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THE RELATIONSHIP BETWEEN CELL ORGANELLES AND CELL WALL THICKENINGS IN PRIMARY TRACHEARY ELEMENTS OF THE CUCUMBER II. QUANTITATIVE ASPECTS*

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SUMMARY

The relationship between the endoplasmic reticulum and the Golgi vesicles and the formation of cell wall thickenings in primary tracheary elements of the hypocotyl of the cucumber was investigated. In order to study the degree to which the endoplasmic reticulum and the Golgi vesicles might contribute to thickening formation, the relationship between the size of the thickening and the density of these cell organelles in the cytoplasm near the thickening was studied quantitatively. It appeared that the density, and hence the contribution, of the endoplasmic reticulum and the Golgi vesicles reaches an optimum in the mid-stage of the process of thickening formation.

The endoplasmic reticulum and, to some extent, the Golgi vesicles occur in the cytoplasm between the cell wall thickenings in higher densities than in the cytoplasm facing the thickenings. This supports the hypothesis that cell wall substances brought by the endoplasmic reticulum and the Golgi vesicles pass through the plasmalemma between the thickenings and are then transported through the primary cell wall towards the sites of the thickenings.

Lomasomes seem to be involved in the initiation of the thickenings. It seems likely that cell wall substances pass through the plasmalemma by way of the lomasomes. The substances are then transported sideways through the primary cell wall and the thickenings arise at each side of the vanishing lomasomes.

1. INTRODUCTION

In mature tracheary elements, cell wall thickenings are found. The thickening consists of rings or spirals which are formed by the deposition of pectic substances, hemicelluloses, cellulose, lignin, and protein against the primary cell wall. The formation of this wall thickening has been the subject of many electron microscopical studies recently reviewed by ROBERTS (1969) and by TORREY, FOSKET & HEPLER (1971). For several cell organelles, such as the endoplasmic reticulum, Golgi vesicles, microtubules, and lomasomes, indications are found that they contribute somehow to this formation. In a recent study (GOOSEN-DE ROO 1973), it has been confirmed that endoplasmic reticulum, Golgi vesicles, microtubules, and lomasomes are involved in thickening formation in tracheary elements in the hypocotyl of the cucumber.

It was also pointed out that, during thickening formation, the morphology

* Dedicated to Professor Karstens on the occasion of his retirement.

of the endoplasmic reticulum and the Golgi vesicles changes; that the topography of the microtubules also changes; and, that the lomasomes are present only during the initiation of the thickenings. This may be an indication that the nature of the function of these organelles varies during the process of cell wall thickening formation or, in the case of the lomasomes, that their role in the thickening formation is restricted to a certain phase in the morphogenesis of the thickenings. For example, the smooth endoplasmic reticulum which is present in the initial stage of thickening formation later changes into a rough cisternal form. The main function of the smooth vesicular endoplasmic reticulum seems to be the formation of vesicles containing substances involved in the formation of the cell wall thickenings; whereas, for the rough cisternal endoplasmic reticulum, protein synthesis (chiefly enzyme synthesis) might be the most important role. Transport or conduction also seems to be a possible role for the endoplasmic reticulum. The diameter and the contents of the Golgi vesicles change during thickening formation, probably indicating a difference in contents during successive stages in the formation of the cell wall thickenings. During the formation of the wall thickenings, cortical microtubules are restricted to a single row lining those regions of the plasmalemma which surround the developing thickenings. During the initiation of the thickenings microtubules are present along the whole plasmalemma; the same situation is found in the final stage of thickening formation.

Quite another question is whether not only the way in which, but also the degree to which, the contribution of the endoplasmic reticulum and the Golgi vesicles varies during the process of thickening formation. In order to find an answer to this question, the density (number per square micron) of these cell organelles in the cytoplasm of tracheary elements was studied quantitatively in relation to thickening formation. The present study concentrates in particular on the possible roles of the endoplasmic reticulum and the Golgi vesicles. In addition, the differences in densities of the endoplasmic reticulum and the Golgi vesicles in relation to the developing cell wall thickenings has been studied. As reported earlier, the cortical microtubules have a clearly non-random distribution during thickening formation. They lie in a single row close to the plasmalemma and their distribution is easy to ascertain during the formation of the thickenings. The endoplasmic reticulum and the Golgi vesicles, however, are more dispersed in the cytoplasm. To study their distribution in the cytoplasm relative to the developing thickenings, quantitative methods are required. If present during thickening formation, regularities in the distribution of cell organelles might correspond with the pattern visible on the surface of the protoplast under the light microscope which has been described by CRÜGER (1855), DIPPEL (1867), BARKLEY (1927), and SINNOTT & BLOCH (1945). This pattern consists of more densely granular protoplasm associated with an increased streaming of the protoplasm at these sites. The pattern resembled the cell wall thickening pattern deposited against the primary cell wall. When the material used in this study was subjected to light microscopic examination, a similar pattern was observed on the surface of the protoplast.

2. MATERIAL AND METHODS

2.1. Culture of the plants and electron microscopy

Tracheary elements from still elongating hypocotyls of the cucumber (*Cucumis* 'Sporu-Origineel' from de Ruyter and Son, Bleiswijk, Holland) were studied with the electron microscope. The culture methods for the plants and the electron microscopical methods used were described in detail in a previous paper (GOOSEN-DE ROO 1973). In short, the seeds of cucumber were germinated for 24, 48, 72, or 96 hours. The fixatives for electron microscopy were glutaraldehyde diluted in a sodium cacodylate buffer followed by a post-fixation with osmium tetroxide in a sodium veronal acetate buffer or in a sodium cacodylate buffer.

2.2. Quantitative approach

The stages in the process of the formation of cell wall thickenings were indicated as in the preceding paper (GOOSEN-DE ROO 1973). The stages are based on the size of the cell wall thickenings in radial, or approximately radial, sections through tracheary elements. In the various classes the density of the endoplasmic reticulum and of the Golgi vesicles in the vicinity of a cell wall thickening was determined. In class 0 the same was done for an area of the same size facing the thickening of a neighbouring tracheary element. This area is probably the area in which the thickening will be formed, because thickenings in neighbouring cells nearly always correspond with each other (*plate I*). The areas of cytoplasm studied are represented in *fig. 1*. Areas 1 and 3 represent the cytoplasm between the thickenings; area 2 the cytoplasm facing the thickenings. The region composed of areas 1, 2, and 3 is considered as one observation field (in the tables and figures indicated as N). The densities of the endoplasmic reticulum and of the Golgi vesicles in the cytoplasmic areas of two cell wall thickenings lying next to each other in the same cell were not determined. The density of the endoplasmic reticulum was determined in two ways:

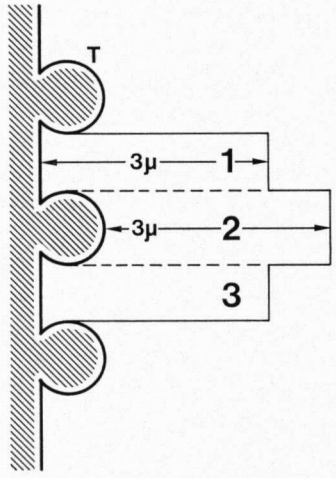

Fig. 1. Schematic representation of a radial section through a tracheary element. The numbers 1, 2, and 3 indicate the area studied; 2 is the area facing the thickenings (T); 1 and 3 represent the area between the thickenings. The hatched areas represent cell wall material.

a. By counting the separate cisternae of the endoplasmic reticulum ($E.R._c$) and the irregularly vesicular shaped endoplasmic reticulum ($E.R._v$) in the whole region (1 to 3) followed by division of the resulting number by the number of square microns of the area. This value gives the number per square micron and will be called E.R. density-score.

b. By measuring the length of the cisternae of the cisternal endoplasmic reticulum in microns. This value, which is also given per square micron, is called the E.R. density-length.

In class 0 the $E.R._v$- and $E.R._c$ density-scores were also determined separately, but the E.R. density-length was only determined for the $E.R._c$. This was because of the fact that length measurement of the irregular shaped vesicular E.R. was not possible. The density of the Golgi vesicles was also determined by counting the number of Golgi vesicles in the same area. Finally, the densities of the endoplasmic reticulum and of the Golgi vesicles were separately determined in the region composed of areas 1 and 3 and in area 2 (*fig. 1*). The observations were made on different cells in the same stage of thickening formation. The number of cells studied per class were 6, 6, 8, 7, and 3 in classes 0, 1, 2, 3, and 4, respectively. The number of observations (N) in the various classes (from 0 to 4) were 24, 34, 53, 27, and 23, respectively.

2.3. Statistical analysis

For the statistical testing of the various hypotheses, non-parametric statistical tests were used (SIEGEL 1956). A Kruskal-Wallis one-way analysis of variance was used for comparing the cell organelle densities between the various classes, in order to decide if the densities in these classes came from different populations. A median test was used for determining whether the average densities of cell organelles in two independent groups differed in central tendencies. Significance was considered at the 5% level.

3. RESULTS

3.1. Distribution of densities over the various classes

3.1.1. Endoplasmic reticulum

Figure 2 gives the densities of the endoplasmic reticulum in the five stages of formation of the thickenings. The E.R. density-score is highest in class 2; this density is an optimum. The E.R. density-length also seems to reach an optimum in class 2, but not as clearly as in the other method of density determination.

A Kruskal-Wallis one-way analysis of variance shows that the density-score as well as the density of the length varies significantly ($p < 0.001$) in the five classes. *Figure 2* gives for class 0 the E.R. density-score and the E.R. density-length of the cisternal endoplasmic reticulum only. The median test comparing the classes two by two shows that, in the case of the E.R. density-score, there is no significant difference between class 0 and class 1. Between class 0 and the other classes there is a significant difference in E.R. density-scores ($p < 0.001$). The E.R. density-score of the vesicular endoplasmic reticulum, which is present

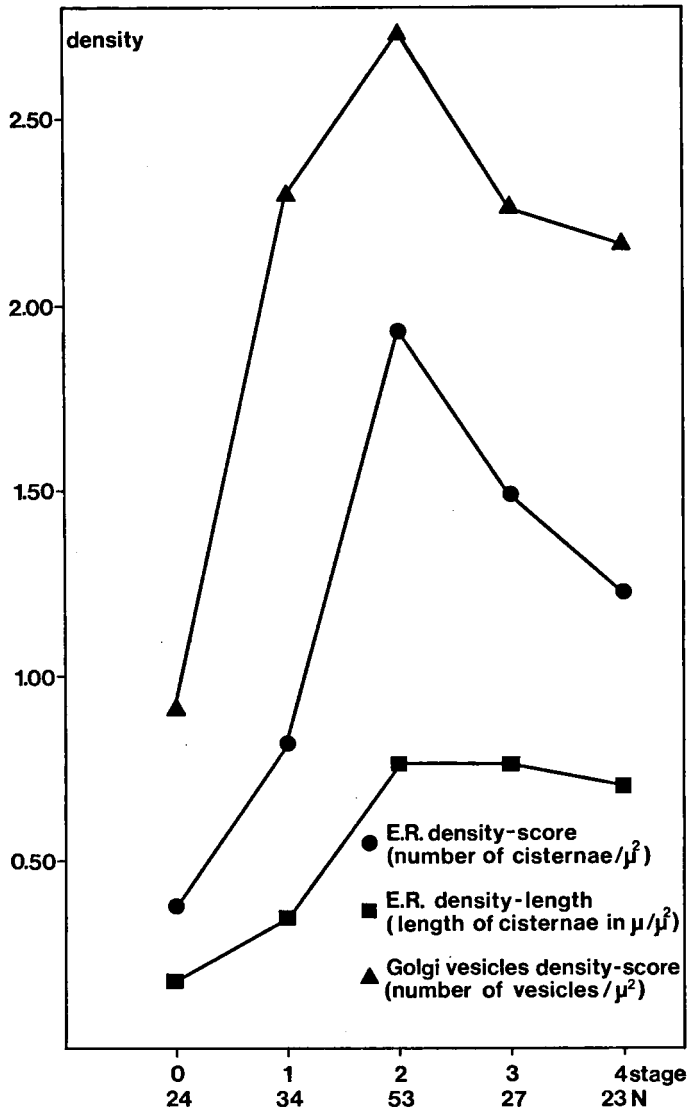

Fig. 2. Densities of the endoplasmic reticulum and densities of Golgi vesicles in the various size classes (stages) of the thickenings. N is the number of observations. The data given are the average values.

only in class 0, is 1.23. This score has not been added to the score of the cisternal endoplasmic reticulum, as the two types of endoplasmic reticulum are markedly different (GOOSEN-DE ROO 1973). The E.R. density-length in class 0 differs significantly from all the other classes ($p < 0.001$). The E.R. density (-score and -length) in class 1 differs significantly from the E.R. densities in classes 2

($p < 0.02$), 3 ($p < 0.01$), and 4 ($p < 0.01$). Among the remaining classes no significant differences in either E.R. density are found.

3.1.2. Golgi vesicles

Figure 2 gives the density-score for the Golgi vesicles. Like the density-score of the endoplasmic reticulum, the density-score of the Golgi vesicles also shows an optimum which again is reached in class 2.

A Kruskal-Wallis one-way analysis of variance indicates that the densities in the five classes represent different populations ($p < 0.001$). Comparison of the classes two by two gave the following results. The density-score of the Golgi vesicles in class 0 differs significantly from the densities in all the other classes ($p < 0.001$). Among the densities of Golgi vesicles in the other classes no significant differences are found.

3.2. Distribution of densities in the cytoplasm surrounding a thickening

3.2.1. Endoplasmic reticulum

The distribution of densities of the endoplasmic reticulum in the peripheral cytoplasm in the various stages of thickening formation was studied. The results are given in *table 1* (E.R. density-score) and *table 2* (E.R. density-length). The E.R. density-score for class 0 in *table 1* does not include the vesicular

Table 1. E.R. density-scores between and facing the thickenings. N is the number of observations, p the probability. The data given are the average values.

class	E.R. density-score between the thickenings	E.R. density-score facing the thickenings	N	p
0	0.38	0.42	24	$p < 0.05$
1	0.83	0.83	34	$p < 0.05$
2	2.27	1.39	53	$p < 0.02$
3	1.79	1.31	27	$p < 0.01$
4	1.55	0.95	23	$p < 0.02$

Table 2. E.R. density-lengths between and facing the thickenings. N is the number of observations, p the probability. The data given are the average values.

class	E.R. density-length between the thickenings	E.R. density-length facing the thickenings	N	p
0	0.19	0.17	24	$0.70 > p > 0.50$
1	0.33	0.29	34	$0.20 > p > 0.10$
2	0.89	0.54	53	$p < 0.01$
3	0.94	0.62	27	$0.20 > p > 0.10$
4	0.99	0.49	23	$p < 0.01$

endoplasmic reticulum. In all stages the endoplasmic reticulum is unequally distributed in the cytoplasm between and facing the thickenings (*table 1*). The E.R. density in the cytoplasm between the thickenings is higher than in the cytoplasm facing the thickenings, even though, as in class 1, the average values in the cytoplasm between and facing the thickenings are the same. The data from the E.R. density-lengths (*table 2*) gave similar results, although this time in stages 0, 1, and 3 the distribution of the endoplasmic reticulum is not significantly different in the cytoplasm between and facing the thickenings. Only in stages 2 and 4 there is significance that more endoplasmic reticulum is present between than facing the thickenings.

3.2.2. Golgi vesicles

Table 3 gives the results for the density distribution of the Golgi vesicles. Like the endoplasmic reticulum, the Golgi vesicles reach a higher density-score between the thickenings than in the cytoplasm facing the thickenings. In none of the classes is there a significantly unequal distribution of Golgi vesicles in the cytoplasm between and facing the thickenings.

Table 3. Density-scores of Golgi vesicles between and facing the thickenings. N is the number of observations, p the probability. The data given are the average values.

class	Golgi vesicles density-score between the thickenings	Golgi vesicles density-score facing the thickenings	N	p
0	1.09	0.75	24	$0.70 > p > 0.50$
1	2.53	2.09	34	$0.20 > p > 0.10$
2	2.51	3.00	53	$0.70 > p > 0.50$
3	2.43	2.31	27	$0.70 > p > 0.50$
4	2.35	2.11	23	$p = 1$

4. DISCUSSION

4.1. Endoplasmic reticulum

The densities of the cisternal endoplasmic reticulum in class 0 and the endoplasmic reticulum in the other classes (always chiefly cisternal) show an optimum in class 2. The morphology of the cisternal endoplasmic reticulum in the various classes does not differ very much (GOOSEN-DE ROO 1973); nearly all the cisternal endoplasmic reticulum is rough. When the results are taken together, the two methods by which the density of the endoplasmic reticulum was determined give some information about the form of the endoplasmic reticulum membrane system. The average length of the endoplasmic reticulum per average number of endoplasmic reticulum cisternae for the various stages in the formation of the thickenings is given in *table 4*. The length of the endoplasmic reti-

Table 4. Quotient of the E.R. density-length and E.R. density-score in the various size classes of the thickenings. N is the number of observations. The data given are the average values.

	0 N = 24	1 N = 34	2 N = 53	3 N = 27	4 N = 23
E.R. density length per E.R. density-score	0.47	0.43	0.40	0.52	0.58

culum cisternae, as cut in the radial sections, increases starting in stage 2. This probably indicates a less branched system of endoplasmic reticulum membranes in the later stages of thickening formation.

The principal function of the rough cisternal endoplasmic reticulum is generally assumed to be protein synthesis. A possible role in transportation has also been mentioned. This would involve the transport of molecules, and especially the conduction of Golgi vesicles, along its flattened and elongated structures towards the cell wall (GOOSEN-DE ROO 1973). The amount of cisternal endoplasmic reticulum might be an indication of the degree of protein synthesis and the degree of guided transport. If that is the case, it seems feasible that at some stages during the formation of the thickenings more endoplasmic reticulum is required to carry out these functions. In stage 2, especially, much endoplasmic reticulum seems to be required.

In class 0, i.e., before the formation of cell wall thickenings takes place, the endoplasmic reticulum is present in two forms; smooth vesicular, and rough cisternal, the former in a much larger amount than the latter. In class 1 only a few vesicular shaped structures are present and these are no longer smooth. The density of cisternal endoplasmic reticulum in class 0 is very low and fits in with an optimum curve of the E.R. densities in the various classes. An interesting point is what happens with this smooth vesicular endoplasmic reticulum. Study of electron microscope photographs reveals the simultaneous occurrence of three events. A certain fraction of the vesicular endoplasmic reticulum seems to give rise to vesicles involved in thickening formation, as suggested earlier in the morphological part of this study (GOOSEN-DE ROO 1973) and illustrated in *plate II, fig. 1*. A second occurrence is that a portion of the smooth vesicular endoplasmic reticulum flattens out into cisternal shaped endoplasmic reticulum and becomes rough after attachment of ribosomes to its outer membranes (*plate II, fig. 2*). Finally, a fraction of it seems to blend into small vacuoles (*plate II, fig. 3*).

In the cytoplasmic areas between the thickenings in all of the stages the density of endoplasmic reticulum is higher than in the areas facing the thickenings (*plate III, fig. 1*). The cisternae of the endoplasmic reticulum between the thickenings almost always lie perpendicular to the plasmalemma (*plate III, figs. 2* and *3*). In the literature some situations are described in which the endoplasmic reticulum is said to have a function in masking sites of the cell wall where no cell wall material is to be deposited. For example, during the process of the forma-

tion of the cell wall of pollen the endoplasmic reticulum is located opposite the future apertures in the cell wall (HESLOP-HARRISON 1963). Another example is given by NORTHCOTE & WOODING (1966): during the formation of the sieve plate in sieve elements of the phloem the endoplasmic reticulum covers the sites where the sieve pores arise. A role in the thickening formation in xylem elements was also suggested by PORTER (1961). The endoplasmic reticulum between the developing cell wall thickenings is thought to prevent the deposition of cell wall material between the thickenings. We can not agree with this function of the cisternae of the endoplasmic reticulum in the formation of cell wall thickenings in tracheary elements, although, when density determination is made by counting cisternae in all classes, the density of the endoplasmic reticulum cisternae is higher between the thickenings than facing them. After determining the density by length measurement, however, we do not find a larger amount of endoplasmic reticulum between the thickenings than facing them in class 0 and in class 1. This is not in accordance with the view of PORTER (1961), since class 0 and class 1 are the classes where a masking role of the endoplasmic reticulum between the thickenings would have been very important. It is in these stages that the initiation of the thickenings occurs. But more important in connection with this problem are the following considerations: for a masking role, a parallel orientation of the endoplasmic reticulum cisternae to the plasmalemma seems to be more effective than the perpendicular orientation observed in the present experiments. In the examples of a probable masking role which have been mentioned, this parallel orientation to the plasmalemma is always found. The location and orientation of the endoplasmic reticulum reported here suggest rather a function in the transport of substances such as pectin, hemicelluloses, lignin, and protein from the cytoplasm to the sites between the developing cell wall thickenings. PICKETT-HEAPS & NORTHCOTE (1966) also suggest a role of the endoplasmic reticulum in directing materials from the cytoplasm to the cell wall. They also find endoplasmic reticulum concentrated at areas in which there is little wall growth; that is, between the thickenings. They suggest two alternative ways in which the endoplasmic reticulum might contribute to transportation:

a. by directing or channeling material to the "active sites of wall growth" (the thickenings), or
b. by excluding material from other sites.

Study of the distribution of the endoplasmic reticulum gives support to the earlier assumption (GOOSEN-DE ROO 1973) of a transport and conducting role for the cisternae of the endoplasmic reticulum which was based on the morphology of the endoplasmic reticulum. The transport of molecules and the guiding of vesicles from the cytoplasm to the sites between the developing thickenings suggest an indirect role of the endoplasmic reticulum in the determination of the pattern of the cell wall thickenings.

4.2. Golgi vesicles

With the exception of class 0, the densities of Golgi vesicles do not differ very

Table 5. Densities of Golgi vesicles, average diameter and average surface of the Golgi vesicles and % surface consisting of Golgi vesicles per μ^2 protoplasm. N is the number of observations. The data given are the average values.

	0 N = 24	1 N = 34	2 N = 53	3 N = 27	4 N = 23
Golgi vesicles density-score (number of vesicles/μ^2)	0.91	2.29	2.74	2.26	2.17
Average diameter (2r) of Golgi vesicles in Å	396	1199	1560	679	837
Average surface (πr^2) of Golgi vesicles in μ^2	0.0012	0.0113	0.0191	0.0036	0.0055
% Surface consisting of Golgi vesicles per μ^2 protoplasm	0.11	2.59	5.23	0.81	1.19

much in the various stages of thickening formation. The highest density is found in class 2. This result is similar to the optimum distribution of the endoplasmic reticulum. Although the number of Golgi vesicles does not differ very much in the various stages, the morphology (the diameter and electron density of the contents) of the vesicles differs in these stages (GOOSEN-DE ROO 1973). The average diameters of the Golgi vesicles in the various stages differ greatly (*table 5*). Assuming that the size of a vesicle is determined by the total amount of substance a vesicle contains, the degree of the contribution to the thickenings varies considerably in the successive stages. The average surface (πr^2) of a Golgi vesicle in μ^2 and the part of protoplasm consisting of Golgi vesicles (Golgi vesicles density-score multiplied by the average surface of a vesicle given as % per μ^2) in the various stages are given in *table 5*.

There are no significant differences in the densities of Golgi vesicles present in the cytoplasm between and facing the thickenings in any of the classes. There is a trend, however, that in the cytoplasm between the thickenings more Golgi vesicles are present (*plate IV, figs. 1* and *2*). This observation supports the idea of the deposition of cell wall substances (pectin, hemicelluloses, lignin, and protein) involved in cell wall synthesis between the developing thickenings, as suggested in the discussion on the distribution of the endoplasmic reticulum.

5. CONCLUSION

Concerning the first question of this paper: studying the degree to which the endoplasmic reticulum and the Golgi vesicles contribute at various stages of thickening formation, the following can be said. In the various stages of the thickening process, variation in the amount of the endoplasmic reticulum and the Golgi vesicles in the vicinity of the developing thickenings was found. In stage 2 there is an optimum in the amount of endoplasmic reticulum and the number of Golgi vesicles. This probably indicates an active role for both cell organelles in the formation of the thickenings at this stage.

The second question of this paper refers to the distribution of the endoplasmic reticulum and the Golgi vesicles in relation to the pattern of the cell wall thickenings. The density determination of the endoplasmic reticulum in the cytoplasm between and facing the thickenings gives evidence for a regular distribution of the endoplasmic reticulum in a pattern corresponding with the thickening pattern. Although no significant results were found for the Golgi vesicles, the data show the same trend; more Golgi vesicles in the cytoplasm between the thickenings. These two types of organelles might be responsible for the pattern on the surface of the protoplast in differentiating tracheary elements, as can be observed under the light microscope.

The pattern of the distribution of the cortical microtubules differs from the distribution of the endoplasmic reticulum and the Golgi vesicles. The distribution of the cortical microtubules is the opposite of the endoplasmic reticulum and the Golgi vesicles distribution. Especially in the cytoplasm facing the thickenings, the microtubules are present. In stage 4, however, the microtubules are evenly distributed along the plasmalemma. It is difficult to determine what the pattern under the light microscope really represents: the microtubule pattern or the pattern of both the endoplasmic reticulum and the Golgi vesicles. From the light microscopical investigations of the authors referred to in the introduction (Crüger, Dippel, Barkley, and Sinnott & Bloch), it is not clear whether the dense granular protoplasm areas correspond with cytoplasm areas between or facing the future thickenings. A study in which both light microscopical and electron microscopical preparations of plasmolysed cells were compared was aimed at this particular problem and will be published later.

6. Hypotheses on the role of some cell organelles in thickening formation

In the first paper (Goosen-de Roo 1973) it was assumed that endoplasmic reticulum, Golgi vesicles, microtubules, and lomasomes play a role in the formation of the thickenings in tracheary elements. In principle, there are three different ways in which a cell organelle can contribute to thickening formation:

1. Synthesis of cell wall substances.
2. Transport of cell wall substances to the plasmalemma.
3. Determination of the site of deposition of cell wall substances.

In the thickenings, pectic substances, hemicelluloses, cellulose, lignin, and protein are present. Therefore, the manner in which a cell organelle contributes can also be specified in accordance with the biochemical substance involved.

In electron microscopical preparations, cellulose is visible in the primary cell wall and in the thickenings as microfibrils. In radial sections through the tracheary elements the cellulose microfibrils in the thickenings are arranged in concentric circles having a common point of contact with the primary wall. The microfibrils in the thickenings seem to be deposited by apposition. A role of microtubules in directing the orientation of cellulose microfibrils was established by Hepler & Fcsket (1971). Of the cell wall substances reported as being found

in the thickenings, only lignin can be made visible with certainty for electron microscopy. Fixation with $KMnO_4$ specifically stains the lignin in the cell wall (HEPLER, FOSKET & NEWCOMB 1970). From the photographs it can be seen that the lignification process starts from the sites of attachment of the thickening to the primary cell wall and extends little by little through the whole thickening (HEPLER & NEWCOMB 1963). *Plate V, fig. 1* illustrates this process. It is a photograph from a study in which the glutaraldehyde-osmium fixation and $KMnO_4$ fixation were compared. $KMnO_4$ (0,5% in distilled H_2O) was employed for 6 hours at room temperature.

In stage 0 the sites of deposition of the first cell wall substances for the thickenings are to be determined. In this stage lomasomes and microtubules are present along the plasmalemma. The microtubules lie in a single row along the plasmalemma, but they are absent around the membrane of the lomasomes. The distribution of the lomasomes in relation to the sites of attachment of the thickenings in neighbouring cells is rather irregular. In relation to the neighbouring thickenings, however, two different positions of the lomasomes can be distinguished. These two possibilities (A1 and B1) are given in *fig. 3*; the lomasomes are situated either opposite to the cell wall thickenings (A1) or between the cell wall thickenings (B1) in the neighbouring cell. Type B1 occured more often than type A1 in the material studied (*plate V, figs. 2* and *3*). These two locations represent two alternative hypotheses for the initiation of the thickenings. When we consider the distribution of the endoplasmic reticulum in stage 0, we see that the few cisternae of the endoplasmic reticulum lie statistically significantly more in the cytoplasm opposite that between the thickenings of the neighbouring cell

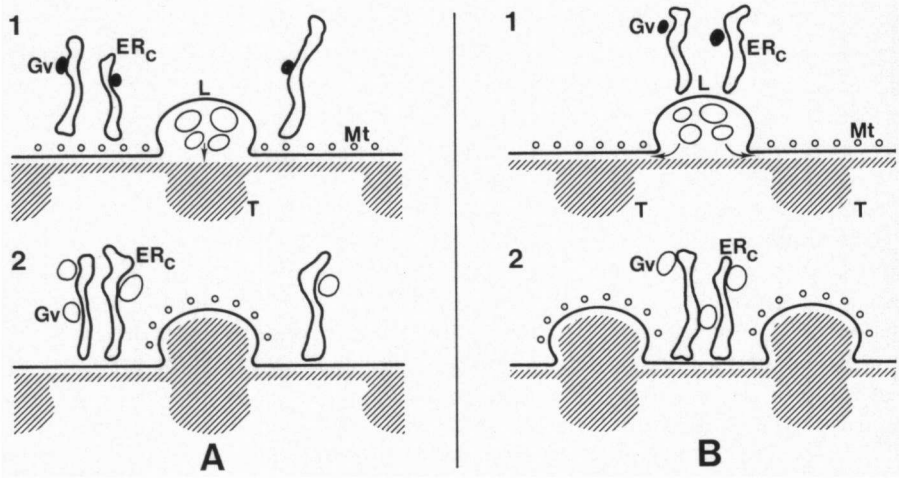

Fig. 3. Initiation and formation of cell wall thickenings as proposed in the two alternative hypotheses (A and B), based on the two types of lomasome localization. The hatched areas represent cell wall material, the arrows indicate the transport route of cell wall material.

than in the cytoplasm opposite that facing the thickenings of the neighbouring cell. The Golgi vesicles seemed to be distributed in a similar way. Microtubules in the initial stage of thickening formation are not found around the lomasomes. During thickening formation, microtubules are found only around the cell wall thickenings. In situation A1 (*fig. 3*) the endoplasmic reticulum and the Golgi vesicles would be present in the same areas as the microtubules; that is, the site opposite the areas between the thickenings in the neighbouring cell. In situation A2 the endoplasmic reticulum and the Golgi vesicles, and on the other hand the microtubules are located in different areas: a. the endoplasmic reticulum and the Golgi vesicles are found in the areas corresponding with the sites between the neighbouring thickenings, and b. the microtubules are located around the meanwhile developed cell wall thickenings of the neighbouring cell. Therefore, in case A the endoplasmic reticulum and the Golgi vesicles would remain at their original sites, while the microtubules would change their position during thickening formation. In situation B1 the endoplasmic reticulum and the Golgi vesicles would be present in the area corresponding with the sites between the cell wall thickenings in the neighbouring cell; the microtubules at the sites corresponding with the areas opposite the cell wall thickenings in the neighbouring cell. In situation B2 the cell organelles remained in the same position as found in B1. Therefore, the endoplasmic reticulum and the Golgi vesicles always show an alternative position with regard to the microtubules. During thickening formation the endoplasmic reticulum and Golgi vesicles always occupy positions alternate to those of the microtubules.

In these experiments it was assumed that during the process of thickening the endoplasmic reticulum conducts Golgi vesicles to the sites between the thickenings. No microtubules are present at these sites. The orientation of the cellulose microfibrils in the thickenings (interpreted as an indication for the appositional way of cellulose deposition) in addition to the orientation of the microfibrils mirroring the orientation of the microtubules, support the assumption that the cellulose precursors are deposited at the sites where microtubules are present. We have also previously suggested that the microtubules form a barrier for vesicles containing cell wall precursors for pectic substances, hemicelluloses, lignin, and protein. The parallel orientation of the microtubules with respect to the plasmalemma, together with the relatively small distance between the microtubules, suggest this barrier function. The cell wall substances mentioned above are probably deposited in stage 0 at the sites of the lomasomes and between the developing thickenings in the following stages. Hypothesis A requires that cell wall material present in the lomasomes is deposited at the same site; that is, exactly beneath the lomasomes (arrow in *fig. 3*, A1). In hypothesis B the cell wall material present in the lomasomes "moves" sideways in two directions (arrows in *fig. 3*, B1).

With regard to hypothesis A, the report of CLOWES & JUNIPER (1968) on the distribution of microtubules in xylem tracheary elements is briefly considered. These authors gave an interpretation in schematic drawings of four stages in the development of a xylem vessel which was based chiefly on information from

PICKETT-HEAPS & NORTHCOTE (1966) and PICKETT-HEAPS (1967), but also on the reports of WOODING & NORTHCOTE (1964) and CRONSHAW & BOUCK (1965). In the first stage (procambial cells) the microtubules are randomly distributed along the plasmalemma. In the second stage the microtubules are concentrated between the developing cell wall thickenings. They are situated around the cell wall thickenings in the third stage, which makes it necessary that the microtubules change their position. In fact, with regard to the thickenings they exchanged their position with the endoplasmic reticulum. In the fourth stage the cell wall thickenings are fully developed and protoplasm is no longer present. Neither Clowes & Juniper nor Pickett-Heaps & Northcote gave a possible mechanism for this striking exchange of position between the microtubules and the endoplasmic reticulum. It is possible that the microtubules disappear from their original sites and that new microtubules are formed at the new sites. Perhaps the small cell wall thickenings in the second stage of CLOWES & JUNIPER (1968) are identical with the lomasomes in stage 0 which are described here. In that case, if the lomasomes are considered as small cell wall thickenings, the microtubules are indeed located between the "thickenings".

Hypothesis B seems to be the more likely considering the following observations (a and b) and suppositions (c and d):

a. during thickening formation, both the endoplasmic reticulum and the Golgi vesicles are always situated at sites different from those of the microtubules,
b. the distribution of cell organelles as given in *fig. 3*, B1 occurs more often than the distribution in *fig. 3*, A1 in the material studied,
c. the deposition of cell wall material by the lomasomes in the case of hypothesis B seems to be more complicated than in hypothesis A. However, it must be recalled that the cell wall is liquid-like and transportation sideways, therefore, seems to be not unrealistic. Besides, on the basis of our observations a similar route is assumed for cell wall substances (with the exception of cellulose) during the later stages of thickening formation.
d. Hypothesis A would require a complicated movement or reconstruction of microtubules and a transport route for cell wall substances (again, with the exception of cellulose) different from that in later stages.

Summarizing, the possible roles of the cell organelles studied in the formation of the thickenings in tracheary elements can tentatively be described as follows:

1. The endoplasmic reticulum plays a role in the synthesis of enzymes involved in cell wall synthesis and in the synthesis of the hydroxyproline-rich cell wall protein. The endoplasmic reticulum might also play a part in the transport of various molecules through the cisternae and in conducting Golgi vesicles along its surface to the sites between the developing thickenings.
2. The contents of the Golgi vesicles contribute to the formation of the thickenings.
3. The microtubules play a role in the determination of the initiation sites of the cell wall thickenings and in the orientation of cellulose microfibrils. Furthermore, they form a barrier for Golgi vesicles containing enzymes and precursors for cell wall substances, with the exception of cellulose.

4. The lomasomes determine the initiation sites of the cell wall thickenings and contribute to the formation of these thickenings by depositing cell wall material.

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List of abbreviations used in the text, figures, tables, and photographs

D	dictyosome	PCW	primary cell wall
ER	endoplasmic reticulum	Pl	plasmalemma
ER_c	cisternal endoplasmic reticulum	Pr	polyribosomes
ER_v	vesicular endoplasmic reticulum	R	ribosomes
Gv	Golgi vesicle	RER	rough endoplasmic reticulum
L	lomasome	S	starch
Li	lipid body	SER	smooth endoplasmic reticulum
M	mitochondrion	T	cell wall thickening
Mt	microtubule	Va	vacuole
P	plastid		

List of fixation and staining used per photograph

	Fixation	Staining		Fixation	Staining
Plate I	2	U/R	Plate IV, fig. 1	2	U/V
Plate II, fig. 1	1a	U/V	fig. 2	2	U/R
fig. 2	1a	U/V	Plate V, fig. 1	$KMnO_4$	U/R
fig. 3	1a	U/V	fig. 2	1a	U/R
Plate III, fig. 1	2	U/R	fig. 3	1a	U/R
fig. 2	1b	U/R			
fig. 3	2	U/R	Explanation, see GOOSEN-DE ROO (1973)		

Plate I

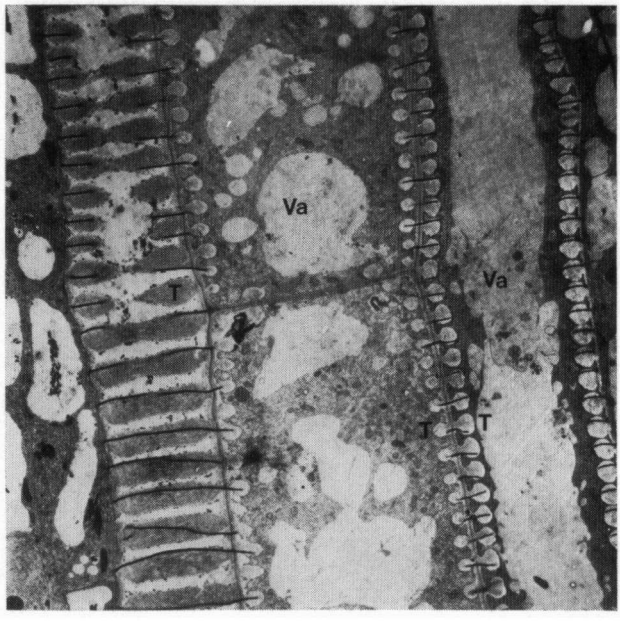


Plate I. Radial section through two tracheary elements; the left tracheary element is sectioned tangentially. The stages of these cells are from the left to the right 5, 1, and 3, respectively. The thickenings in neighbouring cells correspond with each other.
The dark bands on the thickenings are artifacts caused by sectioning, × 1,550.

Plate II

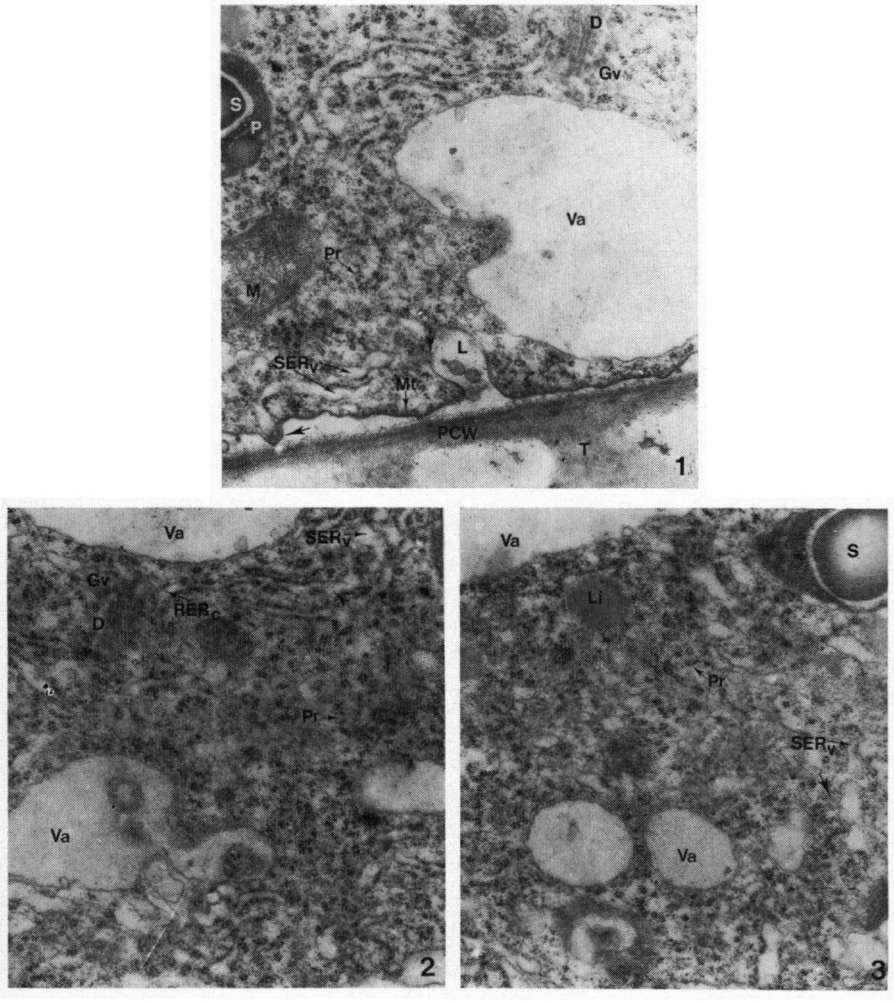


Plate II, fig. 1. Class 0: cytoplasmic area next to the cell wall. The vesicular shaped endoplasmic reticulum is in contact (large arrows) with the plasmalemma at two places, suggesting that the contents of the vesicles between the plasmalemma and the primary cell wall are of endoplasmic reticulum origin, × 19,800.

Plate II, fig. 2. Class 0: vesicular shaped endoplasmic reticulum that seems to flatten out to form cisternal endoplasmic reticulum (large arrow), × 20,600.

Plate II, fig. 3. Class 0: vesicular endoplasmic reticulum probably forming small vacuoles (large arrow), × 20,600.

Plate III

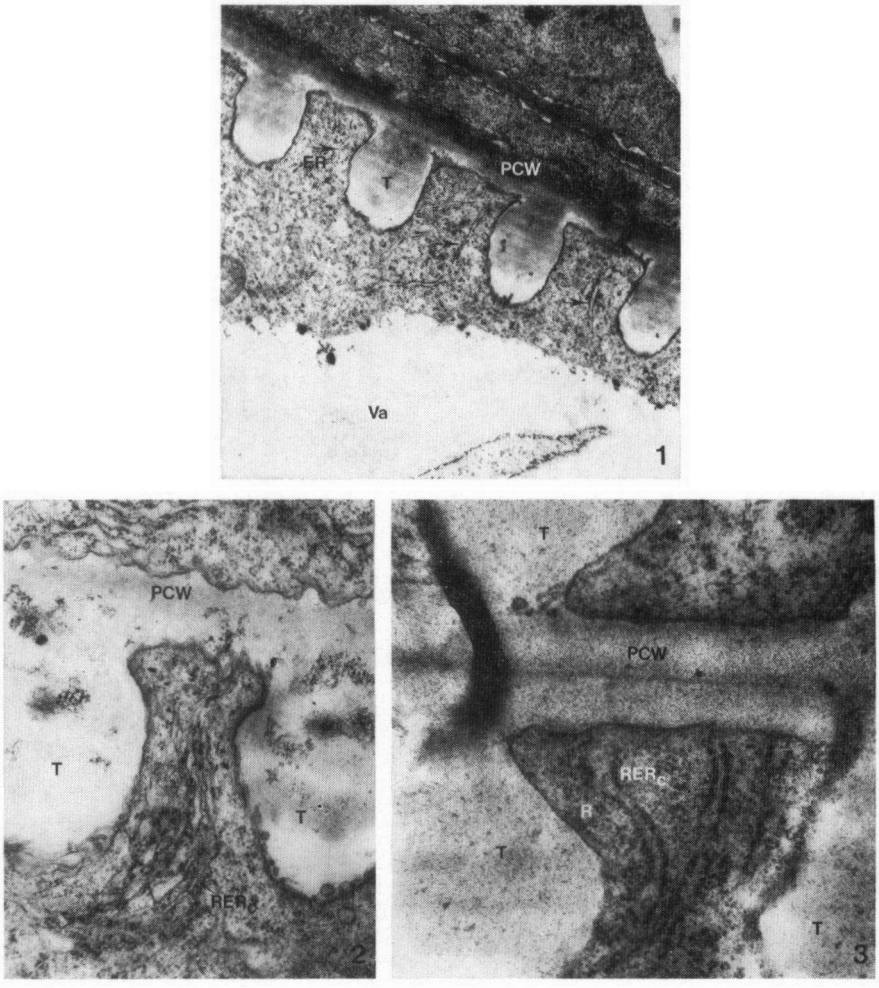


Plate III, fig. 1. Class 1: part of a tracheary element with some cell wall thickenings. The cisternae of the endoplasmic reticulum are present between the thickenings and oriented perpendicular to the primary cell wall (arrows). A part of the central vacuole is visible, × 8,700.

Plate III, fig. 2. Class 2: cytoplasmic area between two cell wall thickenings. The rough endoplasmic reticulum cisternae have an orientation perpendicular to the plasmalemma between the thickenings. The electron-opaque areas on the cell wall thickenings are caused by lead contamination, × 27,200.

Plate III, fig. 3. Class 3: small part of cytoplasm between two cell wall thickenings. The cisternae of the endoplasmic reticulum are oriented perpendicular to the plasmalemma between the thickenings. The dark band on the cell wall is an artifact caused by sectioning, × 29,200.

Plate IV

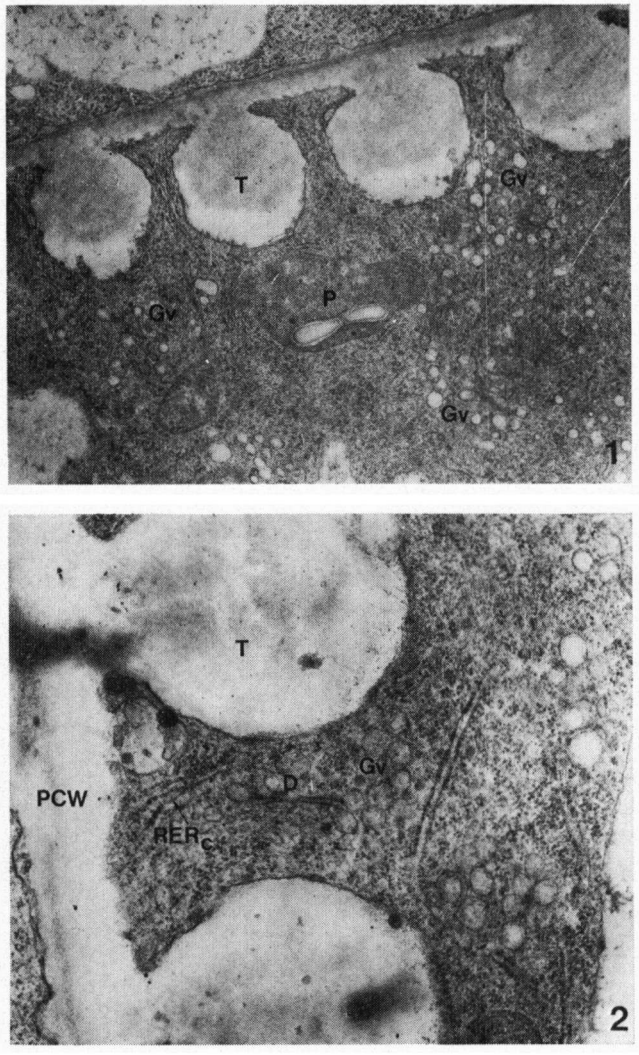
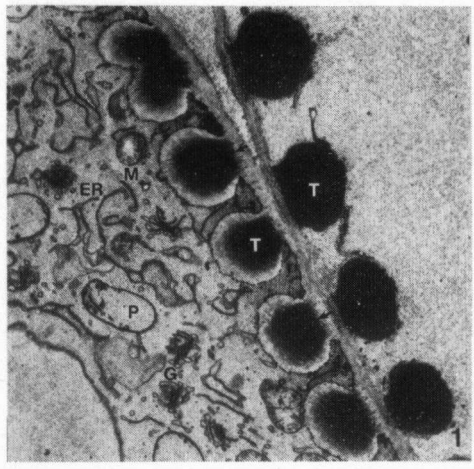
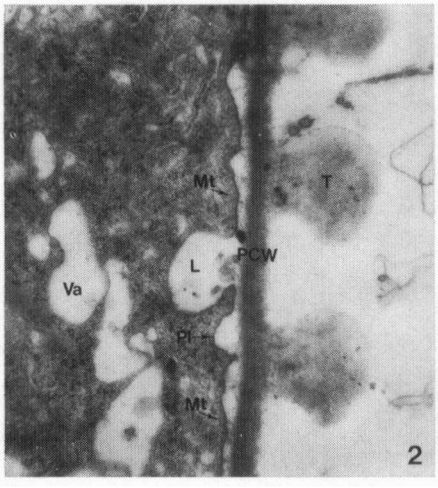


Plate IV, fig. 1. Class 2: general view of a tracheary element, many Golgi vesicles are visible. The areas between the thickenings (fig. 1: areas 1 and 3) have a greater density of Golgi vesicles than the areas facing the thickenings (fig. 1: area 2), × 10,900.
Plate IV, fig. 2. Class 2: area of cytoplasm between two cell wall thickenings. A dictyosome and Golgi vesicles are visible in this area, × 25,700.

Plate V





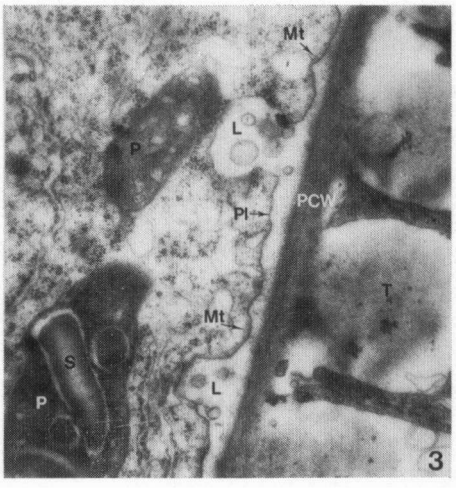


Plate V, fig. 1. Two neighbouring tracheary elements after $KMnO_4$ fixation. In the cell on the right the thickenings are totally lignified; in the cell on the left lignification is still in progress. This process starts from the point of attachment of the thickenings to the primary cell wall (arrows), × 3,000.

Plate V, fig. 2. Two neighbouring tracheary elements, the cell on the left represents stage 0; the cell on the right, stage 2 (this cell is plasmolysed). A lomasome lies opposite the site between two cell wall thickenings in a neighbouring cell. Along the plasmalemma surrounding the lomasome no microtubules are present. Note the double row of microtubules, × 19,600.

Plate V, fig. 3. Two neighbouring tracheary elements; the cell on the left represents stage 0. Two lomasomes are visible. They are localized more or less opposite the sites between the cell wall thickenings in the neighbouring tracheary element. Note the distribution of microtubules, × 23,000.