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The self-incompatibility response in *Papaver rhoeas* pollen causes early and striking alterations to organelles

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Abstract

Self-incompatibility (SI) in *Papaver rhoeas* is accompanied by a cascade of signalling events that result in the rapid arrest and eventual death of the pollen tube. We have used rapid freeze fixation, freeze substitution and transmission electron microscopy to provide the first description of changes to pollen at the ultrastructural level during SI in this species. Our studies reveal that dramatic alterations to the morphology of mitochondria, Golgi bodies and ER occur within 1 h of SI induction. Similar symptoms have also been observed during programmed cell death (PCD) in some cell types. These include: the conspicuous condensation of the vegetative and generative nuclei, the swelling and loss of cristae in mitochondria and the disappearance of Golgi bodies. Some of the early alterations to the mitochondria and Golgi bodies observed at 1 h, almost certainly occur when cells are still alive. Other events, such as nuclear condensation, occur later and coincide with DNA fragmentation and the loss of cell viability. Our observations suggest that the SI response in *P. rhoeas* pollen may potentially involve a type of PCD.

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Abbreviations: ER, endoplasmic reticulum; GM, growth medium; HR, hypersensitive response; MAPK, mitogen-activated protein kinase; PCD, programmed cell death; SI, self-incompatibility; TEM, transmission electron microscopy

Introduction

In *Papaver rhoeas* (the field poppy), a gametophytic self-incompatibility (SI) system operates.[1] A series of studies have shown that in *P. rhoeas* the stigmatic S protein acts as a signalling ligand that triggers an intracellular signalling cascade(s) within the pollen.[2–11] This includes transient increases in cytosolic free Ca^{2+} ($[Ca^{2+}]_i$),[2,12,13] and activation of several protein kinases resulting in phosphorylation of several pollen proteins,[5,7] including a putative mitogen-activated protein kinase (MAPK).[11] We have obtained good evidence that the actin cytoskeleton is a target for these SI-specific signals. Rapid and dramatic alterations to the pollen actin cytoskeleton have been observed and characterized.[14–16] It has also recently been demonstrated that these changes involve actin depolymerization of a very rapid and also sustained nature.[17] Intriguingly, long-term F-actin depolymerization has been identified as a feature of apoptosis.[18–20] Preliminary data suggesting that a programmed cell death (PCD) signalling cascade may be stimulated in the SI response was provided by evidence that DNA fragmentation, generally considered a hallmark in PCD, is stimulated in SI challenged pollen tubes.[21] This, together with similarities between the *P. rhoeas* SI system and the hypersensitive response (HR), which triggers a PCD cascade, has led to the idea that PCD may be triggered by the SI response.

Cells die in essentially two ways, by necrosis, also called accidental death, or in a manner programmed by the cell as part of a developmental process: PCD. PCD is an energy requiring process, whereas necrosis does not involve active processes such as novel protein synthesis. The term apoptosis is often used synonymously with PCD, even though it identifies a specific kind of PCD that is associated with characteristic ultrastructural symptoms. Since the first description of apoptosis in 1972,[22,23] studies revealing the cell ultrastucture have remained one of the most reliable methods for classification of cell death.[24] Generally, apoptosis in animal cells is associated with several morphological and biochemical events (reviewed in Häcker[25], Clarke,[26] and Kerr and Harmon[27]). Not all, but some, of these features are also distinctive for PCD in plant cells.[28] Biochemical events include: cleavage of DNA into nucleosomal fragments, caspase activation, cytochrome *c* leakage from mitochondria, activation of key antiapoptotic genes, such as *BCL2*. Chromatin condensation and shrinkage of the nucleus are observed in many plant cell types undergoing PCD.[28,29] Like animal cells cytoplasmic condensation and shrinkage usually accompany PCD.[28] Other morphological changes frequently associated with PCD include the swelling or condensation of mitochondria and the dilatation of endoplasmic reticulum (ER).[30–33] Necrosis can be distinguished clearly from PCD at the ultrastructural level. Chromatin disappears progressively and the cells frequently swell and rupture since membranes lose their integrity.[22] Although the distinction can be made between PCD and necrosis, it is now generally assumed that both forms of cell death constitute two extremes of a continuum (reviewed in Kroemer *et al.*[34] and Susin *et al.*[35]).

Necrosis can be observed without PCD, but it is generally accepted that necrosis can occur at the end of a PCD cascade, as the cell finally dies.

It is well established that plants undergo PCD, which has been shown to be triggered during the HR to pathogens[36–38] and various forms of terminal cellular differentiation, organ abortion and senescence.[28,38–40] The process of sexual reproduction in flowering plants usually leaves one of the two partners dead or dying. In the case of a compatible interaction between pollen and receptive pistil, the penetration of the pollen tube triggers the degeneration of cells forming the transmitting tissue of the pistil.[41,42] In SI, in an incompatible reaction, the male partner, the pollen is killed before it effects fertilization.[8,43] The process of pollen rejection in SI is a good system in which to study cell death in plants, as along with a variety of species-specific incompatibility processes, there is a range of different ways to die for these unsuccessful pollen.[44]

Although there is circumstantial evidence that PCD may be operating during SI in *P. rhoeas*, DNA fragmentation alone is not a sufficient criterion for identifying PCD. In order to explore the possibility of PCD stimulation during the SI response further, we have investigated whether any of the cellular changes associated with SI in pollen tubes are characteristic for this type of cell death. Here we describe ultrastructural alterations observed after SI challenge, using rapid freeze fixation-freeze substitution. This revealed striking and quite rapid ultrastructural alterations that may corroborate earlier findings that indicated a possible involvement of PCD.

Results

In order to characterize the processes involving alterations of the cell ultrastucture during the SI response of *P. rhoeas*, we have used transmission electron microscopy (TEM) after rapid freeze fixation and freeze substitution. This has enabled us to assess the SI events triggered in incompatible pollen tubes of *P. rhoeas* at the ultrastructural level for the first time. Pollen that was grown *in vitro* was challenged with S proteins and fixed by rapid freeze fixation at time points of 1, 2, 3, 4 and 5 h after challenge (see Material and Methods for full details). Over the time-period examined, no changes to the general outer morphology of the pollen grain or tube were observed after SI challenge. At the light microscope level, the cytoplasm became increasingly dense and granular,[45] and callose was accumulated in the pollen grain and tube.

Examination of control pollen grains and pollen tubes with TEM showed a relatively electron translucent cytoplasm that was densely filled with mitochondria, Golgi bodies, ER, small vacuoles, vesicles and conspicuous bodies with a vesicular contents and a diameter of ca 1–1.3 μm (Figure 1a). Since we are not sure that the latter are identical with the plastids containing starch inclusions described by Cresti *et al*.,[46] we will refer to these elements as 'vesicular bodies'. Mitochondria appear rather electron-dense and show dense packing of the well-developed cristae as described earlier[46] (Figure 1b).

At 1 h after SI induction, between 50 and 80% of the pollen had germinated in the control samples. As expected, the incompatible samples were inhibited and only a few (less than 10%) pollen grains had germinated. When pollen germination had occurred in incompatible pollen, pollen tubes generally did not achieve lengths above 200 μm, which was considerably less than that achieved for compatible or untreated pollen that were generally at least 400 μm long by this time.

In incompatible samples the pollen tube cytoplasm had an opacity comparable to that of control pollen. However, most of the incompatible pollen grains showed considerable structural differences to the control samples (Figures 1a–d). At 1 h after SI challenge the mitochondria in many of these pollen grains were swollen, and they showed a slight reduction of cristae (Figures 1c,d). They also appeared very electron translucent (Figure 1d), in contrast to the electron-dense mitochondria in the control samples (Figure 1b). At this time point, some pollen looked similar to unchallenged grains, whereas the entire mitochondria population of other pollen grains was swollen. Generally, all mitochondria in an individual pollen grain showed the same symptoms. At this time point, within an individual pollen grain, intermediate stages showing intact and dilated mitochondria side by side in one individual grain were not observed. This seems to suggest that a certain threshold is required, whereupon a simultaneous swelling of the complete mitochondrial population in an individual cell is triggered.

In contrast to control pollen tubes, in which the ER is mostly flat and aligned parallel with the longitudinal axis of the tube (Figure 2a), SI-affected pollen tubes (Figure 2b) and pollen grains (Figure 2c) showed dilated ER cisternae that were curved or folded. They also appeared to have changed their localization and arrangement, and were observed to be wrapped around other organelles (Figure 2c). Furthermore, distinctive structures (Figure 1d) appeared, which had not been present in unchallenged pollen grains. We are not aware of the presence of similar structures described for other cell types. We speculate that these structures might have derived from the Golgi bodies, as there was no sign of anything recognizable as these organelles in these cells. The conspicuous vesicular bodies mentioned earlier (Figure 1c) appeared much more electron-translucent after SI challenge, in contrast to their darker appearance in control pollen grains (Figure 1a and c). An indication of callose deposition was observed in few pollen grains (data not shown). No obvious actin filaments were detectable, which might substantiate previous data measuring actin depolymerization, but this could equally be due to technical limitations, as detecting actin filaments using TEM is difficult.

At 2 h after SI induction, large callose depositions adjacent to the cell wall were detected (Figure 3a). Although organelles were still detectable in the cytoplasm they were mostly deformed (Figure 3b). The ER was considerably swollen. Golgi bodies were not recognizable at all at this time point. Mitochondria showed two types of aberrations. The first type showed extreme swelling, accompanied by a reduction of cristae (Figure 3c). These mitochondria were frequently observed to appear to be fused together. The second type of morphological aberration detected in mitochondria was blebbing or local ballooning of the cristae and/or of the outer membrane (Figure 3d). Both aberrations could be present in the same cell (Figure 3e).

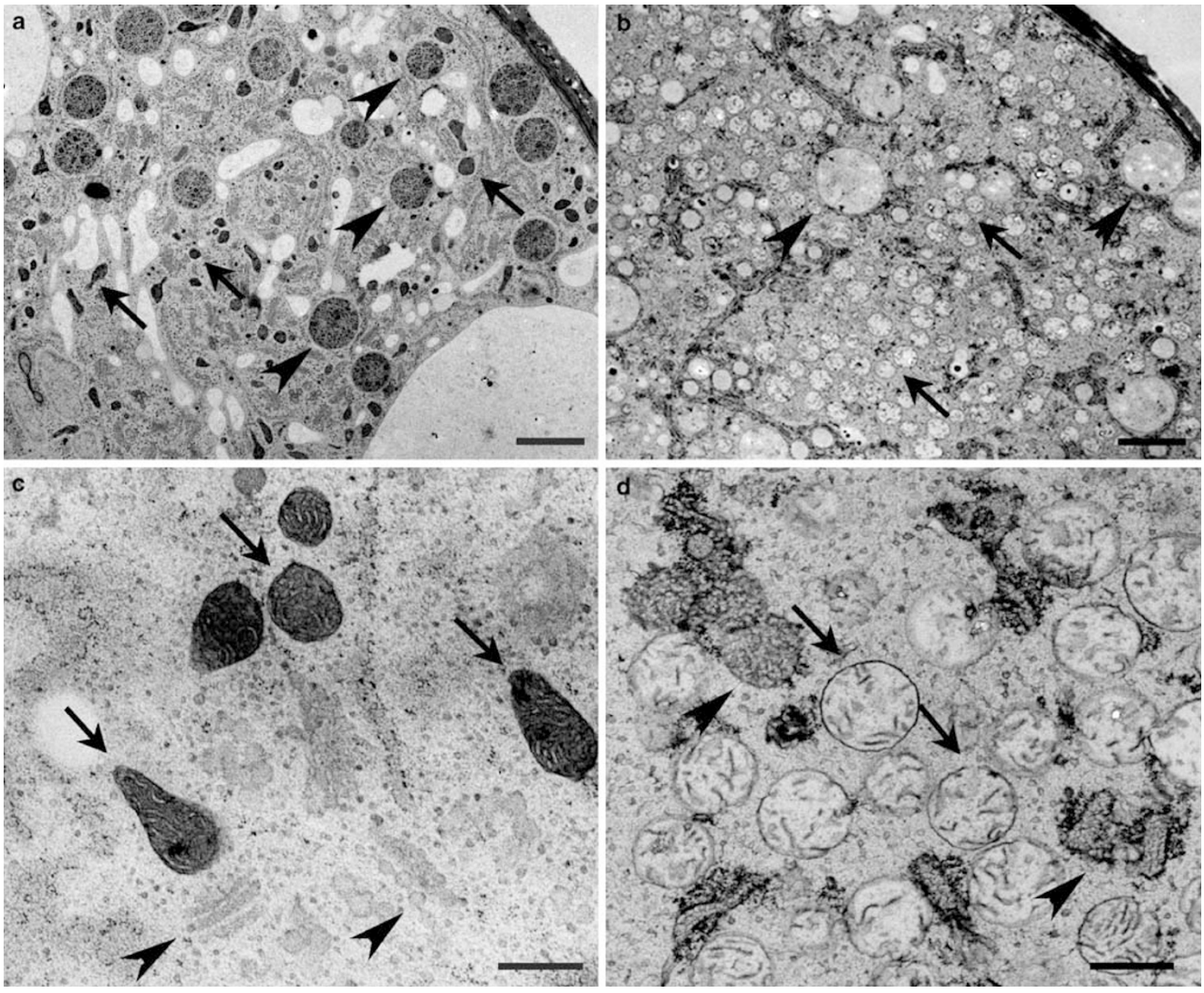
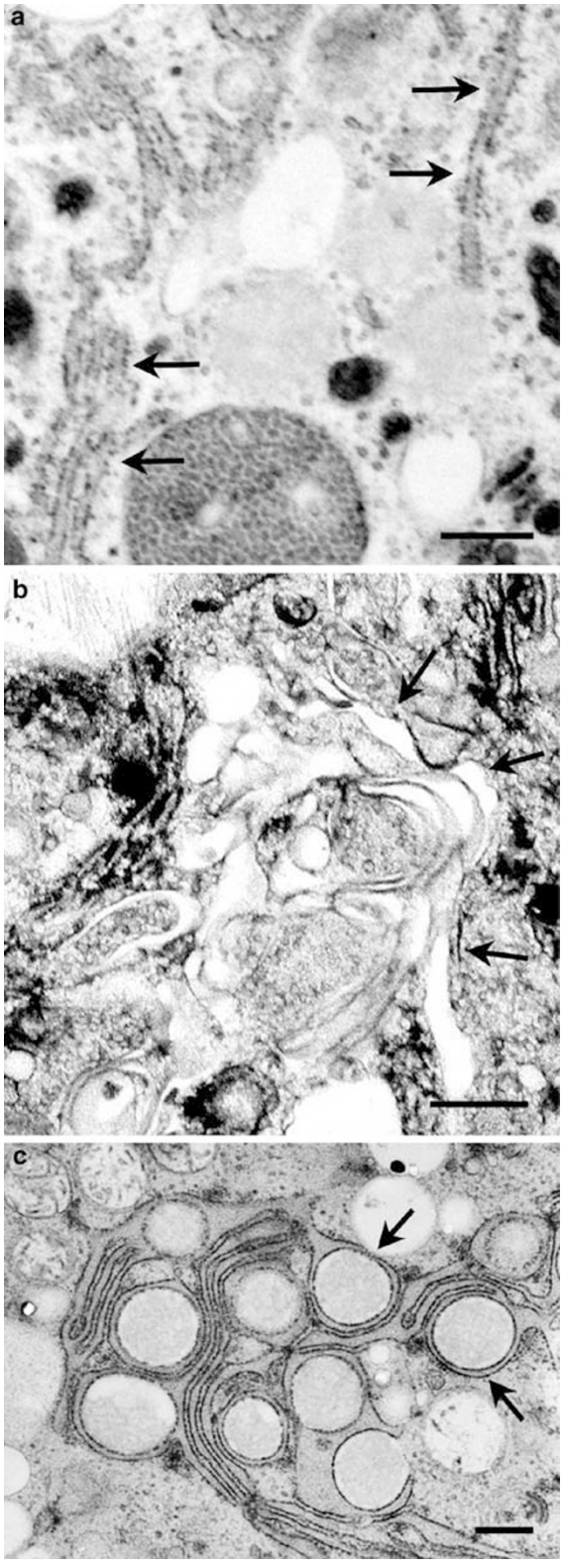


Figure 1 Mitochondria exhibit gross ultrastructural changes 1 h after SI induction. Pollen tubes were grown *in vitro* for 1 h in the absence (**a**,**c**) or presence (**b**,**d**) of S proteins. (**a**) An unchallenged pollen grain illustrates organelle conformation typical for living and active cells. Electron-dense mitochondria (arrows) are detectable; smaller and larger vacuoles, and conspicuous larger vesicular bodies (arrowheads) are also evident. (**b**) SI challenged pollen grains exhibit swollen mitochondria (arrows) and electron translucent vesicular bodies (arrowheads). (**c**) Mitochondria in unchallenged grains often have an oval shape and densely arranged cristae (arrows). Numerous Golgi bodies are present in the cytoplasm (arrowheads). (**d**) Mitochondria in SI challenged grains are swollen and have assumed a spherical shape (arrows). They show a reduction of cristae. The darker, distinctive structures with fuzzy surface might be dilated Golgi bodies (arrowheads). Bars = 2 μm (**a**,**c**), 0.5 μm (**b**,**d**)

At 3 h post-SI induction the mitochondrial blebbing had become more prominent. All cells showed this symptom that appeared in both electron-dense (Figure 4a) and swollen mitochondria (Figure 4b). ER cisternae remained dilated, and appeared the same as at earlier time points (Figure 4c) and undefined membranous aggregates appeared (Figure 4b). Since the formation of vacuoles is thought to be an indicator for apoptotic death, we attempted to ascertain if vacuoles increased in pollen during progression of SI. However, it was impossible to establish if the vacuoles shown in Figure 4d, which were typical for this and later time points, were an effect of the SI reaction. This was because unchallenged pollen grains often had numerous smaller vacuoles, and germinated control pollen grains and tubes at later time points develop large vacuoles (not shown) away from the mainly cytoplasmic tip region as part of the normal process of pollen tube tip growth.[8,47]

At 4 h after SI induction, a general disintegration of cellular structures was apparent in the cytoplasm. Vacuoles, dilated ER cisternae and large vesicular bodies were still discernable to some degree, but most other cell components had apparently degenerated (Figure 5a). In contrast to the control pollen tubes at the same time point, mitochondria were almost impossible to identify in SI challenged grains. The degeneration process eventually led to a condensation of the cytoplasm into an electron-dense, heterogeneous mass, which was very obvious at 6 h (Figure 5b). Callose depositions at the inside of the cell wall were extremely conspicuous at this time point. Both the vegetative nucleus and the nucleus of the generative cell started to become more electron-dense and less

homogenous at 4 h and they were extremely condensed at 6 h (Figure 5c). This contrasts strongly with the rather electron translucent nuclei in control pollen (Figure 5d).



Discussion

Our TEM studies of *P. rhoeas* pollen undergoing the SI response have revealed striking changes to the morphology of a number of organelles, in particular, the dilation of mitochondria, Golgi bodies and ER. Rapid freeze fixation is a method that is considered to result in excellent organelle preservation that is superior to that after chemical fixation. This has allowed us to observe various morphological alterations stimulated by SI in poppy pollen grains and tubes. We undertook these studies initially to examine if there were any ultrastructural alterations stimulated by the SI response that might suggest a possible involvement of PCD. This was suspected because of the many similarities between the signalling components activated in the poppy SI system and the HR elicited by plant pathogens, which is known to involve PCD. In the poppy SI system early signalling events include increases in cytosolic free Ca^{2+} ($[Ca^{2+}]_i$), Ca^{2+} influx, activation of several protein kinases, including a putative MAPK, p56.[48] All of these components have been implicated or shown to play a role in PCD in the HR system. Furthermore, we had evidence of DNA fragmentation being triggered by the SI response.[21] In addition to the DNA fragmentation, changes to the actin cytoskeleton also implicate the possibility of PCD in the SI response, as the highly sustained actin depolymerization that accompanies SI[17] has been observed in several animal cells undergoing apoptosis.[18–20] Our electron microscopy data describing the morphological changes in pollen undergoing the SI response revealed none of the morphological hallmarks that typically characterize necrosis, such as loss of membrane integrity, cellular swelling or rupture. Even though we cannot rule out the possibility of secondary necrosis, our data are not inconsistent with the hypothesis that PCD may occur in SI challenged pollen tubes.

Major ultrastructural changes

Nuclear condensation

In animal cells, cytoplasmic condensation and shrinkage always accompanies apoptosis, and in plant cells these morphological features are also usually observed.[28] Perhaps the best evidence for putative PCD occurring in pollen tubes undergoing the SI response is, therefore, the strong increase in electron density of the nucleus, indicating condensation of the chromatin. In animal cells changes in the nuclear morphology such as condensation, blebbing of the nuclear membrane, and segmentation are important symptoms that indicate the apoptotic nature of cell death and PCD.[49] Chromatin condensation is also frequently detected in plant cells undergoing PCD.[28,29] Interestingly, our data reveal that

Figure 2 Alterations to the ER are detectable 1 h after SI induction. (**a**) Typical control pollen grown for 1 h *in vitro*. ER cisternae in normally growing pollen tubes are mostly flat and aligned parallel with the longitudinal axis of the cell (the axis is oriented vertically in the figure). (**b**) Pollen tubes 1 h after SI induction exhibit ER cisternae that are dilated and arranged in curved and irregular shapes. There is no visible alignment with the longitudinal axis of the cell (oriented vertically in the figure). (**c**) In pollen grains 1 h after SI induction, the ER is dilated. Its configuration has altered and it appears to be wrapped around other organelles and vacuoles. Bars = 0.5 μm

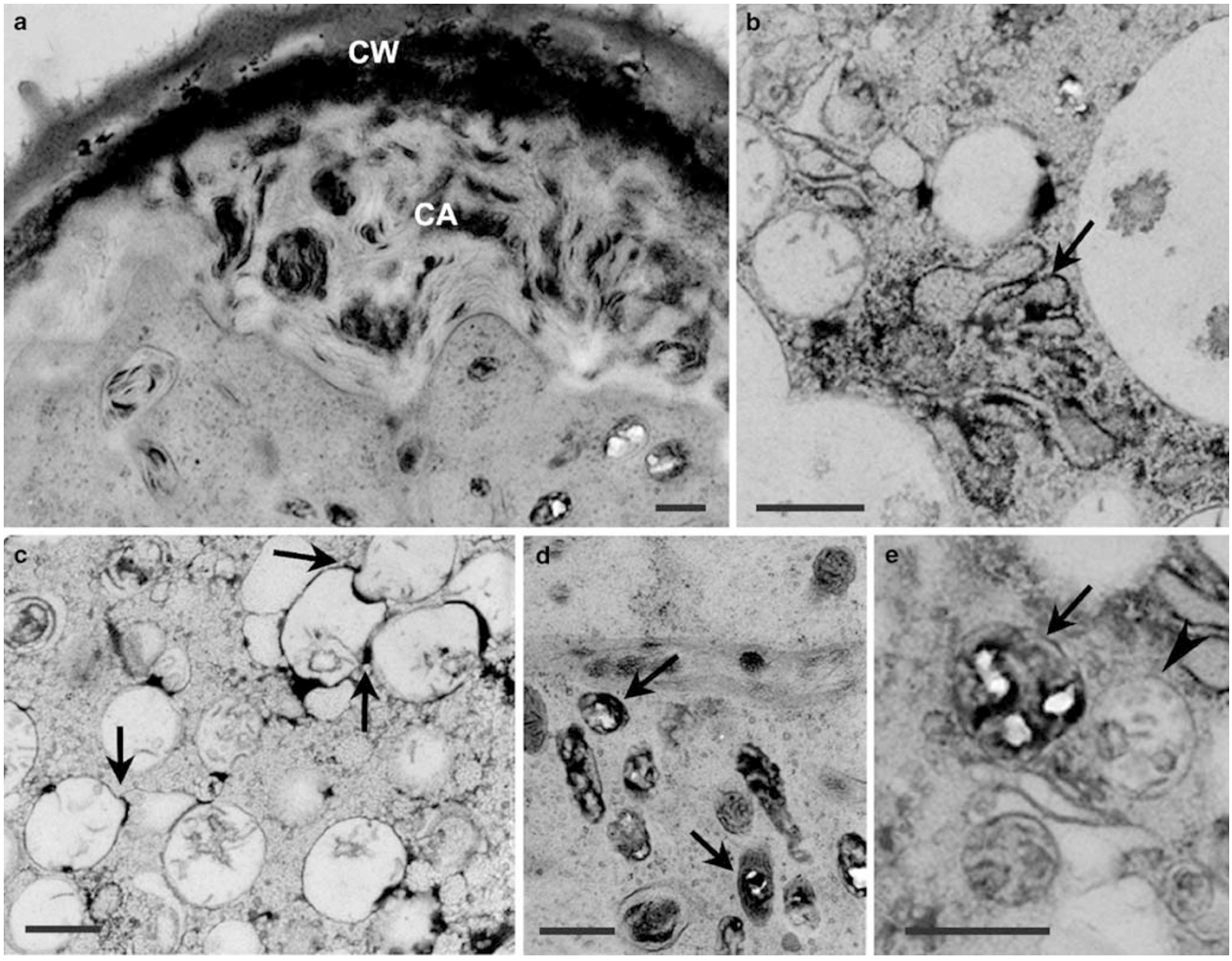


Figure 3 Changes to the ultrastructure in pollen grains 2 h after SI induction. (**a**) Deposition of callose (CA) at the inside the cell wall (CW). (**b**) ER cisternae are dilated (arrow). There are two populations of mitochondria: swollen and condensed (**c**,**d**). (**c**) Mitochondria are extremely swollen and show loss of cristae. Frequently they seem to fuse with each other (arrows). (**d**) Mitchondria exhibit blebbing of the inner and/or outer membrane (arrows). (**e**) Condensed (arrow) and swollen (arrowhead) mitochondria were detectable in the same cell, albeit this situation being rare. Bars = 0.5 μm

the time course of nuclear DNA condensation in incompatible poppy pollen corresponds exactly to when DNA nicking was observed.[21] In both instances, the first indication for nuclear alterations appeared at 4 h after challenge and at 6 h the reaction was well underway. Together with the fact that by this time mitochondria and Golgi bodies are not discernable, this indicates that the cells are in a 'post-mortem' phase. This fits well with data showing loss of viability at 4 h after SI induction observed in earlier studies.[21]

Mitochondria

Within 1 h of SI induction, dramatic changes to the mitochondria were observed in most pollen grains. This included swelling, loss of cristae, loss of electron density in the matrix, and blebbing. The former three alterations exhibit similarities with the swelling of mitochondria and/or rupture of their outer membrane described for several animal systems undergoing apoptosis.[30–33,50,51] Mitochondrial swelling has also been observed in plant cells undergoing PCD.[52] A swollen mitochondrial morphology strikingly similar to the one observed in SI challenged poppy pollen has been observed in microspore mother cells in male sterile sunflower,[53] and in the tapetal cells in sunflower or maize lines with cytoplasmic sterility.[54,55] Since there is good evidence that PCD is triggered in these cells,[56] the implication is that mitochondrial swelling may be associated with PCD. Thus the swollen mitochondria observed in SI-induced pollen may potentially be a consequence of PCD being triggered. Most early studies agree that mitochondria remain intact structurally until late in the process of apoptosis[23,57] and swollen mitochondria were considered to be typical for cells undergoing necrosis (as reviewed in Kroemer *et al*.[34]). However, concomitant with the recognition of the important role of mitochondria in the apoptotic signalling process, structural changes of this organelle have been observed in a number of cases of apoptosis[30–33,50,51] Although these alterations are not generally considered as 'key' or 'hallmark' features of apoptosis or PCD, this phenomenon has been observed in a number of cell types undergoing apoptosis and PCD.

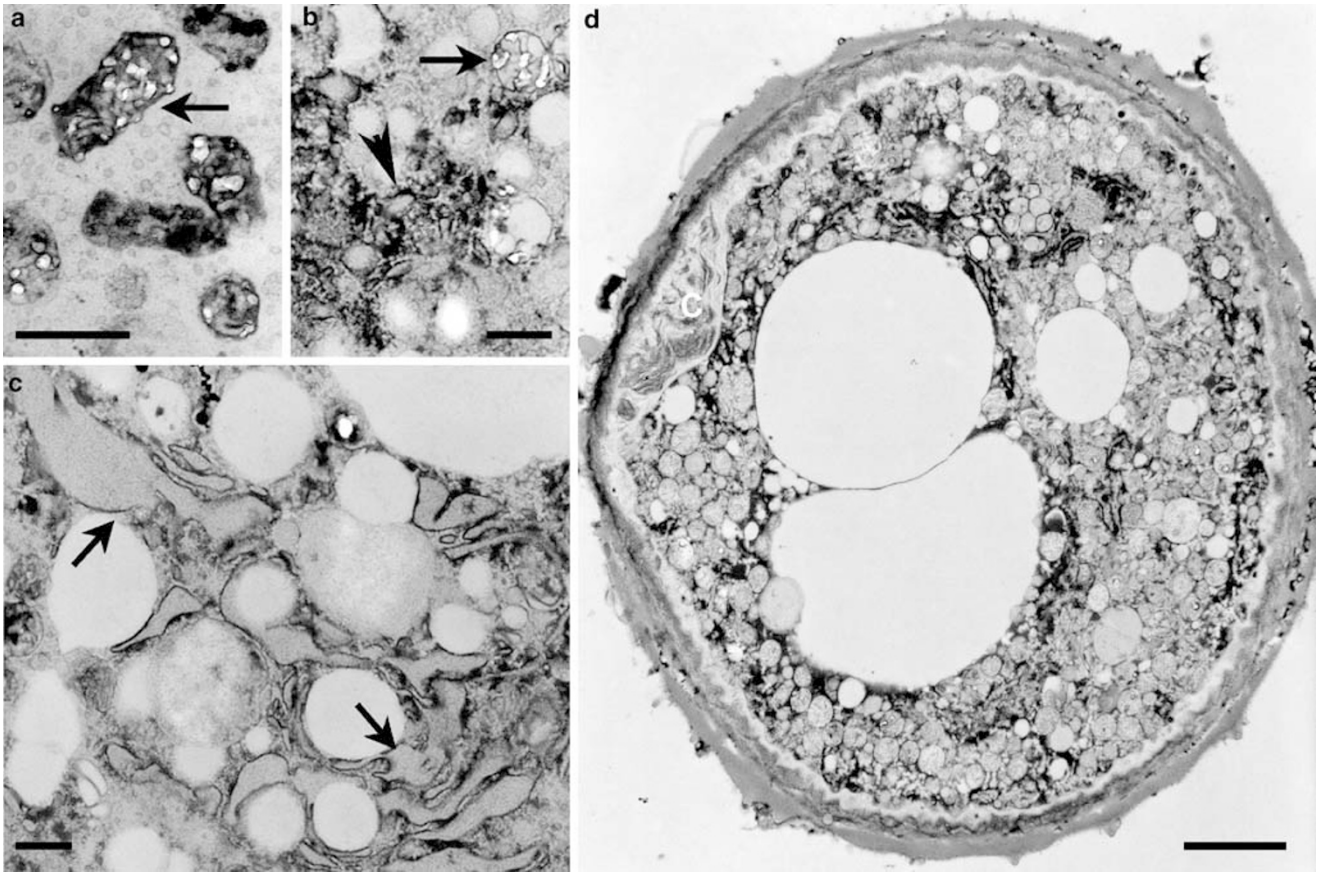


Figure 4 Ultrastructure of poppy pollen grains 3 h after SI challenge. (**a**) Mitochondria show extreme blebbing of the cristae or the outer membrane. This symptom appears in condensed and in swollen mitochondria as in (**b**) (arrow). (**b**) Amorphous membranous structures (arrowhead) and a swollen mitochondrium with detectable blebbing (arrow). (**c**) ER cisternae are extremely dilated (arrows). (**d**) The cell is vacuolated and shows callose depositions (C) between the plasma membrane and the regular cell wall. Bars = 0.5 μm (**a–c**), 2 μm (**d**)

The biochemical processes taking place in the mitochondria in apoptotic cells have been postulated to represent the 'point of no return' in the cell death signalling cascades in animal cells,[34,58] indicating their critical role in the orchestration of PCD (for reviews see Green and Reed[59] and Gottlieb[60]). Our observation that mitochondrial swelling occurs relatively early in SI challenged poppy pollen suggests that PCD may be occurring in this system, though our ultrastructural observations clearly have to be followed up by biochemical studies. We have preliminary data, using Mitotracker Red CMXRos, which accumulates in functional mitochondria, that indicate that a significant number of pollen tubes were still viable at 1 h.[17] This suggests that although the mitochondrial morphology is affected, activity may not be at this time point, though more detailed studies are required to confirm this.

Golgi bodies and ER

Our studies indicate that considerable structural changes to the Golgi bodies occur relatively early in the SI response. Within 1 h of SI induction these organelles either disappear from the cytoplasm of SI challenged poppy pollen grains or are not recognizable as such. It is not evident from the micrographs whether the Golgi cisternae have completely disappeared, or whether they are swollen to a degree that makes them unrecognisable. This is a common phenomenon in apoptosis[61,62] and it confirms observations made on plant cells undergoing PCD.[54] Although the dilation of ER observed after SI induction is not generally regarded as a 'hallmark' feature of apoptosis or PCD, it is, however, observed in a number of apoptotic animal cells.[25,63,64] Observations made on plant cells undergoing PCD also show this phenomenon. Degenerating tapetal cells,[54,65] microspore mother cells in male sterile *Helianthus*[53] or differentiating xylem vessels,[52] all show evidence of ER dilation. This suggests that this feature may be a useful indicator of apoptosis and PCD in certain cell types. Although there is not much data on the involvement of Golgi and ER in apoptosis/PCD, recent data from animal cells,[66] implicate a possible involvement of these organelles in this process.

Callose deposition

Deposition of callose is observed in several plant cell systems undergoing PCD. It accompanies the HR triggered by an invading pathogen[67–69] and the activation of PCD signalling pathways by fumonisin B1.[70] Callose deposition is also present on the wall of the pollen mother cell in cytoplasmic male sterile lines of common bean.[71] In self-incompatible

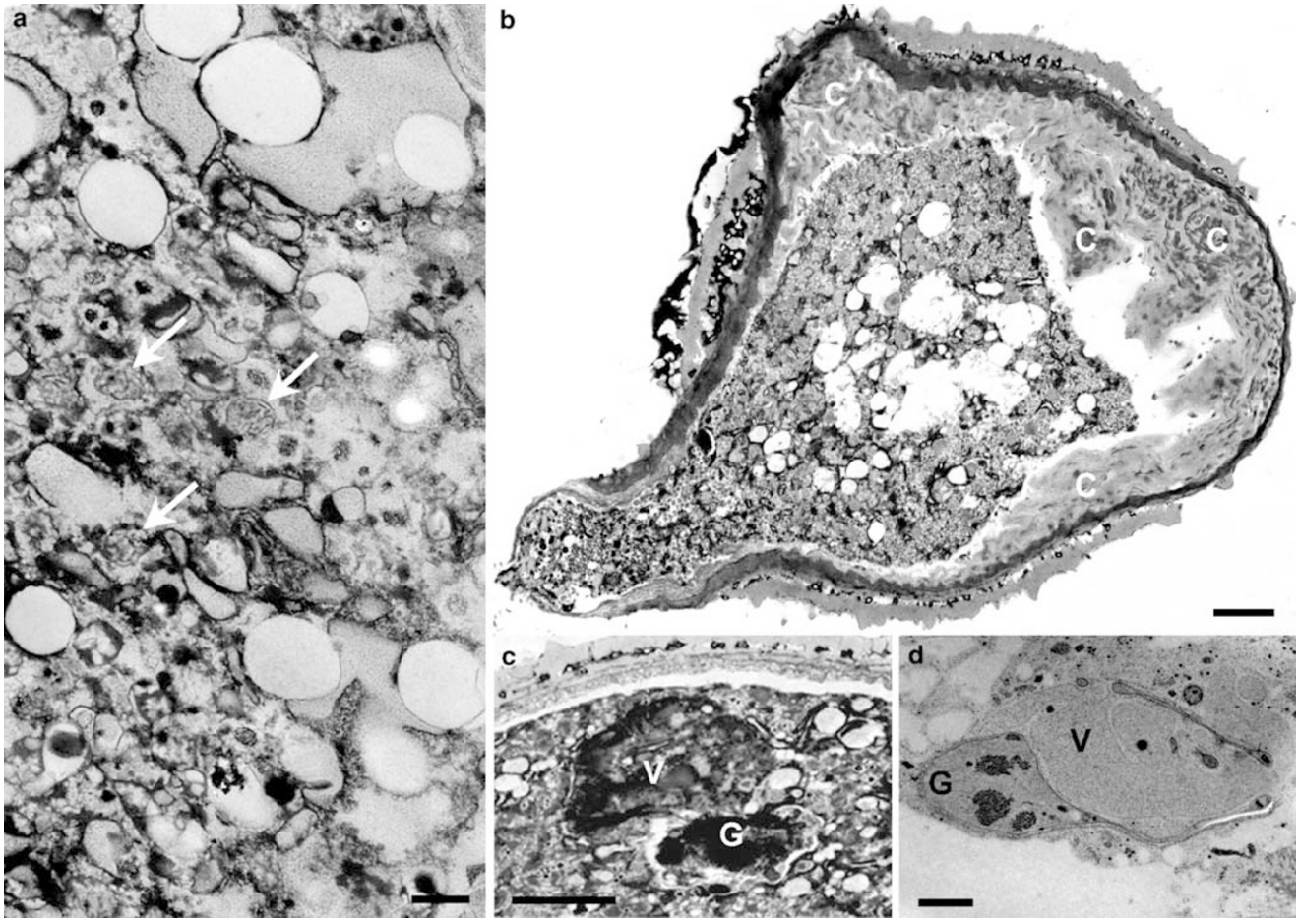


Figure 5 Further changes to the ultrastructure of poppy pollen grains at later time points. (**a**) At 4 h after SI challenge, degeneration of cytoplasm is evident and organelles are almost indistinguishable. Arrows indicate possible remainders of mitochondria. (**b**) At 6 h after SI challenge, this peripheral section shows the massive accumulation of callose (C) at the inside of the pollen grain cell wall. (**c**) At 6 h after SI challenge, both generative (G) and vegetative (V) nucleus show extreme condensation. (**d**) At 6 h of control incubation, the control situations show the electron-translucent and rather homogeneous texture of nuclei in an unchallenged pollen tube. Bar = 0.5 μm (**a**), 2 μm (**b**–**d**)

pollen tubes, aberrant or increased callose deposition seems to be typical not only for poppy but also for other species (see Geitmann,[44] Geitmann *et al*.[72] and Pacini[73] and references therein). However, the phenomenon is unlikely to be a cell death specific process but is rather a more general side effect, as already suggested previously.[44] It is likely that callose production is a pathway that is not easily disturbed and that, if maintained in cells in which the normally extremely fast growth is inhibited, results in huge depositions of the polysaccharide. Interestingly enough, our ultrastructural study showed that callose deposition continues far beyond the point of time when Golgi bodies have become undistinguishable, thus indicating that the membrane located callose synthases remain active for extended periods of time.

The chronology of SI-induced events

The first ultrastructural alterations observed during the SI response in *P. rhoeas* pollen are observed surprisingly early, and thus are initiated within 1 h. These structural changes can be placed within the time course of SI-induced events, which is summarized in Figure 6.

Very early events appear to be involved in the inhibition of pollen tube growth, which is arrested within a few minutes of SI challenge. An increase in cytosolic $[Ca^{2+}]_i$ involving Ca^{2+} influx[10] results in the phosphorylation of p26 via a CDPK within 90 s.[7,11] Alterations in the actin cytoskeleton and F-actin depolymerization are stimulated within 60 s.[15,17] Intriguingly, the level of depolymerization is far larger than that required for inhibition, and is sustained for at least an hour. Since sustained actin depolymerization has been observed in animal cells undergoing PCD, this suggests that PCD may be triggered in poppy pollen.[74] Studies of pollen viability, which give some indication of cellular metabolic activity, during the SI response, tested with fluorescein diacetate, an established marker for pollen viability, indicate that the viability of incompatible pollen undergoing SI is not significantly different at 0, 10, 20 and 40 min ($P = 0.154$), although there was a highly significant reduction in viability at 60 min ($P < 0.001$). In total, 57% $\pm$ 7.9 of pollen tubes remained viable up to this time point compared to 89.6% $\pm$ 6.9 in the control samples.[17] Although we did not quantify pollen tubes and organelles studied using TEM, the majority of them appeared to be affected morphologically at

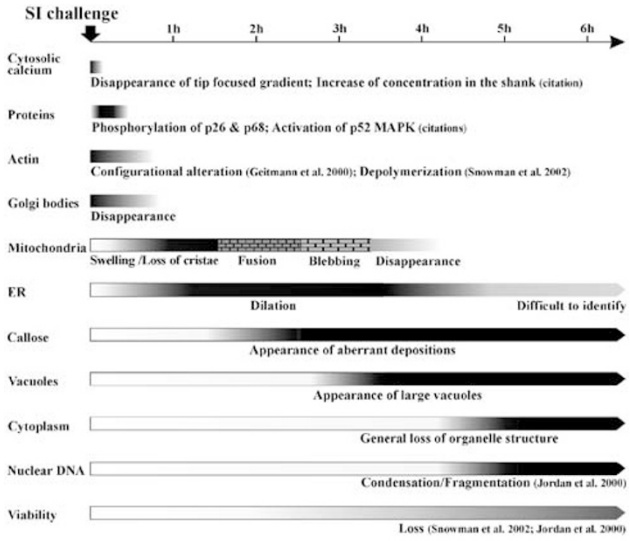


Figure 6 Time course of events occurring after SI challenge of poppy pollen. This overview attempts to place the morphological markers of SI alongside the other markers previously identified as SI-induced events. The disappearance of Golgi bodies and morphological changes in mitochondria and ER are the first alterations apparent at ultrastructural level. Viability assays indicate that the majority of pollen tubes/grains are viable at 1 h after SI challenge. Degeneration of the cytoplasm, condensation of nuclei and large depositions of callose occur later in the time course, and probably indicate 'post-mortem' events

60 min. This suggests that, albeit being dramatic, the morphological changes occurring within 1 h have not resulted in the complete arrest of cellular functioning. On the contrary, the relatively early appearance of morphological alterations in mitochondria and ER suggests that they may play an active role in the process leading to cell death, though at present we have no biochemical evidence for this. Preliminary data indicate leakage of cytochrome *c* from mitochondria occurs within 1 h of SI induction (SG Thomas and VE Franklin-Tong, unpublished data). A crucial question that remains to be answered is whether death is actively caused via a caspase-like activity, or whether a bioenergetic catastrophe caused by the loss of the electrochemical gradient across the inner mitochondrial membrane and a declining ATP production leads to secondary necrosis (reviewed in Kroemer *et al.*[34] and Green and Reed[59]). However, the two are not necessarily mutually exclusive. In support of the former are preliminary data that indicate that a caspase-like activity may be triggered during the SI response (SG Thomas and VE Franklin-Tong, unpublished data). However, we also have evidence that inactivation of p26, which an inorganic pyrophosphatase, is likely to be inactivated by increases in $[Ca^{2+}]_i$, which would be predicted to result in a decrease, albeit temporarily, in ATP production (Rudd *et al.*, unpublished data; Rudd and Franklin-Tong[11]).

We also observe the complete degeneration and total loss of distinguishable mitochondria, Golgi and the condensation of cytoplasm and nucleus between 4–6 h, which most likely indicate the completion of cell death. These morphological data provide evidence that the cell enters a 'post-mortem' phase at $\sim$4 h after SI induction, which correlates nicely with data from Jordan *et al.*[21] that indicate that cell viability is lost around 4 h.

Ultrastructural changes in other SI systems

Comparison of our ultrastructural studies with other similar studies of SI responses in other plant species indicates that although PCD might potentially be implicated in poppy SI, it is clearly not in other species. Other gametophytic SI systems, such as those present in the Solanaceae, are characterized by a much later onset of ultrastructural changes in the pollen tube, often 4–8 h after pollination and are accompanied by different ultrastructural symptoms (Geitmann[44] and references therein). In *Brugmansia suaveolens* the ER does not dilate but forms circular structures followed by membrane fusions. Incompatible pollen tubes in this species were found to rupture.[44] The outer, pectinaceous pollen tube cell wall thickens in this and other species.[75,76] We did not observe these phenomena in incompatible poppy pollen tubes. The observation that stigmatic S-RNases elicit very slow inhibition of pollen tube growth that takes many hours, indicates that cell death in Solanaceae is a nonprogrammed necrotic event subsequent to RNA degradation (see Geitmann[44] for a detailed discussion). Other plant families, such as the Liliaceae and the Brassicaceae, show different biochemical mechanisms, neither of which appear to be based on PCD.

If PCD should be confirmed to be the mechanism of SI in *P. rhoeas*, it raises a fundamental question: 'Why is it necessary to let the male partner of an incompatible sexual interaction die in an active and energy-consuming way, if the main goal is the prevention of self-fertilization?' This goal could be achieved by the simple cessation of pollen tube growth before it reaches the ovule. The subsequent death and degeneration of the pollen tube are not strictly necessary to prevent fertilization. Are the morphological changes observed purely of the 'bystander type'? Are they only the fortuitous occurrences of the specific SI signalling events? What advantage is there if the flower causes the complete demise of the unwanted pollen? One scenario that might explain this radical solution is the fact that, albeit their growth being arrested, pollen tubes might trigger the degeneration of the female transmitting tissue and thus initiate precocious death of (parts of) the flower, thus leading to wilting before it has been fertilized by compatible pollen. Alternatively, one might speculate that a flower can only accommodate a limited number of pollen tubes, and incompatible pollen tubes would occupy precious space and/or use available nutrients that should be kept available for the correct male partner.

In conclusion, our ultrastructural studies clearly demonstrate some relatively rapid alterations in the ultrastructure/morphology of organelles in pollen of self-incomaptible pollen. The data, while not completely conclusive, corroborate earlier findings that indicate that a form of PCD is stimulated by the SI response in poppy pollen.

Materials and Methods

Plant material

Plants of *P. rhoeas* L. (Shirley variety) segregating for known SI genotypes (S_1S_3, S_2S_4, S_4S_6) were used for the experiments involving S proteins (see Franklin-Tong *et al.*,[77]). Pollen was stored at -20°C, over silica gel, until required.

In vitro growth of pollen tubes

Pollen was hydrated in humid chamber at room temperature for at least 30 min before use. Hydrated pollen was sown on a thin layer of growth medium (GM), comprising 100 μg ml^{-1} H_3BO_3, 300 μg ml^{-1} $Ca(NO_3)_2$ H_2O, 100 μg ml^{-1} KNO_3, 200 μg ml^{-1} $MgSO_4 \cdot 7H_2O$, 50 μg ml^{-1} sucrose), solidified with 0.5% low-gelling-temperature agarose (BDH Limited, Poole, UK) prepared on pieces of dialysis tubing. Pollen was germinated at 25°C, the pieces of dialysis tubing were placed on a thick layer of 3% agarose in GM to prevent desiccation. The whole setup was placed in a humid chamber.

Production of recombinant S protein in *Escherichia coli*

Recombinant S proteins S_1e and S_3e were prepared as described in Kakeda *et al.*[78] The purified proteins were kept at −70°C and dialyzed overnight against GM before use. Dialyzed S proteins were kept at 4°C. The concentration of S proteins after dialysis was determined using the protein assay kit BCA-200 (Pierce).

SI challenges of *in vitro* growing pollen tubes

For the SI challenges, pollen from plants of *S* genotype S_1S_3 or S_1S_1 (for incompatible reactions) and S_2S_4 or S_4S_6 (for fully compatible reactions) were grown on pieces of dialysis tubing covered with agarose GM as described above (S_1S_1 seeds were obtained after selfing of immature flower buds). Active concentrations of the recombinant S proteins S_1e (20 μg ml^{-1}) and S_3e (30 μg ml^{-1}) were incorporated in the agarose layer on which the pollen was spread as described in Geitmann *et al.*[15]

The advantage of this *in vitro* method as opposed to *in vivo* pollinations was first, the ability to precisely assess the time point of germination, and second, it was possible to fix the pollen by rapid freezing, which results in superior ultrastructural preservation compared to chemical fixation. Inclusion of S_1e or a combination of S_1e and S_3e recombinant proteins in the cultivation medium resulted in the prevention of germination or arrest of pollen tube growth at very early stage in S_1S_1 or S_1S_3 pollen, respectively. This demonstrates that these pollen–protein combinations are incompatible and that the effect of isolated S protein mimics the *in vivo* situation. Two sets of control experiments were carried out: S_2S_4 pollen grown on medium containing S_1e and S_3e recombinant proteins (compatible pollen–protein combination) as well as S_1S_1 or S_1S_3 pollen grown on medium not containing any S proteins. In all cases germination took place around 30 min after sowing the hydrated pollen on the solidified medium. In all control set-ups pollen tube growth proceeded uninhibited during the time period assessed here. For both incompatible and compatible situations, several time points were assessed; times are given as interval between sowing of pollen and rapid freeze fixation. Because of the 30 min delay between sowing and germination, in the 1 h sample pollen tubes had been growing for ca 30 min, in the 2 h sample for 1 h and 30 min and so on.

Sample preparation for TEM

Pieces of dialysis tubing (2 × 2 cm^2) carrying pollen were rapidly frozen by pouring liquid propane over the sample, and subsequently transferring it to liquid nitrogen. The dialysis tubing was broken into small pieces, which were transferred into incubation vials used for freeze substitution. Freeze substitution was carried out in 1.7 or 2% osmium tetroxide in dried acetone. Vials were kept in a freeze substitution system (FreasySub, Cryotech) and brought to room temperature after passing the following temperature steps: 24 or 36 h at –90°C, 10 h at –60°C, 10 h at –30°C with intermediate periods of 3 h for change of temperature, respectively. The fixation medium was then replaced gradually for Spurr's epoxy resin (Serva, Heidelberg, Germany) over 48 h. Polymerisation of the embedded samples took place at 70°C. Samples were cut in a Reichert Ultracut, ultrathin sections (ca 90 nm) were collected on formvar coated copper grids and stained with lead citrate (Reynolds) and uranyl acetate. Observations were made in a JEOL 1200 EXII transmission electron microscope (TEM) operated at 80 kV.

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