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Micro-Vesicles, Pyriform Vesicles and Macro-Vesicles Associated with the Plasma Membrane in the Root Hairs of *Vicia hirsuta* after Freeze-Substitution

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After freeze-substitution, micro-vesicles were found only in close proximity to the plasma membrane. Macro and pyriform vesicles were found throughout the cytosol, but also 'packaged' close to the plasma membrane, the package delineated by electron transparent outlines similar to the endoplasmic reticulum. These outlines appeared to be continuous with nearby endoplasmic reticulum and were always associated with Golgi bodies and microtubules. Micro-vesicles were found only in grazing sections of the plasma membrane made between the apical dome and the region of the nucleus, where the cell is the most cytoplasmic, and only in close proximity to the plasma membrane. Micro-vesicles were also found in close proximity to microtubules as well as other vesicle types. From the results it is suggested that pyriform and micro-vesicles may have specialised roles in root hair tip growth.

Key words : Freeze-substitution — Macro-vesicles — Micro-vesicles — Nod-factors — Plasma membrane — Pyriform vesicles

Root hairs are tip-growing cells that grow by the addition of cell wall material delivered to the tip inside vesicles. Vesicles in root hairs are mainly found close to the tip region, where there is a distinct 'froth' of vesicles that are presumably emanating from the endomembrane system. In the cytoplasm-rich region between the nucleus and the tip, there is a large amount of endoplasmic reticulum and Golgi bodies that form the production and modification centre of cell wall precursors. The nucleus follows the growing tip at a set distance, presumably because continuous transcription of the genes for proteins involved in cell wall production is needed. Indeed, the cytoplasm in this area is dense with ribosomes, suggesting that translation is a continuous process (see e.g. Ridge 1988, 1990a, and figures in this paper).

The flow of vesicles and cell wall precursors to the tip is apparently controlled by the cytoskeleton (Lloyd *et al.* 1987), and the flow of vesicles within the apical dome may be controlled by a calcium gradient (Jaffe 1982). The cytoskeleton is also involved in maintaining the integrity of the hair shape (Lloyd 1984) and in positioning of the nucleus, which follows the tip as it grows. Excess membrane from the tip is likely recycled by a clathrin-based system at the base of the dome (Ridge 1993, see also Emons and Traas 1986).

Recent advances in the knowledge of root hair biology have resulted from the use of dry cleaving and freeze fracturing (e.g. Emons and Traas 1986), by rapid-freeze, freeze-substitution for electron microscopy (e.g. Emons 1987, 1988, Ridge 1988, 1990a) and by drug studies aimed at determining the role of the cytoskeleton (Lloyd *et al.* 1987, Ridge 1990a). The root hairs of legumes are particularly important to study because they are the entry point for *Rhizobium* in its symbiosis with many of the world's major legume food crops. Ideas on root hair growth and *Rhizobium* infection are summarized and modeled in Ridge (1993).

After freeze-substitution treatment, several kinds of vesicle have been found in root hairs, viz : smooth vesicles, coated vesicles, clathrin-coated vesicles and the unusual pyriform vesicle (Ridge 1988). It is possible that the smooth and coated vesicles are involved in the transport and deposition of cell wall substances to the tip, and can be categorised as secretory vesicles. However, clathrin-coated vesicles are clearly involved in membrane recycling (see discussion in Emons 1987, and in Ridge 1988, 1993). A role for pyriform vesicles has yet to be deduced by experimentation.

In this paper I report the occurrence of another kind of vesicle, defined as a micro-vesicle, that is found only at the plasma membrane, and the occurrence of macro-vesicles and pyriform vesicles in discrete compartment-like zones or 'packages' close to the plasma membrane.

Material and Methods

Seed germination

Vicia hirsuta seed was treated with concentrated sulphuric acid for 45 min (they have a very hard seed coat), rinsed ten times, and then left in water for several hours. Swollen seeds were left to germinate on damp absorbent

paper in a sealed dish, in the dark, for 3 days at room temperature. Seedlings with well-developed hairs were used for rapid freezing.

Electron microscopy

The methods of Ridge (1988, 1990b) were used, which were essentially as follows: Roots were cut at about 1 cm from the root tip, held by very fine tweezers at the cut end, and frozen by rapidly plunging into liquid propane from a height of about 50 cm. The liquid propane was prepared by condensing propane gas into an aluminium container cooled with liquid nitrogen to about −196C. After freezing, specimens were transferred to vials containing 0.3% OsO_4 in dry acetone over molecular sieve (Bio-Rad type 3a) and left for 3 days in a −80C freezer. The containers were then transferred to −20C for 8 hr, given 2 changes of clean dry acetone over 4 hr, and then warmed to room temperature over 4 hr. Samples were infiltrated by gradually increasing the concentration of Spurr's resin over 2 days plus another day at 100%, and embedded at 60C. Sections (*ca* 70 nm) were stained with 2% uranyl acetate in 50% ethanol for 15 min followed by lead citrate for 5 min and viewed in a JEOL 1200DX electron microscope at 80 kv.

Results

Grazing sections of root hairs taken between the nucleus and the apical dome revealed distinct compartment-like zones or 'packages' of vesicles at the cytoplasm/plasma membrane interface (Figs. 1-3). The packages were found to be delineated by electron transparent outlines similar to the endoplasmic reticulum and always associated with Golgi bodies (dictyosomes) (Fig. 1). From consecutive serial sections the packages were found to be of irregular shape. The vesicle population in these packages consisted mostly of secretory vesicles (which are electron transparent) but with associated pyriform vesicles (electron dense); coated vesicles were never found in the packages. Secretory vesicles, which are always spherical, were found to have the greatest diameter of approximately 120 nm, while pyriform vesicles, which are spherical but have distinct 'tails', have a maximum diameter of approximately 95 nm. Macro-vesicles and pyriform vesicles were also found within the cytoplasm of the cell, mostly within the apical dome of the root hair tip.

Microtubules were found inside the packages in close association with the vesicles, either adjacent to the plasma membrane and inside the package, or deeper in the cytoplasm next to the package (Figs. 2 and 3). Microfilaments were not found within or close to the packages, although they were found in other parts of the cell and often within contact distance of the plasma membrane. The packages were not found at the extreme tip of the hair, where there is a high density of secretory vesicles that subtend the plasma membrane and where organelles are usually absent. The exact distribution of the packages was not studied, except that they were only found in the cytoplasm-rich region between nucleus and tip.

A population of micro-vesicles was found in grazing sections made at a high angle (close to parallel), and only close to the plasma membrane in the region between the nucleus and apical dome (Fig. 4). They were not found endoplasmically (i.e. within the cytoplasm), and they could not be found on sections made at right angles to the plasma membrane. The micro-vesicles were not uniform in shape or perfectly spherical (Fig. 4) but of largest dimensions of approximately 50 nm (compare to the spherical secretory vesicles in Fig. 4). Because electron microscopy sections are generally about 70 nm in thickness, it is likely that many complete micro-vesicles were seen in one section. If this were to be the case, then the micro-vesicles seen in Fig. 4 range in size of from 15 to 50 nm, and are thus, in their smallest dimensions, close to the size of ribosomes after the same rapid-freeze treatment. Micro-vesicles were found in close association with macro-vesicles, as well as with microtubules (Fig. 4).

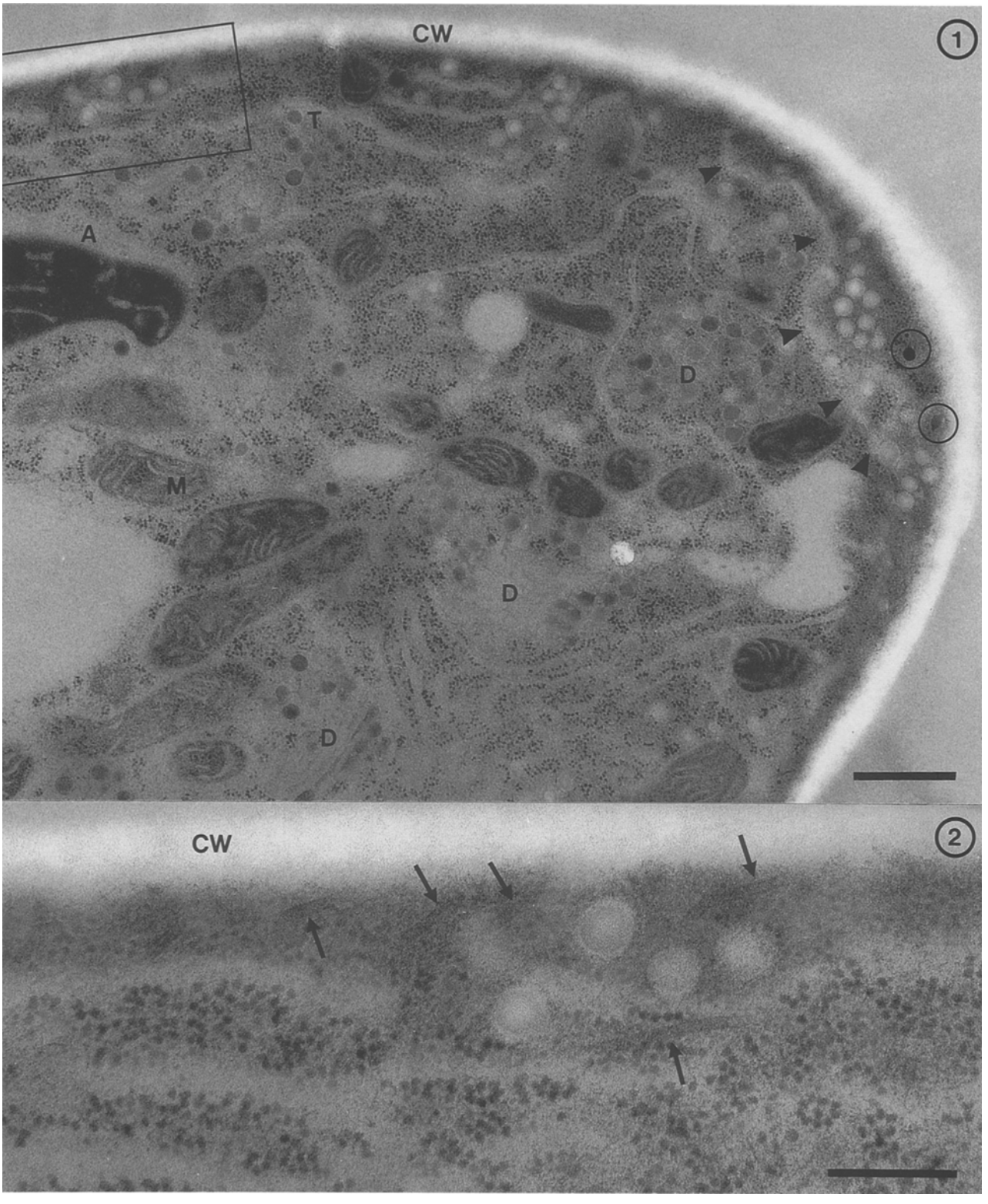
Discussion

Vesicle 'packages'

Macro-vesicles and pyriform vesicles have already been described, but they have not previously been shown to be 'packaged' close to the plasma membrane. Pyriform vesicles were first described in root hairs (Ridge 1988) and as far as I know have never been described for any other organism. Observations of the packages close to the membrane, associated with both Golgi and microtubules, leads me to suggest that the packages possibly move to the tip in close association with the plasma membrane, guided by the microtubules and supplied with vesicles by the Golgi, perhaps moving as a unit towards

Fig. 1. Low magnification view of a grazing section of a root hair cell between the nucleus and the apical dome of *Vicia hirsuta*. Vesicles packaged close to the cell wall are confined within an 'outline' similar to the endoplasmic reticulum (arrowheads point to an example) and continuous with it. Note the proximity of Golgi bodies. Examples of pyriform vesicles, which are electron dense, are circled; secretory vesicles are electron negative. The rectangular section at the top of the Figure is enlarged in Fig. 2. Note the excellent preservation of polyribosomes and mitochondrial ribosomes after freeze-substitution, and the coated vesicles in the area designated as possible *trans*-Golgi network. The cell wall and membrane are not stained. CW=cell wall, T=possible *trans*-Golgi network, A=amyloplast, D=dictyosome, M=mitochondrion. Bar=500 nm.

Fig. 2. High magnification view of the upper section rectangle of Fig. 1. Arrows indicate microtubules associated with a small vesicle 'package' close to the membrane. Bar=200 nm.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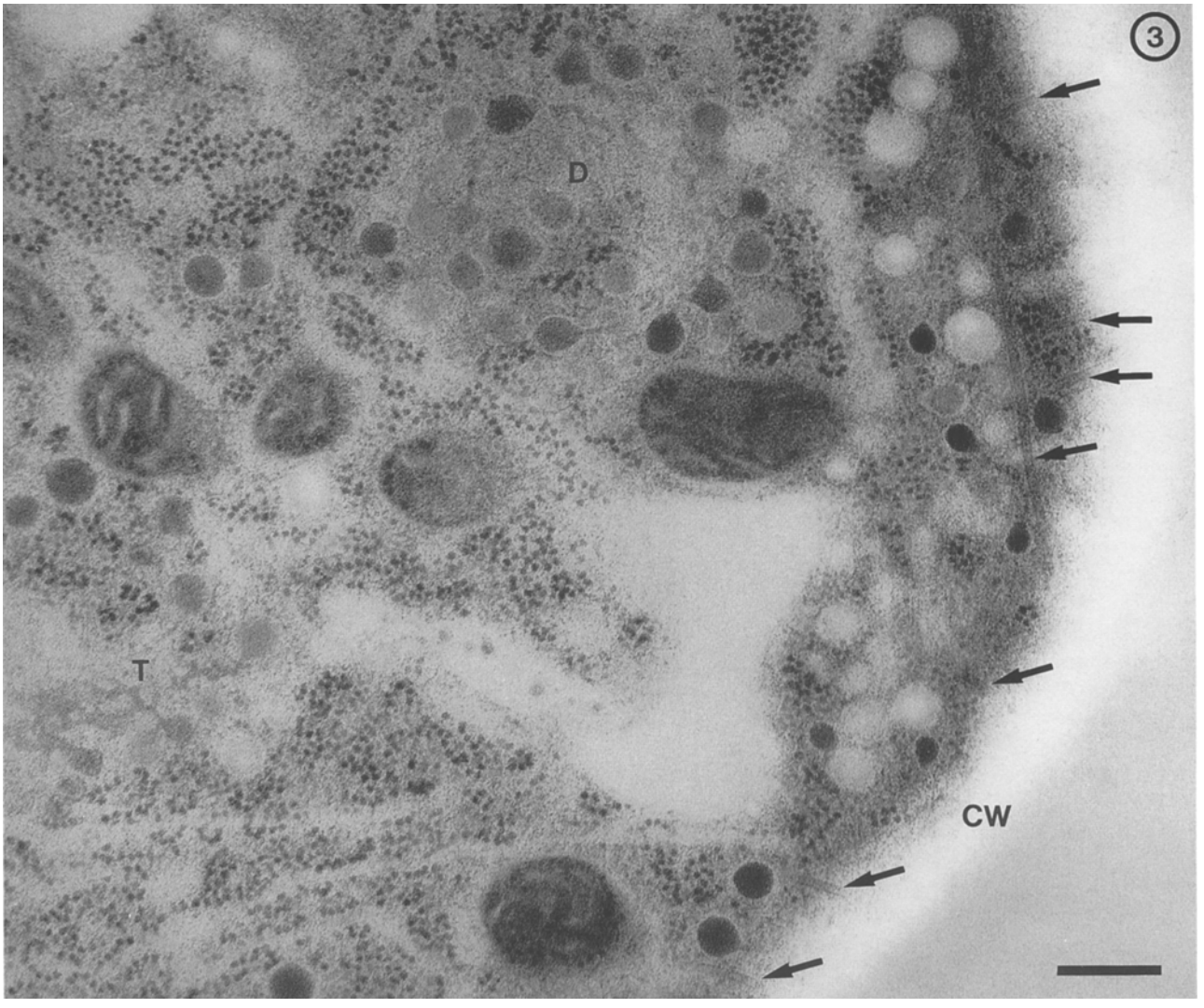

Fig. 3. High magnification view of grazing section to show dense packing of vesicles in a 'package' next to the plasma membrane. This section is consecutive to that shown as Fig. 1. Note the presence of numerous microtubules (arrows) within and close to the package, the mixture of secretory and pyriform vesicles, and the close proximity of a Golgi body (dictyosome). The plasma membrane and cell wall are unstained. CW=cell wall, D=dictyosome, T=*trans*-Golgi network. Bar=200 nm.

the tip. The plasma membrane of many types of cell is a dynamic 'organelle' in its own right, in which many kinds of proteins and other membrane-bound molecules are able to move. It is possible therefore that the plasma membrane is responsible for movement of these packages to the tip, which is the inevitable direction of all secretory vesicles in plant tip-growing cells. Clearly also, pyriform vesicles may have a specialised role to play in the organisation and fate of other kinds of vesicle, although it is equally valid to say that they may have a role in other aspects of the cell, such as the delivery of special molecules such as those required for signal transduction.

Micro-vesicles

This is the first description of micro-vesicles in root hairs. Micro-vesicles have been described for fungal mycelium, where they are also called chitosomes (Ruiz-Herrera *et al*. 1977). Bartnicki-Garcia (1990) gives a value of 40-70 nm in diameter for conventionally-fixed material, and states that freeze-substituted fungus gives significantly smaller values. The results presented here show that *Vicia hirsuta* root hair micro-vesicles range in size from 15 to 50 nm, which is in the range of the dimensions suggested by Bartnicki-Garcia.

As in fungal hyphae, micro-vesicles in root hairs may have a different function to the larger macro-vesicles. Fungal micro-vesicles are known to be involved in the

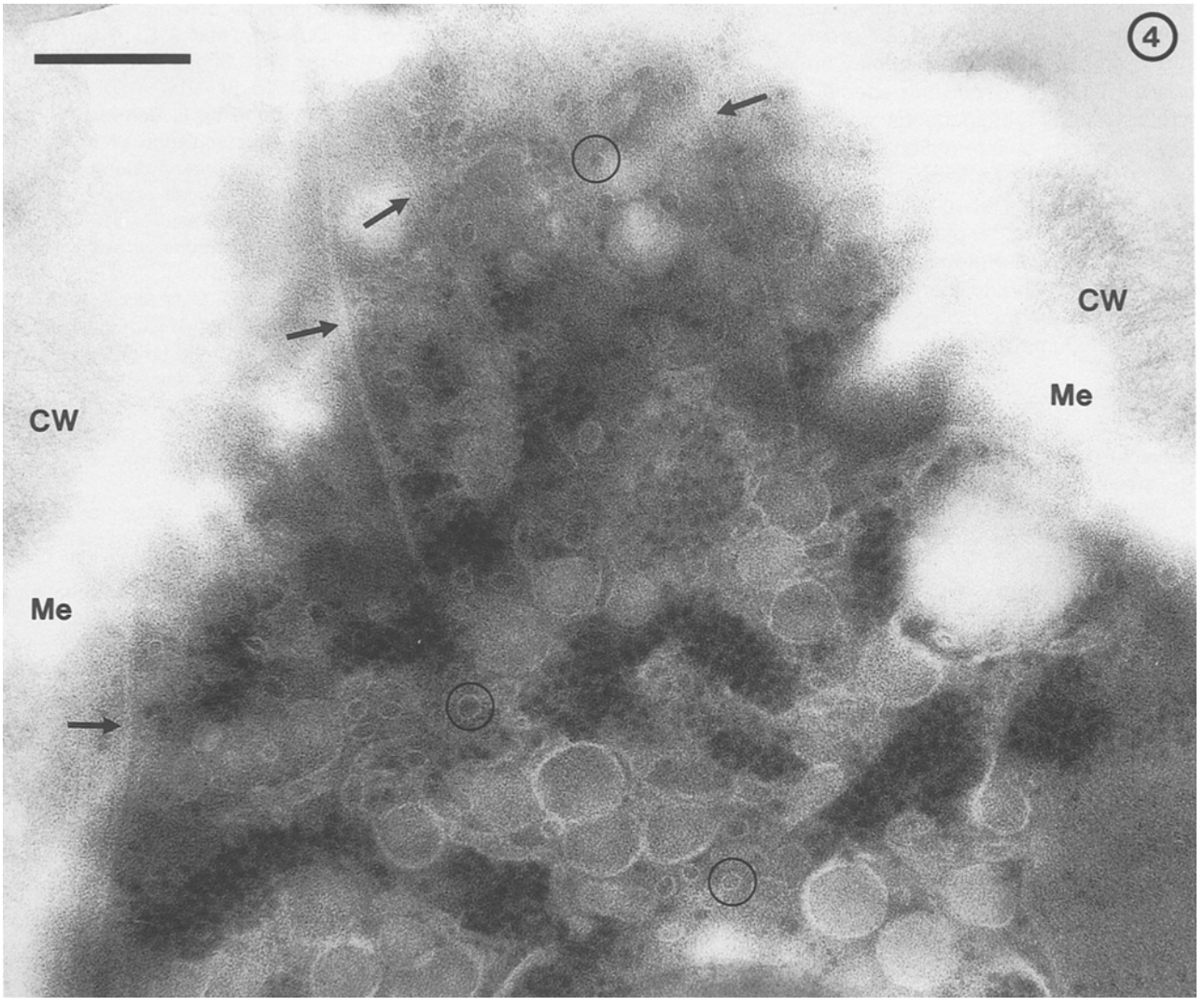

Fig. 4. High magnification view of a grazing section next to the plasma membrane showing micro-vesicles close to the plasma membrane and in association with macro-vesicles. Examples of micro-vesicles are indicated with circles, and microtubules with arrows. The size and shape of the micro-vesicles indicate that they are not transverse sections of the tails of pyriform vesicles. Note the dense packing of ribosomes in this section, indicating that there is a high level of mRNA translation, probably into nearby endoplasmic reticulum, and suggesting that this is an area active in protein movement through the endomembrane system. CW=cell wall, Me=plasma membrane. Bar=200 nm.

delivery of chitin synthetase, which is synthesised at the plasma membrane-cell wall interface (Bartnicki-Garcia 1990). If root hair micro-vesicles have a parallel role, then it could be possible that these micro-vesicles carry substances with special tasks to the root hair tip. For example it is known that plants produce chitinases (Boller *et al*. 1983, Schlumbaum *et al*. 1986). Indeed, Krause *et al*. (1994) have made a cDNA bank from mRNA isolated from *Vigna unguiculata* root hairs after stimulation by *Rhizobium* Nod-factor, and have found, amongst others, cDNAs coding for chitinase I, III and IV.

It is well established in the literature that Nod-factors are the signal molecules that initiate the *Rhizobium*/legume symbiosis (for review see Fisher and Long 1992), and it is therefore clear that the Nod-factors must interact with a signal receptor protein that is most likely to be located on the plasma membrane. Yet at the growing tip of root hairs, where *Rhizobium* attaches and eventually invades, membrane is constantly being deposited and flows away back down the apical dome for recycling by a clathrin-based system. The supply of any molecules involved in recognition and transmission of a signal molecule must be constantly renewed, and I suggest that the various vesicles found in root hair tips may have such different and

specialised roles. That is, spherical macro-vesicles deposit cell wall precursors for growth of the tip, but pyriform and/or micro-vesicles may be involved in different roles, such as depositing special domains of membrane containing proteinaceous molecules that transduce signal messages by interaction with external signal molecules, such as Nod-factors.

The close association of micro-vesicles with microtubules, close to the plasma membrane, also indicates that microtubules are probably guiding the flow of micro-vesicles, presumably to the hair tip.

Further work on the biological role of specialised vesicles in the legume root hair is needed.

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