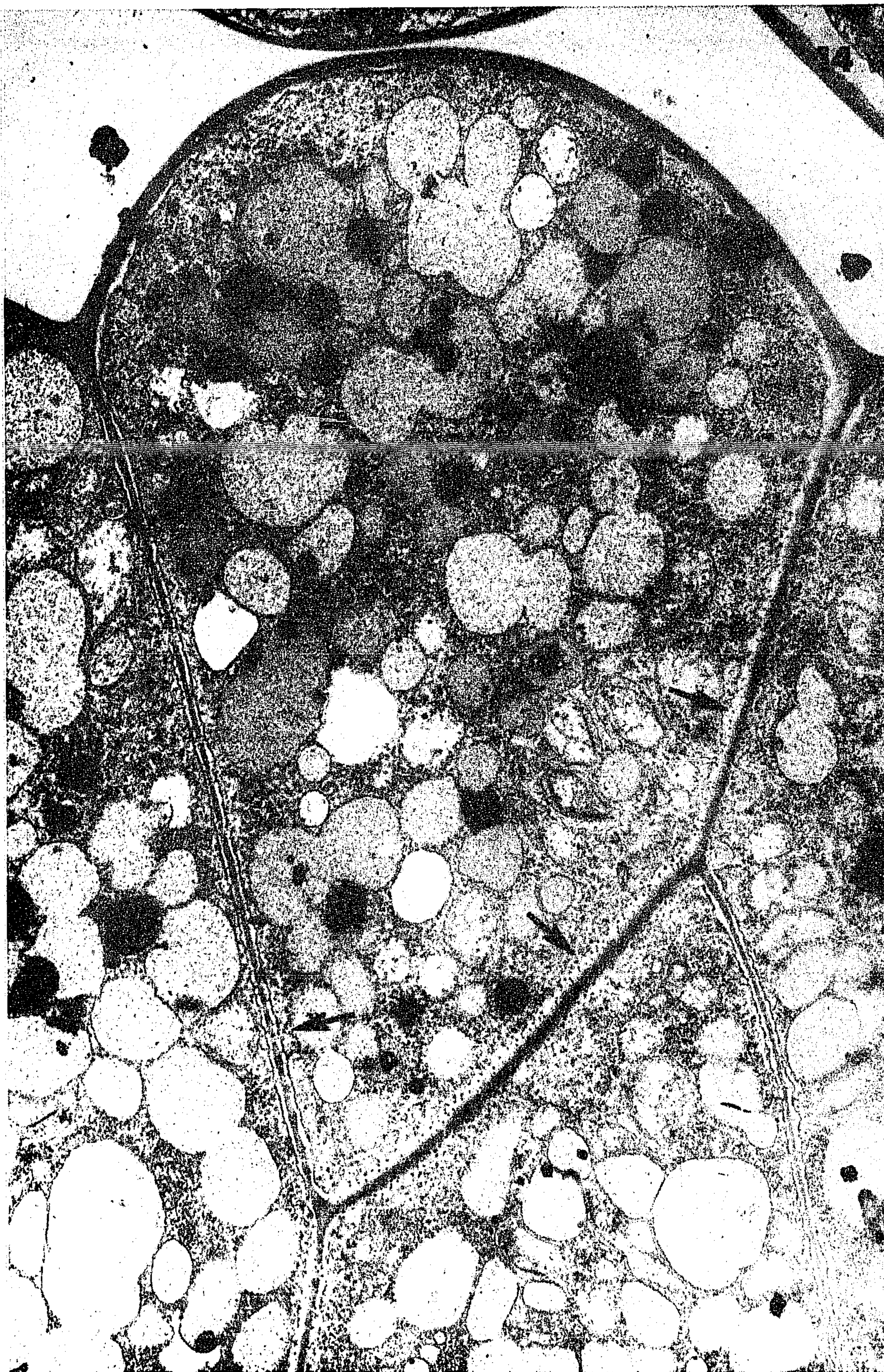


Figure 15. TEM through the two-celled leaf primordium (leaf one) of Plagiochila arctica. The two cells are typically meristematic in appearance. A dense cytoplasm contains the nuclei and the various organelles, i.e., plastids, mitochondria, and vacuoles. The vacuoles are mostly small. Some appear empty while others contain various globular or granular like substances. A cuticle (Arrow) is evident on the outer walls of both the two celled leaf primordium and the next outer leaf wall in the upper right hand corner. A number of lipid inclusions are also present. 7750x.

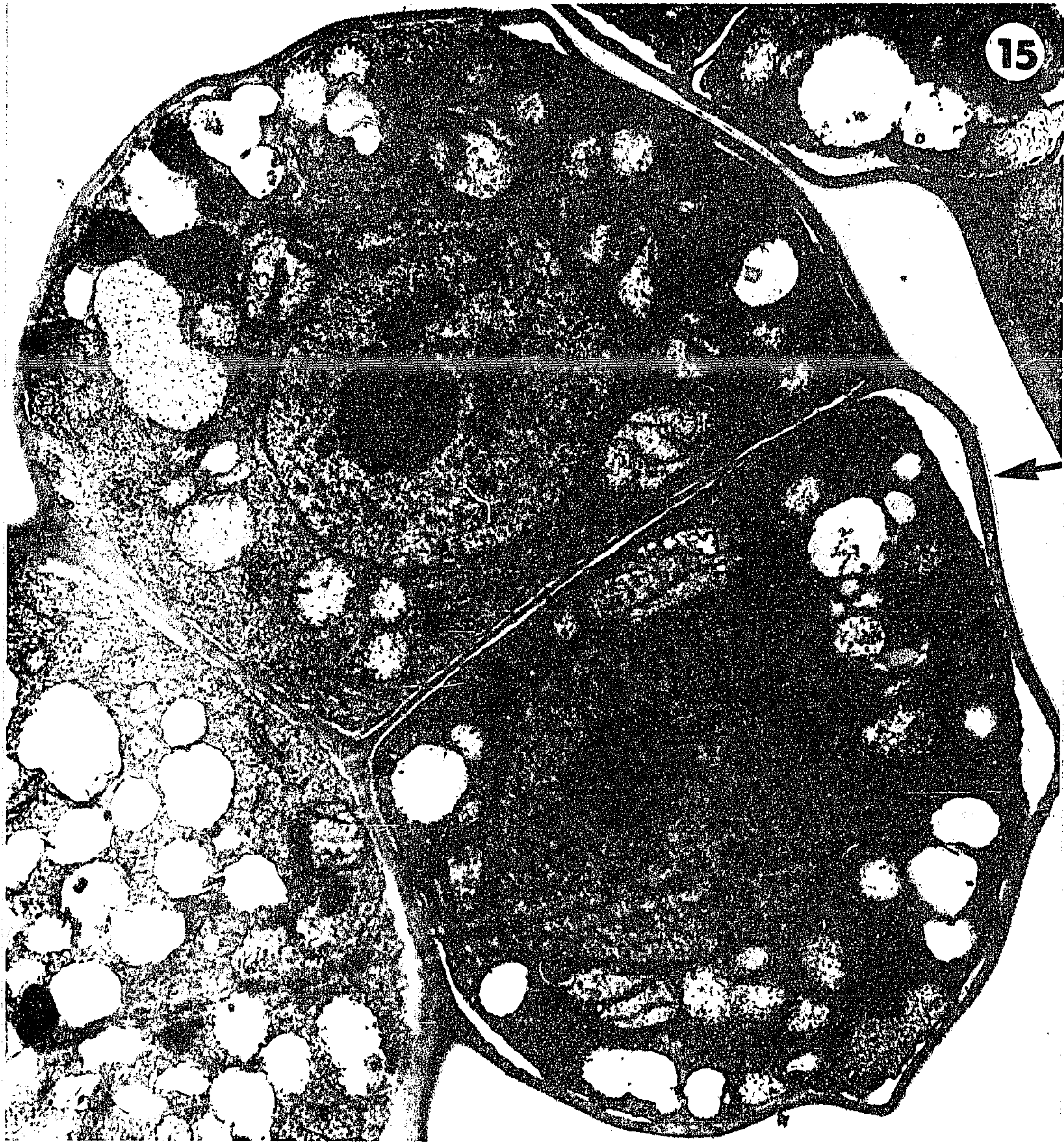

15

Figure 16. TEM through the two celled leaf primoridium. Plastids and mitochondria are present as well as a Golgi complex (G). Note the dumbell shaped chloroplast (Ch) apparently prior to dividing to form two chloroplasts. Plasmodesmatal connections (Arrows) both in longitudinal and cross section are evident. Some vacuoles appear empty while others contain small globular inclusions One such vacuole (V) is indicated. A lomasome (L) area is present. 9300x.

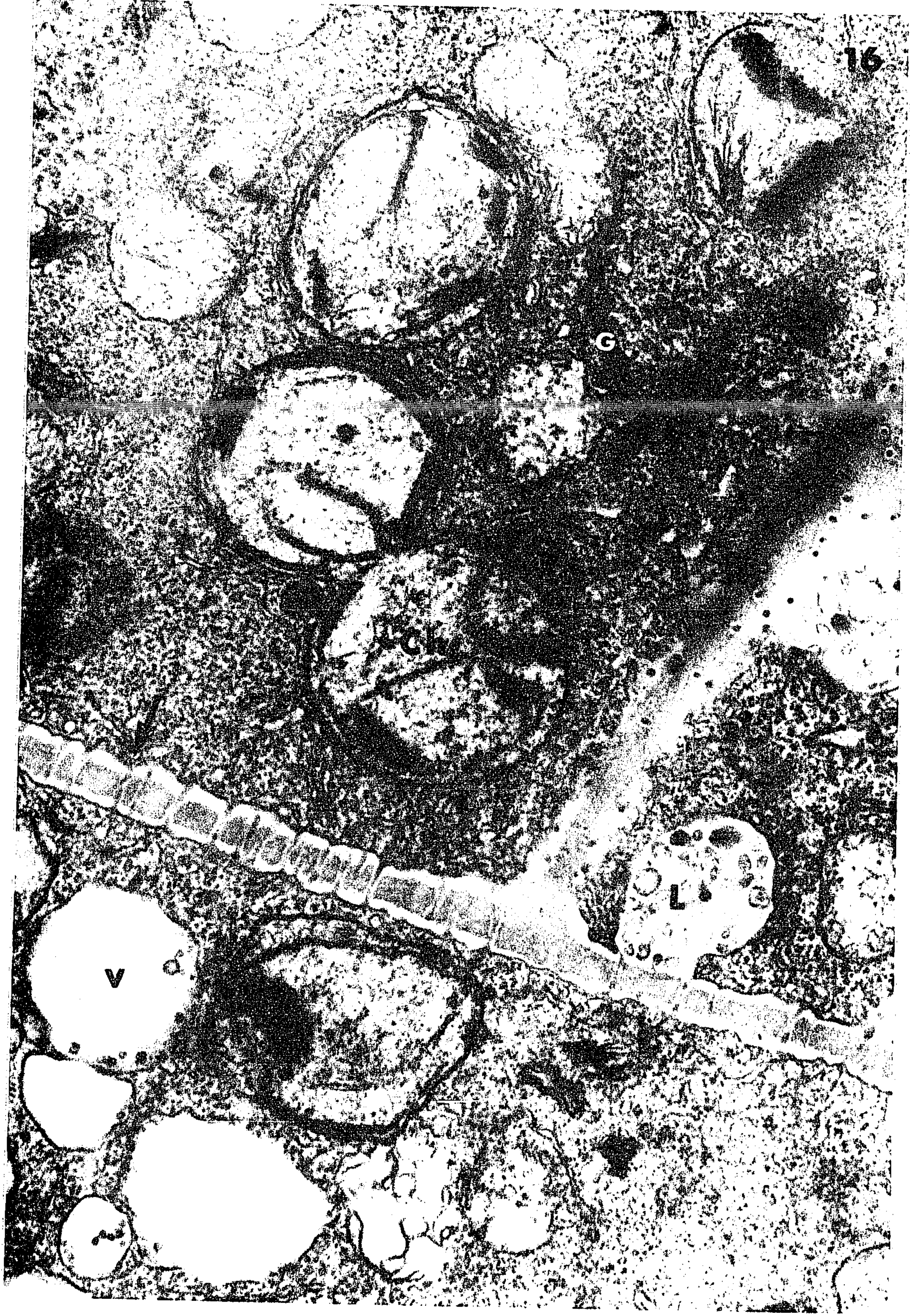

16
G
L
V

Figure 17. TEM of lomasome (L) areas presumably adding material to the cell wall. Many of the globules within the lomasome appear membrane bound. Note the continuance of the plasma membrane around the lomasome. A mitochondria is also present as are vesicles presumably Golgi derived. Plasmodesmata (Arrows) are seen both in longitudinal section and cross section through the two walls in the section. The cytoplasm is dense and typically meristematic in appearance. 50,000x.

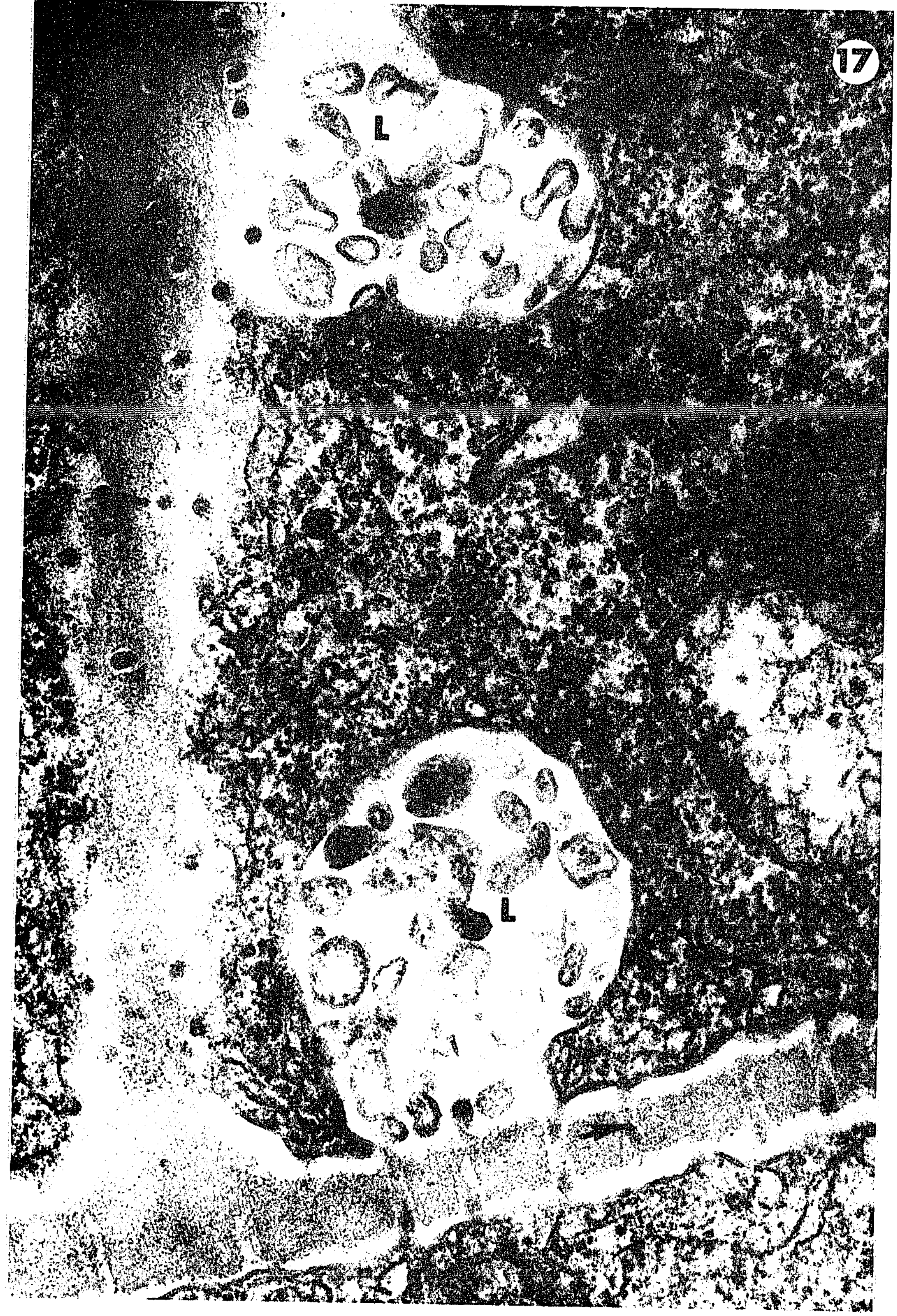

17
L
L

Figure 18. TEM cut parallel to the surface of a young leaf of Plagiochila arctica. Cell division is apparently active at this stage. Note the cell walls. There is variability in the thickness. The cells in the upper portion of the micrograph have a much thinner wall between the two prominent nuclei indicating a more recent division and formation of the wall than those to the lower left portion of the cell. The cells on the left side of the micrograph are still in the stage of wall formation. A phragmoplast is forming in each of these to divide the cells in two. 7500x.

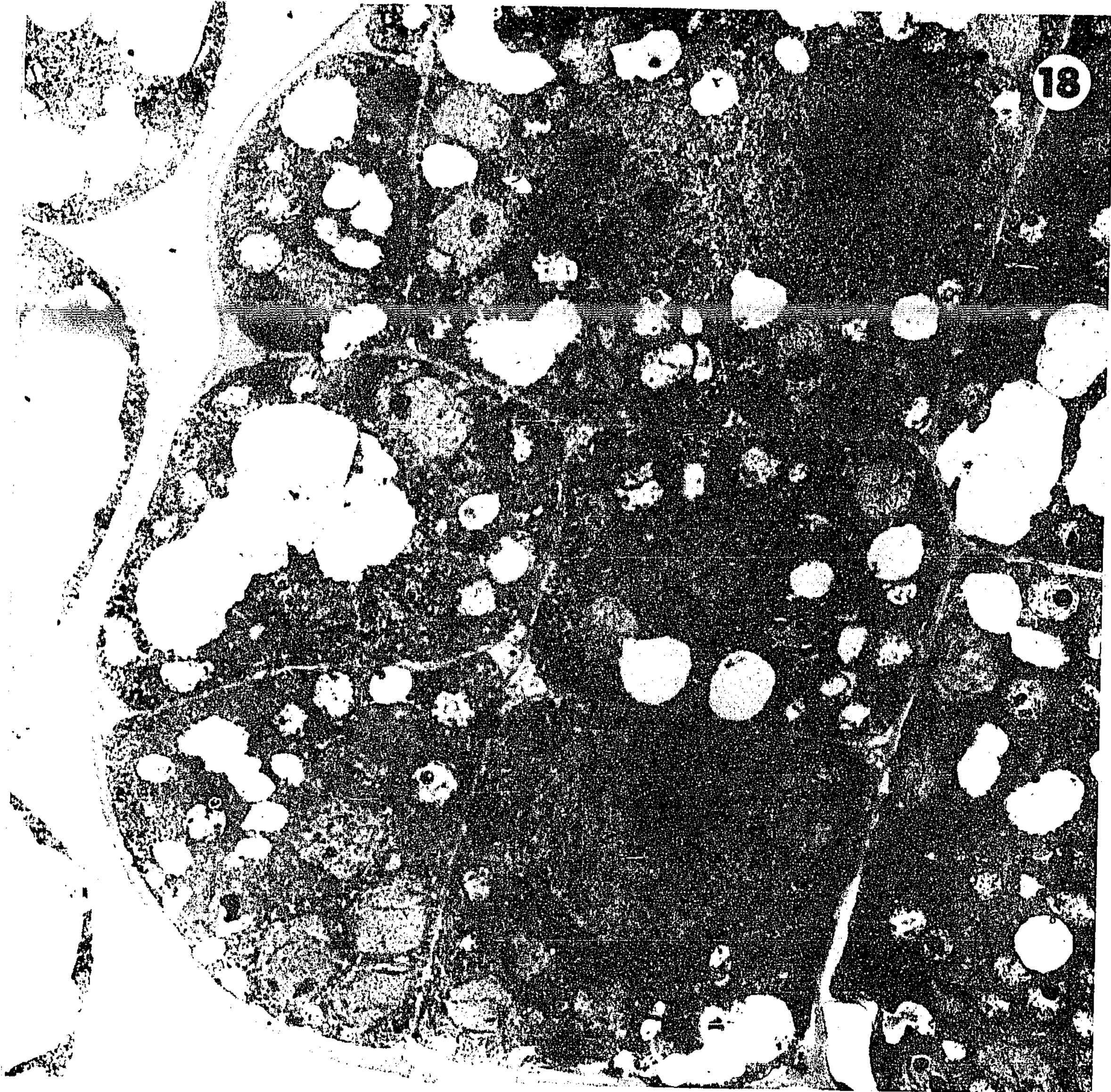

18

Figure 19. Enlargement of portion of figure 18. TEM of cell wall formation. The cell plate being directed by the phragmoplast is evident forming in the center of the cell and extending toward both outer walls. Many Golgi-derived vesicles (Ve) are present as are mitochondria (Mi). Microtubules (Arrow) appear to be directing wall formation. Numerous vacuoles are present. 9300x.

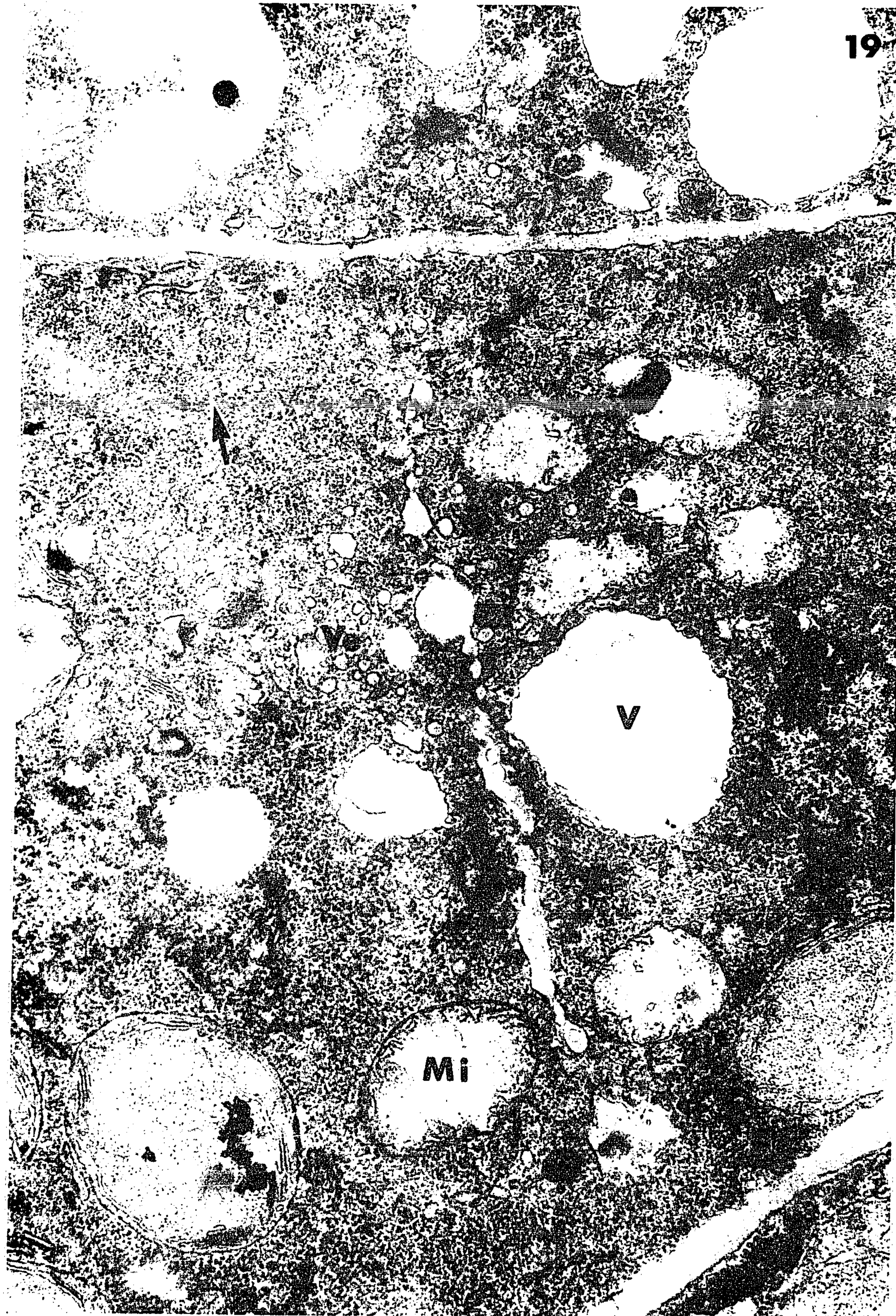

19
V
Mi

Figure 20. Enlargement of a cell from figure 18. TEM of new cell wall formation. Note the forming cell plate (Small paired arrows), vesicles, mitochondria and microtubules (Arrow) which are apparently directing wall formation. A lomasome (L) area is present where three cells meet. 8500x.

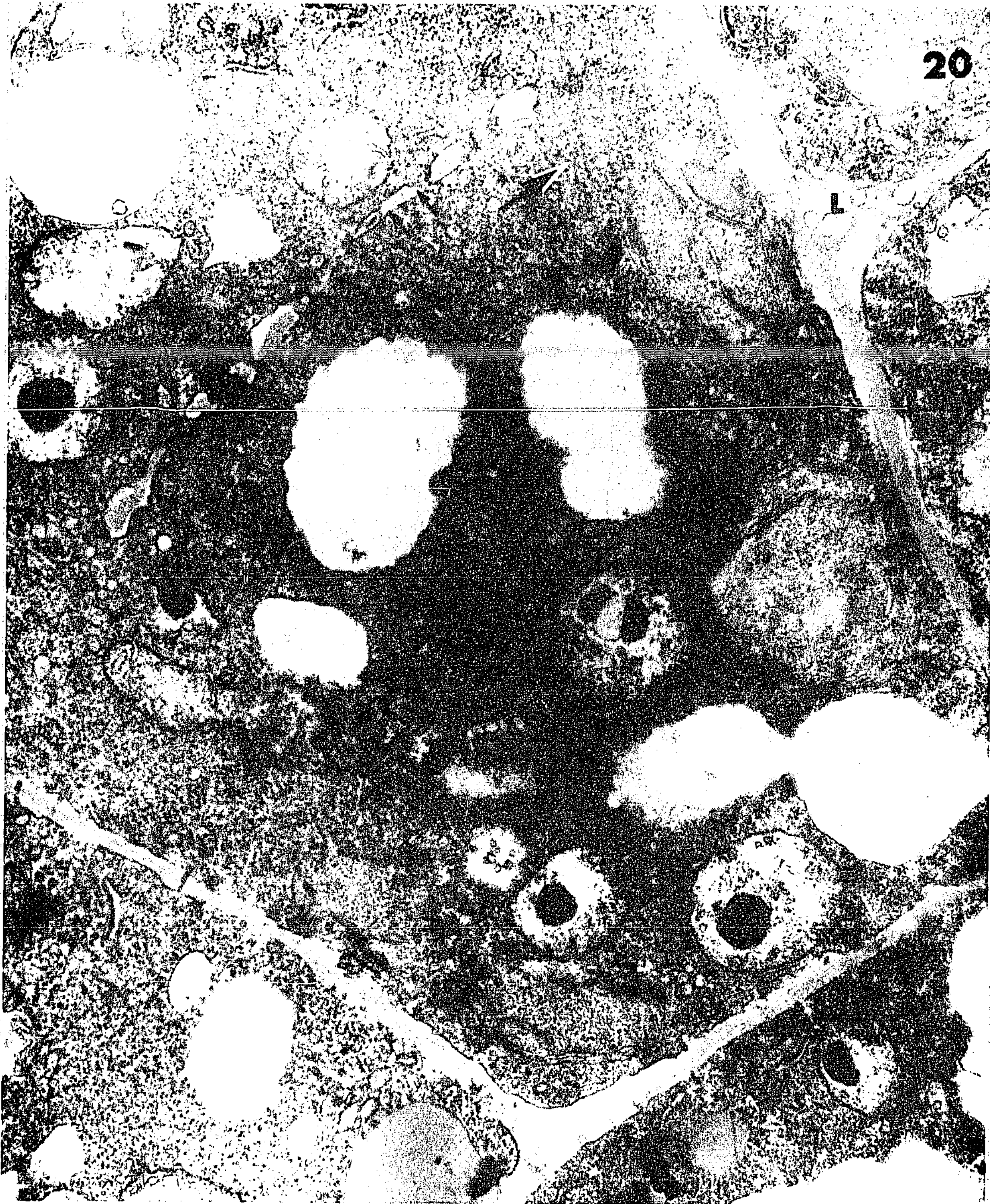

20
L

Figure 21. TEM of portion of phragmoplast area forming the cell wall. Cell plate formation is evident (Paired white arrows). Note the microtubules (Single arrows) present in the area of the phragmoplast and also beyond the ends of the phragmoplast apparently directing the formation of the cell wall. Many vesicles are also present as are mitochondria (Mi). 25,000x.

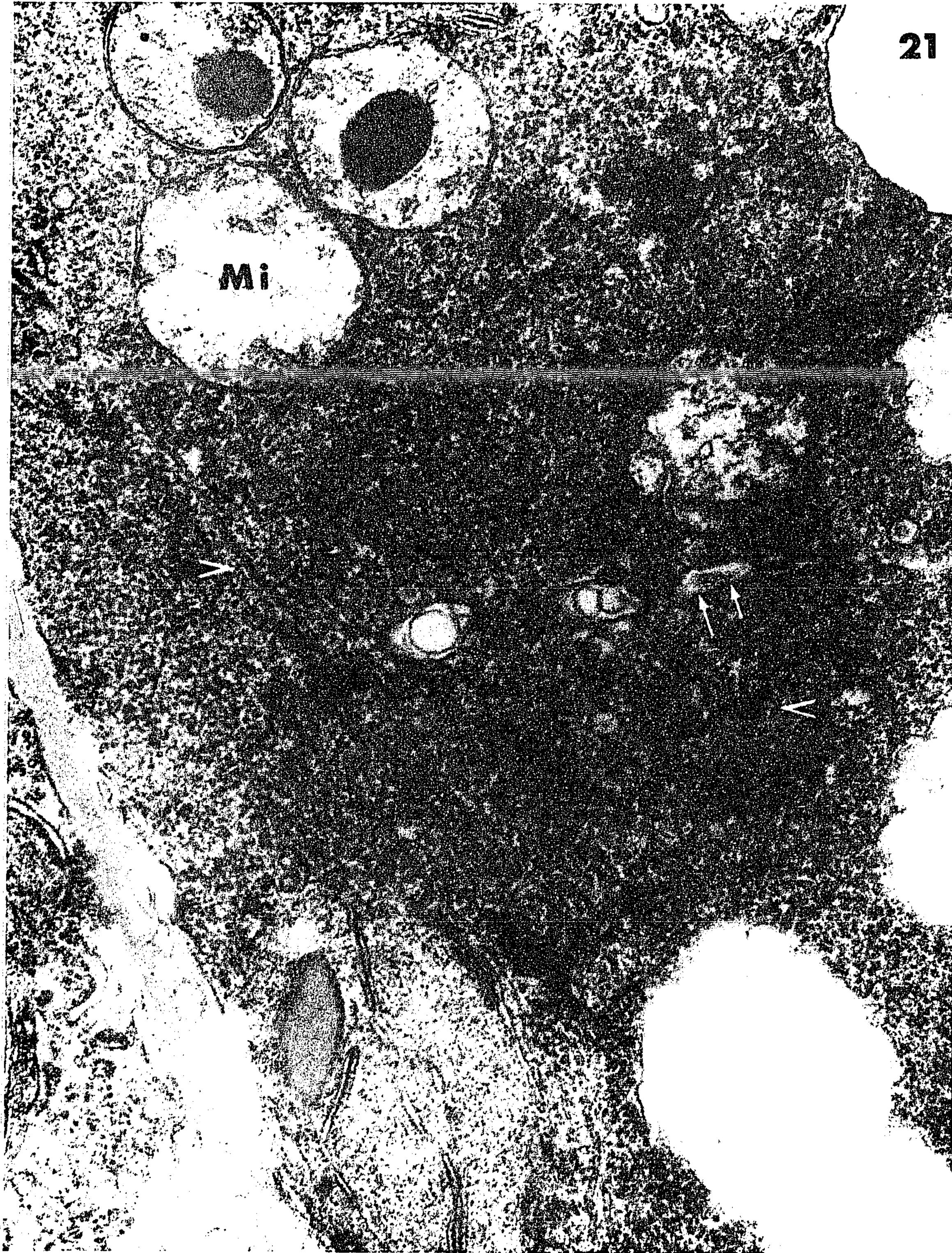

21
Mi

Figure 22. TEM of the forming cell plate (Paired white arrows) and the microtubules (paired black arrows) directing its further development. They are aligned at right angles to the forming plate. Many vesicles (Ve) are also present. 25,000x.

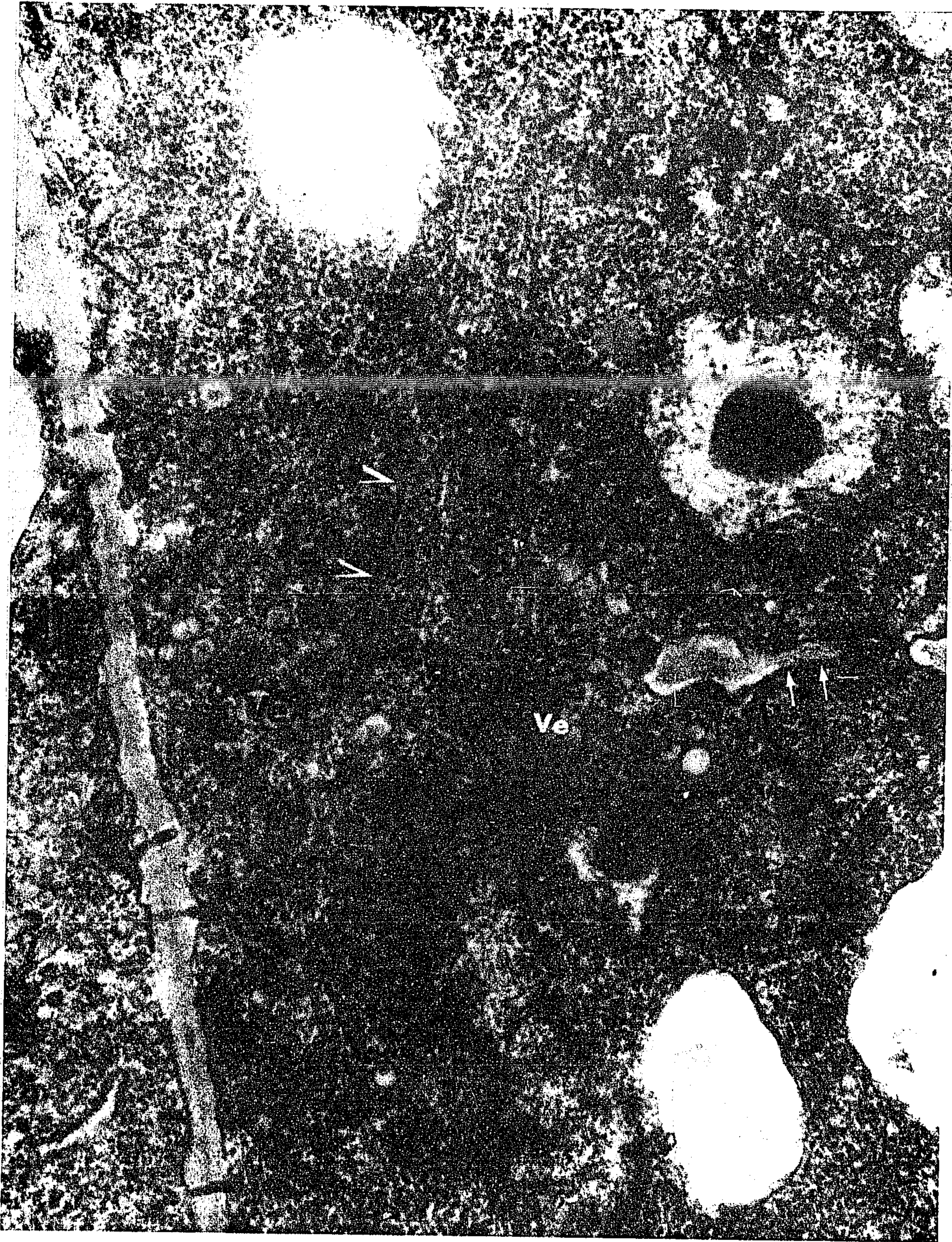

Ve

Figure 23A. TEM of cortical microtubules (Arrows). Many are aligned along the cell wall, frequently in groups of three of four. 50000x.

23A

Figure 23B. Cortical microtubules (Arrows) in longitudinal section and plasmodesmata (Pl) in cross section. 50000x.

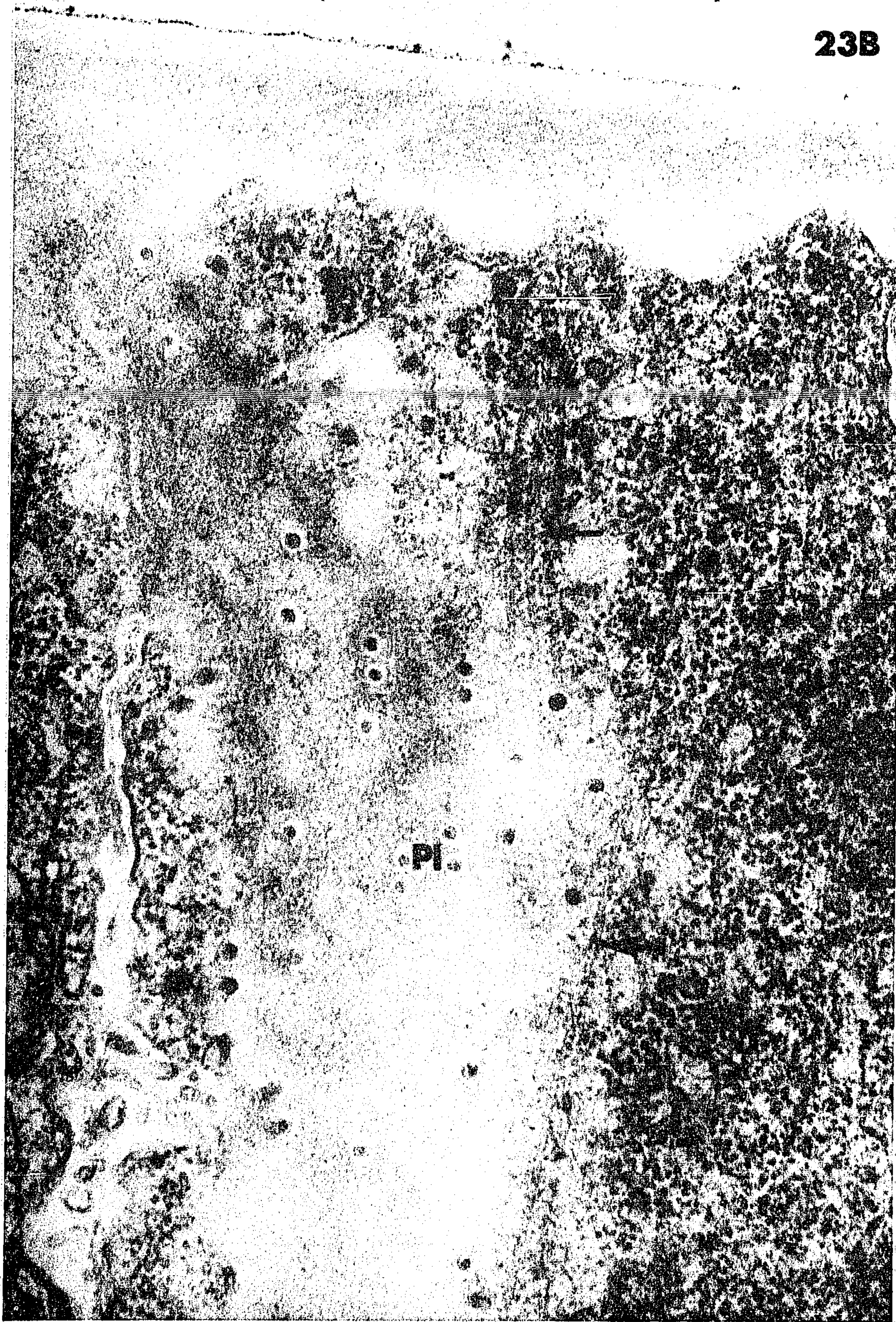

23B
Pl

Figure 24. TEM of young leaf of *Plagiochila arctica*. Typical plant organelles are present including developing chloroplasts (Ch), mitochondria, golgi and vacuoles (V). Note the various inclusions in the vacuoles. In the lower right vacuoles (V) there are a waffle or grid-like inclusion (Arrow), a lipid body and a membranous inclusion. In the upper right portion of the micrograph a small vacuole (v) contains round globular-like inclusions. 9300x.

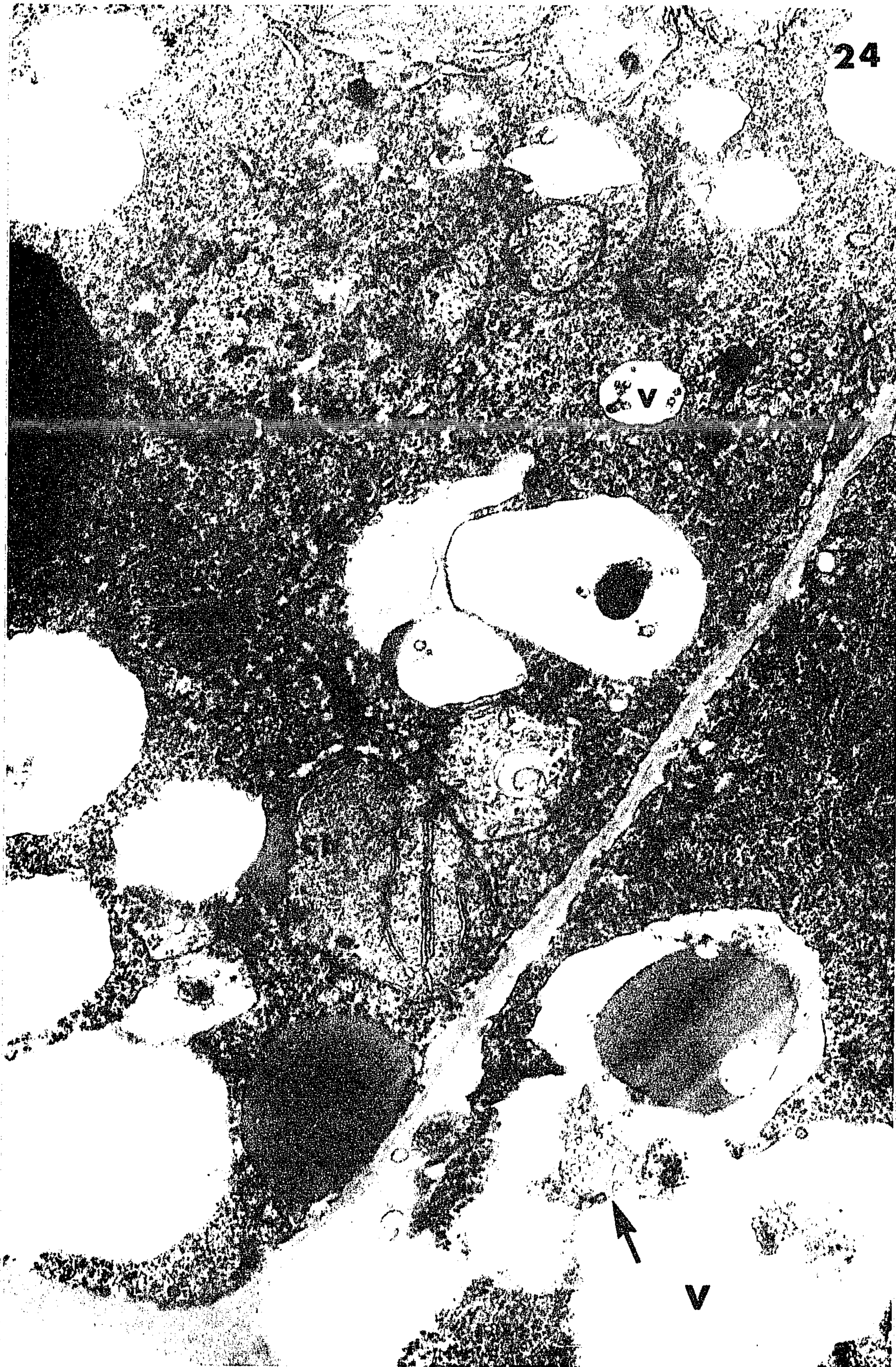

24
V
V

Figure 25. TEM through portion of young leaf showing increased wall thickenings at corners where cells meet. Note the lomasome (L) area. Some of the vacuoles (V) appear empty while others contain globular like material. The plastids still contain few thyllakoids. The mitochondria also contain few cristae. A Golgi complex is present. 9300x.

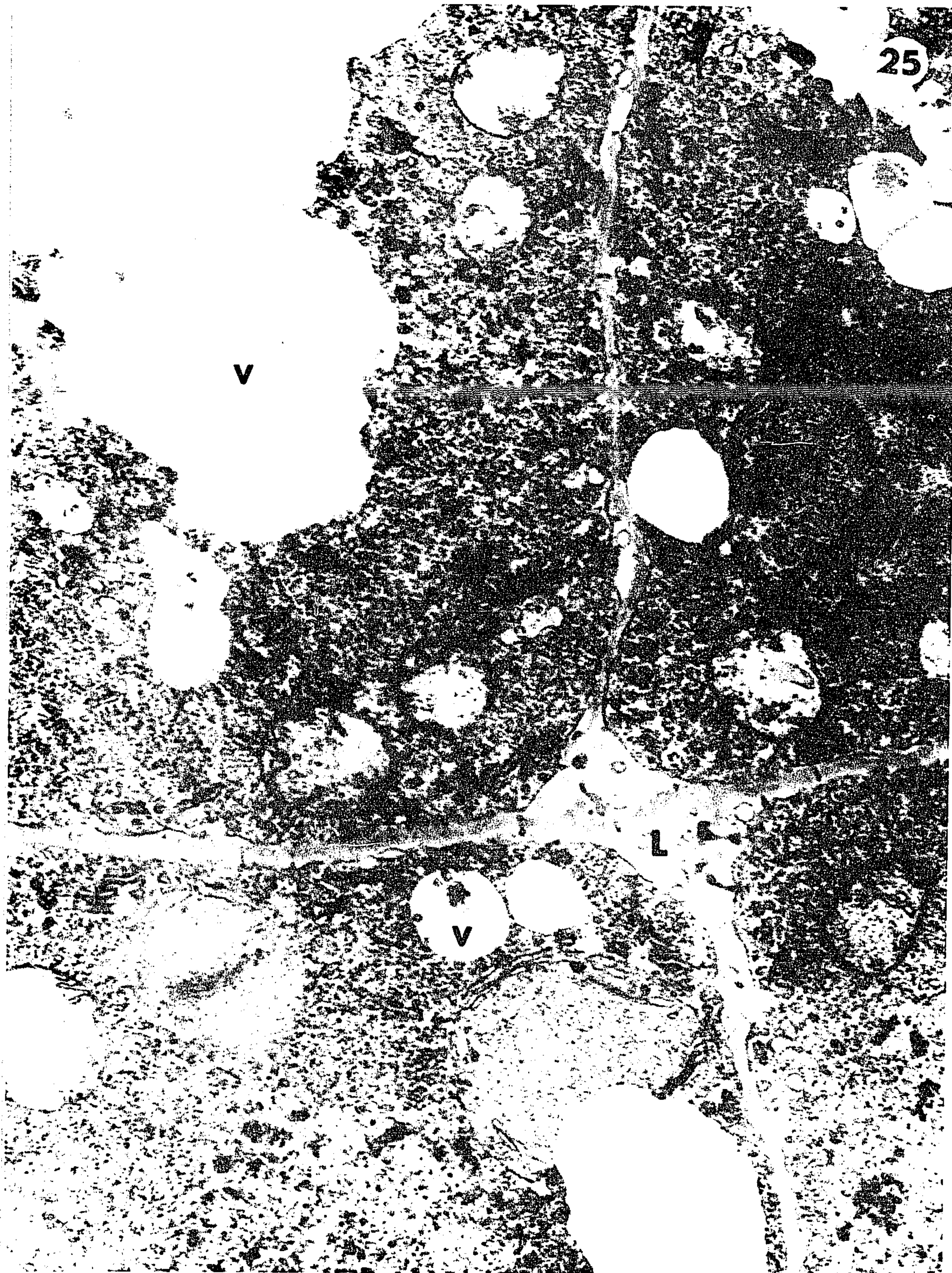

25
V
L
V

Figure 26. TEM of cross section through three cells of a young leaf. The cytoplasm is dense and typically meristematic in appearance. The prominent nuclei (Nu) are evident as are plastids (Pl), mitochondria (Mi) and vacuoles (V). Note the evident thickness in the walls between the cells and the thickened corners of the cells. The cuticle (Arrows) is more apparent as it has been pulled loose in a number of areas. 8500x.

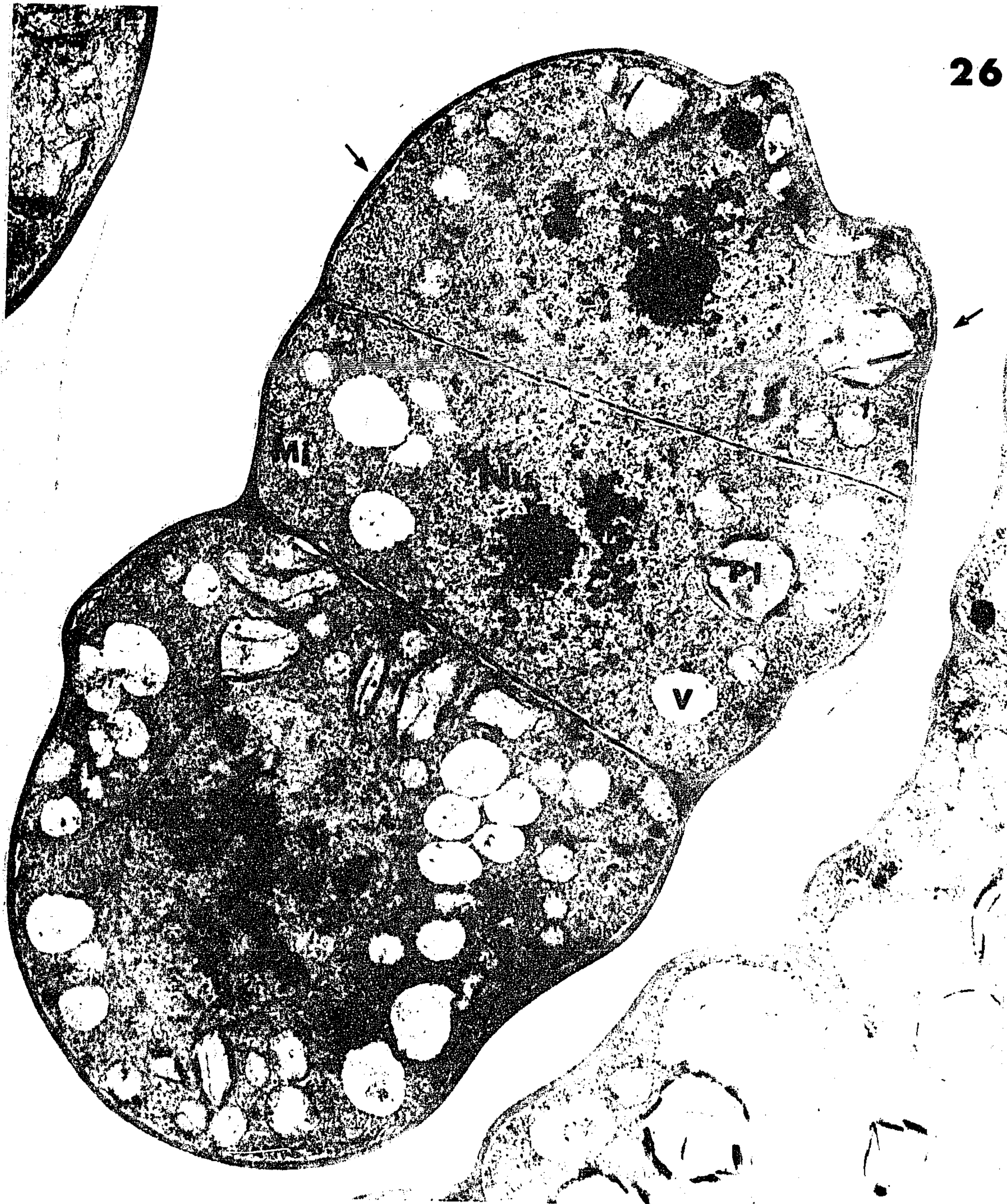

26
Mi
Nu
Pl
V

Figure 27 . TEM through a young leaf. Most apparent is the abundance of chloroplasts. Note the enlarged chloroplast (Ch) and the position of the grana stacks. Apparently it is preparing to divide. 21000x.

Nu
Ch

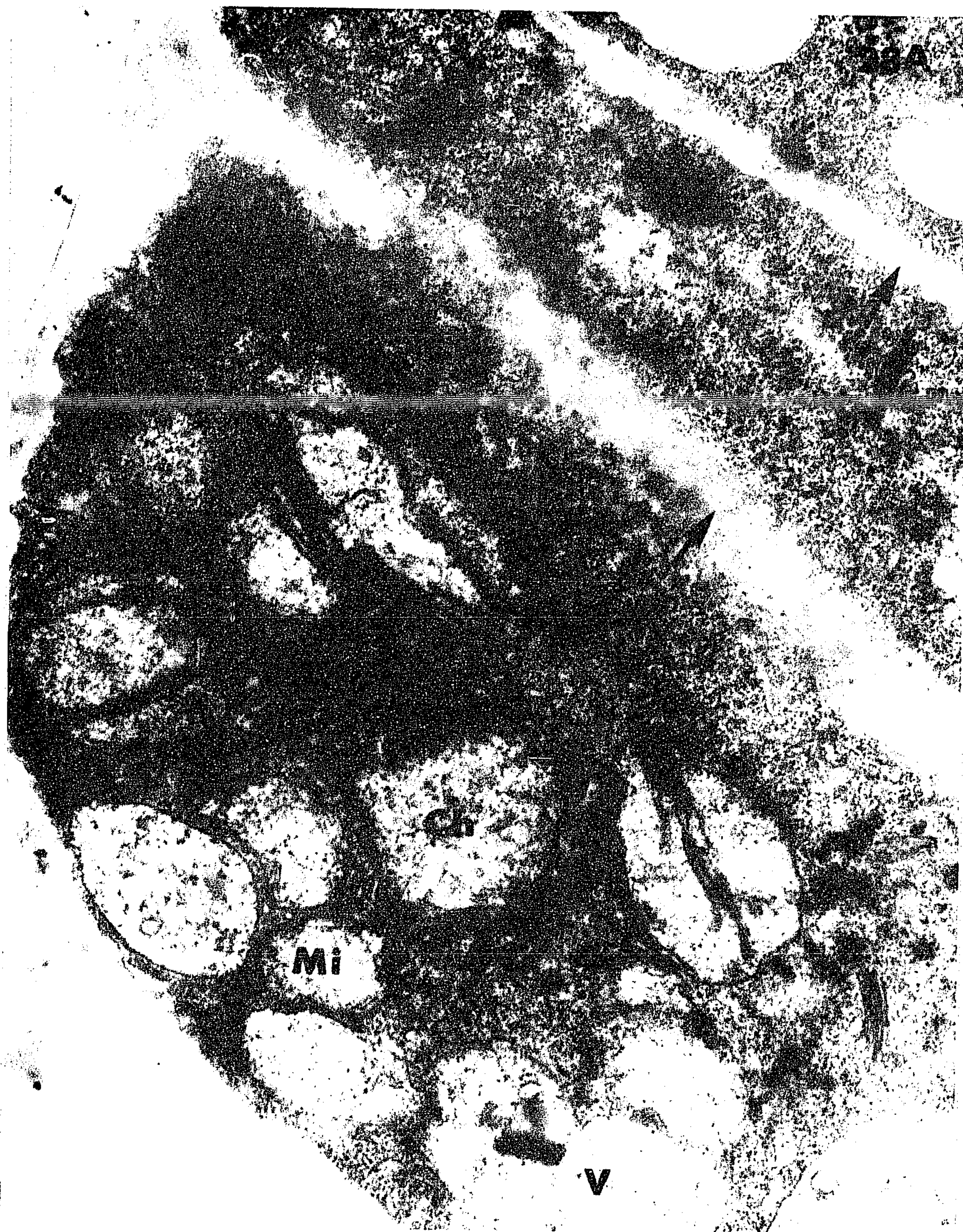
Figures 28A-D.

A. TEM through a young leaf. Chloroplasts (Ch), mitochondria (Mi), vacuoles (V) and Golgi are present. Plasmodesmatal connections (Arrows) are visibile both in cross and longitudinal section. 21700x.

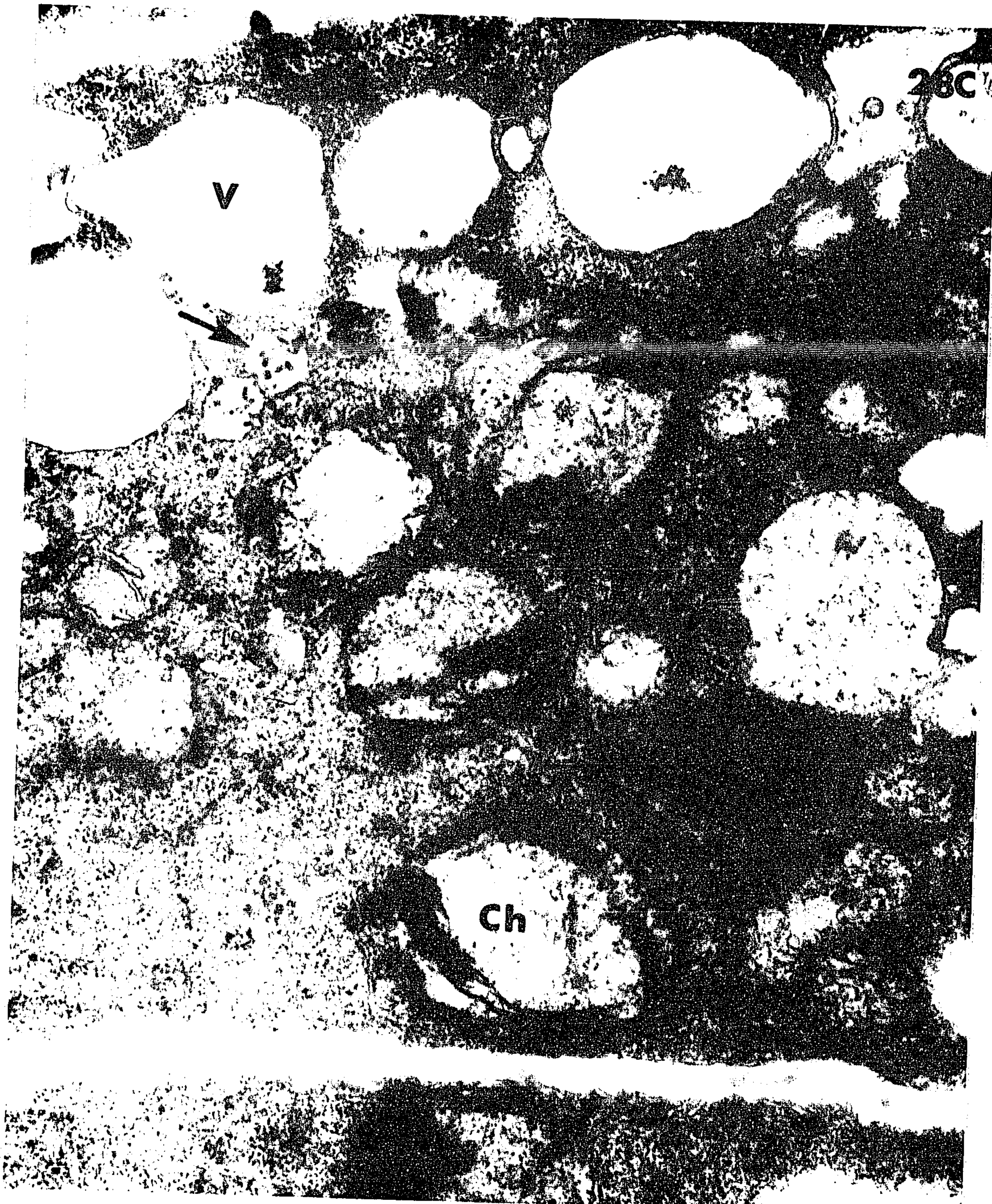
B. TEM through a young leaf. Chloroplasts (Ch)are most prominent. In some the grana are more stacked. The stroma still occupies the major portion of the plastid. Mitochondria are present as are Golgi (G) A lomasome (L) area is seen. Vacuoles (V) are present. 21700x.

C. TEM through a young leaf. Chloroplasts (Ch) , mitochondria, endoplasmic reticulum and vacuoles (V) are present. Two small vacuoles (Arrow) containing globular inclusions are visible. 21700x.

D. TEM through a young leaf. Chloroplasts (Ch), mitochondria (Mi), and vacuoles (V) are seen. Numerous plasmodesmata (Arrow) cut in cross section are visible in the cell wall. 21700x.


Mi
V

28B
V
Ch
L


28C
V
Ch

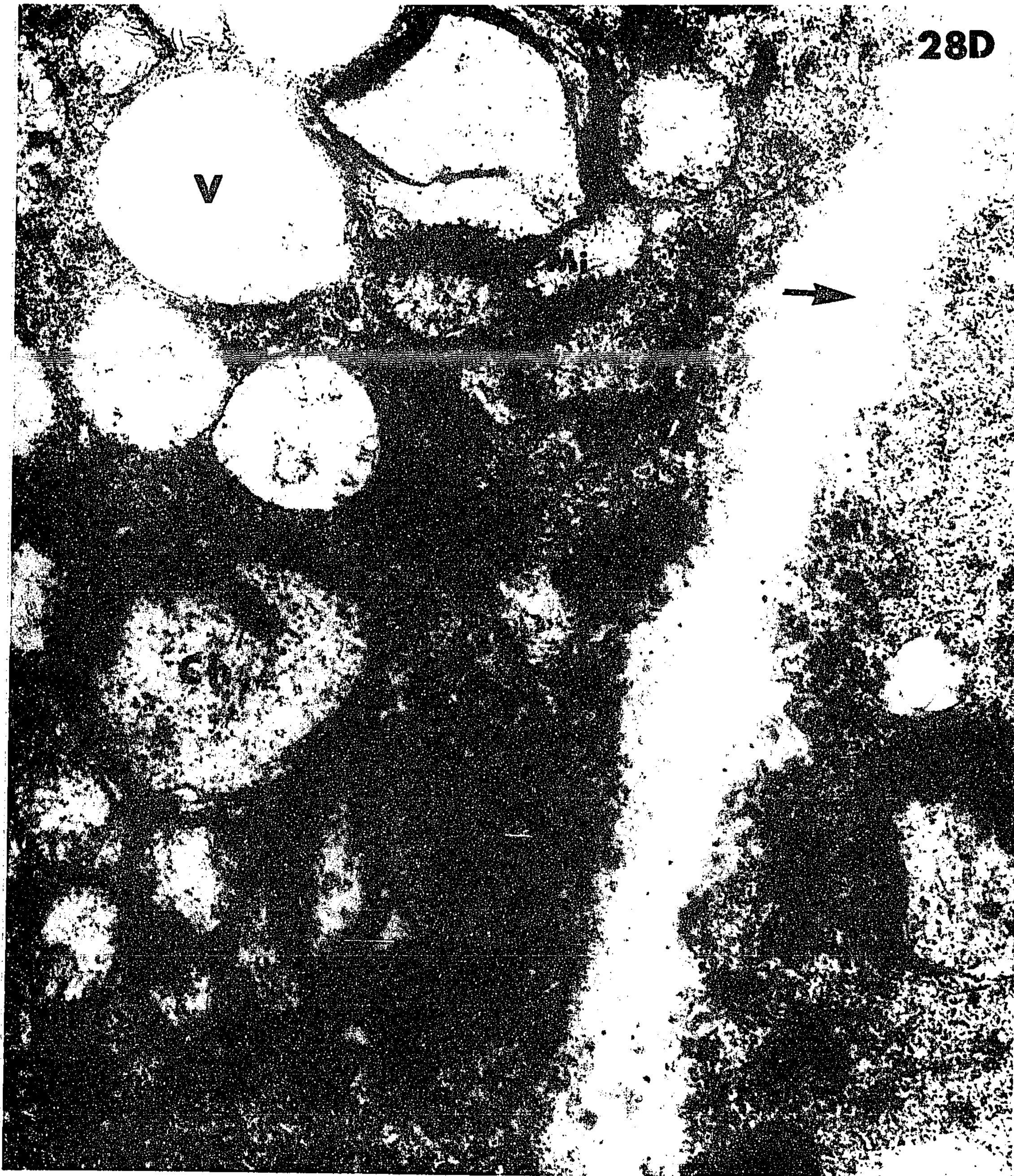

28D
V
Mi

Figure 29. TEM through a leaf at the 5-6 leaf stage of development. The nuclei (Nu) are still prominent. The chloroplasts (Ch) show increasing stacking of grana. Vacuoles (V) are coalescing to form larger vacuoles. Mitochondria are present (Mi). Corner thickening is evident. 7500x.

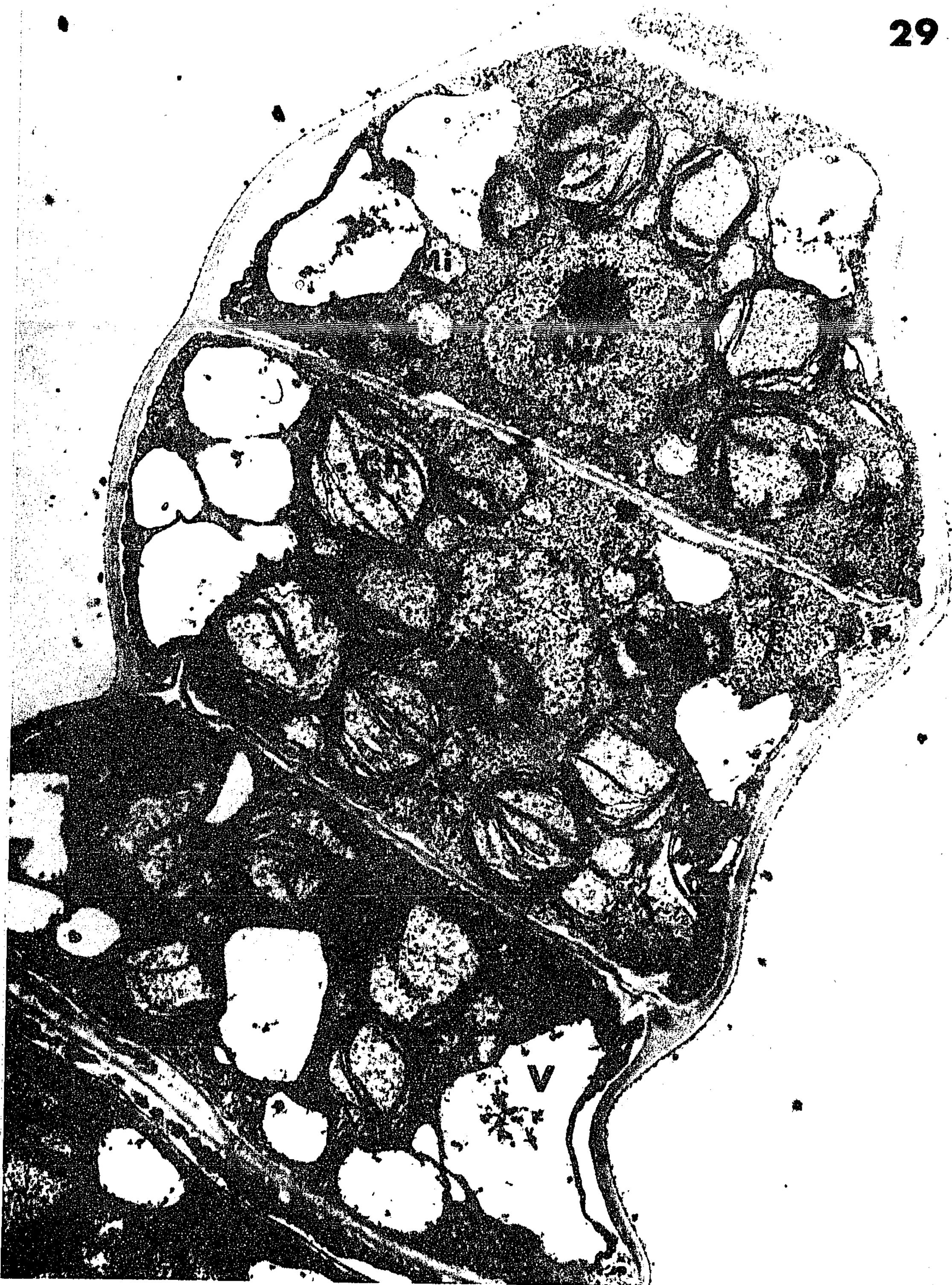

29
mi
V

Figure 30. TEM through a leaf. Several chloroplasts are most present. One chloroplast (Ch) is elongated and beginning to acquire a dumbell shape. Note the orientation of the grana. Apparently this plastid is preparing to divide. The nuclei (Nu) of the two cells can be seen. Plasmodesmatal connections (Arrows) both in longitudinal and cross-section are evident. 25000x.

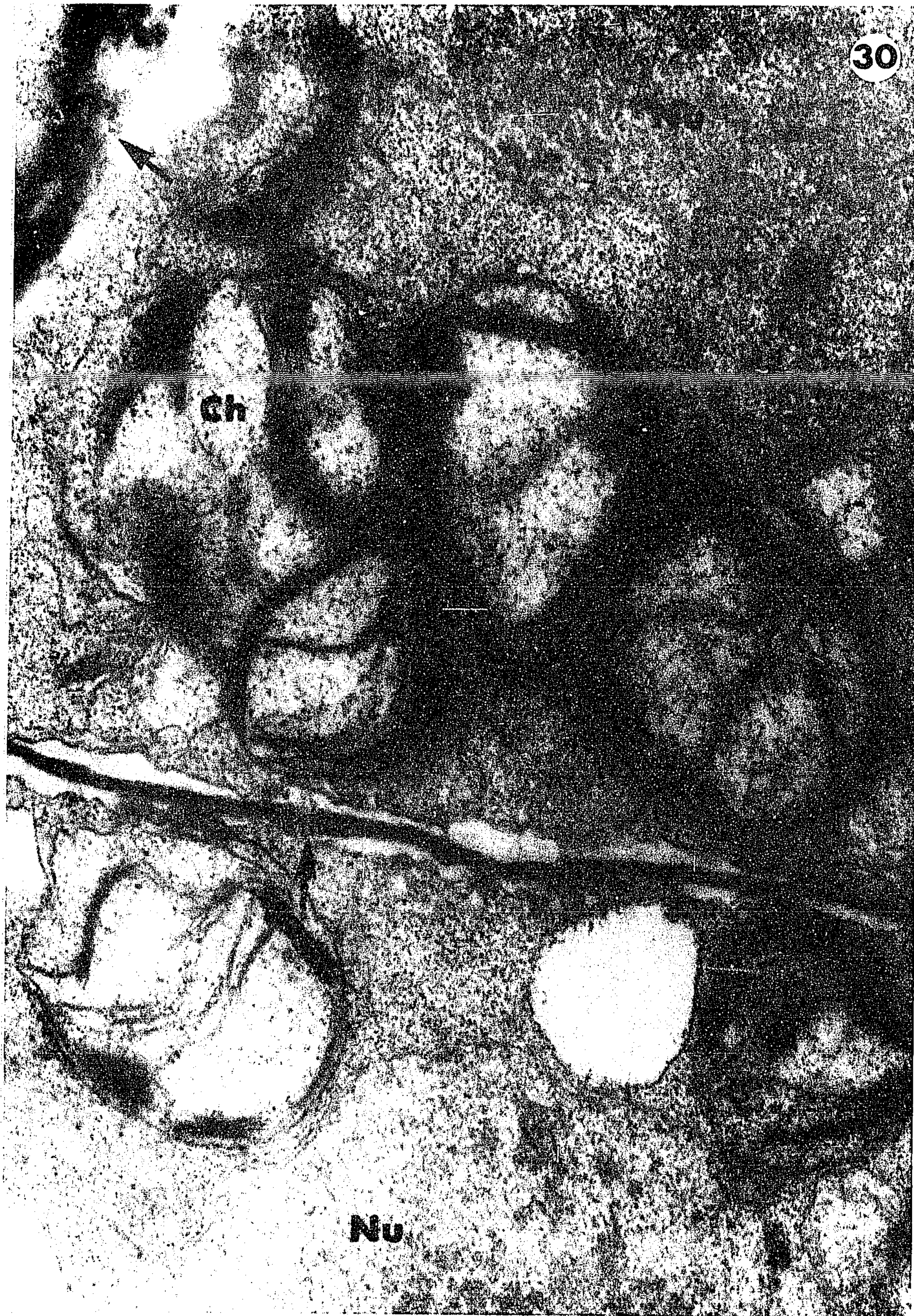

30
Ch
Nu

Figure 31. TEM through a portion of a leaf showing cell plate formation. Within the phragmoplast area (Arrows) many small vesicles can be seen apparently coalescing to form the future cell wall. The cytoplasm continues to appear dense. Vacuoles are seen some apparently having fused to form larger vacuoles. 25000x.

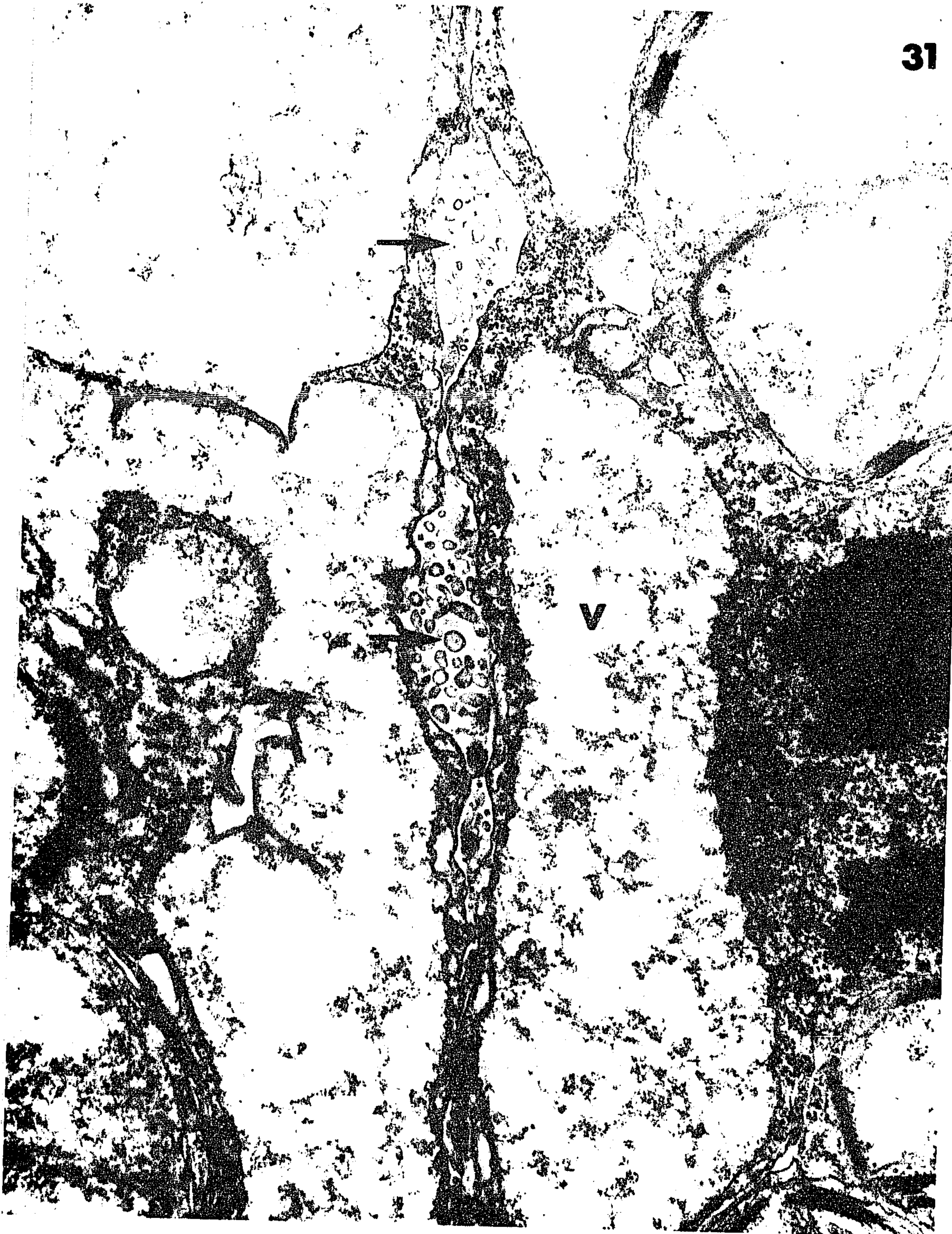

31
V

Figure 32. TEM of a leaf 7 stage. The cells visible show the various aspects of the leaf that can be shown at this stage of development. One cell has been sectioned to show the centrally located nucleus (Nu) , the next has the nucleus barely visible, the third is above the nucleus. Many other organelles that are contained within the typical cell at this stage, i.e. oil bodies (OB), chloroplasts (Ch), mitochondria, vacuoles (V) and a lomasome (L) area can be seen in these cells. 7500x.

32

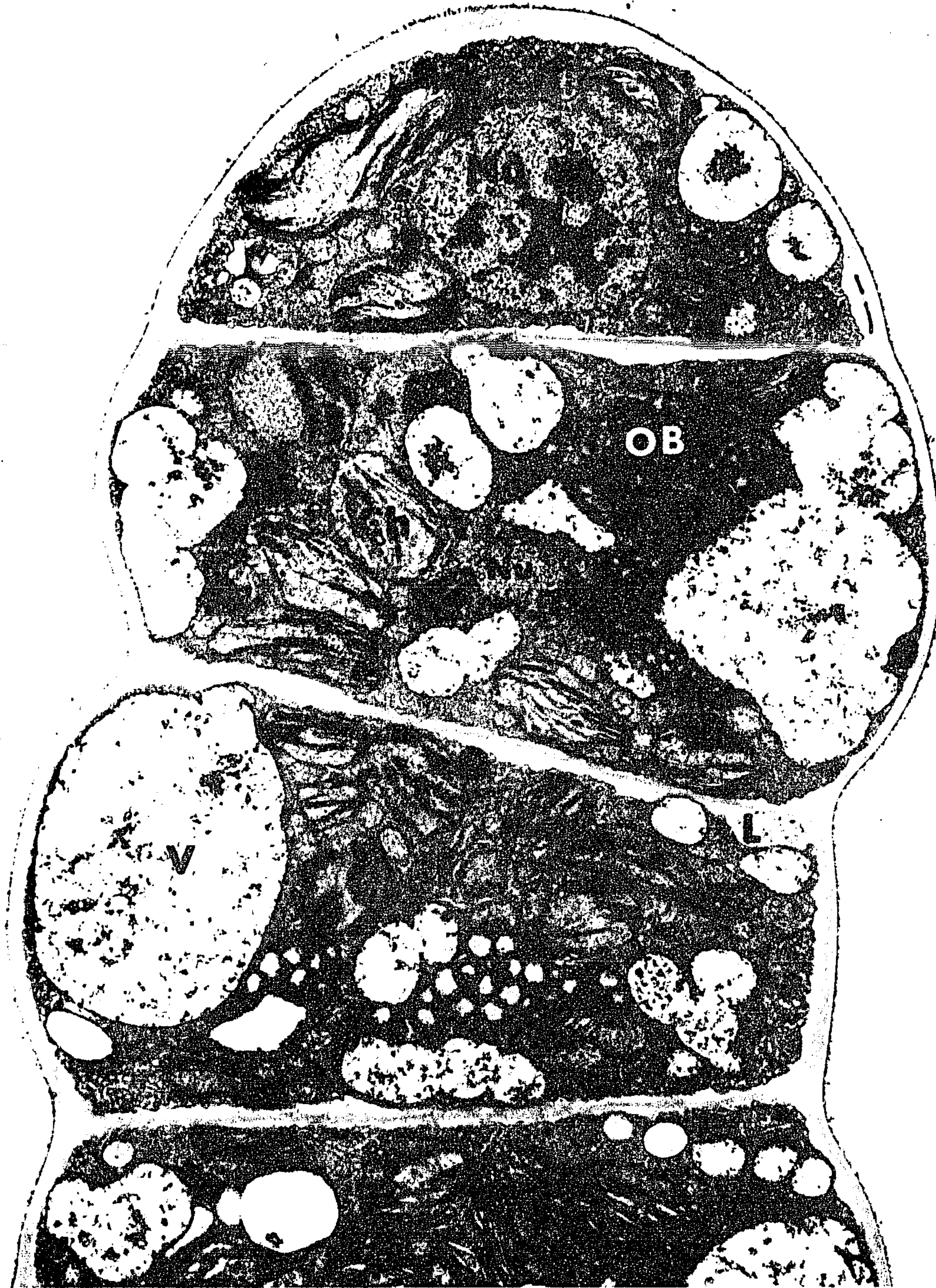


Figure 33. TEM of single cell of P. arctica at the leaf seven stage. Most noticeable are the large vacuoles (V), oil bodie (OB), chloroplasts (Ch) and the centrally located nucleus (Nu). 10000x.

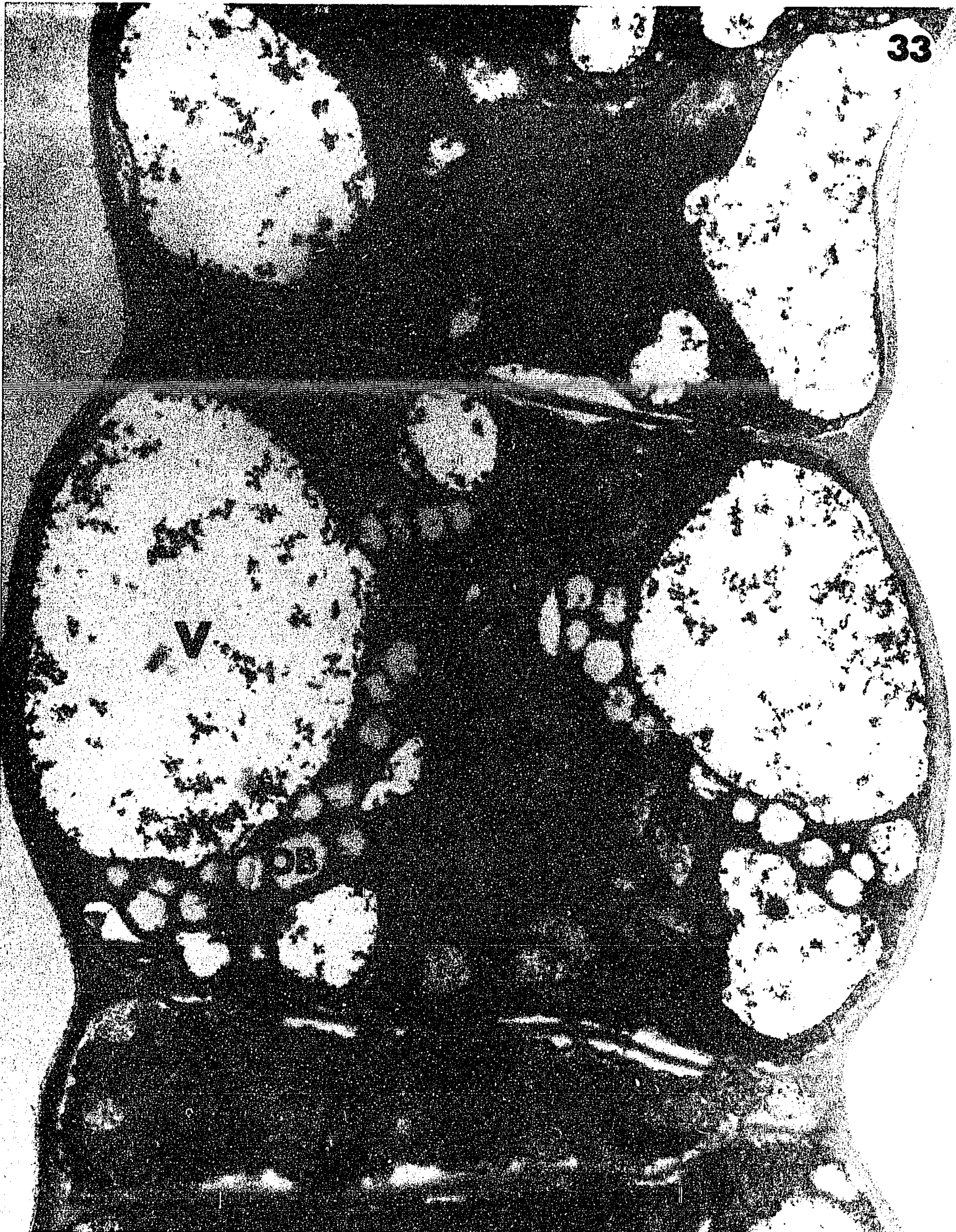

33
V
OB

Figure 34. TEM of partial cell of _P. arctica_. At this magnification it can be seen that the oil bodies (OB) are contained within a membranous enclosure (sac). The vacuoles (V) are large and comprise most of the cell. Some fibrilular material continues to be present in the vacuoles. Chloroplasts (Ch) contain well developed grana and starch grains. What is left of the cytoplasmic area is still dense and endoplasmic reticulum (ER) is still evident. 18500x.

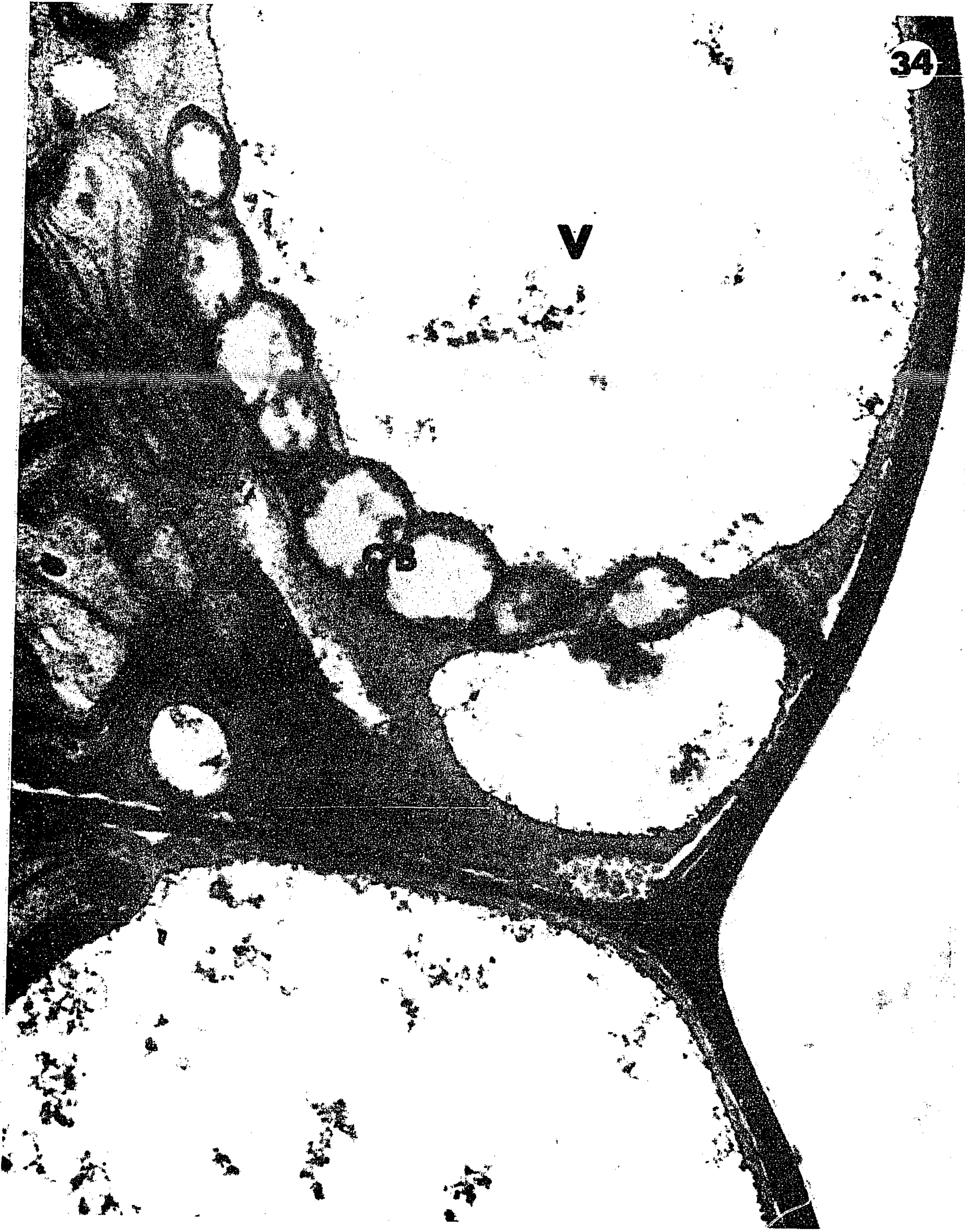

34
V

Figure 35. TEM of the outer cell wall area. Membraneous inclusions (Double arrows) can be seen in the lomasome area between the plasma membrane and the cell wall (CW). Other small particles (Single arrow) usually seen in the lomasome area are also present. Endoplasmic reticulum is present in the dense cytoplasmic area. Portions of large vacuoles can be seen. The cell wall has a fibrillar appearance. A cuticle is visible. 50000x.

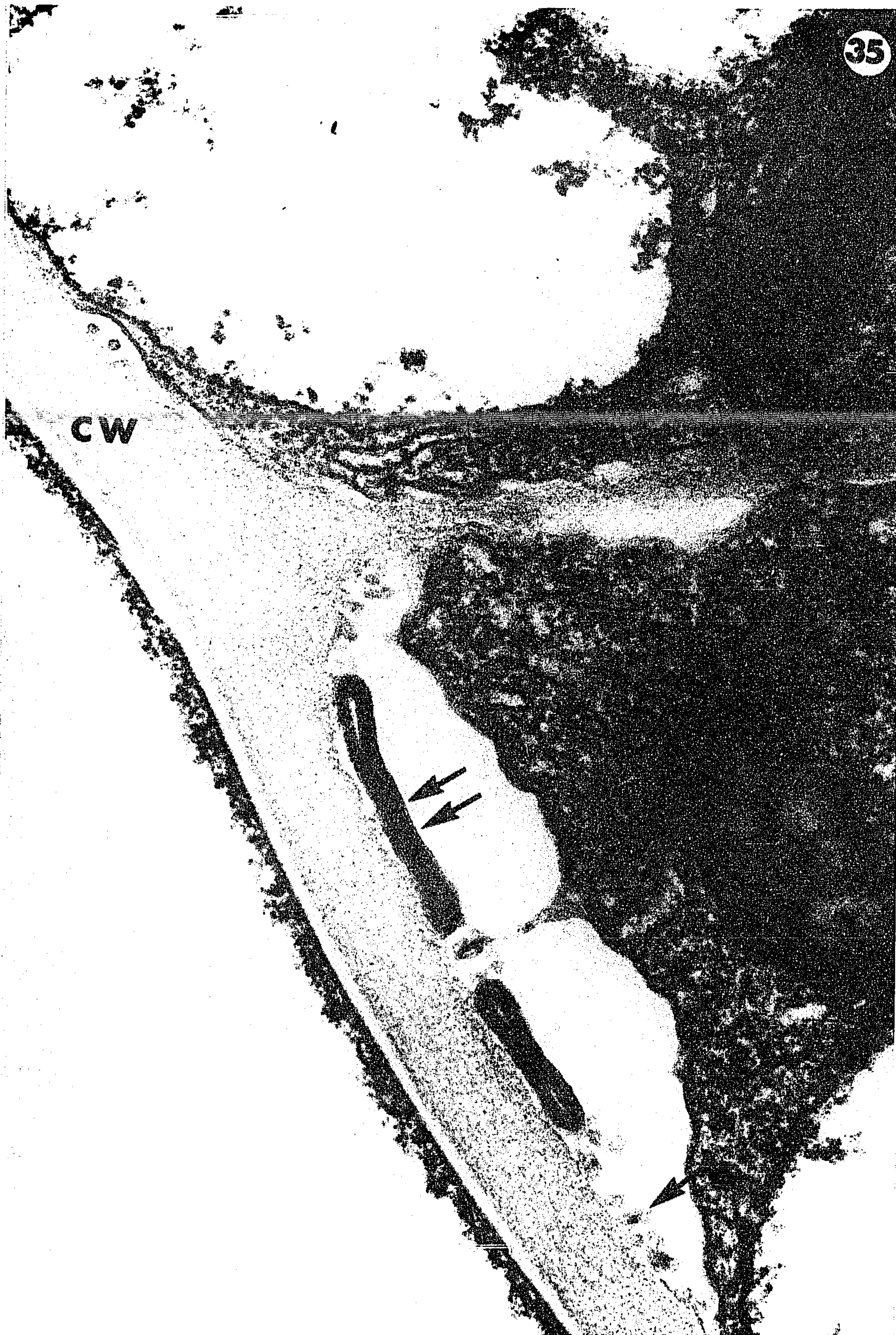

35
CW

Figure 36. TEM of whole cell at the leaf eight stage. Most of the smaller vacuoles have coalesced in what appears to be two large vacuoles (V). Chloroplasts (Ch) with starch grains are present. Oil bodies (OB) are visible. 7500x.

36
Ch
V
OB

Figure 37. TEM of whole cell at the leaf eight stage. Most of the smaller vacuoles have coalesced in what appears to be two large vacuoles (V). Oil bodies (OB) containing large spherical oil droplets are visible. Chloroplasts (Ch) with starch grains are seen. 7500x.

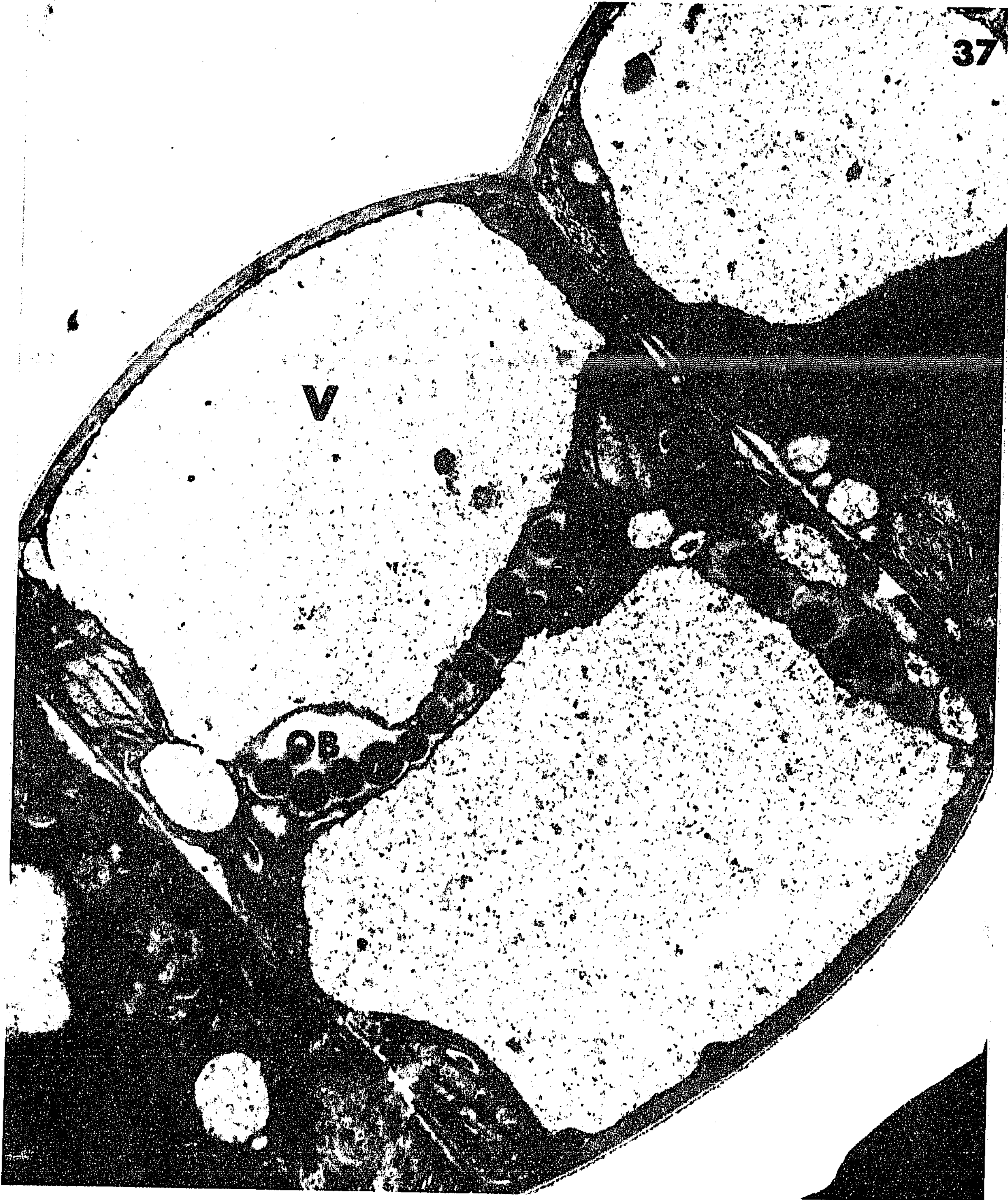

37
V
OB

Figure 38. TEM of a portion of the cell showing inner walls and some of the cytoplasmic contents. The chloroplasts (Ch) contain starch grains and well developed thyllakoids. Vacuoles (V) appear empty or contain membraneous or vesicular components. Mitochondria (Mi) are visible. Endoplasmic reticulum (ER) is present. In some of the wall area plasmodesmatal connections (Arrows) are visible. Increased wall thickening can be seen where three cells meet. 25000x.

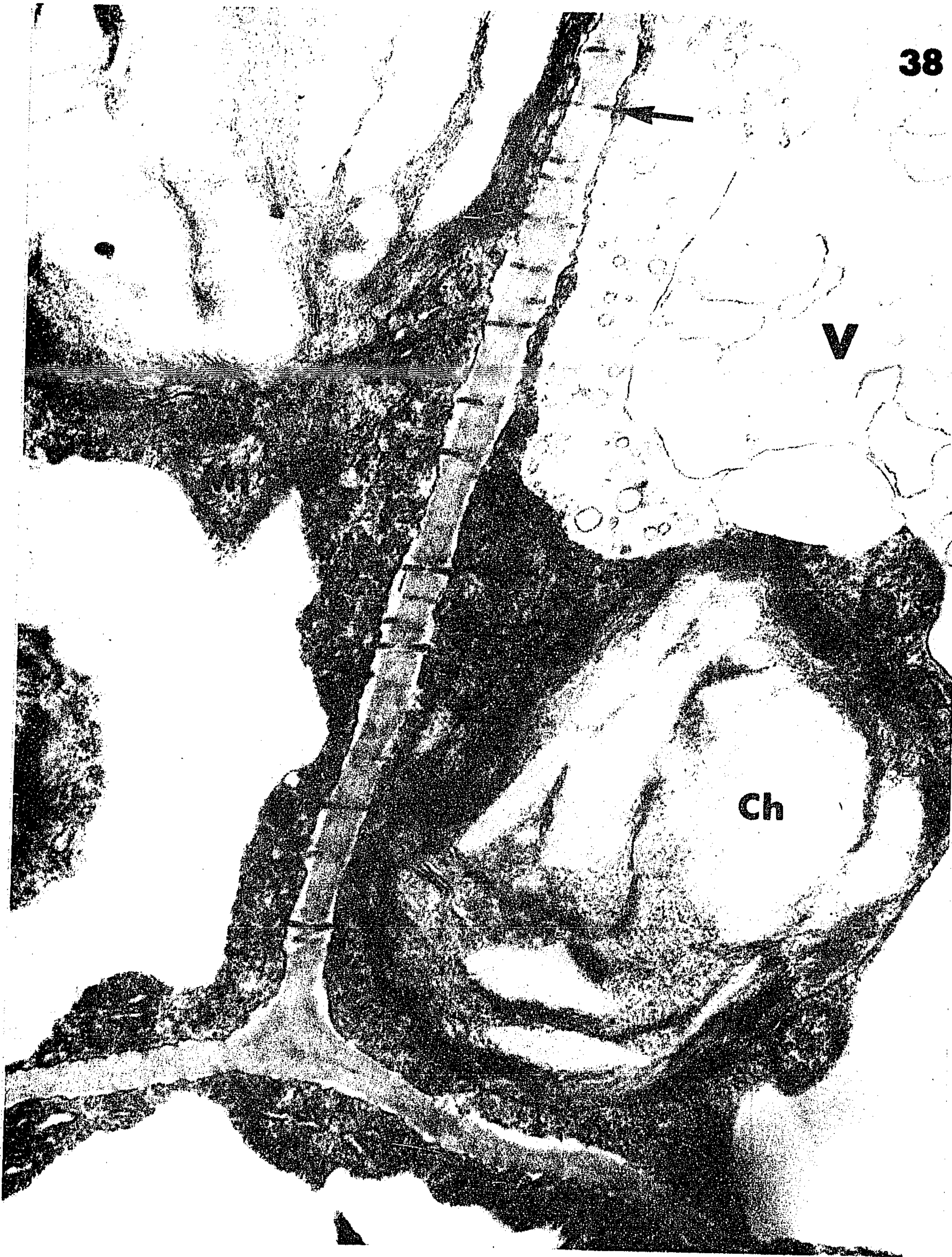

38
V
Ch

Figure 39. Tem of a portion of a leaf eight cell. Most prominent is a chloroplast (Ch) with numerous starch grains and well developed thyllakoids. Some vacuoles appear empty while in others there are vesicular appearing bodies. An elongated mitochondria (Mi) is visible as are lipid inclusions (Li). Endoplasmic reticulum is visible. Plasmodesmatal connections (Arrow) can be seen in one wall section. Increased wall thickening is apparent where three cells meet. 25000x.

39
Mi
Ch
Li
V

Figure 40. TEM through a portion of a leaf eight cell. Well developed chloroplasts (Ch) include starch grains (S), thylakoids (Th) and lipid incluisons (Li). The mitochondria (Mi) contain cristae. Endoplasmic reticulum (ER) is evident in the cytoplasm. Nuclear pores (Arrow) can be seen in one portion of the nucleus (Nu). 25000x.

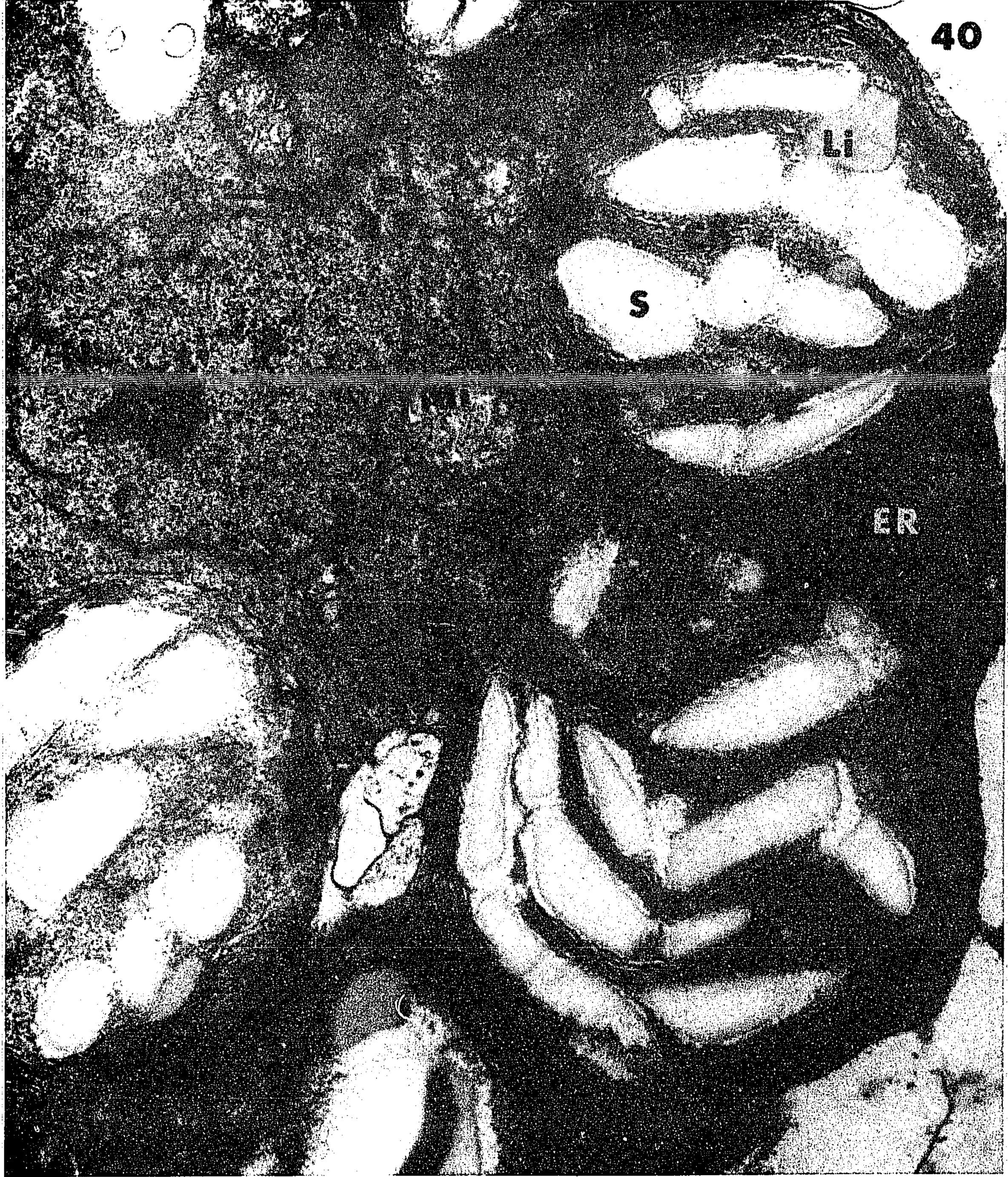

40
Li
S
ER

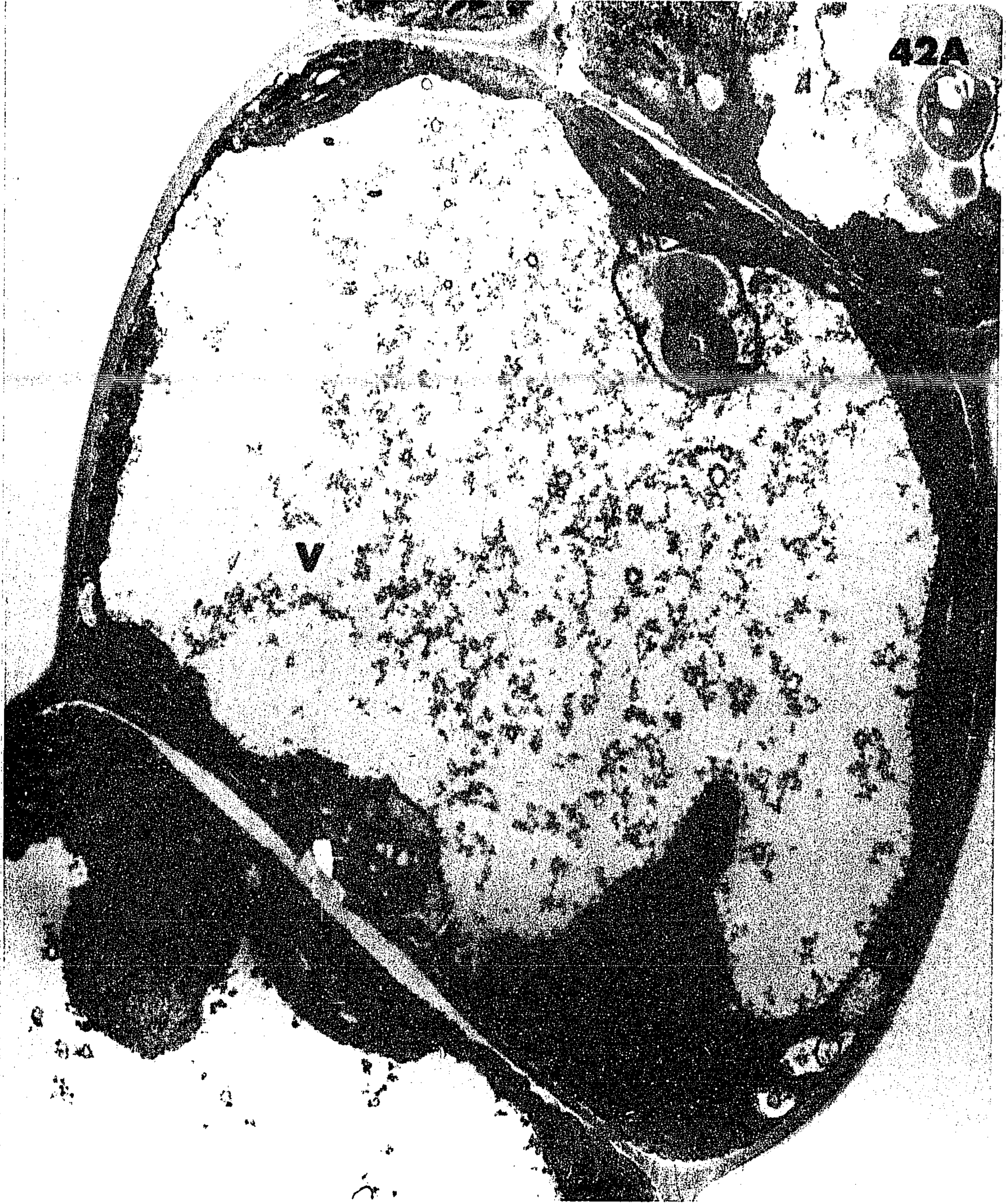
Figure 41. TEM of a portion of a wall between two adjoining cells of leaf eight. Most apparent is the groups of cortical microtubules (Arrows) along both sides of the cell wall. They appear in groups of two, three or four. 49000x.

41

Figure 42 A&B.

A. TEM through a whole cell at leaf stage nine. Most of the cell is occupied by the large central vacuole (V). The cytoplasmic contents surround this central vacuole. The nucleus (Nu), chloroplasts (Ch), mitochondria and oil bodies (OB) are visible in the reduced cytoplasmic area. Thickened corners between adjacent walls are evident. 7500x.

B. TEM through another cell of a leaf nine stage. Most of the cell is occupied by the large central vacuole (V). The band of cytoplasm containing the remaining cell organelles surrounds this large vacuole. The nucleus (Nu), chloroplasts (Ch), and mitochondria (Mi) are visible in this cytoplasmic area. 7500x.


42A
V

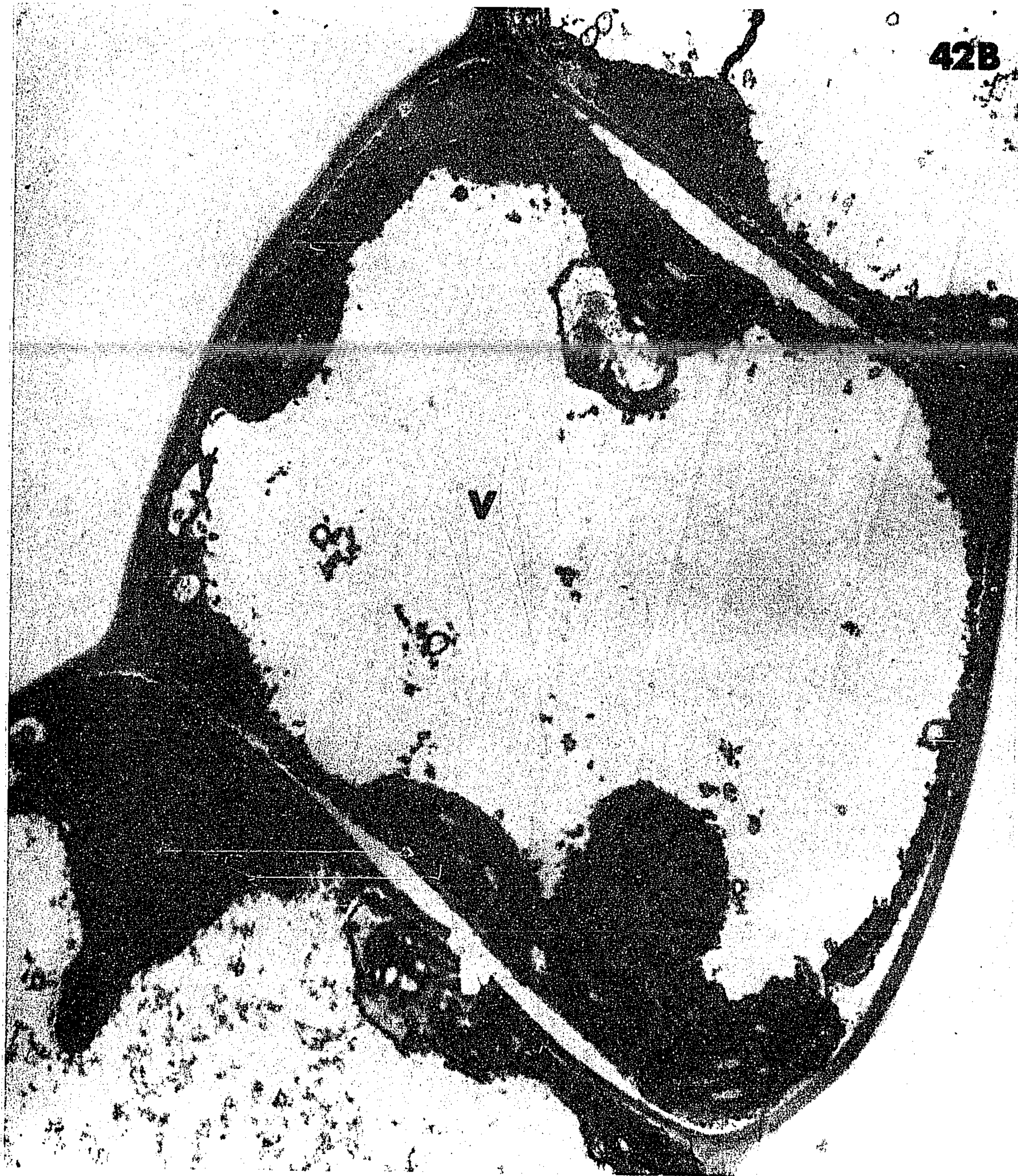

42B
V

Figure 43. TEM of a portion of several large vacuolated (V) cells in a portion of the leaf cut parallel to the leaf surface. The cytoplasmic contents are mainly appressed to the cell walls. Numerous chloroplasts (Ch) containing starch grains are visibile. Cell wall corners are thickened where three cells meet (Arrow). 7500x.

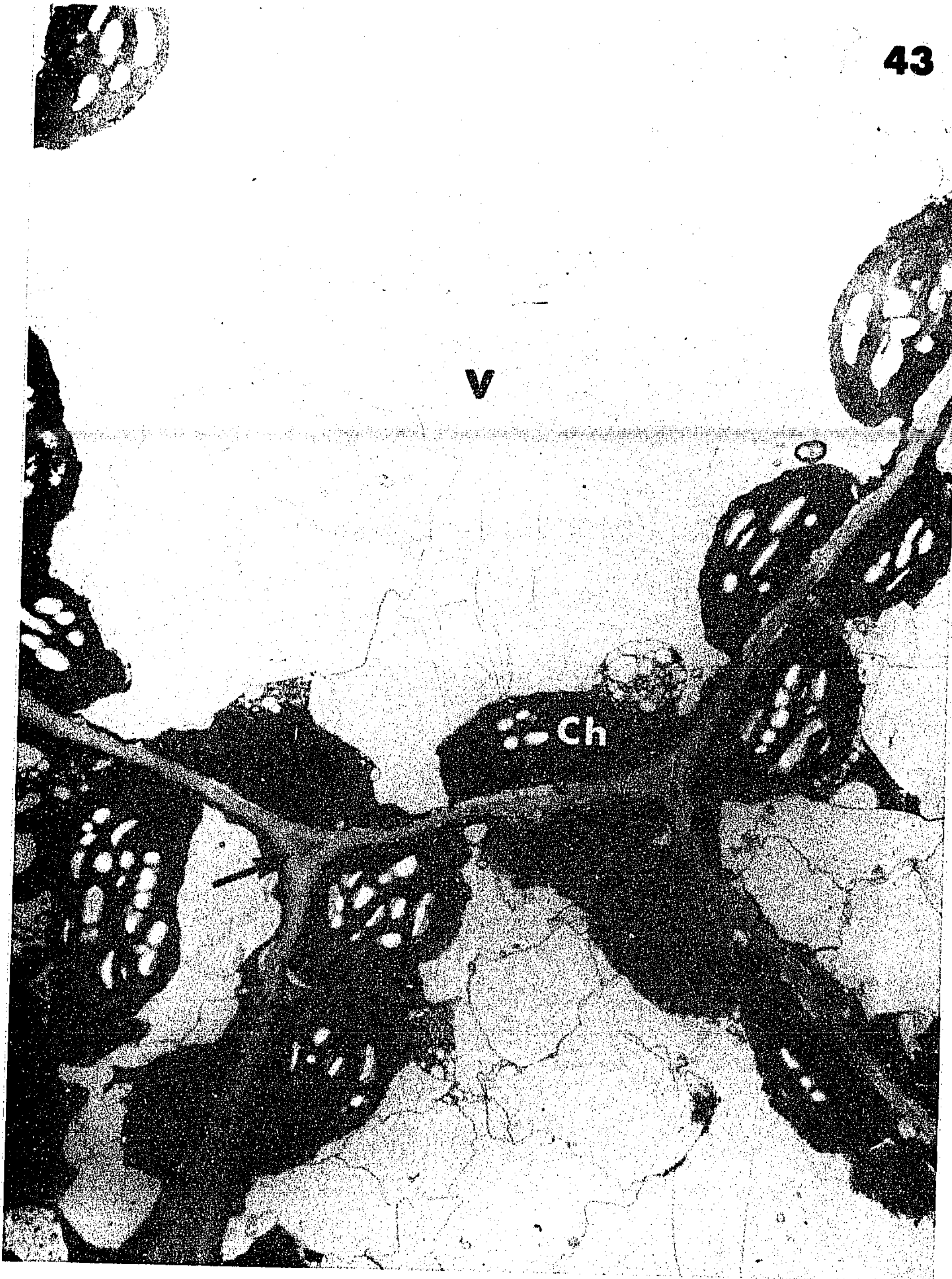

43
V
Ch

Figure 44. TEM of the cell wall area between two adjacent cells. The nucleus (Nu) with a well developed nuclear membrane (White arrow) is visibile in one cell. Both cells contain well developed chloroplasts (Ch) containing starch grains (S) and grana stacks. Mitochondria (Mi) are present. Endoplasmic reticulum is visible. A small lomasome area (Black arrow) can be seen. 21700x.

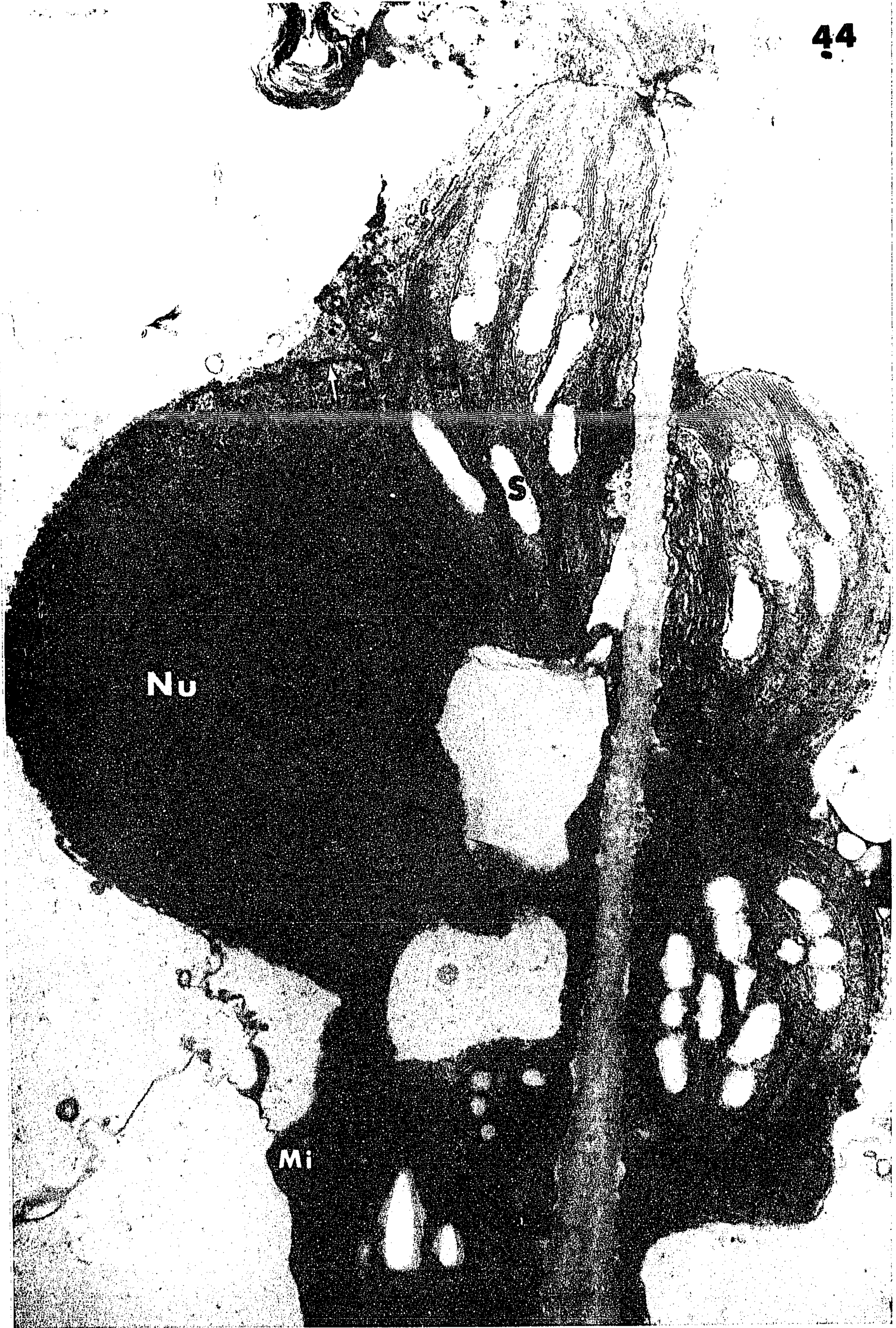

44
S
Nu
Mi

Figure 45. TEM of a portion of a cell wall area between two adjacent cells. Most prominent are the chloroplasts (Ch) containing starch grains (S) and well developed thyllakoids (Th) in grana stacks. Mitochondria (Mi) contain numerous christae. Golgi (G) and Golgi vesicles are present. 21700x.

45
G
Mi
Ch
S

Figure 46. TEM through a portion of the leaf showing parts of two cells at the outer wall boundary. The thickened area between the two cells at the outer cell wall (CW) boundary is evident. The visible chloroplast (Ch) contains numerous starch grains (S) and well-developed grana stacks. Mitochondria (Mi) are visible. Portions of the large vacuoles (V) are seen. Also small vacuole like compartments containing what appear to be small oil droplets (Arrow) are present. These may be precursors to newly forming oil bodies. Endoplasmic reticulum is present. 21700x.

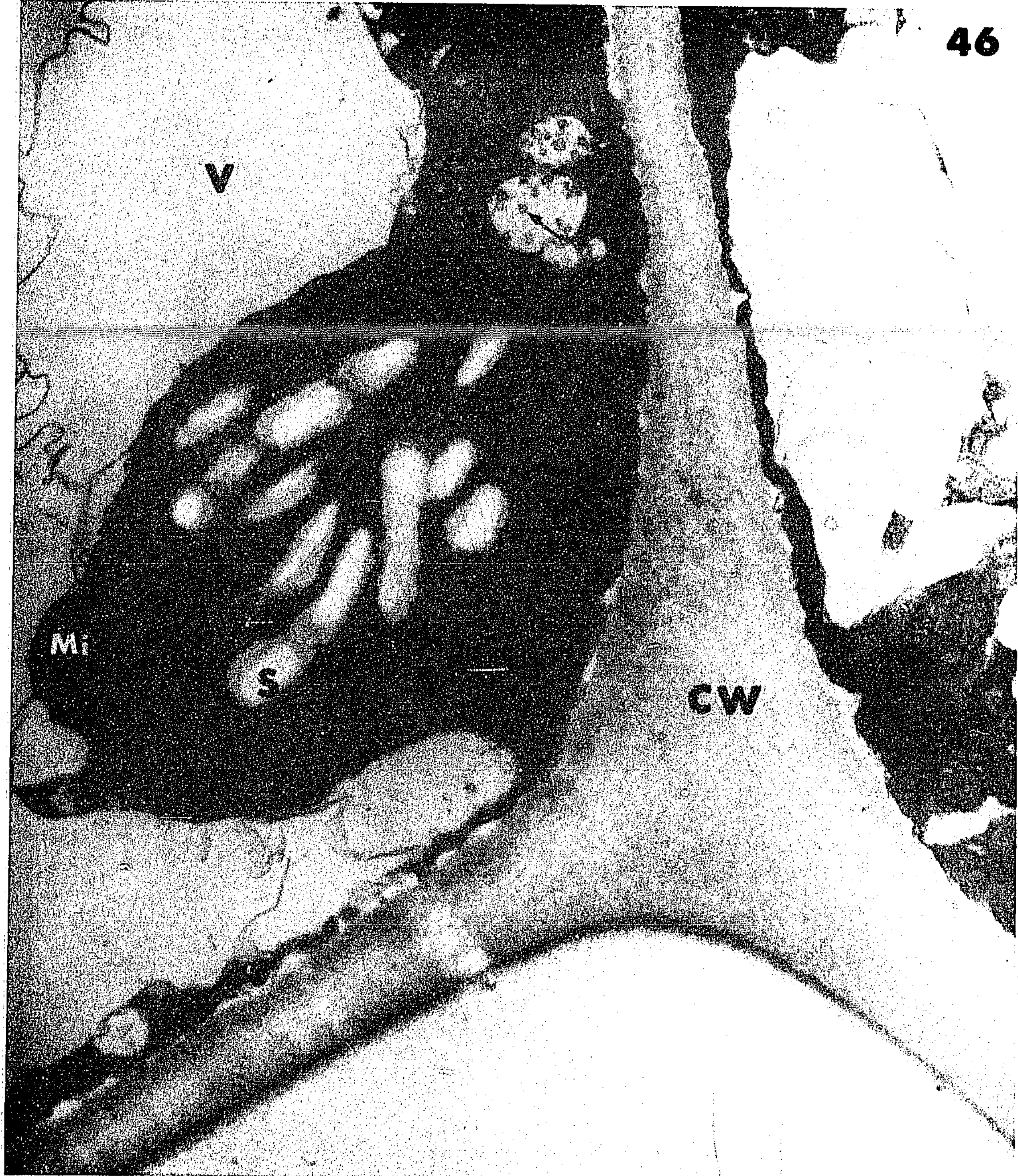

46
V
Mi
S
CW

Figure 47. TEM through a portion of a leaf showing the cell wall (CW) between two cells and parts of the bands of dense cytoplasm containing the organelles of the cells. In one cell a portion of the nucleus (Nu) can be seen. It has a well-developed double membrane (Single arrow). The chloroplasts (Ch) contain starch grains (S) and well developed thyllakoids (Th). Mitochondria (Mi) with christae are present. Endoplasmic reticulum can be seen. A group of cortical microtubules (Double arrows) can be seen in one cell. 21700x.

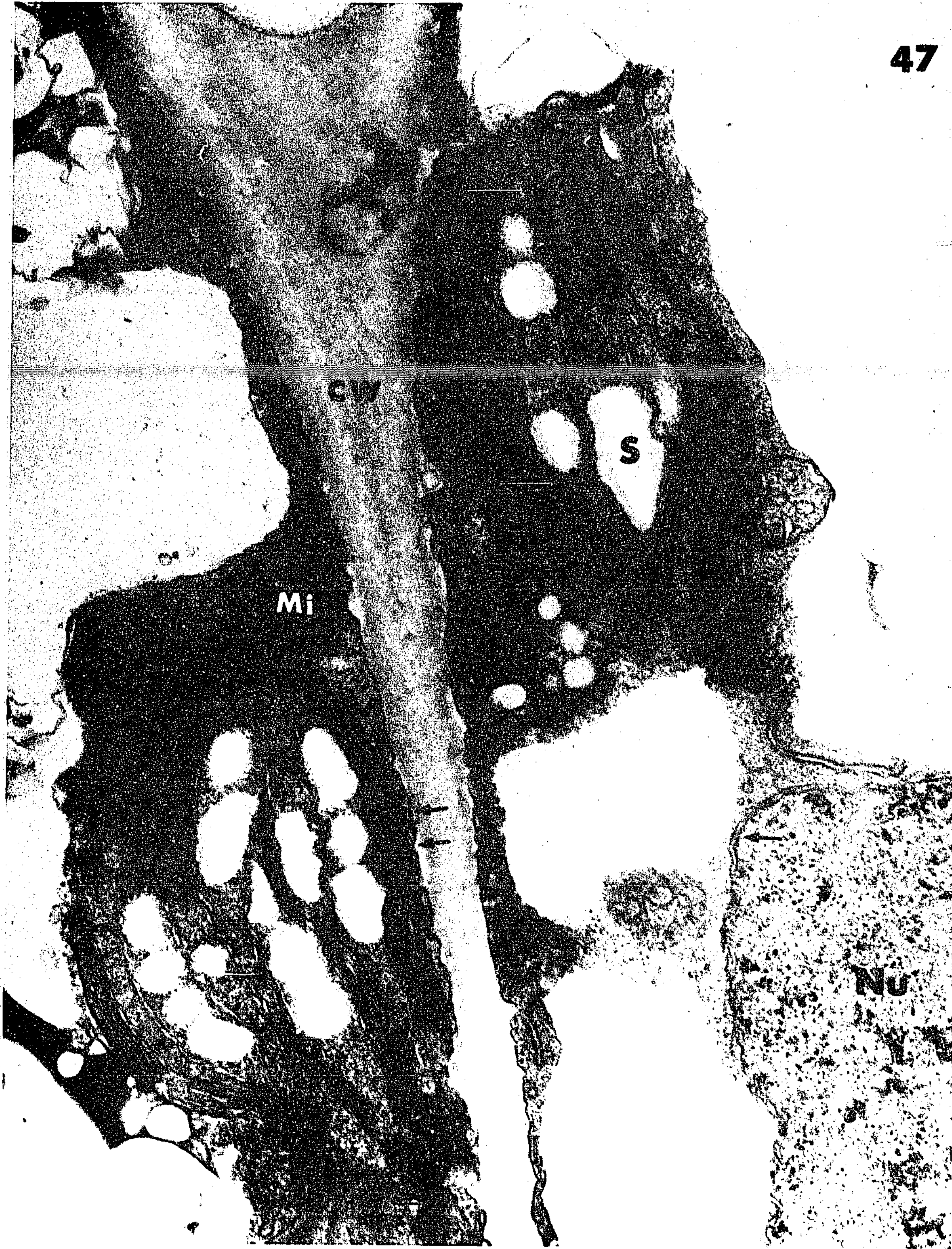

47
CW
S
Mi
Nu

Figure 48. TEM through a portion of a cell. The prominent chloroplast (Ch) contains numerous starch grains (S) which occupy most of the stromal space of the chloroplast. Well developed thyllakoids (Th) can also be seen. Mitochondria are present. The remaining cytoplasmic area continues to appear dense containing endoplasmic reticulum, and many small vesicles. 25000x.

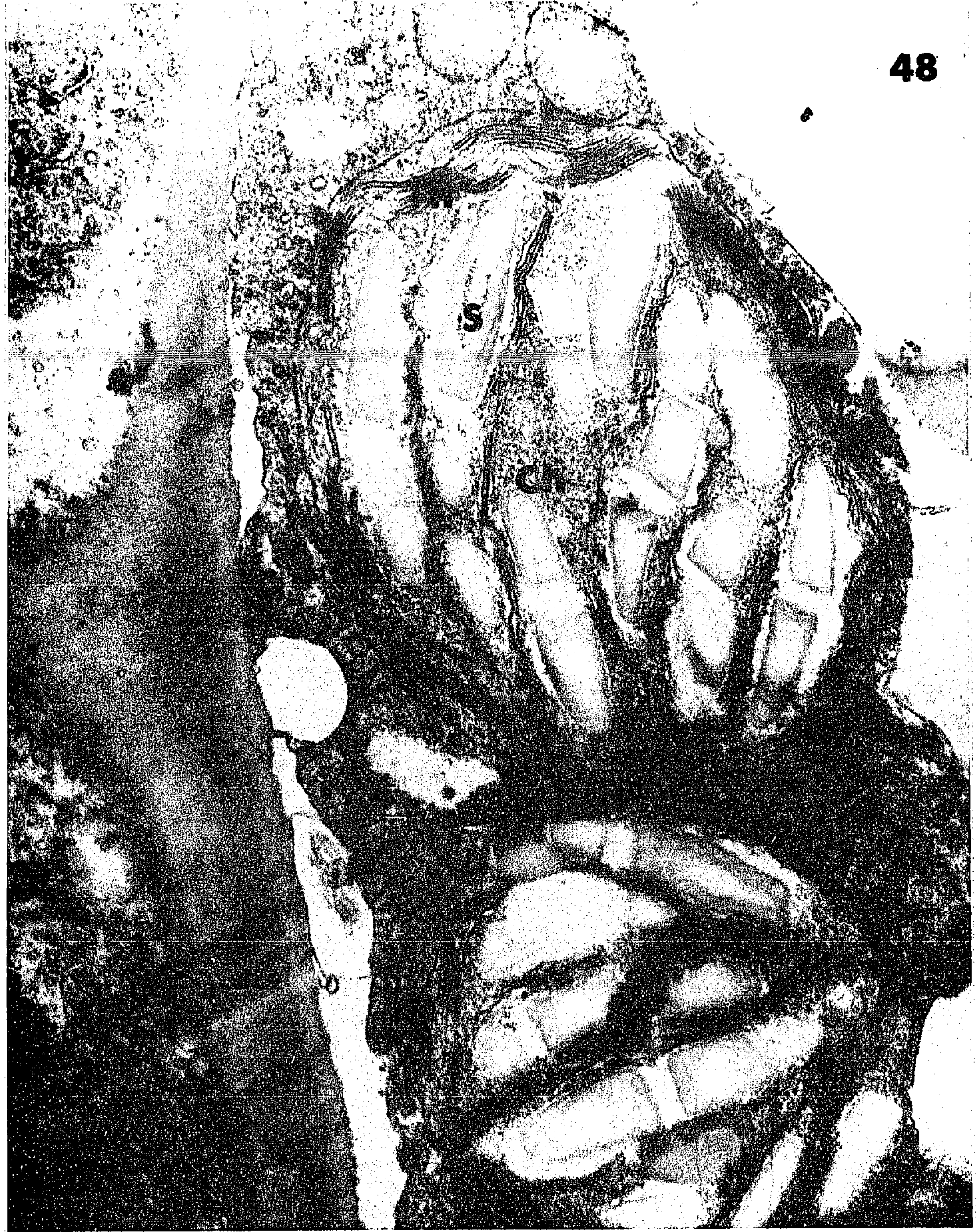

48
S
Ch

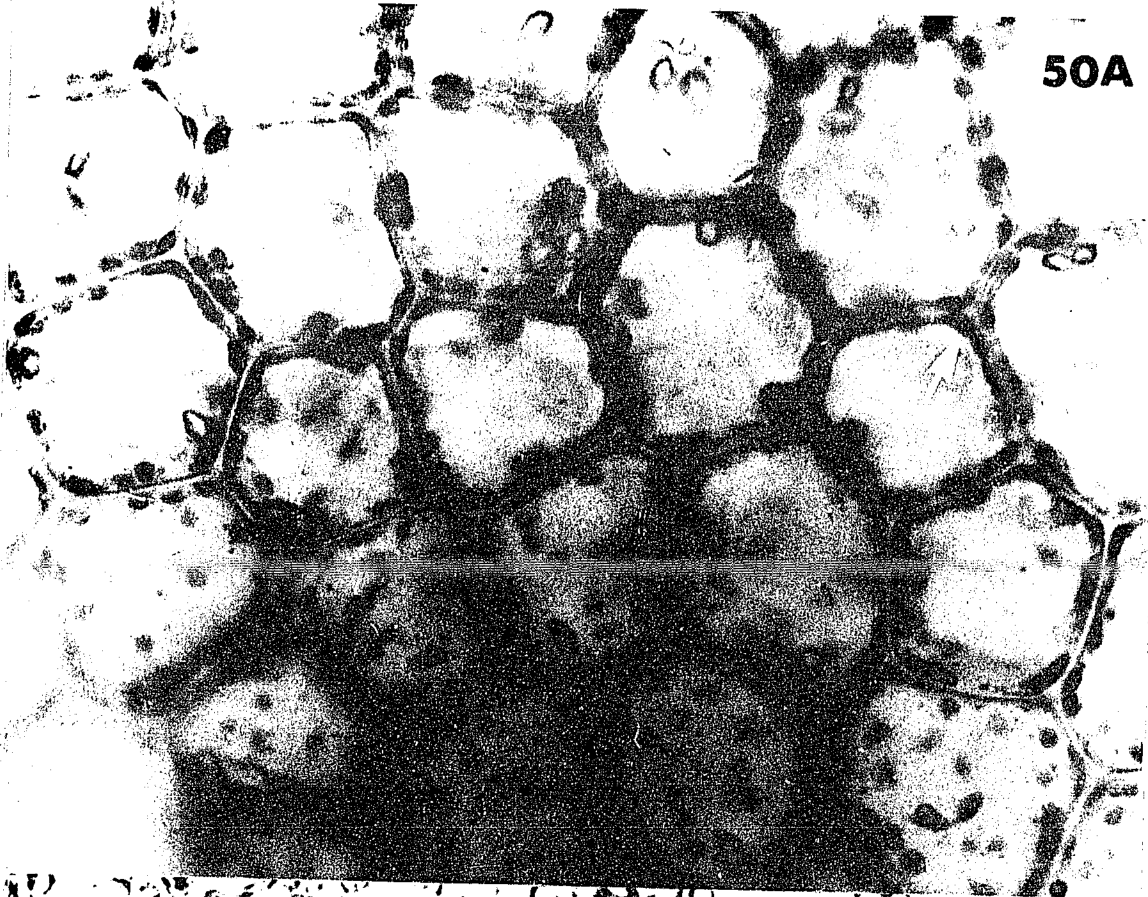
Figure 49. TEM of a cell wall area showing the continued presence of cortical microtubules (Arrow). A well-developed mitochondria (Mi) is also visible. 50000x.

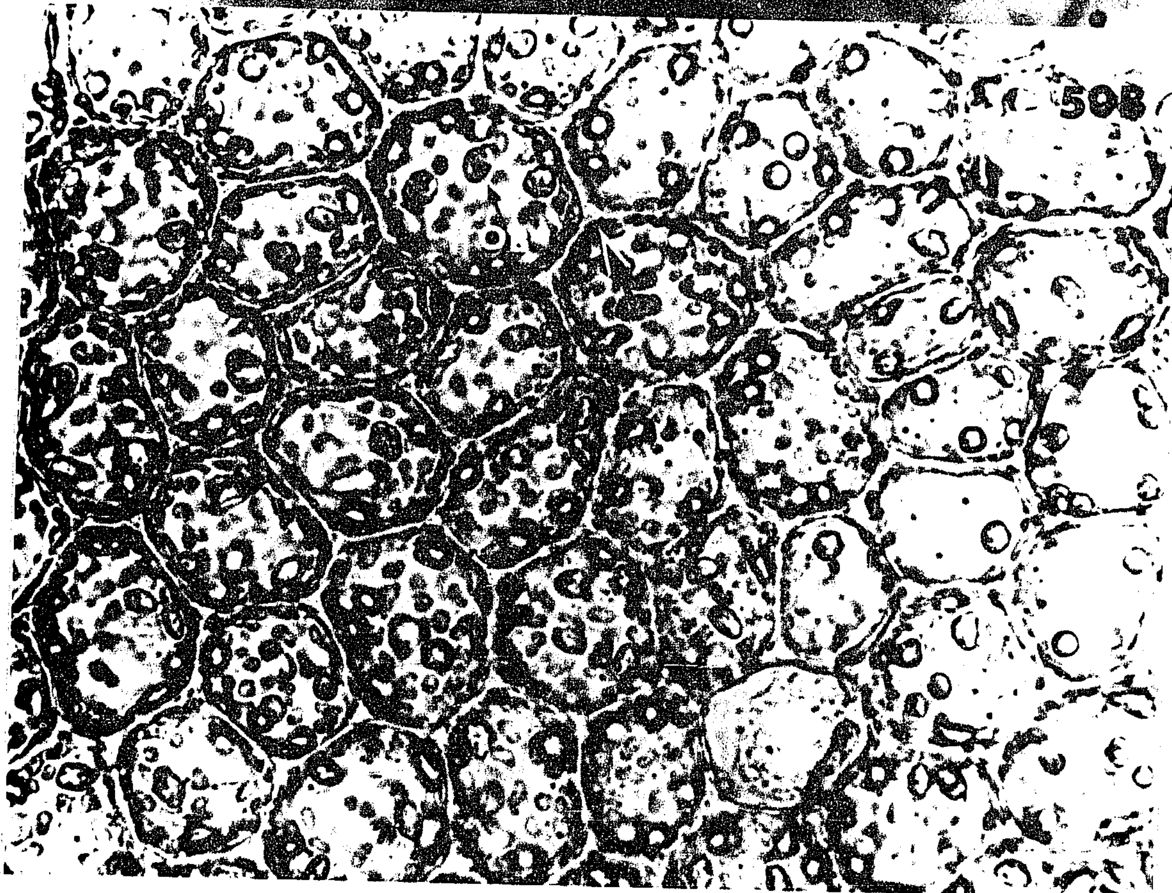
49
CW

Figures 50 A-D. Photomicrographs taken with the light microscope of live, intact, unfixed leaf sections. These all show examples of corners where three or more cells meet that are thickened. This feature of bryophytes is recommonly referred to as being collenchymatous (Schuster, 1980; Smith, 1990). All at same magnification.

A. Plagiochila arctica. Large arrows indicate thickened corners. The small white arrow indicates an oil body.

B-C. Plagiochila spp. Large arrows indicate thickened corners. Small arrows indicate oil bodies (OB).


50A


50B

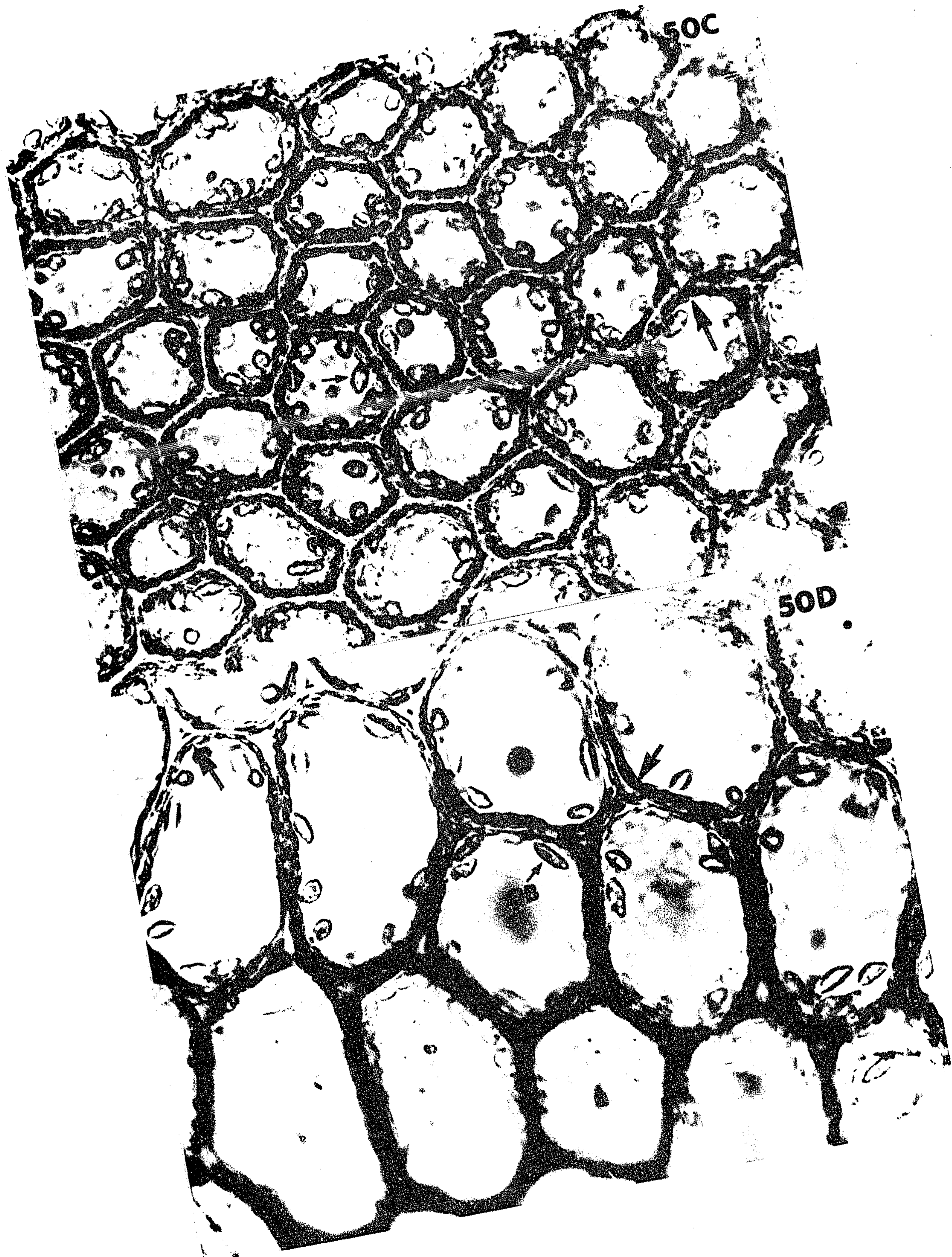
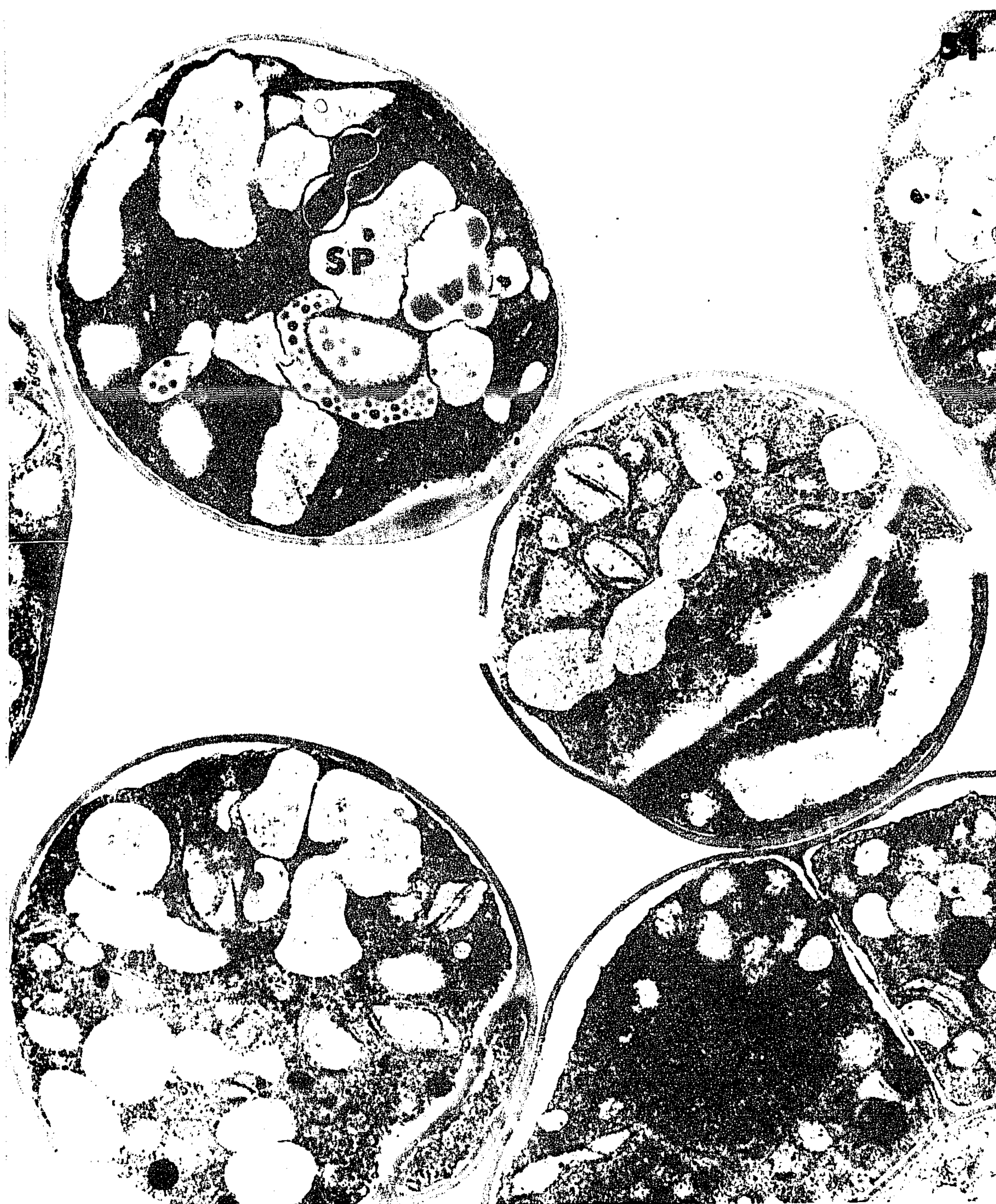

50C
50D

Figure 51. TEM through slime papillae (SP). The cytoplasm is dense and contains all the organelles typically found in a normal leaf cell. Visible are vacuoles, chloroplasts and oil bodies. The wall is surrounded by a cuticle. 7500x.



54
SP

Figure 52. TEM of a longitudinal section through a slime papilla. It is apparent that the cytoplasm is very dense and contains all the typical organelles found in a leaf cell. Note the numerous Golgi (G), mitochondria (Mi) and developing plastids (Pl). Endoplasmic reticulum and a portion of the nucleus (Nu) is evident. The vacuoles are typical of those found in leaf cells. In some vesicular inclusions are seen which may be precursors to oil bodies. 25000x.

52

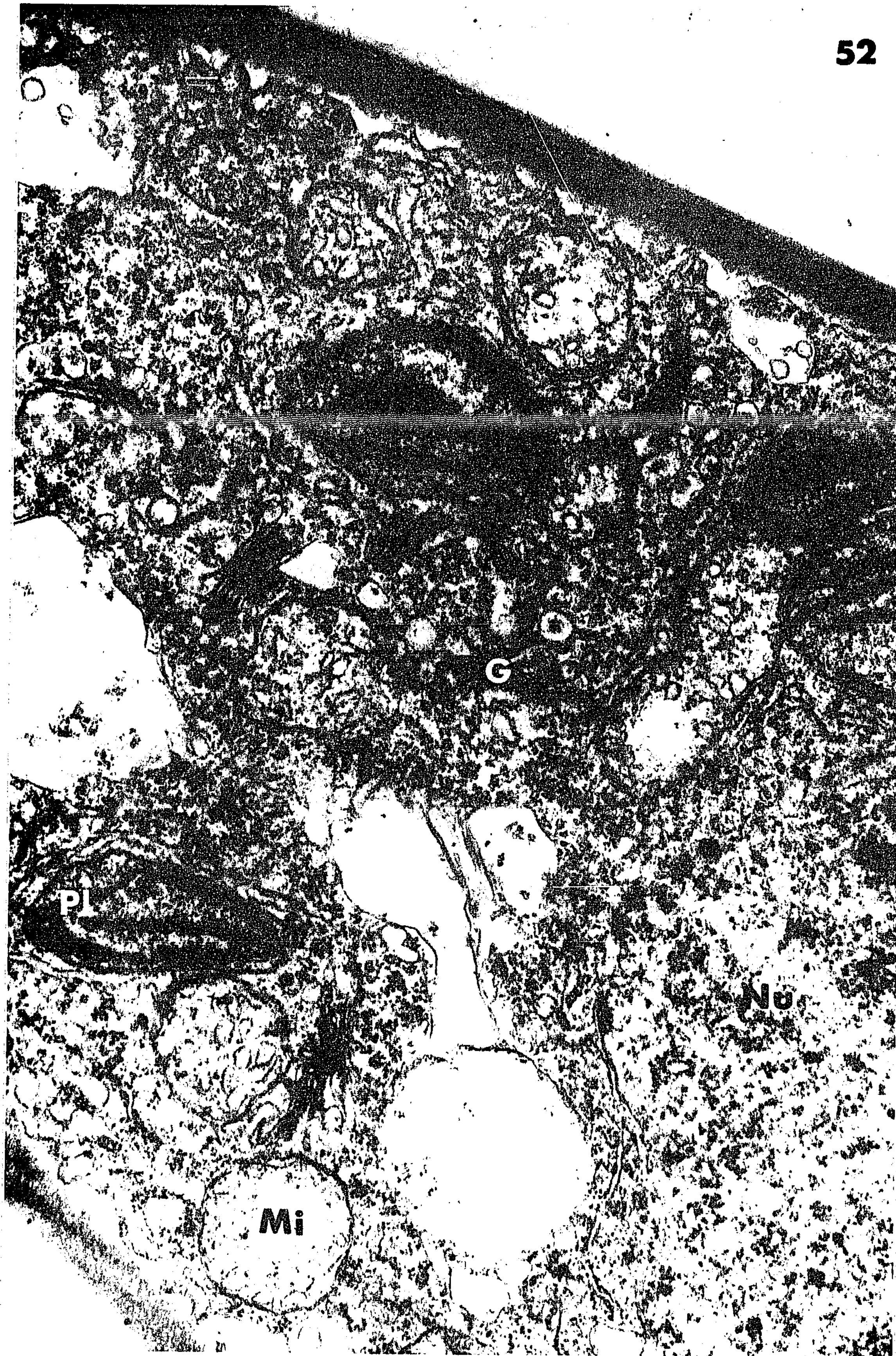


Figure 53. TEM of another area of a slime papilla. This is the basal portion of the slime papilla. Developing plastids (Pl), endoplasmic reticulum (ER) and Golgi (G) are present. Of more interest perhaps is the grid-like inclusion (Arrow) seen in the vacuole (V). Other vesicular like inclusions are also visible in the vacuoles. A plasmodesmatal connection can be seen between the slime papillae and the adjoining cell. The cuticle is evident. 25000x.

53
PI
V

Figure 54. TEM of an "older" slime papilla. Note the thickened cell wall (CW) and the degeneration of the internal cytoplasmic area. The cuticle is indicated by an arrow. 7500x.

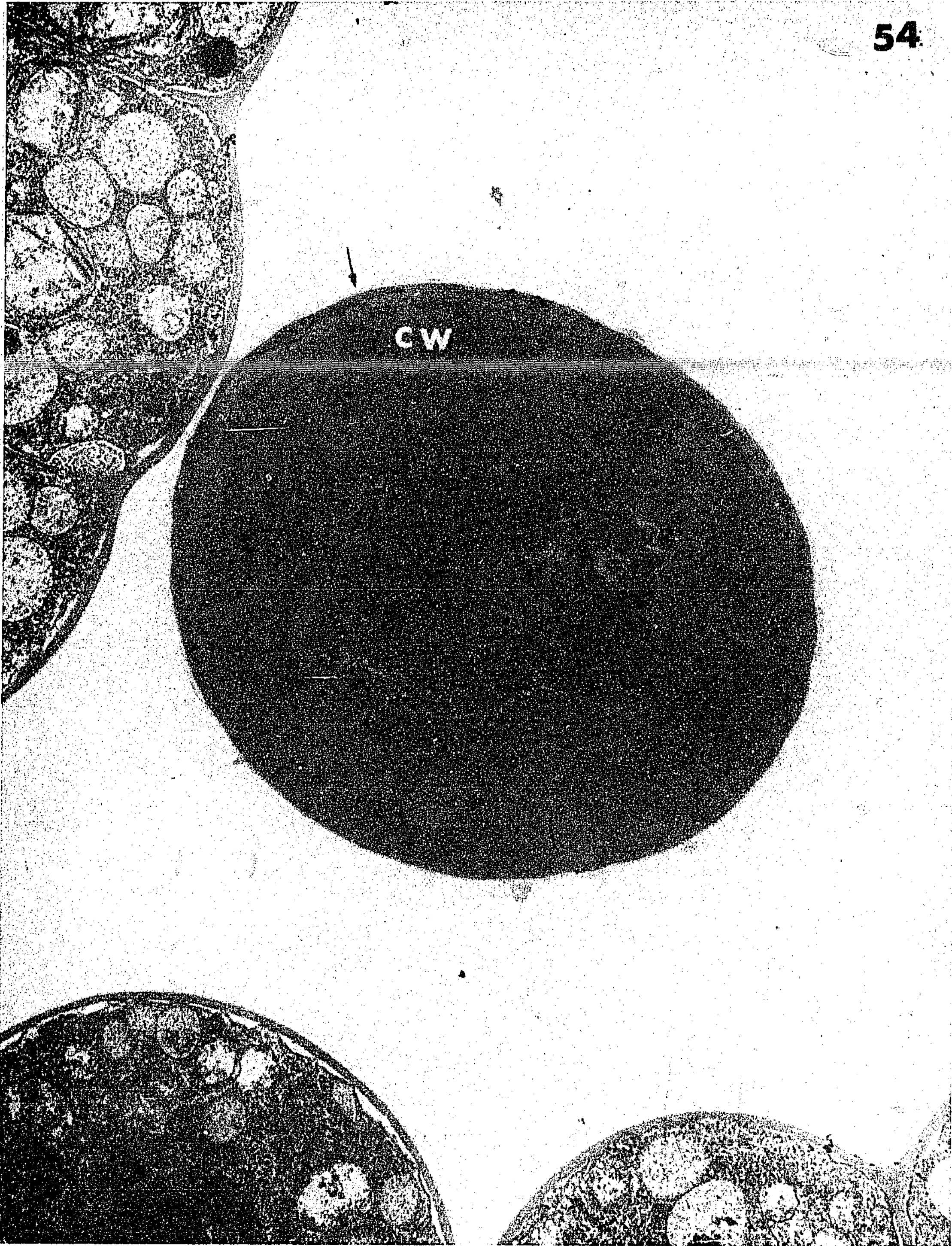

54
CW

Figure 55. TEM of a portion of a mature slime papilla. Although the cell contents appear to be degenerating the vacuoles are still evident as are some of the membranes and lipid inclusions in the cytoplasmic area. A number of lomasome like inclusions are still seen within the wall. 50000x.

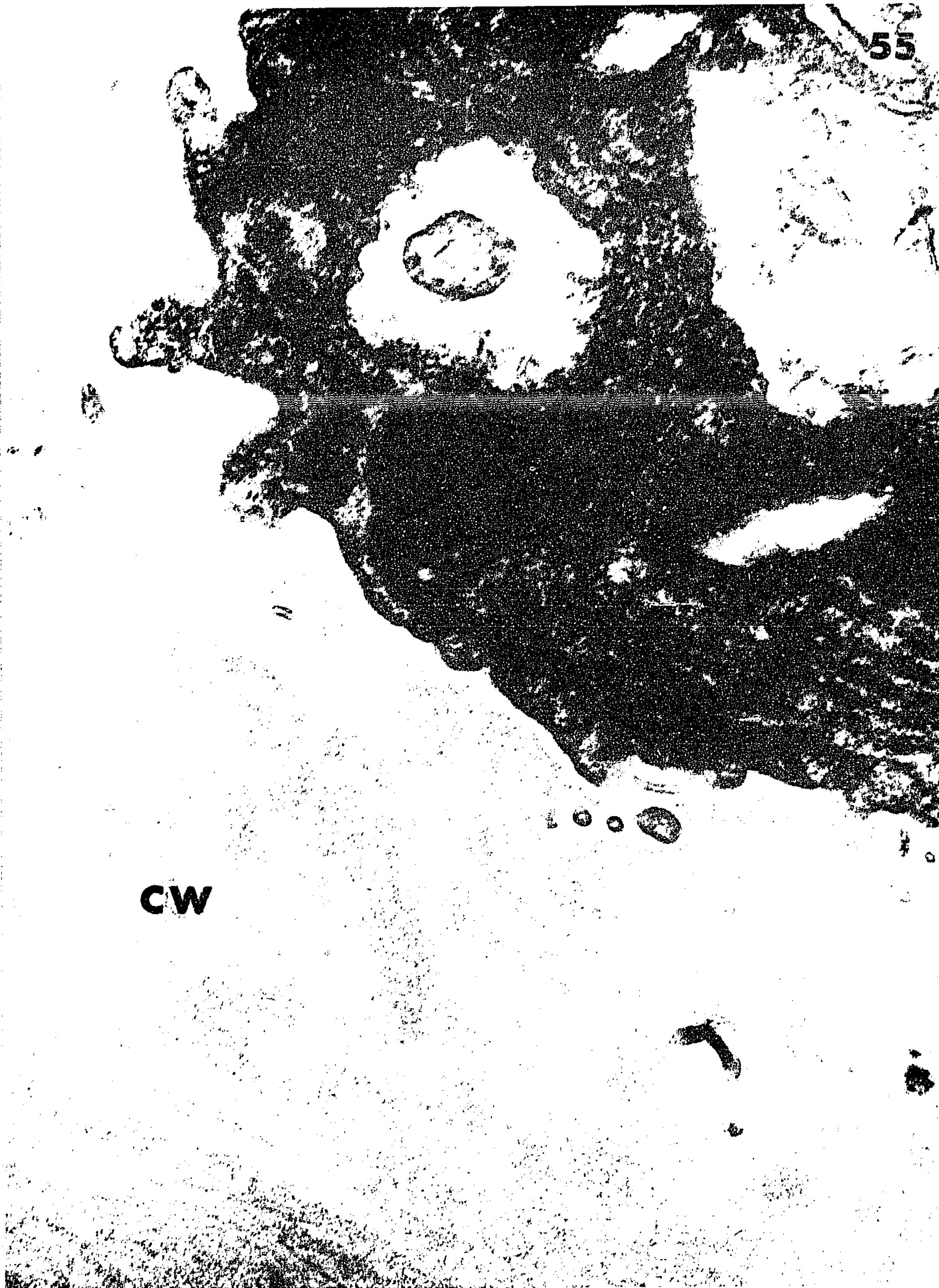

55
CW

Figure 56. TEM of the cell wall (CW) of a mature slime papilla. The wall is layered and "sculptured". A cuticle is visible. 50000x.

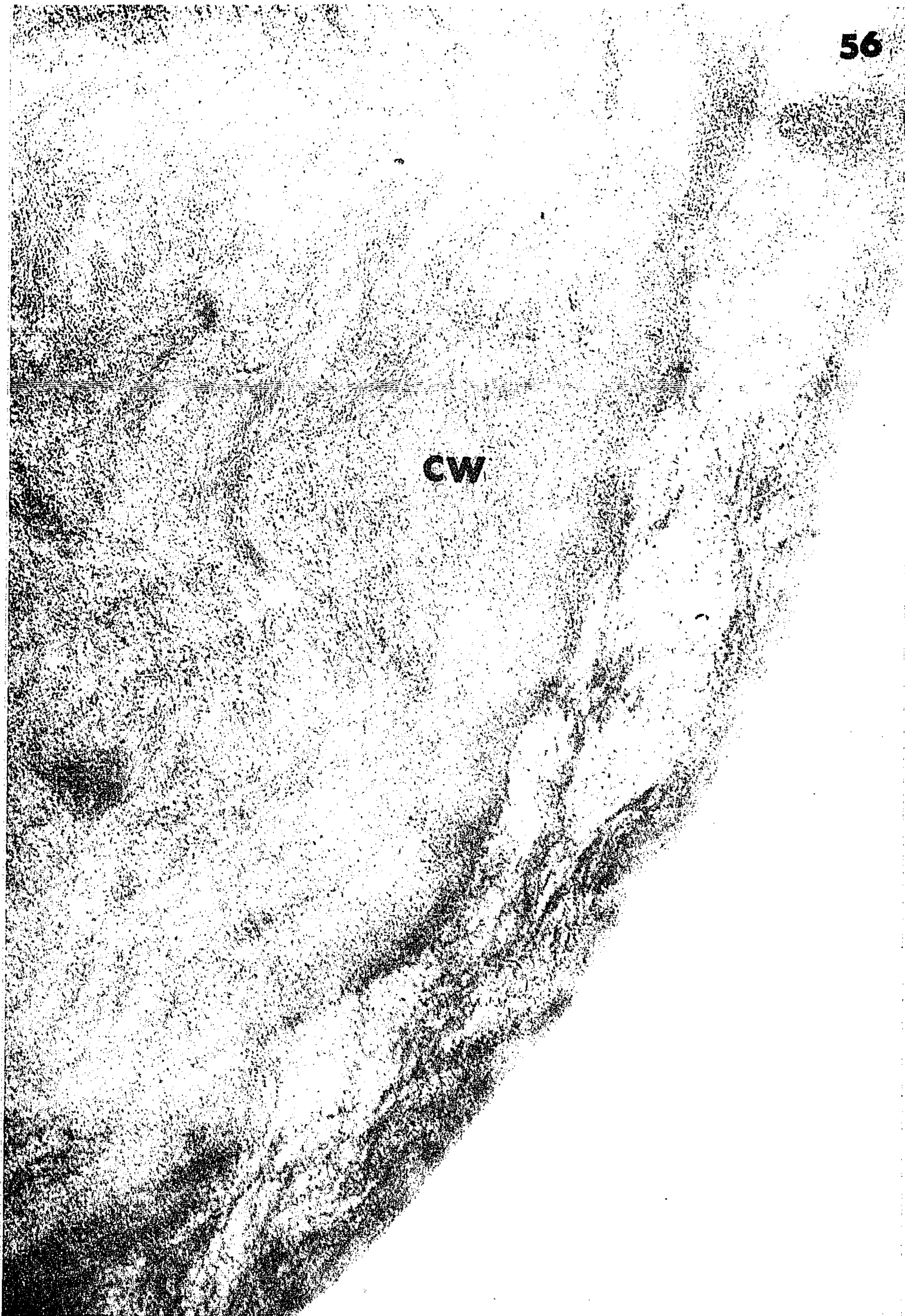
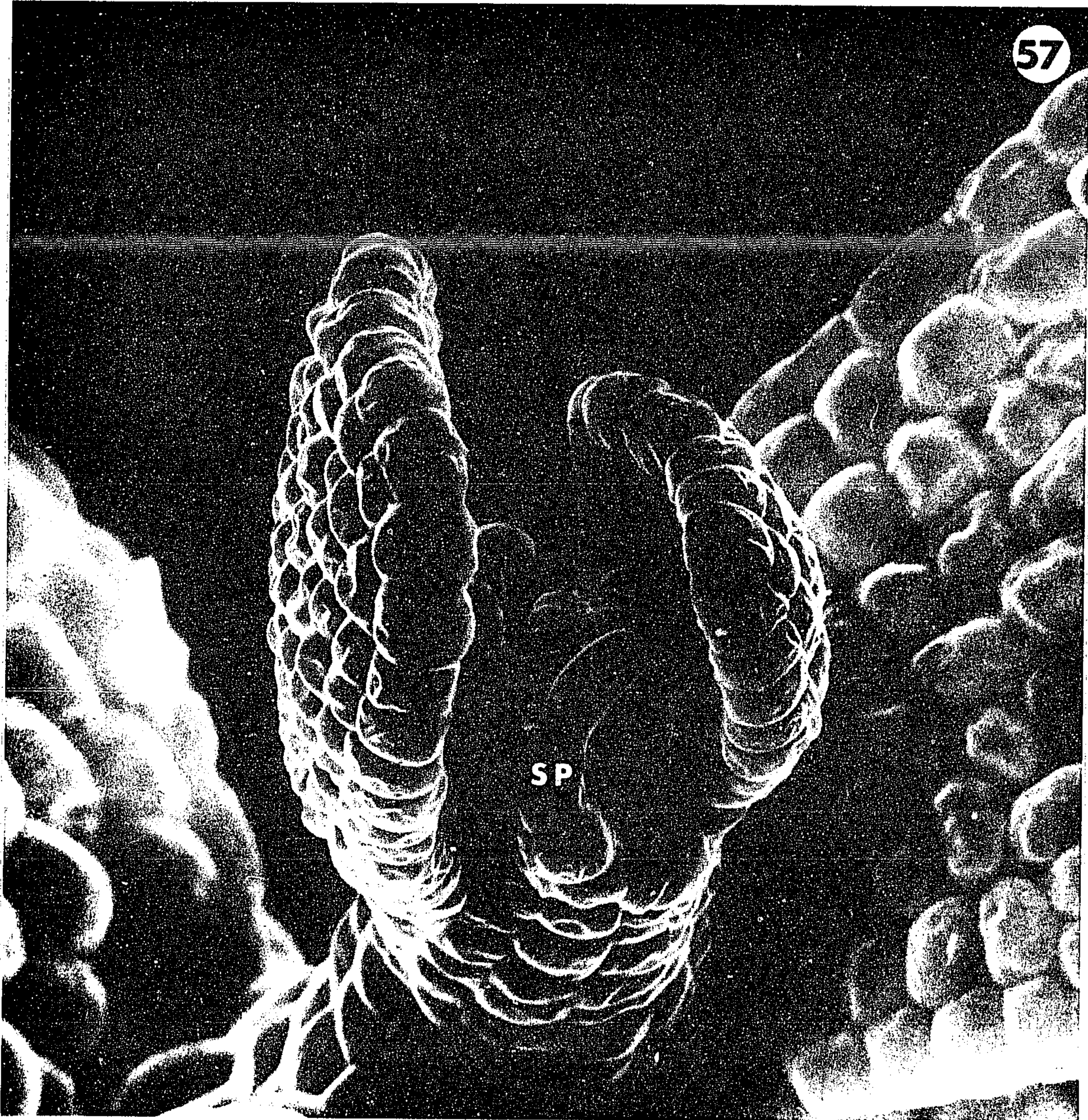

56
CW

Figure 57. SEM of ventral side of the stem. The apical area, containing the apical cell, two-celled leaf primordium, and young leaves can be seen. The single slime papilla (SP) has elongated but clearly has not developed as fully as the adjoining leaves. 350x.


57
SP

Appendix I

Culture Media.

The nutrient medium on which the plants used in this investigation were cultured consisted of a combination of macronutrients, micronutrients, a supplementary carbon source, and a buffering agent.

I. Macronutrients	grams/liter
$(NH_4)SO4$	0.5
$Ca(NO_3)_2.4H_2O$	1.0
$MgSO_4.7H_2O$	0.25
KH2PO4	0.25

This corresponds to formulation F in Basile (1978).

II. Micronutrients	grams /liter
$Fe(NH_4)_2SO_4 . 6H_2O$	7.00×10^{-3}
$MnSO_4 .H_2O$	3.04×10^{-3}
$ZnSO_4.7H_2O$	2.20×10^{-3}
$CuSO_4 . 5H_20$	3.94 x10-4
$(NH_4)_6Mo_70_{24} . 4H_2O$	7.36×10^{-5}
$CoSO_4.7H_2O$	2.38×10^{-4}
H_3BO_3	$5.72 \times ^{10-}$
$Na_3VO_4.16H_2O$	4.62×10^{-5}

This correspond to formulation F' in Basile (1978).

III. Supplementary carbon source

The inorganic nutrients were supplemented with 1% w/v glucose (10 grams/liter) in order to increase the rate of growth and development of the inoculum.

IV. Buffering agent

A buffering agent, 2-(N-morpholino)-ethanesulphonic acid (MES buffer) was added at 0.05 M to combat rapid pH shifts common in ammoniun salt-containing media.

V. pH adjustment

The pH of the medium was adjusted to 4.8 with NaOH .

VI. Sterilization

The culture medium was then cold sterilized through 0.20 μm Nucleopore polycarbonate membrane filters (Nucleopore Corp., Pleasanton, Ca).

Appendix II

Solutions for Electron Microscopy

I. Fixatives and buffers.

A. Glutaraldehyde fixative.

1. A 50% w/w stock solution of glutaraldehyde (Fisher Scientific, Fair Lawn, N.J.) was diluted to a 3% fixation solution in cacodylate buffer, or

2. 6 ml of 50% glutaraldehyde solution was added directly to the culture flasks containing 10 ml of culture medium to make a 3% fixation solution.

B. Sodium cacodylate buffer.

A 0.1 M solution (pH 6.8) of sodium cacodylate buffer was prepared by adding 10.7 gms of sodium cacodylate ($C_2H_6AsNaO_2.3H_2O$) to 500 ml deionized H_2O. The pH was adjusted to 6.8 with HCl.

C. Osmium-tetraoxide (OsO_4) post-fixative.

One gram of osmium tetraoxide was dissolved in 50 ml of deionized water and kept as a stock solution of 2% at 4^oC. When needed for post fixation, the osmium tetraoxide was brought to room temperature and mixed in a 1:1 ratio with cacodylate buffer to make a 1% solution.

II. Post stains.

A. Uranyl acid in methanol (Stempak and Ward, 1964).

Fifteen grams of hydrated uranyl acetate ($UO_2(CH_3COO)_22H_2O$) were dissolved in 50 ml of absolute, acetone-free methanol with a magnetic stirrer for 8 hrs.

The solution was then filtered into a small vial, closed and

stored in a light proof box (or wrapped in foil) at 4 C.

B. Lead citrate (Reynolds 1963).

1.33 gms of lead nitrate ($Pb(NO_3)_2$) and 1.76 gms of sodium citrate ($Na_3(C_6H_5O_7).2H_2O$) were added to 30 ml deionized water, which had been previously boiled to remove CO_2, in a 50 ml volumetric flask.

The mixture was shaken for one minute, then allowed to cool for one-half hour with intermittent shaking.

Eight ml of 1.0 N NaOH was added and the solution was mixed gently. Previously boiled H_2O was then added to the flask to make 50 ml. The stain was then decanted into small vials and stored in a dark box at 4^oC.

Before using it was ascertained that no precipitate had formed.

III. Embedding media.

A. Spurr Low Viscosity Embedding Media (Polysciences, Inc., Warrington, PA).

The medium was freshly prepared as needed. The formula for a firm block was used as follows:

10.0 gm Vinylcyclohexene dioxide (VCD)

6.0 gm Diglycidyl ether of polypropyleneglycol (D.E.R. 736)

26.0 gm Nonenyl succinic anhydride (NSA)

After gently mixing the first three ingredients, the catalyst was added -

0.4 gm Dimethylaminoethanol (DMAE)

The complete medium was then thoroughly but gently mixed.

B. Epon 812 (Polysciences, Inc., Warrington, PA).

Stocks of Epon 812 mixture A and B were prepared and stored

at 4°C.

Mixture A.

62 ml Epon 812

100 ml Dodecanyl succinic anhydride (DDSA)

Mixture B.

100 ml Epon 812

89 ml Nadic methyl anhydride (NMA)

When needed, the stocks were brought to room temperature. Mixture A and B were combined in a 3:2 ratio and 0.2 ml of DMP-30, the polymerization catalyst for Epon, was added for every 10 ml of Epon mixture.

The mixture was stirred thoroughly, but gently to avoid bubble formation, before use.

Appendix III

Fixation, dehydration and embedding procedures for transmission electron microscopy (TEM).

I. Fixation

Tissue was fixed by either of two methods:

A. Tissue fixed in situ.

1. To cultures grown in 10 ml KnM 4.8 medium, 0.6 ml 50% glutaraldehyde was added at room temperature. The plants were fixed for 1-2 hrs at room temperature. - or

B. Tissue fixed in 3% glutaradehyde in cacodyate buffer.

1. A 3% glutaraldehyde solution was made up in 0.1 M cacodylate buffer. Plants were removed from the culture medium and placed in a small vial contaning the 3% glutaraldehyde solution in 0.1 M cacodylate buffer. Plants were fixed for 1-2 hrs at room temperature.

The remaining steps were the same for both sets of plants.

2. Cultures were rinsed 3x in cacodylate buffer (pH 6.8) for 15-30 min each rinse.
3. Cultures were post fixed in 2% osmium tetraoxide (OsO4) for 1hr.
4. Cultures were rinsed in cacodylate buffer 1x for 15-30 min.

II. Dehydration

1. Cultures were incubated in 50% ethanol (ETOH) for l5-30 min.
2. Tissues were incubated in 70% ETOH for l5-30 min.
3. Contents of the culture flasks were then poured out into a petri dish and examined under a dissecting microscope. A

suitable number of plants were then selected and transferred to a small vial in 70% ETOH and the dehydration process was continued.

4. Tissues were incubated in 95% ETOH for 1 hr.
5. Tissues were incubated in 100% ETOH for 15-30 min.
6. Tissues were incubated in 100% ETOH for 15-30 min.
7. Tissues were incubated in 100% ETOH for 15-30 min.

Dehydration and embedding was then continued in a fume hood.

8. Tissues were incubated in propylene oxide (PO_4) which had been brought to room temperature for 15-30 min.
9. Tissues incubated in PO_4 for 15-30 min
10. Tissues incubated in PO_4 for 15-30 min.

Plants were then prepared either for embedding in EPON 812 or Spurrs' embedding medium.

IIa. For EPON 812 the following schedule was used -

11. Tissues transferred to 25% EPON:75% PO_4 for 30 min.
12. Tissues transferred to 50% EPON:50% PO_4 for 1 hr.
13. Tissues transferred to 75% EPON:25% PO_4 for l hr.
14. Tissues transferred to 100% EPON for l hr.

IIIa. Embedding of EPON tissue

Flat embediment molds (Energy Beam Sciences, Agawam, MA) were prepared and labeled.

1. A small amount of embedding medium (EPON) was added to each mold. The individual plant was then carefully placed at the tip end of the mold with the apex located at the top. The

mold was then filled with additional EPON to fill the mold.

2. The molds were placed in a dessicator for 24 hrs.

3. The EPON embedded tissue was then polymerized according to the following schedule:

35 C - 24 hrs.

50 C - 24 hrs.

65 C - 3 days

4. The molds were then allowed to cool to room temperature and the polymerized blocks containing the EPON embedded plants were removed.

IIb. For fixation in Spurr's, the above schedule was followed through step 10. Then the following schedule was used -

Rather than transfer the tissue directly to a Spurr's solution, part or all of the solution was removed each time and Spurr's was added to make the following concentrations.

11. 25% Spurrs' for 30 min.

12. 50% Spurrs' for 1 hr.

13. 75% Spurrs' for 1 hr.

14. 100% Spurrs' for l-2 hrs.

IIIb. Embedding of Spurr tissue.

Flat embediment molds (Energy Beam Sciences, Agawam, MA) were then prepared and labeled.

1. A small amount of embedding medium (Spurrs') was added to each mold. The individual plant was then carefully placed at the tip end of the mold with the apex located at the top. The mold was then filled with additional Spurrs' to fill the mold.

2. The molds were placed in a dessicator for 24 hrs.

3. The Spurrs' embedded tissue was then polymerized in a 70 C. oven for 24 hrs.

4. The molds were then allowed to cool to room temperature and the polymerized blocks containing the Spurrs' embedded plants were removed.

Appendix IV

Preparation of material for scanning electron microscopy (SEM).

I. Fixation

1. To axenically grown cultures of Plagiochila arctica Bryhn & Kaal., 0.6 ml 50% glutaraldehyde was added to 10 ml culture medium at room temperature. The plants were kept at room temperature to "fix" for 1-2 hrs.
2. Cultures were rinsed 3x in cacodylate buffer (pH 6.8) for l5-30 min each rinse.
3. Cultures were post fixed in 2% osmium tetraoxide (OsO_4) for l hr.
4. Cultures were rinsed in cacodylate buffer 1x for l5-30 min.

II. Dehydration

1. Cultures were incubated in 50% ethanol (ETOH) for l5-30 min.
2. Tissues were incubated in 70% ETOH for l5-30 min.
3. Contents of the culture flasks were then poured out into a petri dish and examined under a dissecting microscope. A suitable number of plants were then selected and transferred to a mesh container suitable for use in the Samdri-790 Critical Point Dryer. The container, containing the plants and a small piece of filter paper (labeled for identification and also useful during the transfer from fluid to fluid to help prevent drying out of the plant material between transfers) were then transferred to a small vial of 70% ETOH and the dehydration process was continued.
4. Tissues were incubated in 95% ETOH for 15-30 min.

5. Tissues were incubated in 100% ETOH for 15-30 min.
6. Tissues were incubated in 100% ETOH for 15-30 min.
7. Tissues were incubated in 100% ETOH overnite.

A small amount of 100% ETOH was placed in the drying compartment in the Samdri-790 Critical Point Dryer. The containers of plants were added, the compartment closed, and the plants critical point dried with carbon dioxide (CO_2) according to the manufacturer's published directions.

III. Preparation of CPD plants for examination in the SEM.

1. Selection of plants for SEM analysis.

Upon completion of critical point drying, the plants were removed, poured into a filter paper lined petri dish and examined under a dissecting microscope. Those that appeared to be well-fixed and critical point dried without collapse of cells were selected.

2. Copper or aluminum stubs were prepared using double stick tape.

3. Plants were attached to the stubs using a fine forceps so as not to damage the delicate tissue. They were carefully positioned on the double stick tape on the stub so that all aspects of the plant could be seen on one or another of the specimens (i.e. some with the dorsal surface facing upwards, some with the ventral surface facing upwards, and some with the apex facing upwards).

4. The stubs were mounted in a Technics Hummer II and gold coated with 200 nm gold according to the manufacturer's

published directions. A small amount of silver paint was placed on the side of the stub for better contact.

5. Plants were then observed in the SEM - either the JOEL U-3 or the Amray 1830 - and photographed using Polaroid P/N-55 film.

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