# Structure of the generative cell wall complex after freeze substitution in pollen tubes of *Nicotiana* and *Impatiens*

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## Summary

**The mature generative cell in pollen grains and pollen tubes is surrounded by a wall complex that includes two plasma membranes, one facing the generative cell cytoplasm and one facing the vegetative cell cytoplasm, and usually some intervening wall material. After conventional chemical fixation, the two plasma membranes are very uneven and often appear to be joined, giving the impression that numerous plasmodesmata connect the vegetative and generative cells. These areas alternate with swollen, distorted areas, which give the wall complex the appearance of being composed of a chain of vesicles. Utilizing rapid freeze fixation and freeze substitution, we have re-examined the ultrastructure of the generative cell wall complex from pollen tubes grown *in vitro*, and the differences are striking. The two plasma membranes are very smooth and closely appressed to a layer of wall material. Occasionally the wall complex contains swollen areas, or varicosities, and these may contain pockets of lightly stained material, but again the surrounding plasma membranes are tightly appressed to these areas. Plasmodesmata are not seen, but this does not eliminate the possibility that they may exist at an earlier stage of development.**

Key words: freeze substitution, generative cell, pollen tube, rapid freeze fixation, ultrastructure.

## Introduction

The generative cell of pollen exists as a cell within a cell, originating in an asymmetric division of the microspore. The wall that is cut off after this division continues to grow until eventually the generative cell becomes detached from the original microspore wall and is free within the vegetative cell cytoplasm (e.g. see Heslop-Harrison, 1968; Sanger & Jackson, 1971; Owens & Westmuckett, 1983). The generative cell cytoplasm is thus bound by a complex structure that includes its own plasma membrane (PM) and the vegetative cell PM. The presence or absence of intervening wall material between the two PMs has been a matter of some controversy (see Rosen, 1968; Gorska-Brylass, 1970; Owens & Westmuckett, 1983). In ultrastructural studies of conventionally fixed material, the PMs of the surrounding complex appear uneven and sinuous. Swollen spaces in the complex alternate with regions where the two PMs appear to be joined, often creating the impression that the generative cell wall consists of a string of vesicles and that abundant plasmodesmata connect the generative and vegetative cell cytoplasm (e.g. see Burgess, 1970; Jensen *et al.* 1974; Cresti *et al.* 1984, 1985). The suggestion has also been made that in the conifer *Taxus* the spermatogenous cell wall arises *de novo* by the fusion of such vesicles (Pennell & Bell, 1986).

Recently we have reinvestigated the structure of the generative cell wall complex in material that has been prepared by rapid freeze fixation and freeze substitution (RF–FS). The RF–FS procedure allows one to stabilize cytoplasmic structure within a few milliseconds through vitrification of the water in the cell. Subsequently the material is chemically fixed at low temperatures with a minimum of distortion. The

technique thus permits one to obtain a more life-like image of cellular ultrastructure than has been possible by the use of conventional chemical fixation.

The images of the generative cell wall after RF–FS are dramatically different from those obtained by chemical fixation. Most evident is the fact that the wall is remarkably even and uniform, and lacks the swollen vesicular appearance portrayed previously. These new findings are presented in this brief report.

## Materials and methods

### *Chemical fixation*

Fresh pollen grains of *Nicotiana alata* were sown in Brewbaker & Kwack's (1964) medium containing 15% sucrose; the pollen tubes were harvested 3 h after sowing. The materials were gently centrifuged, fixed, embedded, and stained both conventionally and by the Thiery method as reported by Cresti *et al.* (1984). Observations were made at 80 kV with a JEOL 100B electron microscope.

### *Rapid freeze fixation and freeze substitution*

Freshly collected pollen from a greenhouse-grown *N. alata* was sown on the surface of liquid culture medium containing 50 mM-PEG 400 (Zhang & Croes, 1982), 1 mM-$CaCl_2$, and 0·01% $H_3BO_3$. Two to three hours after sowing, pollen tubes were subjected to rapid freeze fixation and freeze substitution as described by Lancelle *et al.* (1986). Pollen from *Impatiens wallerana* was sown as described above except that 10% sucrose was substituted for the PEG 400, and cells were frozen 45 min to 1½ h after sowing. After substitution was complete, the samples were rinsed in several changes of acetone, followed by methanol. *En bloc* staining was then carried out at room temperature in 5% uranyl acetate in methanol for 2 h. After rinsing in methanol followed by acetone, infiltration and embedding were carried out in Epon–Araldite resin. Sections were stained for 3 min in Reynold's lead citrate before examination at 80 kV on a JEOL 100CX electron microscope.

## Results

For the purpose of comparison Figs 1 and 2 show the generative cell wall complex of *N. alata* after conventional chemical fixation. Typically the two PMs are markedly sinuous in profile, and the whole complex appears to be composed of a chain of vesicles (Fig. 1). The vesicles are offset by numerous regions in which the two PMs come close together or where there are structural bridges across the wall. These have been interpreted as channels or connections between the vegetative and generative cells. An indication that polysaccharides occur in the 'vesicular' spaces between the two PMs is shown in preparations stained with the Thiery reagent (Fig. 2).

In contrast to the images obtained from conventionally fixed material, those obtained from cells of both *N. alata* (Figs 3, 4) and *I. wallerana* (Fig. 5) after RF–FS are markedly different. First, the entire aspect of the generative cell wall complex is of a structure that is very even and uniform. There are occasional swollen areas or varicosities (Fig. 3) but these contain stained material and are not the blown-out, lightly stained vesicles that one sees in chemically fixed material.

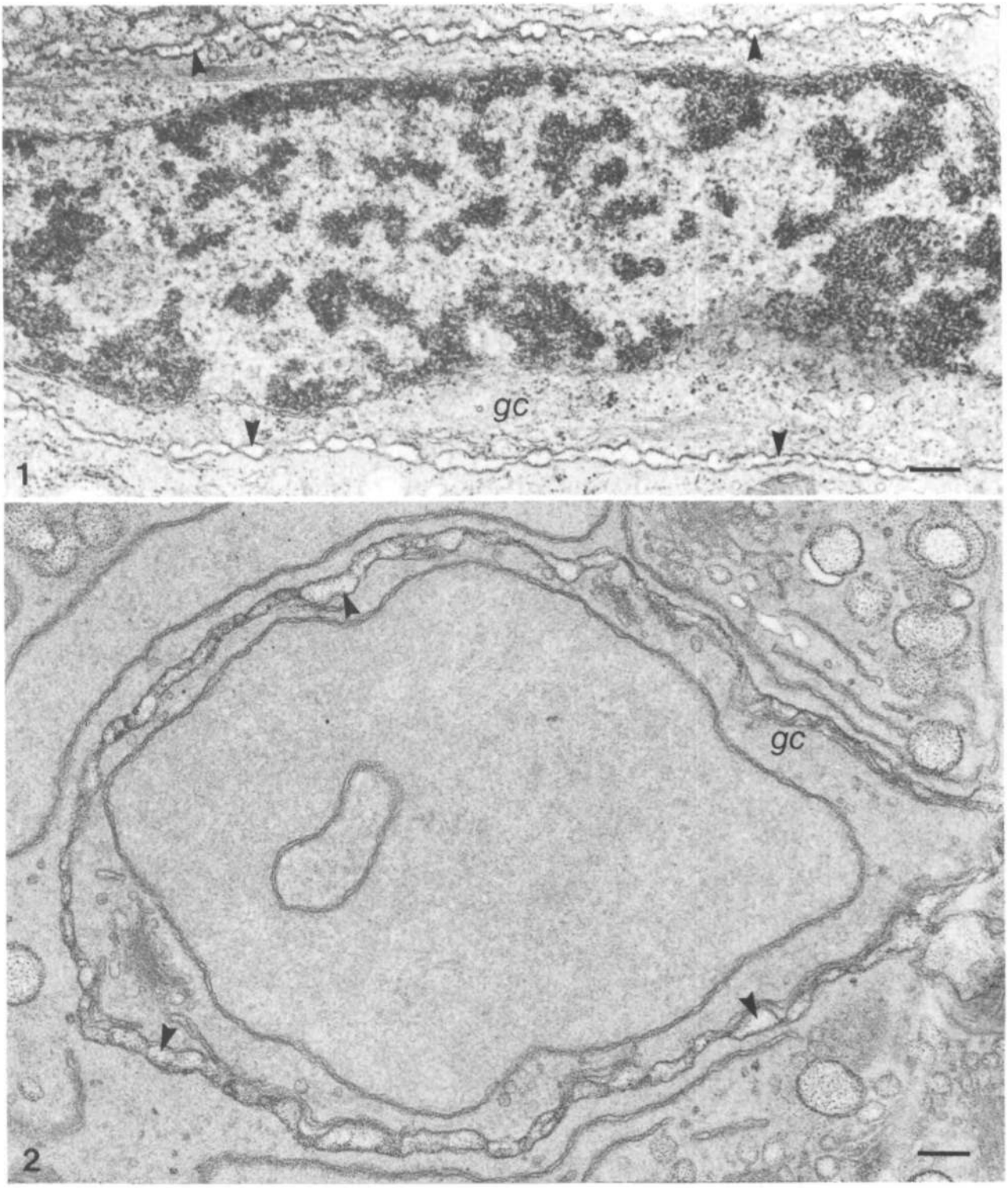
The two PMs that demarcate, respectively, the generative and vegetative boundary of the cell wall are clearly delineated in high-magnification images (Figs 4, 5). They lack the sinuous profile of chemically fixed material; rather they are very smooth and closely appressed to the intervening wall material. Each has a distinct trilamellate appearance, with the membrane leaflets facing the cytoplasmic surfaces (P side) being much more densely stained than the surface towards the wall or the exoplasmic (E) face. Between the two outer surfaces of the PMs, except where the occasional varicosity occurs, there is a space of relatively uniform width that contains stained granular or partially layered wall material similar in electron density to the outer PM surface. The intervening wall material in *Impatiens* (Fig. 5) is approximately 12 nm thick, half the thickness of the wall layer in *Nicotiana* (Figs 3, 4). In regions of wall containing varicosities the width becomes greater and more intervening wall material is evident. The varicosities sometimes appear to contain pockets of lightly stained material (Fig. 3), but after examining both longitudinal and transverse sections of generative cells, we have failed to detect structural breaks that might represent actual pores or channels between the two cells in either species. Rather, the two PMs seem to form a continuous boundary around the varicosities.

We have not seen structures resembling plasmodesmata, nor have we seen regions of contact between the two PMs. A layer of rough endoplasmic reticulum (ER) may lie close to the vegetative PM of the generative cell wall but we have not seen smooth tubular ER characteristic of somatic plant cells in this zone nor have we observed close incursions of the ER to the PM. In one instance we observed a dense bar in the wall (Fig. 4), which, after examination of serial sections, we interpreted as an artifactual fold and not representative of a real structure.

## Discussion

Among the cellular structures that appear different after RF–FS, when compared with conventional fixation, the generative cell wall complex is one of the most dramatic. When prepared by RF–FS the wall complex is remarkably uniform and even in appearance, and much more compressed than the same structure observed after chemical fixation. Because of the rapidity of freeze–fixation and the ability of this

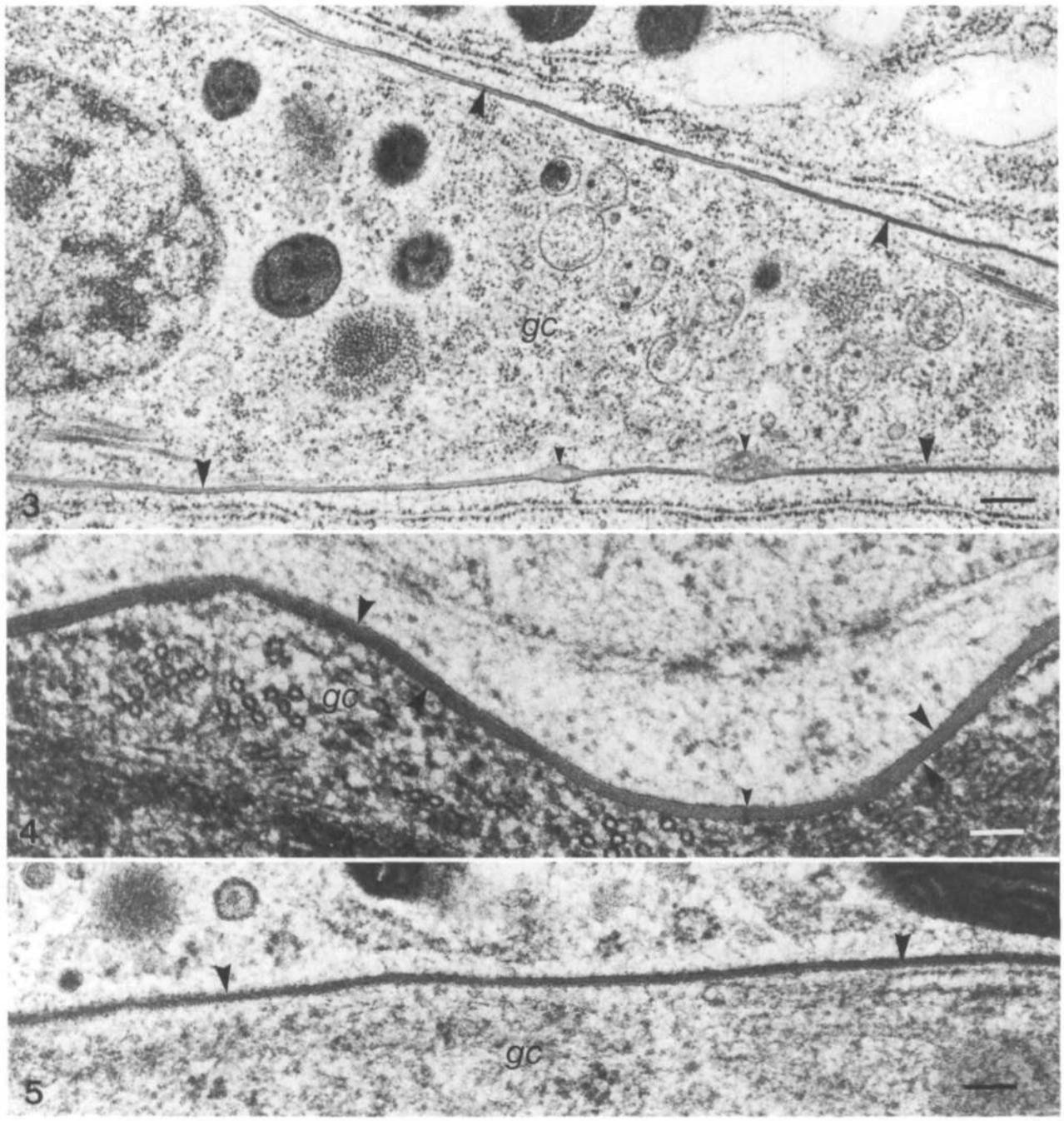
technique to preserve structures that are otherwise lost, we believe that it provides a much more faithful rendering of cytoplasmic structure than conventional chemical fixation. For this reason we assert that the appearance of the generative cell wall complex after conventional chemical fixation is artifact, resulting perhaps from the osmotic swelling of its polysaccharidic components, a suggestion also made by Browning




**Figs 1, 2.** Generative cells (*gc*) of *N. alata* prepared by conventional chemical fixation. The generative cell wall complex (arrowheads) is composed of two plasma membranes and intervening wall material. The two plasma membranes are sinuous in profile, and often appear to be joined, creating the impression that the wall complex is composed of a chain of vesicles. In Fig. 2, Thiery staining indicates that the wall material is composed of polysaccharides. ×35 000. Bar, 0·25 μm.

& Gunning (1977) in their study of freeze-substituted transfer cell walls.

Although the continuous chain of wall vesicles is absent after RF–FS, we note, however, the occasional presence of varicosities. These may contain lightly stained pockets but do not appear to be the result of




**Figs 3–5.** Generative cells (*gc*) from pollen tubes prepared by rapid freeze fixation and freeze substitution.

**Fig. 3.** *gc* from *N. alata* pollen tube has a wall complex (large arrowheads) that is very uniform and even, lacking the sinuous profile or appearance of being composed of a chain of vesicles. There are occasional varicosities (small arrowheads) in the wall, and these may contain pockets of lightly stained material. ×35 250. Bar, 0·25 μm.

**Fig. 4.** This micrograph is also from *N. alata* and shows, at higher magnification, the two plasma membranes (large arrowheads), smooth and closely appressed to the intervening wall layer. In this section only, a dense bar is seen in the wall (small arrowhead), but we conclude that this is an artifactual fold. No other structures resembling plasmodesmata are seen. ×83 800. Bar, 0·1 μm.

**Fig. 5.** The generative cell wall complex from *Impatiens* (arrowheads) also has smooth, closely appressed plasma membranes, and a thinner layer of wall material than is seen in *Nicotiana*. Plasmodesmata are not seen. ×83 800. Bar, 0·1 μm.

osmotic swelling, since the PMs on both sides invariably form a smooth, closely appressed, continuous layer over these structures. They do not appear to represent channels through the generative cell wall; however, their possible function is unknown.

We have shown here that cell wall material is present between the two PMs of the generative cell wall complex in two unrelated species, and that the material is composed of polysaccharides. We also know that the thickness of the wall layer can vary from species to species, with *Impatiens* having a wall half the thickness of *Nicotiana*. Browning & Gunning (1977) showed that chemical fixation resulted in a swollen, very loosely fibrillar appearance of transfer cell wall material, as opposed to the compact fibrillar layer seen after RF–FS. Given this drastic artifactual swelling of the wall that occurs during conventional chemical fixation, it is quite possible that a thin wall layer in a generative cell wall complex such as that found in *Impatiens* would be distorted to the extent that it would be imperceptible, thus leading to the conclusion that wall material is absent, when indeed it may be present. Careful examination of material prepared by RF–FS from diverse species, coupled with more extensive cytochemical tests, are required to settle the question of whether wall material is present between the two PMs in generative cells of all species.

The images obtained after RF–FS do not provide support for the presence of interconnecting pores or channels between the vegetative and mature generative cells in the pollen tube. The PMs on both sides of the wall are always entire, and do not show discontinuities. Furthermore, we do not observe the close appositions of ER to the PM characteristic of the plasmodesmatal structure of most somatic plant cells. However, we do not suggest that communication does not exist between these cells. Ion channels below the limits of resolution employed here could be present, as well as passageways for very small molecules such as water, sugar, amino acids, etc. However, we think it is unlikely that large macromolecules or even molecules of the size ($1000 M_r$) that is known to pass through plasmodesmata could move between vegetative and generative cells.

The isolation from intercellular communication is a phenomenon known to occur in highly differentiated cells. For example, during guard cell maturation the plasmodesmata that formed originally at cytokinesis become plugged or truncated (Wille & Lucas, 1984), thus preventing the transport of dye molecules between epidermal cells and mature guard cells that had been present earlier in development (Palevitz & Hepler, 1985). The generative cell at the stage examined here is also a highly differentiated and specialized cell. From differences in staining properties at different stages (e.g. see Owens & Westmuckett, 1983), it appears that the chemical composition of the generative cell wall may change during development. Whether or not plasmodesmata are present at any of the earlier stages is not known with certainty. It would be interesting to follow the development of the wall to see if at any early stage it had both the structural and functional capacity to move dye molecules.

The double membrane–wall structure of the generative cell is, apparently, retained by the sperm cells following their formation at mitosis. It becomes a question of how this unique boundary layer interacts with the female gametophytic tissue to cause fusion of the sperms with the egg cell and the polar nuclei. Future detailed structural analyses, especially using rapid freezing methods of preservation, could help greatly in elucidating the mechanism of fertilization.

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