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Structural and Functional Aspects of Stomata

I. Developmental Studies in *Polypodium vulgare*

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Abstract. Differential cell wall thickening in developing guard cells of *Polypodium vulgare* L. has been studied with particular reference to guard cell protoplast deformation and the eventual formation of the stomatal pore. Concomitant studies on the development of guard cell chloroplasts and their starch inclusions during ontogeny of the stomatal complex have provided data which have been incorporated into a model to account for the formation of the pore. Guard cell starch inclusions reach a maximum density per unit volume at the same time as the guard cell walls achieve maximum differential thickening. These events coincide with the development of the pore. It is suggested that, whilst pore formation is initiated enzymatically, the mechanical forces required to bring about the separation of the two guard cells are of an osmotic nature derived from starch hydrolysis. The development of the mesophyll in relation to the epidermis is examined in respect of the formation of substomatal chambers.

Key words: Cell walls – Ontogeny, stomatal – *Polypodium* – Starch – Stomata.

Introduction

The development of stomatal complexes has been extensively examined in relation to their ontogenetic derivation and final morphological form (reviewed by Fryns-Claessens and Van Cotthem, 1973), but there is little information available concerning the structure of the guard cells during their development from protodermal origins. Stomatal ontogeny in *Polypodium vulgare* was described by Reuter (1942). This investigation was carried out since only two comprehensive accounts exist on the structure of immature guard cells (Kaufman et al., 1970; Palevitz and Hepler, 1976), and neither of these are on fern species.

Terminologies

The terminologies employed in respect of the ontogeny of the stomatal complex and its eventual morphological form are those used by Stevens and Martin (1978).

The term 'stomatal complex' refers to the complete stomatal apparatus and includes the guard cell pair, the subsidiary cells, and the neighbouring cells. The term 'Stroma' is used in its restricted sense and is applied only to the pore which is formed between a pair of guard cells. The guard cell pair plus its associated subsidiary cells (if present) is commonly subtended by an extended air space in the mesophyll which is identified as the substomatal chamber.

Methods and Materials

The investigation was carried out on young circinately-folded pinnae obtained from *P. vulgare* plants which had originally been collected from a variety of sites in the Plym Forest, Devon. Whole pinna segments were used in the electron microscope studies, whilst detached epidermal strips were used for the light microscope studies.

Electron Microscopy

Pinna segments were vacuum-infiltered with 1,5% glutaraldehyde in 25 mmol l^{-1} cacodylate buffer (pH 7.0), followed by 2% osmic acid in cacodylate buffer for 2.5 h, dehydrated in acetone, and embedded in Spurr Resin (Spurr, 1969).

Silver and gold sections were obtained with an LKB Ultratome III and a Porter Blum MT2-B Ultramicrotome, mounted on coated grids and examined with a Phillips EM 300. No post-staining was employed unless stated to the contrary in the figure legends.

Light Microscopy

Light microscopy was carried out on a Carl Zeiss Photomicroscope II. Pectinaceous materials were tested for by the Ruthenium Red

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method (Johansen, 1940), the hydroxylamine-ferric chloride method (Reeve, 1959), and the Methylene Blue method (Peacock, 1973); carbohydrates were determined with the periodic acid-Schiff's stain (PAS) (Hotchkiss, 1948; McManus, 1948); starch was tested for with I_2KI (Johansen, 1940).

Problems Associated with the Analysis of Thin Sections

The stomatal meristemoid / guard-cell mother-cell is immediately identifiable from other protodermal cells by its dense cytoplasm, small size, and individual shape. However, we have not been able to identify the nature of the protodermal cells immediately adjacent to the stomatal meristemoid/guard-cell mother-cell as either prospective neighbouring or subsidiary cells and, therefore, it has been difficult to distinguish, with certainty, stomatal meristemoids from guard-cell mother-cells. Despite this, it is possible to infer the developmental stage of the meristemoid from its overall size, shape, and spatial relationships with adjacent cells.

The stomatal meristemoid is believed to arise from a vertical asymmetrical division of a protodermal cell very shortly after the latter cell has been cut off from the marginal meristem of the pinna. The inception of the stomatal meristemoid, in this species, is conjectural since we have not been able to observe its formation even after the examination of a large number of protodermal strips. Light microscopy indicates that the young stomatal meristemoid is sub-cuboid in shape and marginally smaller than its adjacent protodermal cells. The first division(s) of the stomatal mersitemoid are also asymmetrical but do not occur in a strictly vertical plane so that the resultant subsidiary cell(s) obliquely subtend the meristemoid immediately following such division(s). At this stage the stomatal meristemoid is sub-circular in paradermal section and lens-shaped in vertical section. The subsidiary cells quickly retract from their partially inferior positions and eventually only subtend the margin of the meristemoid.

After the (last) subsidiary cell has been cut off, the stomatal meristemoid becomes the guard-cell mother-cell and its next and final division is of a symmetrical type which gives rise to the guard cell pair. We consider that this stage is preceded by the guard-cell mother-cell reverting to its sub-cuboid shape (i.e., it is no longer partially subtended by a subsidiary cell), its increased size, and its tendency to tear away from the underlying mesophyll tissue (Fig. 2).

Electron Microscope Observations

The earliest developmental stages observed were as illustrated in Figure 1. The typical lenticular cross-section of the cell suggests that it is a stomatal meristemoid, rather than a guard-cell mother-cell, and, furthermore, it is believed that the osmiophilic ribbons present in the section are chromosomal material gathered at a pole during a mitotic event which would have led to the formation of a subsidiary cell.

It is noteworthy that the chloroplasts of the stomatal meristemoid contain prominent granal lamellae and starch inclusions in contrast to the adjoining protodermal cells. At this stage, there is little sign of vacuolation in the stomatal meristemoid, whilst adjacent protodermal cells are already showing signs of maturation in the form of quite extensive vacuolar systems.

As the stomatal meristemoid develops into the guard-cell mother-cell, the most obvious changes noted were the changes in cross-sectional dimensions and progressive vacuolation (Fig. 2). By this stage, the adjacent neighbouring/subsidiary cells are very highly vacuolated so that almost the entire lumen of the cell is occupied by a single large vacuole with the protoplast and its inclusions of organelles being restricted to the periphery of the cell. Figure 2 also illustrates the endocuticular covering of the inner face of the guard-cell mother-cell beginning to pull away from that which covers the underlying mesophyll tissue.

Immediately following the division of the guard-cell mother-cell, membranous vesicles are laid down in the plane of the future common anticlinal guard cell wall (Fig. 3). As the deposition of the cell wall elements proceeds about these vesicles, the latter enlarge and fuse together. Concomitantly, a wedge of cell wall material commences to be laid down at the junctions of the upper and lower extremities of the developing anticlinal wall with their respective periclinal walls (Fig. 4). At about the same time, the middle lamella of the common anticlinal wall becomes clearly differentiated.

During the maturation process, the wedges associated with the upper and lower extremities of the common anticlinal wall become massively developed (Fig. 5). The wedges can be seen to be formed by the sequential deposition of layers of cell wall material onto the inner face of the thickenings next to the guard cell protoplast. This build up of cell wall material causes a considerable deformation of the guard cell lumen which eventually leads to the nucleus becoming almost wedged between the thickenings of the anticlinal walls (Fig. 5). At the same time, the region of the outer periclinal walls and, to a lesser extent, that of the inner periclinal walls, associated with the wedge-shaped thickenings become depressed below the general levels of these cell walls (Figs. 5 and 9). Following the formation of the wedge-shaped

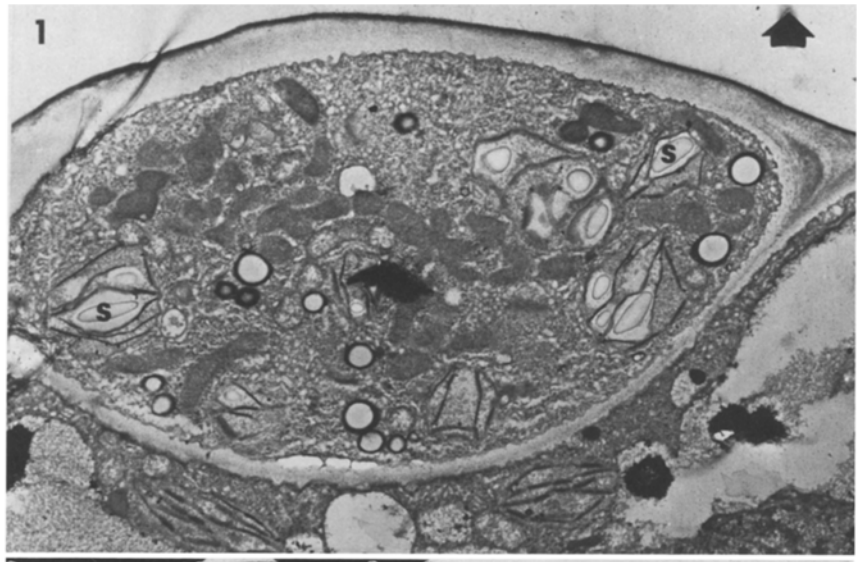


Fig. 1. Vertical transverse section through a stomatal meristemoid. Electron micrograph, ×6400. The solid bold arrow indicates abaxial orientation. The dense protoplast of the stomatal meristemoid contains chloroplasts with well-developed granal stacks and starch inclusions (*s*). The osmiophilic ribbons are believed to be chromosomal material. The protodermal cell subtending the stomatal meristemoid is characterised by the presence of chloroplasts which do not contain starch and a developing vacuolar system

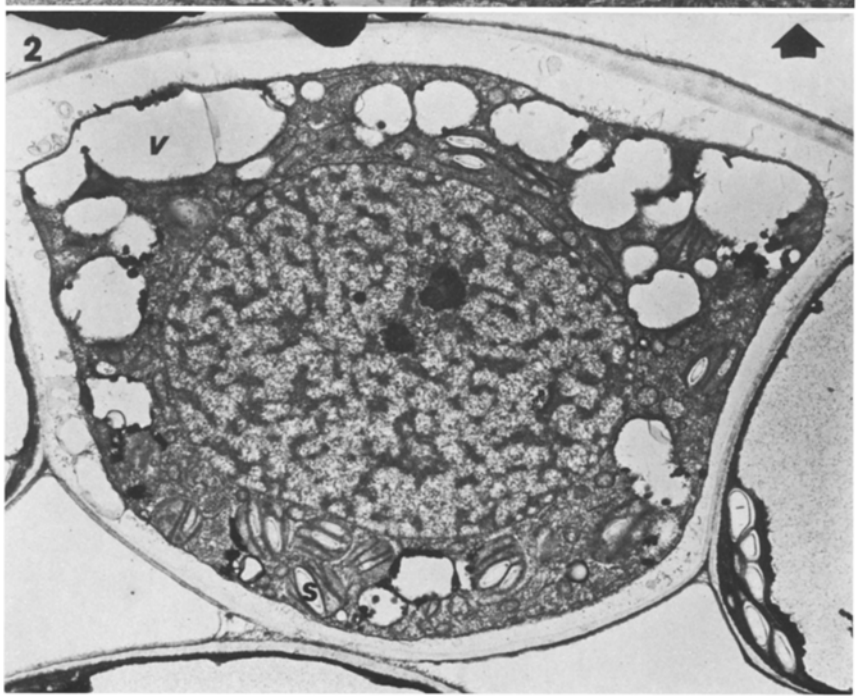


Fig. 2. Vertical transverse section through a young guard-cell mother-cell. Electron micrograph, ×5480. The solid bold arrow indicates abaxial orientation. As in Figure 1, the guard-cell mother-cell contains well-developed chloroplasts with starch inclusions (*s*). By this stage of development vacuolation (*v*) has already commenced. The adjacent mesophyll and protodermal cells are characterised by their large central vacuoles and peripheral protoplasts which contain chloroplasts which, at this stage, exhibit starch inclusions. Note the endocuticle of the guard-cell mother-cell is beginning to tear away from that of the mesophyll

thickenings, the middle lamella region of the common common anticlinal wall commences to separate in the region of the eventual stoma although the guard cell pair remain attached to each other at the extreme apices of the wedges. At the same time, whilst the bulk of the periclinal walls, not associated with wedge formation, continue to develop and thicken as new cell wall material is incorporated into them, those regions of these cell walls immediately adjacent to the wedges appear to become arrested in their development and remain relatively thin (Fig. 5).

During the course of development of the guard cells, the starch content of their chloroplasts continues to increase; an increment which is largely restricted to an increase in the number of starch grains present. However, at the time that the cell wall thickenings achieve maximum development, there is a very noticeable increase in the size of the individual grains (Fig. 6, in which the chloroplasts account for ca. 60% of the area of the protoplast).

Following the formation of the stoma, the guard cell lumen becomes sub-circular in cross-section and the differentially thin areas of the periclinal walls, adjacent to the wedge-like thickenings, become indistinguishable from the rest of the periclinal walls (Fig. 7). During the subsequent maturation of the

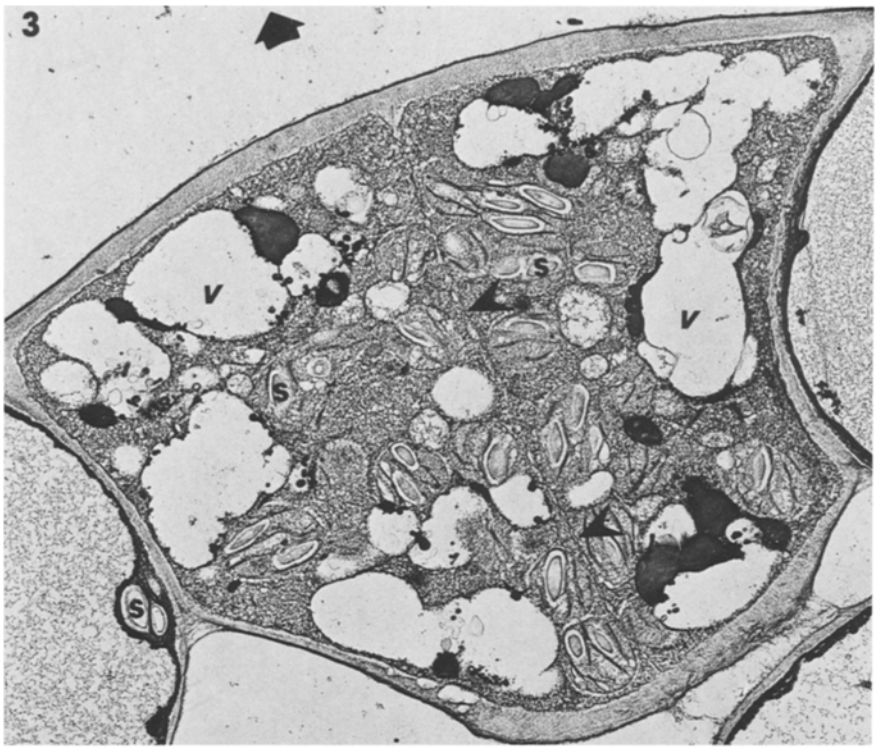


Fig. 3. Vertical transverse section through an immature guard cell complex. Electron micrograph, × 6390. The solid bold arrow indicates abaxial orientation. Both the vacuolar system (*v*) and the starch inclusions (*s*) are rather better developed than in the preceding figure (Fig. 2) of a guard-cell mother-cell. The solid darts indicate the vesicles associated with the early stages of the deposition of the common anticlinal wall. Note the numerous gaps in the vesicular system

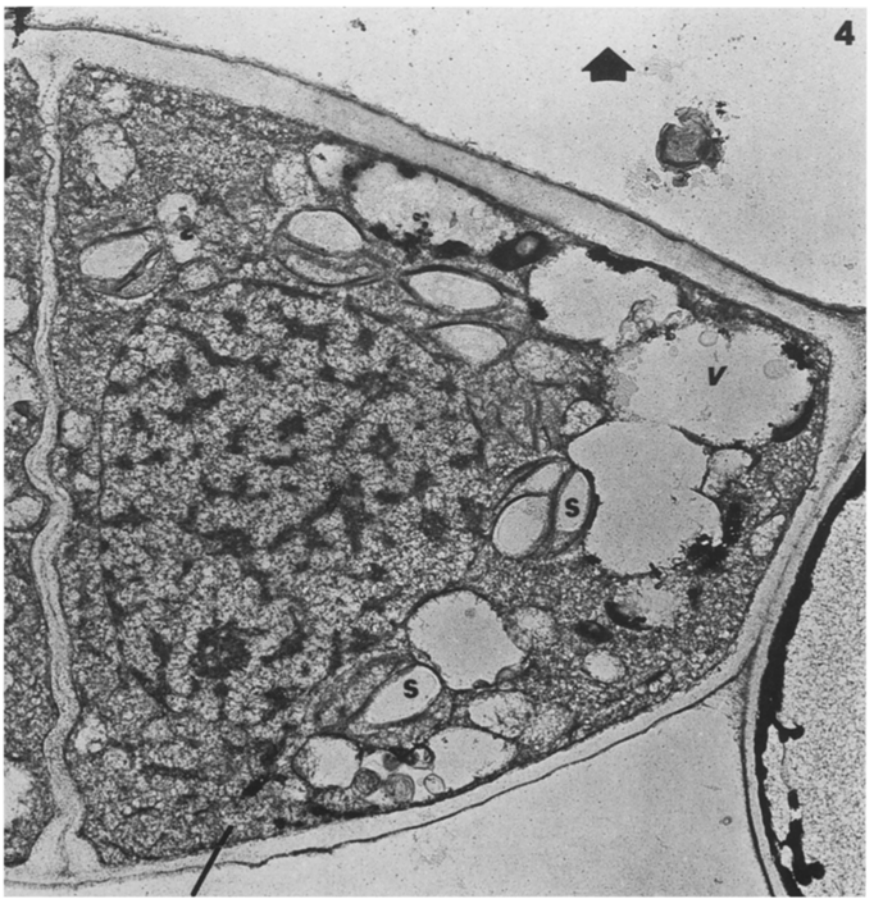


Fig. 4. Vertical transverse section through a single immature guard cell. Electron micrograph, × 8640. The solid bold arrow indicates abaxial orientation; *v* vacuoles, *s* starch inclusions. This micrograph is of a rather later stage than Figure 3 and shows the common anticlinal wall as a solid structure with a well-defined middle lamella which does not extend to the outer faces of the periclinal cell walls. The deposition of the wedge-like thickenings at the upper and lower extremities of the common anticlinal wall has just commenced. The section was post-stained with uranyl acetate and lead citrate

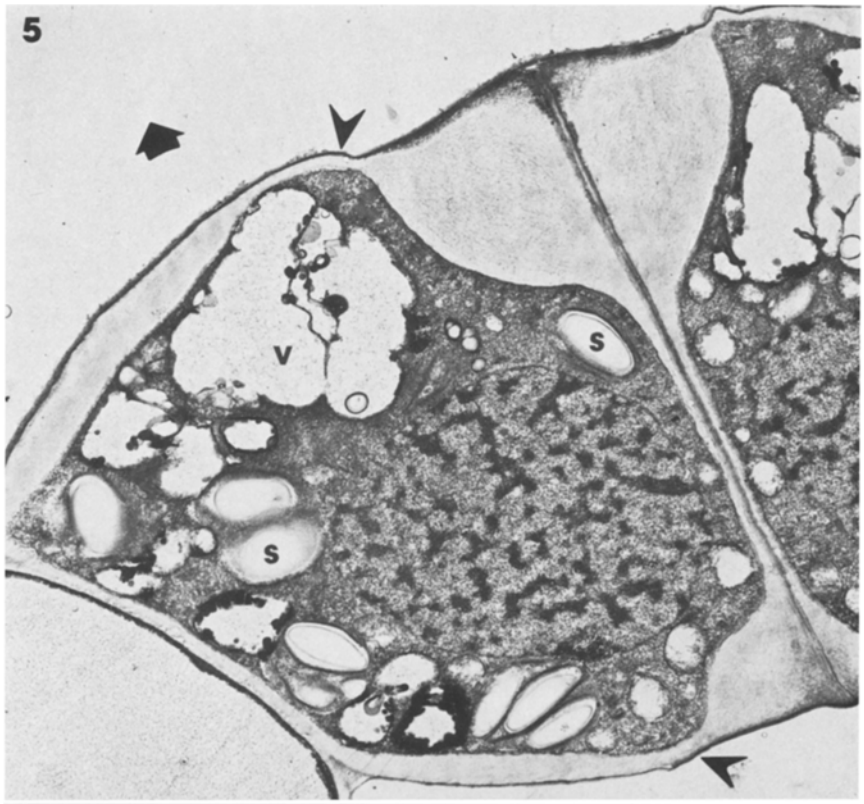


Fig. 5. Vertical transverse section through an immature guard cell complex. Electron micrograph, × 6170— *v* vacuoles, *s* starch inclusions. This section shows the massively developed cell wall ridges at that stage where the middle lamella of the common anticlinal cell walls starts to breakdown. The outer faces of the inner anticlinal walls have already developed a cuticular covering before the guard cell pair completely separate. The wedge-like thickenings are noticeably depressed below the general level of the rest of the periclinal walls and the region of the periclinal walls immediately adjacent to the thickenings are relatively thin (solid darts)

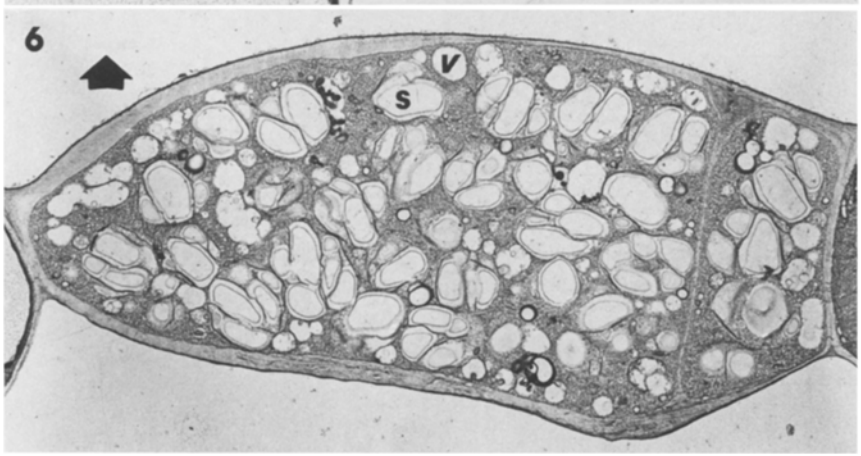


Fig. 6. Vertical obliquely longitudinal section through an immature guard cell complex. Electron micrograph, × 3230. The stage illustrated, which coincides with the actual formation of the stoma, indicates the large increment in starch inclusion bodies (*s*) and the fragmented nature of the vacuolar system (*v*)

guard cells, the proportion of the protoplast volume occupied by the chloroplasts decreases to ca. 50% of the area of the sections studied which is, perhaps, commensurate with a decrease in the size of the individual starch grains to about one half of that observed at the time of stoma formation.

The presence of a cuticle covering both the outer and inner periclinal walls of all the epidermal cells and the external cell wall surfaces of the mesophyll tissue is observable from a very early developmental stage (Fig. 1 and 2). Prior to the actual formation of the stoma, a cuticular layer is laid down adjacent to the middle lamella of the common anticlinal wall of the guard cell pair (Fig. 5). On formation of the stoma, preferential cuticular deposition occurs at the apices of the wedge-shaped thickenings (Fig. 7).

Light Microscopy Observations

Pectinaceous Material

No distinct accumulations of pectic material were observable in the developing guard cell complexes using the techniques listed.

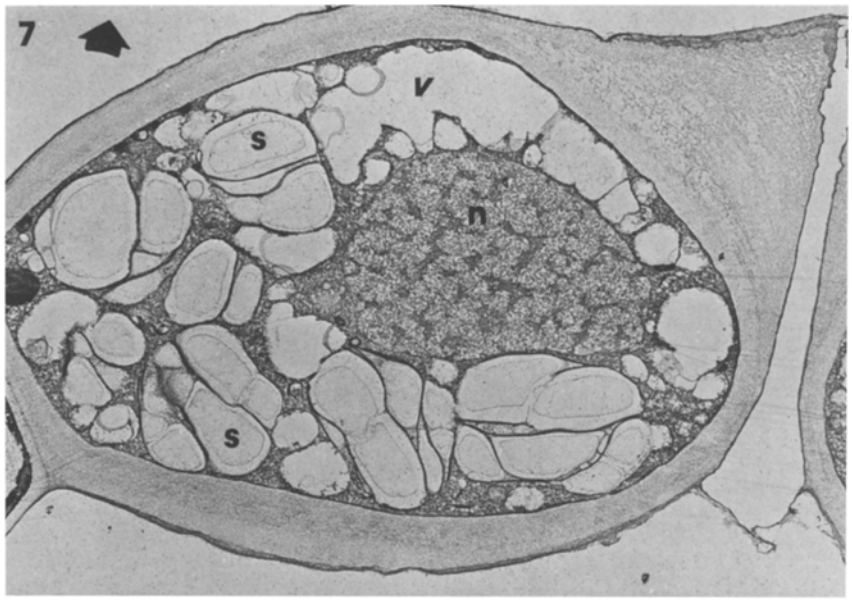


Fig. 7. Vertical transverse section through a single submature guard cell. Electron micrograph, ×5410. The solid arrow indicates abaxial orientation. The section is of a guard cell which has just separated from its pair and is still attached to the latter by a cuticular strand at the internal extremity of the stoma. The state of the chloroplasts, starch inclusions (*s*), and vacuolar system (*v*) is very similar to that found in fully mature tissues. Note the heavy deposition of cuticular material at the apex of the thickened ridge

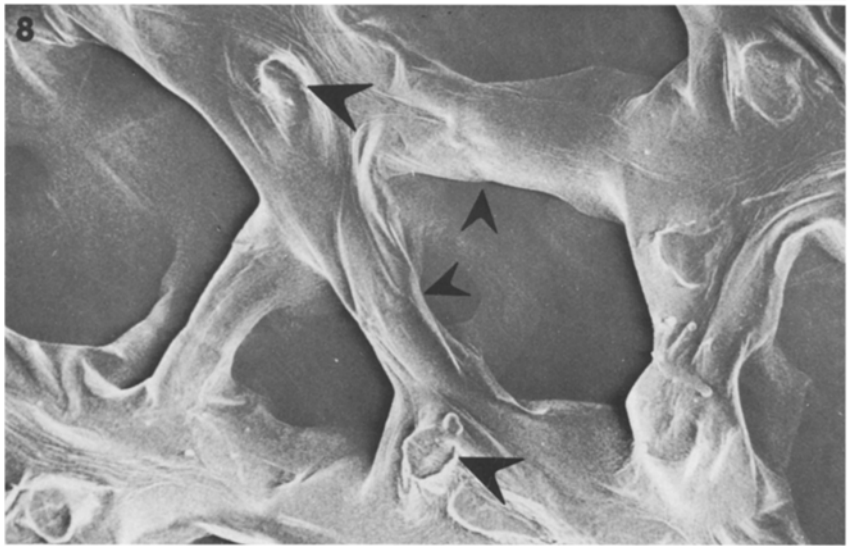


Fig. 8. Scanning electron micrograph of the mesophyll, ×775. The pedicels, upon which the abaxial epidermis rests, are indicated by the large solid darts, and the junctions between adjacent mesophyll cells by the smaller solid darts

Starch

Representative photomicrographs of the I_2KI stain are illustrated in Figures 10–16 inclusive.

Prior to the division of the guard-cell mother-cell, a very weak positive reaction is observable in the chloroplasts of all the epidermal cells (Fig. 10). As the deposition of the common anticlinal guard cell wall is initiated, the staining reaction becomes more positive and polarised within the developing guard cell pair (Fig. 11). This intensification of the reaction continues as the common anticlinal walls develop (Fig. 12), and differential cell wall thickening occurs (Fig. 13). By the time that the stoma is ready to form, the reaction is extremely intense and occludes the entire lumen of the guard cell (Fig. 14). After the appearance of the stoma, the reaction becomes less intense as the chloroplasts become spatially separated from each other (Fig. 15) until at maturity, the individual chloroplasts are almost discernible (Fig. 16). At no stage is a really positive staining reaction obtained from the neighbouring, subsidiary, and ordinary epidermal cell chloroplasts. No evidence was obtained which would indicate that the starch levels varied with either light or dark treatments.

Insoluble Carbohydrates

Representative results of the PAS reaction are illustrated in Figures 17–24 inclusive.

There is virtually no reaction in the meristemoid/

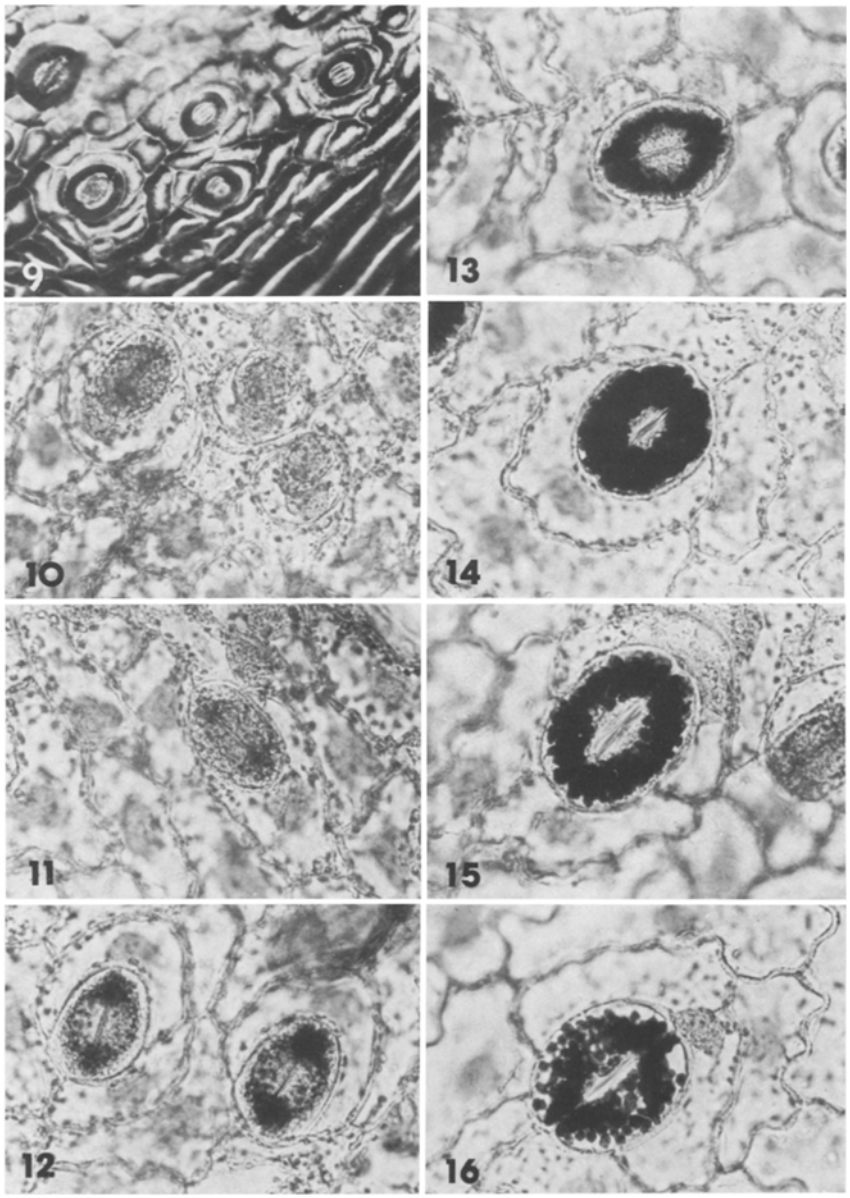


Fig. 9. Reflected light micrograph of developing epidermis, × 165. Note the sunken area of the guard cell complexes in the immediate region of the developing cell wall ridges

Figs. 10–16. Light micrographs of developing isolated epidermal strips treated with I_2KI, × 465

Fig. 10. The two developing stomatal complexes on the right-hand side of the plate are either at the late stomatal meristemoid or early guard-cell mother-cell stage, having each cut off a single horseshoe-shaped mesogene subsidiary cell. All the chloroplasts of both the stomatal and non-stomatal cells show a weak positive reaction

Fig. 11. A newly formed guard cell pair in which the common anticlinal cell wall is only recently formed. The staining reaction is more positive and restricted largely to the poles of the young guard cell complex

Fig. 12. A slightly later stage than that illustrated in Figure 11. The polar staining reaction is becoming more intense

Fig. 13. An even later stage showing an intensification of the reaction

Fig. 14. A guard cell complex in which the thickened-ridges are fully developed and the stoma about to form. The starch reaction largely occludes all detail of the guard cell lumina

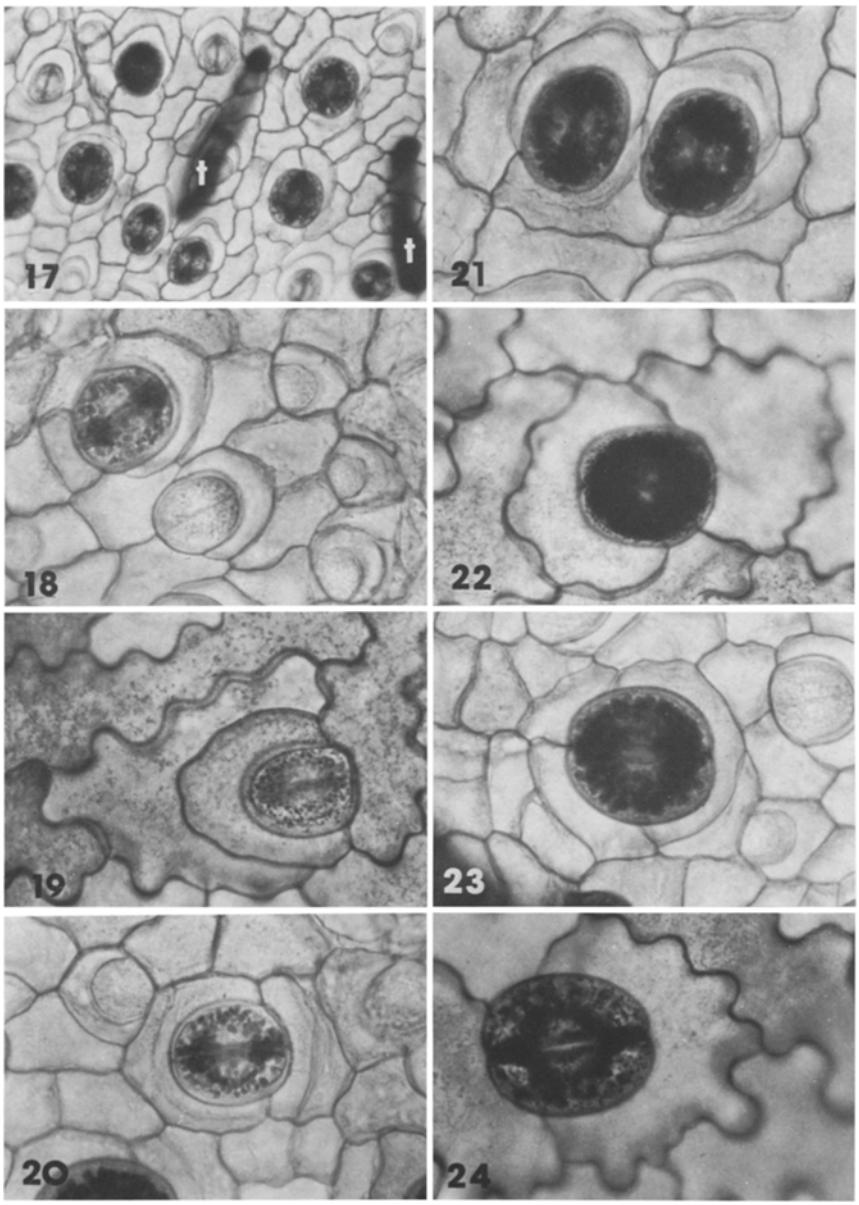
Fig. 15. A submature guard cell complex showing a decrease in the starch content

Fig. 16. A mature guard cell complex

mother-cell prior to the formation of the common anticlinal cell wall. At the time of inception of the common anticlinal wall, small weakly positive sites of reaction appear in the young guard cells (Fig. 18) which become more numerous, larger, and more positive with continued deposition of the cell wall (Fig. 19). At that stage when the guard cell walls commence differential thickening, a certain polarity of staining is observable in the young guard cell pair (Fig. 20). By the time the stoma forms, the entire lumina of the guard cells are obscured by the reaction (Fig. 22). Afterwards, there is an apparent reduction in the reaction (Fig. 23) which continues until the guard cells attain full maturity when it stabilises (Fig. 24). As with the I_2KI reaction, there is no apparent difference between light- and dark-treated mature guard cell complexes. In all the stages studied, no evidence was obtained to suggest that a positive reaction occurs in any of the neighbouring, subsidiary, or ordinary epidermal cells although it was noted that the trichomes react very positively (Fig. 17).

Relationship of the Guard Cell Complex to the Mesophyll Tissue

Observations indicate that both the protodermal and the protomesophyll cells increase their volumes by similar amounts during early developmental stages



Figs. 17–24. Light micrographs of developing isolated epidermal strips treated with PAS reagent. Fig. 17, ×165; Figs. 18–24, ×465

Fig. 17. A micrograph showing most of the developmental stages illustrated in Figures 18–24. Note the positive reaction in the bicelled trichomes (*t*)

Fig. 18. The three stomatal complexes on the right-hand side of the plate are either late stomatal meristemoid or early guard-cell mother-cell stages which show no PAS reaction. The stomatal complex in the lower centre of the plate is of a very young guard cell pair and shows a very weak PAS reaction. The complex in the upper left of the plate is at the same stage as that in Figure 20. (*vide infra*)

Fig. 19. A young guard cell complex (cf. Fig. 11). Note the weak, but distinct, PAS reaction

Fig. 20. A slightly older guard cell complex (cf. Fig. 12). The staining reaction is becoming polarised within the guard cell complex

Fig. 21. An even older pair of guard cell complexes (cf. Fig. 13). The staining reaction is more positive and strongly polar

Note. Both these complexes are atypical. The one on the left is of an aniso-polo-eumesoperigenous type and the one on the right is of an aniso-polo-hemiperigenous type. (Stevens and Martin, 1978)

Fig. 22. A guard cell complex in which the thickened-ridges are fully developed and the stoma about to form (cf. Fig. 14). The PAS reaction occludes all detail of the guard cell lumina

Fig. 23. A submature guard cell complex showing a decrease in the PAS reaction (cf. Fig. 15)

Fig. 24. A mature guard cell complex

so that their relative positions to one another remain constant. Loose endocuticular bonds are established between the two where they make contact. This position is maintained during that time when the stomatal meristemoids are established within the protodermis so that they too become loosely attached to the protomesophyll. As subsidiary cells are cut off by the meristemoid, the area of the protodermis increases at a greater rate than that of the protomesophyll. This results in the cells of the prospective stomatal complex bulging outwards and lifting off from the mesophyll and any attachments which may have been established between the meristemoid and the mesophyll is broken (Figs 2 and 3). This is a characteristic feature of developing pinna and results in the subsidiary cells never developing any direct contact with the mesophyll. The lifting off of the protodermis at the stomatal centres is further aided by the circinately-folded pinnae unfolding in an opposite direction to the direction of protodermal expansion.

The attachment of the stomatal meristemoid to the mesophyll sometimes appears to be greater than that between the mesophyll cells concerned and their neighbouring mesophyll cells. Consequently, it is not unusual to find a small mesophyll cell adhering to the inner face of a meristemoid in detached epidermal strips (Fig. 25). These adhering cells degenerate and disappear before the tissue reaches maturity.

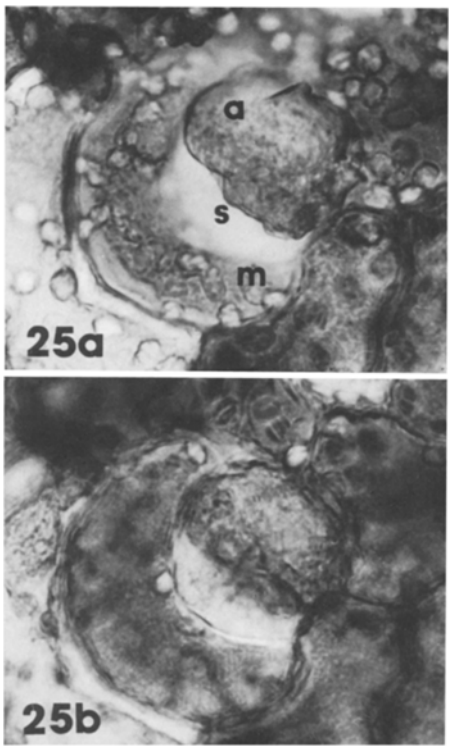


Fig. 25a and b. Substomatal chamber formation in a related fern species, *Phyllitis scolopendrium*. **a** Stomatal complex viewed with the side adjacent to the mesophyll uppermost, ×750. The stomatal meristemoid/guard-cell mother cell (*s*) has already cut off a mesogene subsidiary cell (*m*). An atrophying protomesophyll cell (*a*) has remained attached to the lower periclinal walls of the meristemoid. The micrograph is focussed on the degenerating mesophyll cell. **b** The same complex as in **a**, but focussed on the epidermal tissue

Once the subsidiary cells have been cut off from the stomatal meristemoids, the epidermis expands at a much greater rate than the mesophyll. These unequal growth rates are accomodated, no only by the localised lifting off of the epidermis, but also by the mesophyll cells becoming extended laterally into aerenchymatous-like cells (Fig. 8).

Discussion

Starch Content of Developing Epidermis

The starch inclusions reach their peak concentration per unit volume within the guard cell pair at the time of stoma formation. Subsequently, the starch concentration gradually becomes reduced as the guard cell volume increases.

Electron micrographs indicate that starch is present in appreciable quantities in the stomatal meristemoid and the guard cell mother-cell prior to the latter's division to form the guard cell pair (Figs.1 and 2). The fact that neither PAS nor I_2KI gave any indication of its presence at these developmental stages indicates that the reagents failed to penetrate the immature protoplasts.

An interesting aspect of this study is the fact that the guard cell chloroplasts, in this species, are clearly functional from a very early developmental stage and that this capacity is independant of normal stomatal functioning. Indeed, autofluorescent studies indicated that the plastids of all protodermal cells contain chlorophyll very shortly after being cut off from the pinna meristem and certainly at a developmental stage prior to the stomatal meristemoids being identifiable as such.

Differential Guard Cell Wall Thickening

The electron microscope studies indicate that the differential thickening of the guard cell walls, prior to the formation of the stoma, have a profound effect on guard cell organisation. During the sequential deposition of cell wall material onto the thickening areas, the lumina of the guard cells become progressively restricted in the region of the eventual stoma. Before the wall thickening is inititiated, the vertical cross-section of individual young guard cells can be described as sub-tetragonal (Figs. 3 and 4); as the thickening reaches maximum proportions, the sections become sub-hexagonal in cross-section (Fig. 5); whilst the cross-section of a fully differentiated guard cell after stoma formation is sub-circular (Fig. 7).

The guard cell deformations and variations in starch levels at the time of stoma formation may be related to the mechanism(s) behind the formation of the pore.

Stoma Formation—a Hypothetical Model

The first step in stoma formation is the breakdown of the pectinaceous middle lamella of the common anticlinal wall (Fig. 5) by enzymatic means. It is noteworthy, however, that this breakdown and separation of the two cell wall members does not extend to the surface of the periclinal walls. The reason for this is that the middle lamella stops short of these surfaces (Fig. 4). At this stage, the guard cell pair are firmly attached to each other by their common anticlinal wall at either pole and by a weak connection between adjacent apices of the thickened ridges.

It is suggested that the mechanical pressure required to separate the guard cell pair is of an osmotic type generated by the hydrolysis of some of the starch

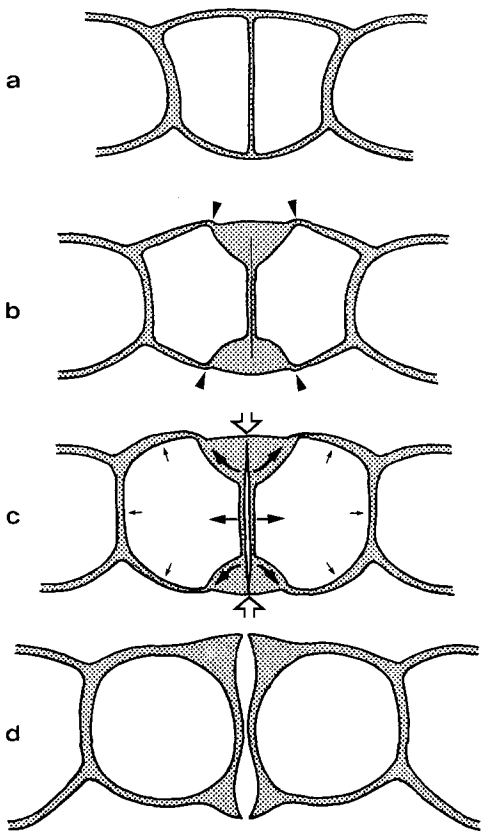


Fig. 26a–d. Model of stoma formation in *Polypodium vulgare*. **a** Young guard cell pair in vertical transverse section after common anticlinal cell wall initiation. **b** Section through the guard cell pair as the thickened ridges are laid down. The darts adjacent to the thickened areas indicate periclinal wall areas which remain comparatively thin. The line down the centre of the common anticlinal wall indicates preparatory enzymatic breakdown of the middle lamella. **c** As turgor builds up in the complex following starch hydrolysis, the cell walls bulge outwards as indicated by the small arrows. Because of constraints on movements of the anticlinal walls, the greatest wall movement will occur in the periclinal walls. These will bulge outwards and cause the thickened ridges to rotate inwards (curved arrows) about their apices (hollow arrows) due to the presence of the thin cell wall areas immediately adjacent to the ridges. At the same time the ventral anticlinal walls of the guard cell will be pulled apart by the rotation of the thickened ridges (straight arrows). **d** A fully differentiated guard cell pair. Note the changes in the cross-sectional shape of the guard cell lumen during these events

inclusions. The developing pressure exerted by the protoplast will be absorbed by the periclinal walls, which since they are unrestricted, will flex outwards. The thin regions of these walls, adjacent to the thickened ridges, will act as hinges and cause the ridges to rotate slightly inwards about their apices which will act as fulcra. The result of such movements will be that the anticlinal walls will pull apart from each other to form the stoma. The foregoing events are summarised diagrammatically in Figure 26.

Relationship of the Stomatal Complex to the Mesophyll

In mesophytes which contain both spongy and palisade mesophyll tissues, two types of substomatal chambers exist. Those associated with the adaxial leaf surface are formed by a space occuring in the otherwise densely packed palisade mesophyll tissue. Those associated with the abaxial leaf surface are less discrete and take the form of extended intercellular gaps in the reticulate network of spongy mesophyll cells (Meidner and Sheriff, 1976; Fig. 2.2). *P. vulgare* has substomatal chambers of the latter type.

During development in *P. vulgare*, the prospective stomatal complexes become lifted off from the mesophyll and only the non-stomatal epidermal cells remain in contact with the mesophyll. The substomatal chambers result from natural air spaces occuring as the protomesophyll becomes extended into its mature aerenchymatous form together with the degeneration of certain protomesophyll cells which would otherwise have developed immediately below a stomatal complex.

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