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Involvement of extracellular calcium influx in the self-incompatibility response of *Papaver rhoeas*

Vernonica E. Franklin-Tong[1,*], Terena L. Holdaway-Clarke[2], Kornelis R. Straatman[3], Joseph G. Kunkel[2] and Peter K. Hepler[2]

[1]*School of Biosciences, University of Birmingham, Edgbaston, Birmingham, B15 [2]TT, UK, and*
[2]*Department of Biology, University of Massachusetts, Amherst, Massachusetts 01003, USA*

Received 21 August 2001; revised 25 October 2001; accepted 30 October 2001.
[*]For correspondence (fax +44 121 414 5925; e-mail V.E.Franklin-Tong@bham.ac.uk).
[3]Present address: Department of Genetics, University of Leicester, University Road, Leicester LE1 7RH, UK.

Summary

We have previously demonstrated that increases in cytosolic free Ca^{2+} are triggered by the self-incompatibility (SI) response in incompatible *Papaver rhoeas* (the field poppy) pollen. However, one key question that has not been answered is whether extracellular Ca^{2+} may be involved. To address this question, we have used an ion-selective vibrating probe to measure changes in extracellular Ca^{2+} fluxes around poppy pollen tubes. Our data reveal several findings. First, we confirm that there is an oscillating Ca^{2+} influx directed at the apex of the pollen tube; we also provide evidence that Ca^{2+} influx also occurs at the shanks of pollen tubes. Second, upon challenge with self-incompatibility (S) proteins, there is a stimulation of Ca^{2+} influx along the shank of incompatible pollen tubes, approximately 50 μm behind the pollen tube tip. This demonstration of SI-induced Ca^{2+} influx suggests a role for influx of extracellular Ca^{2+} in the SI response.

Keywords: self-incompatibility, *Papaver rhoeas*, Ca^{2+} signalling and calcium selective vibrating probe.

Introduction

Self-incompatibility (SI) is one of the most important devices whereby higher plants prevent inbreeding. It is a genetically controlled mechanism that regulates the acceptance or rejection of pollen that lands on the stigma. In *Papaver rhoeas* (the field poppy), SI is controlled by a single, multiallelic, gametophytically controlled *S* locus (Lawrence *et al.*, 1978). Pollen grains carrying *S* alleles that are genetically identical to those carried by the pistil (incompatible) are discriminated from genetically different (compatible) pollen and selectively inhibited, thereby preventing self-fertilization.

Characterization and cloning of *S* genes and components involved in the SI reaction in *P. rhoeas* have begun to elucidate the molecular basis of SI. Several alleles of the stigmatic *S* gene have now been cloned (Foote *et al.*, 1994; Kurup *et al.*, 1998; Walker *et al.*, 1996). They encode small, basic, highly polymorphic S proteins (approximately 14-kDa) that are developmentally expressed and secreted by the stigmatic papillar cells. Tip growth of incompatible pollen of *Papaver rhoeas* is inhibited within a few minutes of encountering the S proteins. Both stigmatic extracts and recombinant S proteins have been shown to have *S*-specific biological activity (Foote *et al.*, 1994; Franklin-Tong *et al.*, 1988). Although the nature of the allelic specificity remains to be elucidated, site-directed mutagenesis has established that certain residues are crucial for the recognition and inhibition of S_1 pollen (Jordan *et al.*, 1999; Kakeda *et al.*, 1998). Further, the available data demonstrate that the stigmatic S-proteins act as signal molecules that trigger a signalling cascade in incompatible pollen tubes that results in their inhibition and includes increased phosphorylation of two soluble proteins, p26 and p68 (Rudd *et al.*, 1996, 1997), alterations in the F-actin cytoskeleton (Geitmann *et al.*, 2000; Snowman *et al.*, 2000) and triggering of nuclear DNA fragmentation (Jordan *et al.*, 2000).

Of special interest is the involvement of Ca^{2+}-dependent signal transduction cascade(s) in pollen tube growth, inhibition and the SI response in incompatible *P. rhoeas* pollen. The importance of cytosolic free calcium (Ca^{2+}_i) for the regulation of pollen tube growth is well known (Franklin-Tong, 1999; Malhó *et al.*, 1994, 1995; Miller *et al.*,

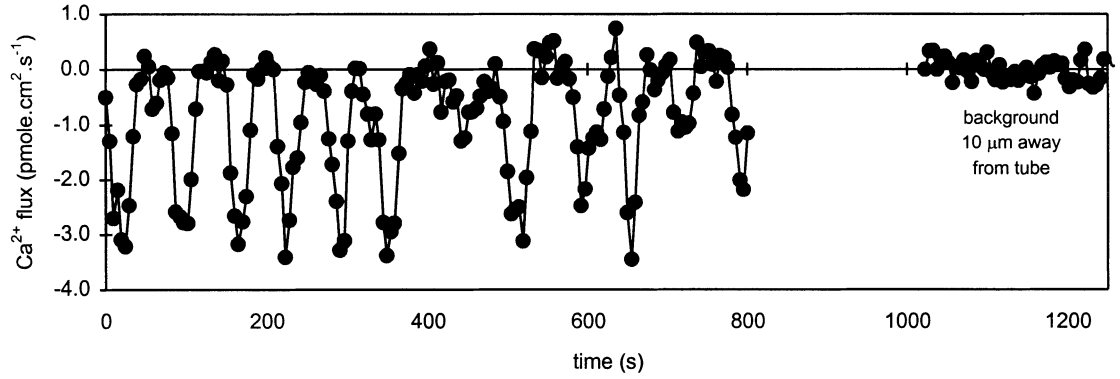


Figure 1. Oscillations of Ca^{2+} influx at the pollen tube tip.
Measurements of oscillating net influx of Ca^{2+} at the apex of a growing pollen tube of *P. rhoeas* were measured using a Ca^{2+}–selective vibrating probe and are displayed graphically here. The plot shows net Ca^{2+} flux (pmol cm^{-2} sec^{-1}), with net influx as a negative reading, and net efflux as a positive reading. The latter part of the plot shows a short background measurement 10 μm away from the pollen tube, in order to demonstrate that the fluxes measured are attributed to the pollen tube, and not noise.

1992; Obermeyer and Weisenseel, 1991; Pierson *et al.*, 1994, 1996; Rathore *et al.*, 1991; Rudd and Franklin-Tong, 1999). Inositol trisphosphate (Ins(1,4,5)P_3)-induced Ca^{2+} release has also been shown to play a role in the regulation of pollen tube growth (Franklin-Tong *et al.*, 1996; Malhó, 1998). Large, transient increases in $[Ca^{2+}]_i$ have been observed in the 'shank' of incompatible pollen tubes (Franklin-Tong *et al.*, 1993, 1995, 1997), implicating a Ca^{2+}-mediated signalling pathway in the SI response. Corresponding decreases in intracellular calcium concentration ($[Ca^{2+}]_i$) in the tip occur concomitantly with SI-stimulated inhibition of tip growth (Franklin-Tong *et al.*, 1997). The nature of the *S*-specific phosphorylation of p26 suggests that a Ca^{2+}-dependent protein kinase requiring CaM-like domains is activated during the SI response, and acts as an intracellular signal mediating the SI response in *P. rhoeas* pollen (Rudd *et al.*, 1996).

While these studies indicate that the arrest of pollen tube growth due to the SI reaction involves increases in $[Ca^{2+}]_i$, the nature and localization of the source(s) of Ca^{2+} involved in generating the SI-stimulated increases in $[Ca^{2+}]_i$ has yet to be determined. The ion selective vibrating electrode (Kühtreiber and Jaffe, 1990; Smith *et al.*, 1994) has previously been used effectively to demonstrate localized influx and efflux of Ca^{2+} in both animal and plant systems, including pollen tubes and root hairs (Cárdenas *et al.*, 1999; Holdaway-Clarke *et al.*, 1997; Kühtreiber and Jaffe, 1990; Messerli and Robinson, 1998; Messerli *et al.*, 1999; Pierson *et al.*, 1994, 1996). This method has several advantages over the more traditional technique of patch clamping for investigation of transmembrane ion fluxes. Most importantly, it is non-invasive, not requiring removal of the cell wall, and so may be used to study tip growth in living cells. Furthermore, in contrast to patch clamping, no unnatural changes in membrane potential are imposed and the natural conditions on both sides of the membrane are maintained during measurement by the extracellular ion selective vibrating probe. Using this approach we have measured extracellular Ca^{2+} fluxes around pollen tubes of *P. rhoeas*, and show here that poppy pollen tubes exhibit novel extracellular Ca^{2+} influxes along their shank that are stimulated during the SI response. This implicates a role for Ca^{2+} influx in the increases in $[Ca^{2+}]_i$ previously reported as mediating the SI response in *Papaver*.

Results

Calcium fluxes in normally growing P. rhoeas *pollen tubes*

We have measured Ca^{2+} fluxes at the extreme apex of growing pollen tubes of *P. rhoeas* using the vibrating probe technique and show, as with other studies, that influx occurs and that it oscillates over time ($n = 5$), for example see Figure 1. Depending on the growth conditions of the pollen tubes, the fluxes varied considerably, though oscillations were still detectable when fluxes were small. The mean maximal Ca^{2+} influx at the peak of the oscillations was 4.64 pmol cm^{-2} sec^{-1} (SEM ± 1.83, $n = 5$).

In order to obtain a more thorough description of the overall pattern of Ca^{2+} current activity in growing pollen tubes, we measured the Ca^{2+} fluxes at various points along the shanks of growing pollen tubes of *P. rhoeas* using 2D mapping ($n = 5$). This approach enabled the mapping of both the magnitude and direction of the Ca^{2+} fluxes. Because we had previously observed increases in $[Ca^{2+}]_i$ in the shank region approximately 50–100 μm behind the tip region during the SI response, we were particularly interested to ascertain if there was any evidence for Ca^{2+}

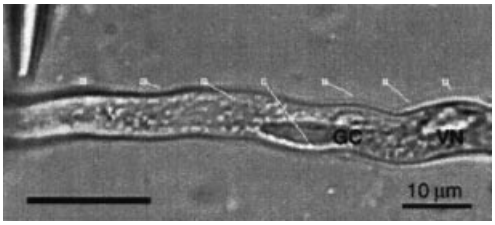


Figure 2. Image of part of a pollen tube of Papaver rhoeas with vector plots of Ca^{2+} fluxes.
Vector plots (indicated by white lines) displaying Ca^{2+} fluxes along the shank of a typical growing pollen tube, measured using the Ca^{2+}–selective vibrating probe. The site of each measurement is indicated by a small box with the vector plot emanating from this; the line indicates the magnitude and direction of the Ca^{2+} fluxes. The unlabelled calibration bar indicated is 10 pmol cm^{-2} sec^{-1}. These Ca^{2+} flux measurements are superimposed upon an image of the pollen tube measured, obtained from the concomitant video recording; the labelled bar indicates scale (10 μm). The vibrating probe tip (to the top left) is approximately 50 μm back from the pollen tube tip, which is to the left of the image. GC indicates the generative cell and VN the vegetative nucleus. Note that the largest Ca^{2+} influx in this region is towards the generative cell.

fluxes in this region of the pollen tube. Figure 2 shows 2-D mapping of Ca^{2+} influxes along part of the length of a typical growing pollen tube shank, concentrating on this area of interest. Three of the average vectors were taken when adjacent to a nucleus. The SEs of these vectors were small, even at a 95% confidence interval (data not shown). We have ascertained that the probe was not measuring markedly different fluxes, by calculating the means and SEs for the fluxes at these points (data not shown). Although some of the fluxes measured seem to be parallel to the tube, there is also clear evidence for influx localized in the region of the nucleus, since the bounding flux vectors, left and right, in Figure 2 are small. There is therefore no strong evidence that the tube-parallel component observed in the longest vector has its source or sink in another region (e.g. the pollen grain, which is to the left in Figure 2). Notably there are relatively large extracellular Ca^{2+} fluxes in this 'shank', particularly in the region occupied by the male germ unit. An inwardly directed Ca^{2+} flux of 4.1 pmol cm^{-2} sec^{-1} was measured directly adjacent to the generative cell in Figure 2. In another pollen tube, a large inwardly directed Ca^{2+} flux of 5.02 pmol cm^{-2} sec^{-1} was measured in the region of the male germ unit. The standard errors on pollen tubes measured in this way were small; for the pollen tube in Figure 2, the average coefficient of variation for the vectors was 11%.

We also performed 1-D vibrating probe measurements of Ca^{2+} fluxes along the shanks of growing pollen tubes of *P. rhoeas* that were several hundred μm long (n = 18), as shown in Figure 3. Typical measurements made along an individual pollen tube are shown in Figure 3(a). There is Ca^{2+} influx at several points along the pollen tube shank. From many measurements made on different tubes (n = 34), as well as along the length of the same pollen tube, it was evident that these Ca^{2+} fluxes were variable in their magnitude. The reason for this is not known, but it may be related to the length of the pollen tube and/or the rate of growth. Figure 3(b) shows that despite the substantial variation in the extent and amplitude of the Ca^{2+} fluxes along individual pollen tube shanks (which explains the large error bars shown), there is clear evidence for influx in the region around 50 μm behind the pollen tube tip. The Ca^{2+} influxes detected along the shank of untreated tubes were generally in the range 1–9 pmol cm^{-2} sec^{-1} (Figure 4). These data, together with the 2D vector data, provide evidence for the presence of Ca^{2+} influx in regions of pollen tube other than the pollen tube tip. In contrast, at 130–170 μm behind the tip there was consistently scarcely any evidence for Ca^{2+} influx (Figure 3b).

Use of Gd^{3+} and La^{3+} to block calcium influx and growth in pollen tubes

We used the lanthanides Gd^{3+} and La^{3+} (Malhó *et al.*, 1995) in an attempt to provide further evidence that the fluxes measured are due to Ca^{2+} movement across the plasma membrane. Addition of 75 μM Gd^{3+} resulted in considerable reduction in apical Ca^{2+} influxes (n = 11), since the tip oscillations were effectively damped out, leveling out at approximately 0–0.5 pmol cm^{-2} sec^{-1}; 100 μM La^{3+} also markedly inhibited influx at the apex (data not shown). We also tested the effect of these agents on Ca^{2+} influx measured 50 μm behind the tip (n = 13). 100 μM La^{3+} and Gd^{3+} markedly reduced Ca^{2+} influx; however, in several examples, between 100 and 500 μM La^{3+} did not completely block the Ca^{2+} influx in this region. As shown in Figure 4, the mean maximal influx at the shank (50 μm behind the tip) before addition of lanthanides was reduced by 50% after treatment with 500 μM La^{3+} and to less than half with Gd^{3+}.

We also examined the concentrations of Gd^{3+} and La^{3+} required for the inhibition of pollen tube growth in an attempt to corroborate the findings described above, with respect to inhibition of Ca^{2+} flux activity. Figure 5 demonstrates that although approximately 40 μM La^{3+} or Gd^{3+} is required for half-maximal inhibition of pollen tube growth, considerably higher concentrations are required for complete inhibition.

Ca^{2+} fluxes at the shanks of pollen tubes during the SI response

Having established the patterns of Ca^{2+} fluxes in normally growing pollen tubes, we challenged growing pollen tubes with incompatible S proteins in order to investigate

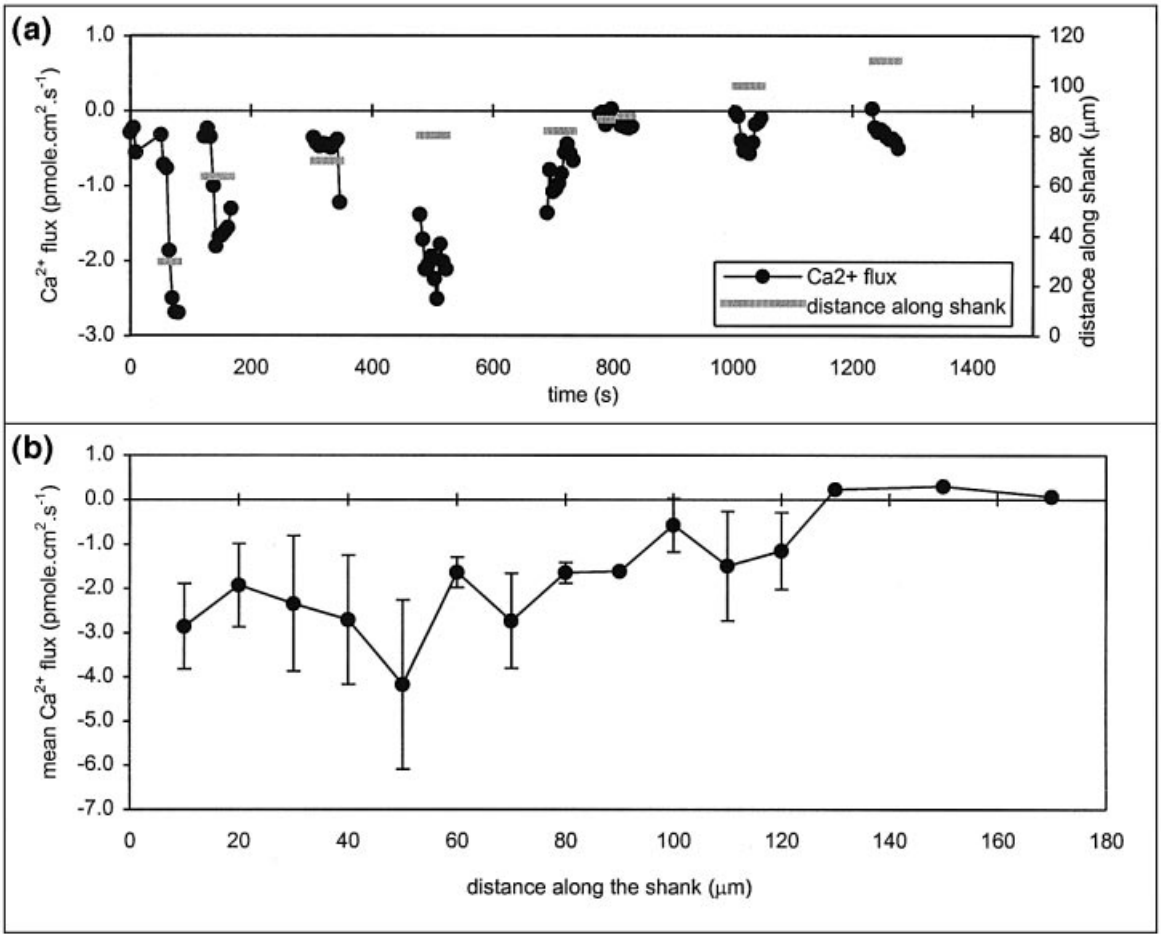


Figure 3. Measurements of Ca^{2+} influx along the pollen tube shank.
The plots show net Ca^{2+} flux, with net influx as a negative reading along the shank of tubes several hundred μm long.
(a) A representative plot of Ca^{2+} fluxes along an individual growing pollen tube of P. rhoeas. Measurements of Ca^{2+} flux were made at various points along the pollen tube shank (indicated by the grey bars and the secondary *y*-axis on the right-hand side). These were made by recording sequentially at a point along the pollen tube, then moving the probe to another location; the times at which recordings were made are indicated by the *x*-axis. The primary *y*-axis shows net Ca^{2+} flux (pmol cm^{-2} sec^{-1}). Note that there is a high level of variability in the level and location of the Ca^{2+} fluxes between the measurements made at different points. However, there is clearly evidence for net Ca^{2+} influx at some points along the pollen tube shank.
(b) Mean Ca^{2+} fluxes measured along the shanks of pollen tubes of P. rhoeas. The means in this figure were obtained from sets of data ($n = 7$) where measurements were made at various points along the pollen tube length. Despite the high variability, there is a region of the pollen tube around 50 μm behind the tip, that consistently exhibits net Ca^{2+} influx (mean influx 4.17 pmol cm^{-2} sec^{-1}, SEM ± 1.92, $n = 7$).

whether there is a role for extracellular Ca^{2+} in the SI response. Using the Ca^{2+}-selective vibrating probe, we chose a region of the pollen tube 50 μm behind the pollen tube tip, because we had previously observed alterations in Ca^{2+}_i using ratio imaging that appeared to be generated in this region of the pollen tube (Franklin-Tong *et al.*, 1993, 1995, 1997). Moreover we had previously noted that this region also appeared to correlate with the general localization of the male germ unit, and we had obtained evidence, presented earlier in this paper, for Ca^{2+} influxes in this region. Figure 6 demonstrates that large Ca^{2+} fluxes that are S-specific ($n = 13$) are triggered along the pollen tube shank. It can be clearly seen in Figure 6(a,b,c) that S proteins may trigger influxes of Ca^{2+} as large as 10–18 pmol cm^{-2} s^{-1} in incompatible pollen tubes 50 μm back from the tip. Challenge of pollen tubes with incompatible S proteins induced an average 13.6-fold increase in Ca^{2+} influx at 50 μm from the tip (Figure 4) (highly significant; $P < 0.001$, *T*-test).

Temporally, an increase in Ca^{2+} influx was detected very soon after addition of S proteins. Although increases were not detected immediately in all cases, in several experiments the first point measured was approximately 2-fold higher than before treatment. Even where increases appeared to be delayed (e.g. Figure 6a), < 10 sec after treatment Ca^{2+} influx was increased almost 4-fold. Ca^{2+} influx increased gradually over the subsequent approximately 4 min (Figure 6a–c). However, detailed analysis

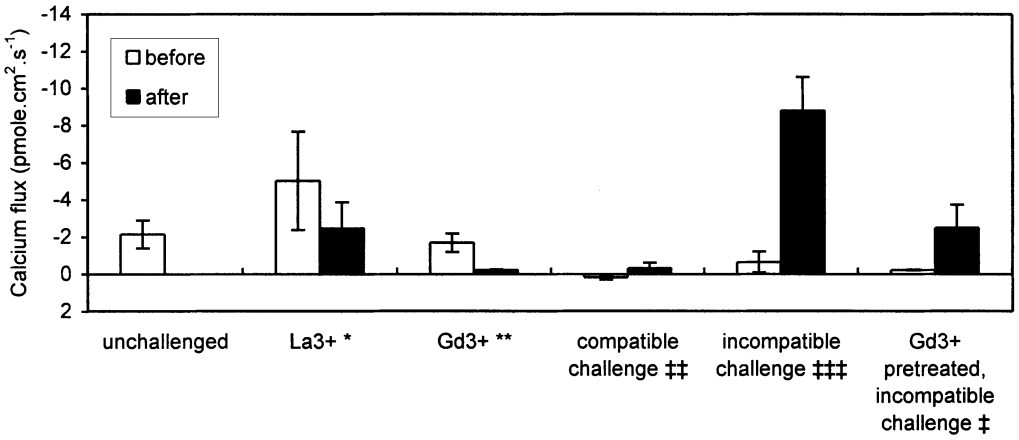


Figure 4. Average Ca^{2+} fluxes before and after treatment with S proteins and Ca^{2+} channel blockers.
Average Ca^{2+} fluxes measured 50 μm behind the tip of *Papaver* pollen tubes before and after 500 μl La^{3+}, 500 μl Ga^{3+}, compatible, incompatible challenge and incompatible challenge of pollen tubes pre-treated with Gd^{3+}. Comparisons were made between before and after measurements of individual pollen tubes treated similarly, and not with the overall mean in untreated pollen tubes.
Significance (*T*-test) levels are indicated as follows: * $P < 0.05$ one-sided; ** $P < 0.005$ one sided; ‡ $P < 0.05$ two-sided; ‡‡ $P < 0.01$ two-sided; ‡‡‡ $P < 0.001$ two sided.

revealed that within this overall trend of an apparently slow increase in influx, Ca^{2+} influx oscillated, sometimes as rapidly as 10–15 sec, usually altering several-fold between oscillations, throughout this period. Ca^{2+} influx subsequently gradually decreased over a further 5–8 min, returning to the original pre-stimulation levels.

Figure 6(d–f) illustrate typical Ca^{2+} fluxes in a fully compatible SI situation. Under these conditions fluxes of only 2–3 pmol cm^{-2} sec^{-1} were observed after challenge, which is far below those detected in response to an incompatible situation. Indeed, this figure is more in line with the mean Ca^{2+} fluxes detected in normally growing, unchallenged pollen tubes. The mean Ca^{2+} influxes for the compatible pollen tubes before challenge with compatible S proteins was significantly different from those measured after the challenge ($P < 0.01$, *T*-test) but at a significance level 10 times lower than observed in the incompatible situation. As Figure 4 shows, the average magnitude of fluxes induced by incompatible S proteins is clearly much larger than the average flux induced by compatible S proteins. Although some low-level Ca^{2+} influx was detected in most cases (as expected for this region of the pollen tube shank), no Ca^{2+} fluxes comparable with the levels measured in the incompatible SI response were detected. This demonstrates the *S*-allele specificity of the alterations in Ca^{2+} fluxes measured at pollen tube shanks in response to S protein challenge.

Ca^{2+} fluxes at the tip of incompatible pollen tubes during the SI response

We also examined what effect the SI response had on Ca^{2+} fluxes at the pollen tube tip. This region of the pollen tube usually has a high level of Ca^{2+} influx activity, which is inhibited when pollen tube growth is blocked (Pierson *et al.*, 1994). We have previously shown that the intracellular apical Ca^{2+} gradient is rapidly dissipated in the SI response (Franklin-Tong *et al.*, 1997). Using the Ca^{2+}-selective vibrating probe, we found that upon addition of incompatible S proteins, there was a gradual reduction of Ca^{2+} influx at the tip, with Ca^{2+} fluxes continuing to oscillate, but decreasing with each oscillation as shown in Figure 7. A few minutes after challenge the mean maximal Ca^{2+} fluxes at the tips of these treated pollen tubes were reduced to around 5% of the mean pre-treatment flux ($n = 3$).

Effect of pre-treatment with La^{3+} and Gd^{3+} on SI-induced Ca^{2+} influx at the shank

Our data demonstrate that incompatible S proteins stimulate Ca^{2+} influx at the shank of the pollen tube. Since approximately 500 μM La^{3+} and Gd^{3+} reduced or blocked Ca^{2+} influx at the shank, we examined if the SI-induced influx is sensitive to lanthanides. Pollen tubes were treated with La^{3+} or Gd^{3+} prior to addition of incompatible S proteins. As shown in Figures 4 and 8, tubes pre-treated with 500 μM Gd^{3+} or La^{3+} had substantially reduced Ca^{2+} influx triggered by the SI response. Although exposure to lanthanides reduced the Ca^{2+} flux prior to challenge, the proportional increase in Ca^{2+} influx induced by an incompatible challenge was similar to that observed in tubes not pre-treated with Gd^{3+} or La^{3+} (11-fold compared with 13-fold). However, as shown in Figure 4, the Ca^{2+} influx, though increased, was far below that detected in the incompatible SI response, and was within the levels of fluxes detected in unchallenged pollen tubes. These data

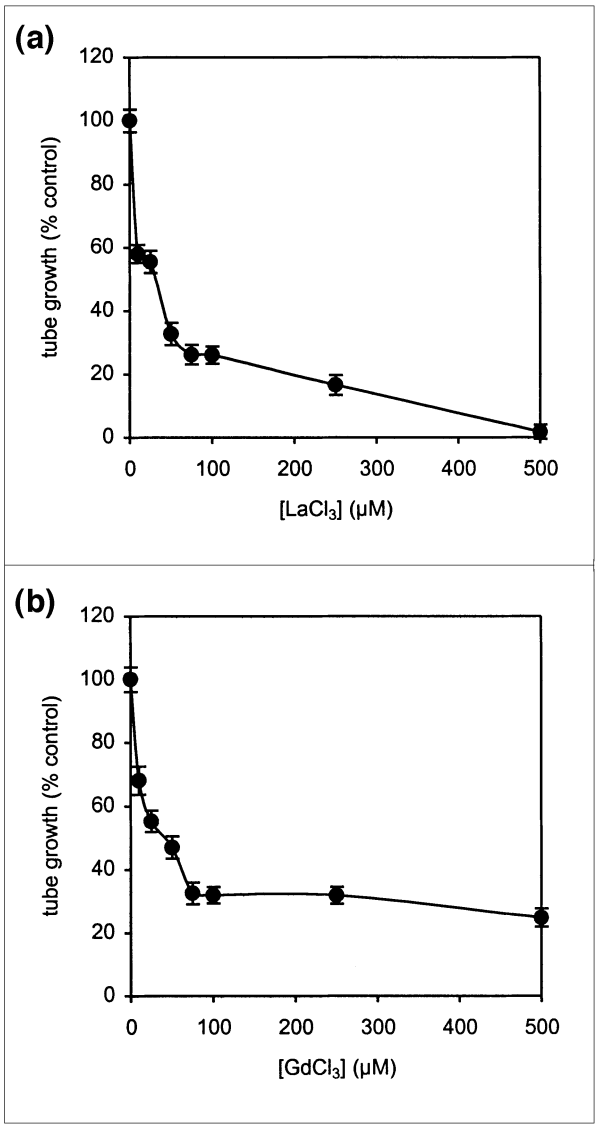


Figure 5. Inhibition of pollen tube growth by La^{3+} and Gd^{3+}.
These plots contain data from seven independent experiments, with $n = 105$ pollen tubes measured for each data point.
(a) Dose–response curve showing inhibition of pollen tube growth by $GdCl_3$. Half-maximal inhibition was obtained at approximately 40 μM.
(b) Dose–response curve showing inhibition of pollen tube growth by $LaCl_3$. Half-maximal inhibition was obtained at approximately 40 μM.

suggest that the SI-response might be mediated by two different kinds of Ca^{2+}-conducting channels, one sensitive to inhibition by lanthanides and the other insensitive.

We obtained data using ratio imaging of pollen tubes that were pre-treated with 500 μM La^{3+} prior to challenge with incompatible S proteins ($n = 8$). Figure 8(c) shows that the increases in intracellular cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) normally stimulated by the SI response (Franklin-Tong *et al.*, 1993, 1997) are virtually completely inhibited by pre-treatment with La^{3+}. Only one pollen tube showed increases in $[Ca^{2+}]_i$ and this was quite abnormal, being delayed (initiating 80 sec versus 1–2 sec after challenge) and short-lived (sustained for approximately 50 sec, rather than 5–10 min). We suspect that this single event was due to an incomplete blocking of channels through which Ca^{2+} enters. These results demonstrate that the Ca^{2+} influx stimulated in the SI response is required for the increases in $[Ca^{2+}]_i$ previously reported, and indicates that Ca^{2+} influx is required for the SI response.

Discussion

The ion-selective vibrating probe provides a powerful method for measuring the magnitude and direction of extracellular ion fluxes in regions adjacent to living cells; it has been especially effective in studies of pollen tubes (Feijó *et al.*, 1995, 1999; Holdaway-Clarke *et al.*, 1997; Kühtreiber and Jaffe, 1990; Messerli *et al.*, 1999; Pierson *et al.*, 1994). Here we have used the ion-selective vibrating probe to investigate Ca^{2+} fluxes associated with growing poppy pollen tubes, with particular reference to the SI response.

Calcium influxes are detected at the pollen tube shank

Our observations show, in agreement with previous studies on lily pollen tubes (Holdaway-Clarke *et al.*, 1997; Messerli and Robinson, 1997), that there is an oscillating Ca^{2+} influx directed at the tip of the poppy pollen tube. We also demonstrate significant influxes of Ca^{2+} at the shank of normally growing pollen tubes. There is clear evidence for localized influx, despite some of the fluxes measured appearing parallel to the tube. Although it is not possible to rationalize the parallel component outside the cell by any diffusion process, it is known that large electrical fields surround the growing pollen tube (Weisenseel *et al.*, 1975). These could potentially interact with the concentration gradient of Ca^{2+} to produce local distortions of the resultant vectors parallel to the pollen tube shaft. Until now, it has been generally considered that there is negligible Ca^{2+} influx except that occurring at the tip (Malhó *et al.*, 1995). Although Weisenseel *et al.* (1975) reported net ion influx at the pollen tube shank, the contribution of Ca^{2+} was not determined, and Kühtreiber and Jaffe (1990) demonstrated only small Ca^{2+} influxes of approximately 1 pmole cm^{-2} sec^{-1} at the shank of a tobacco pollen tube.

Extracellular Ca^{2+} is involved in the SI response

Although increases in $[Ca^{2+}]_i$ are known to be stimulated by the SI response in incompatible poppy pollen (Franklin-Tong *et al.*, 1993, 1995, 1997), one key question remaining has been the source(s) of Ca^{2+}. Here we demonstrate that

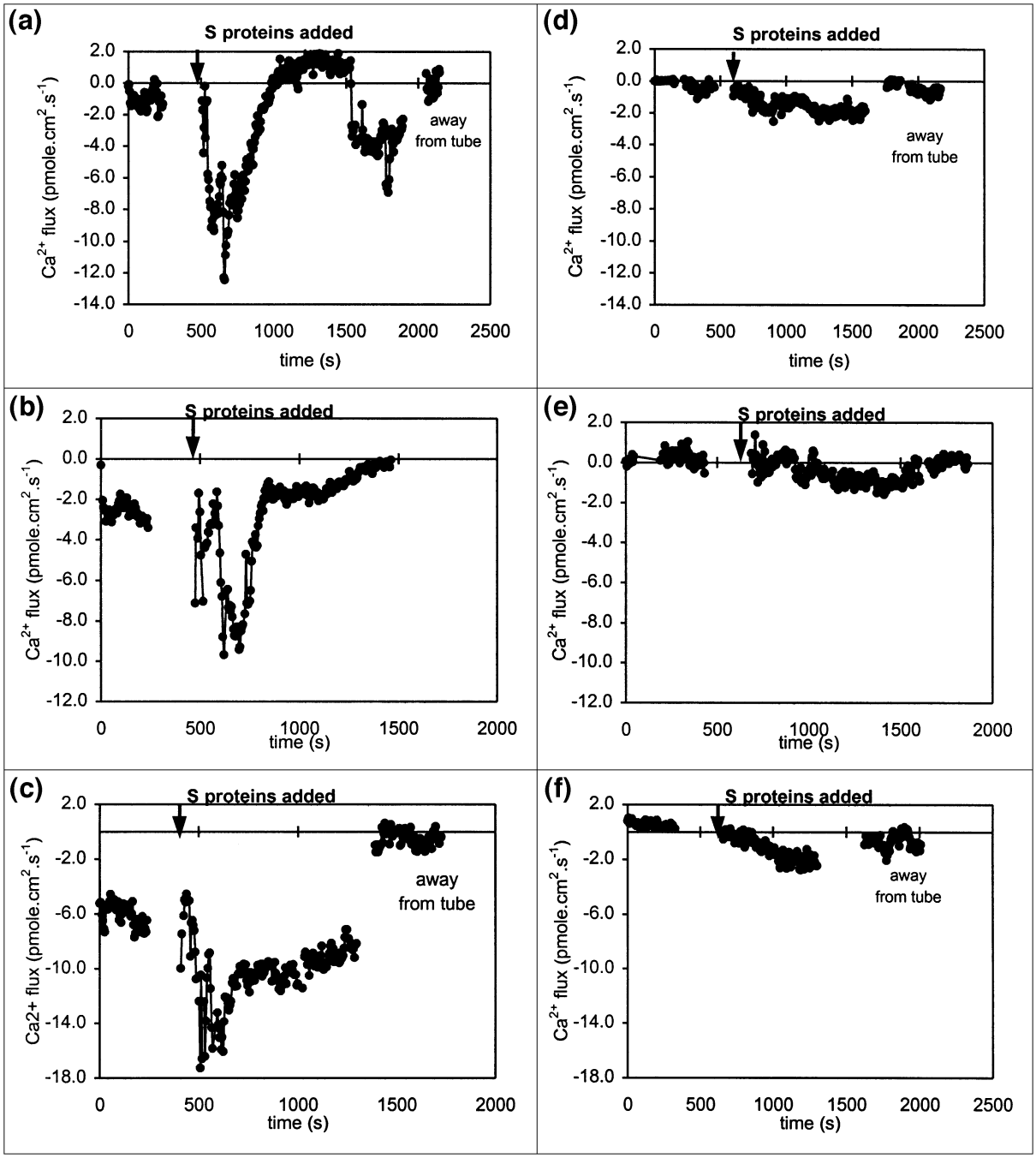


Figure 6. Ca^{2+} fluxes are triggered at the pollen tube shank in the SI response.
The plots show net Ca^{2+} flux, with net influx shown as a negative reading. a, b and c illustrate typical examples of the response detected by the vibrating probe in an incompatible SI response, while d, e and f are typical compatible SI reactions. These comparisons demonstrate the *S*-allele specificity of the Ca^{2+} influxes measured, as the same S proteins were used, and the only differences are due to different *S*-alleles carried by the pollen samples. All measurements were made in the shank region of the pollen tube, at 50 μm behind the pollen tube tip. Measurements before treatment and after addition of S proteins (indicated by arrow) are shown. Some plots indicate readings taken afterwards, at 10 μm away from the pollen tube, in order to give a background reading.

the SI response stimulates an influx of extracellular Ca^{2+}. Our data indicate two alterations in the normal pattern of Ca^{2+} fluxes in the pollen tube: increased influx in a region of the shank, and decreased influx at the tip, as illustrated in Figure 9(a). We used Gd^{3+} and La^{3+}, which are reported to be inorganic Ca^{2+} channel blockers (Marshall *et al.*, 1994), in an attempt to further decipher the observed influxes, and found that both inhibitors blocked Ca^{2+} influx

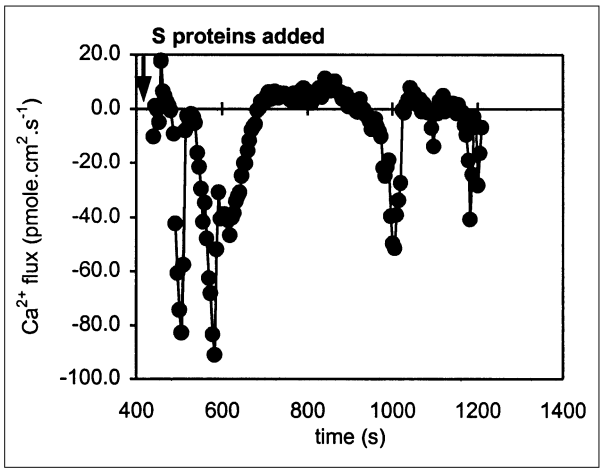


Figure 7. Ca^{2+} influx at the pollen tube tip is inhibited in the SI response. Addition of incompatible S proteins results in Ca^{2+} influx being rapidly inhibited in the apical region of the pollen tube. Measurements at the tip, before and after addition of incompatible S proteins.

in pollen tubes. Furthermore, our ratio-imaging data, showing that La^{3+} blocked SI-induced increases in $[Ca^{2+}]_i$, clearly demonstrates that Ca^{2+} influx is required for these increases in $[Ca^{2+}]_i$.

Although the levels of Gd^{3+} and La^{3+} (100–500 μM) used are high, they are consistent with the 100 μM La^{3+} used by Malhó *et al.* (1995) and Messerli and Robinson (1997) to dissipate Ca^{2+} influx at the tip. In addition, Marshall *et al.* (1994) found that although half-maximal inhibition was obtained at 40 μM La^{3+} or Gd^{3+}, 500 μM La^{3+} or Gd^{3+} did not completely inhibit Ca^{2+} uptake in maize vesicles; in intact plant protoplasts even 1 mM La^{3+} and 1 mM Gd^{3+} did not inhibit Ca^{2+} uptake completely. Thus our observations reflect the findings from other systems. Since more La^{3+} or Gd^{3+} is required to inhibit Ca^{2+} influx in the shank of the pollen tubes than at the tip, this suggests that there might be different types of Ca^{2+}-conducting channels, including non-specific cation channels, in the tips and shanks of pollen tubes. Furthermore, since the proportional increase in Ca^{2+} flux with incompatible challenge is similar, regardless of whether or not tubes were pre-treated with lanthanides, this suggests that the SI-response might be mediated by two different kinds of Ca^{2+} channel, one sensitive to lanthanides and the other insensitive. We conclude therefore that there must be active Ca^{2+}-conducting channels along the shank of the pollen tube (though we have no direct evidence for their presence).

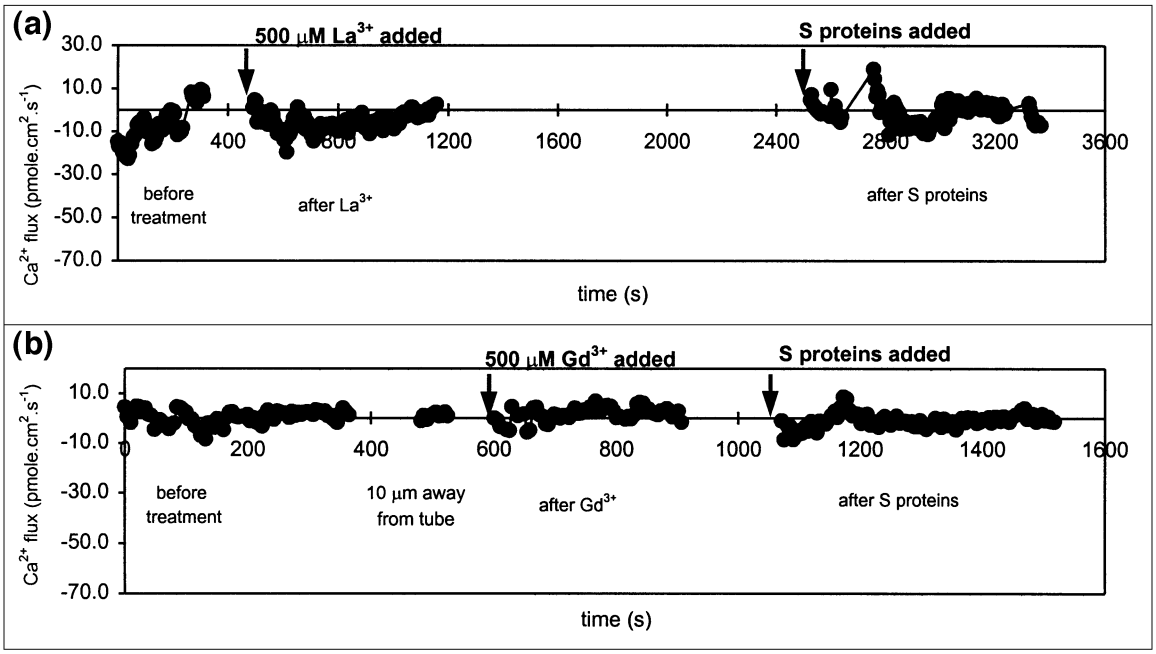
The relationship between Ca^{2+} influx and increases in $[Ca^{2+}]_i$

Although our data show that Ca^{2+} influx is triggered early in the SI response and in line with the timing of the first detectable increases in $[Ca^{2+}]_i$ imaged previously, we did not detect the dramatic and immediate influx that might, perhaps, be expected if the extracellular Ca^{2+} influxes relate directly to the increases in $[Ca^{2+}]_i$. Nevertheless, the initial Ca^{2+} influxes measured are potentially large enough to trigger the initial increases in $[Ca^{2+}]_i$, since Holdaway-Clarke *et al.* (1997) demonstrated that the Ca^{2+} influx required to fuel the large apical gradient was very small. Thus, it is likely that the relatively modest initial Ca^{2+} influxes that occur immediately after SI-induction are sufficient to initiate the increases in $[Ca^{2+}]_i$. However, since localized Ca^{2+} fluxes are reported to remain at their sites of cell entry for many minutes following channel activation (Clapham, 1995; Trewavas, 1999), we favour the view that, although the initial $[Ca^{2+}]_i$ increases involve the extracellular influx reported here, at least some of the elevations in $[Ca^{2+}]_i$ originate from intracellular Ca^{2+} stores, since we previously visualized large changes in $[Ca^{2+}]_i$ that appear to move as a wave through the pollen tube shank (Franklin-Tong *et al.*, 1997). Increases in $[Ca^{2+}]_i$ through influx could be amplified through Ca^{2+}-induced Ca^{2+} release or IP_3-induced Ca^{2+} release as a secondary process, and propagated as Ca^{2+} waves previously observed (Franklin-Tong *et al.*, 1996). Since the magnitude of Ca^{2+} influxes stimulated by SI gradually increased over several minutes, this suggests that this extracellular Ca^{2+} contribution may serve to further ramp up $[Ca^{2+}]_i$. One possible explanation is that only a small amount of influx is required to trigger Ca^{2+} release from intracellular stores, but increasing amounts are needed to sustain and propagate this $Ca^{2+}{}_i$ release.

A model for Ca^{2+} increases in the SI response

Together, our data indirectly implicate a role for intracellular release, but more importantly, directly demonstrate that Ca^{2+} influx is a key early step in the SI response; indeed it may represent the first event. The rapidity of the $Ca^{2+}{}_i$ response is in line with the idea that the SI reaction is a receptor-mediated response, with the S-protein acting as a signal molecule. The notion that the Ca^{2+} influx is likely to represent an early step in the signal transduction cascade is not unprecedented. Transient influx of Ca^{2+} across the plasma membrane has been shown to play a role in the early steps of the pathogen defence response signal cascade (Romeis *et al.*, 1999). Similarly, one of the earliest measured responses to Nod factors by their host legumes is a stimulation of Ca^{2+} influx (Felle *et al.*, 1998; Yokoyama *et al.*, 2000).

We consider it unlikely that the S proteins act directly on Ca^{2+}-conducting channels since S-specificity needs to be encoded in some way, as elevations in $[Ca^{2+}]_i$ do not occur as a consequence of a compatible SI reaction. Our current model (Figure 9b) therefore proposes that there is a membrane-located pollen *S*-receptor that binds S proteins



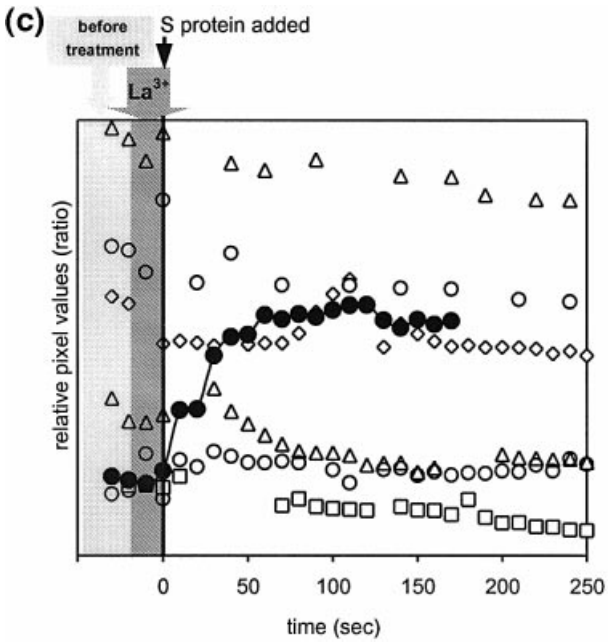


Figure 8. Pre-treatment of pollen tubes with Gd^{3+} and La^{3+} prior to the SI response blocks Ca^{2+} fluxes.
Effects of (a) 500 μM La^{3+} and (b) 500 μM Gd^{3+} on the calcium influxes in pollen tubes in response to incompatible S-proteins. The plots show net Ca^{2+} flux, with net influx as a negative reading.
(c) Ratiometric Ca^{2+}-imaging of pollen tubes response to incompatible S proteins after pre-treatment with La^{3+}. The pale grey area indicates the pre-treatment period. The dark grey area indicates treatment with 500 μM La^{3+}. The arrow and line indicate addition of S proteins. Open symbols indicate ratiometric values of $[Ca^{2+}]_i$ after La^{3+} treatment. The plot with black symbols shows a plot without La^{3+} pre-treatment for comparison.

in an *S*-specific manner. Although we have not identified the pollen *S*-receptor, a pollen-specific integral membrane proteoglycan, SBP, has been shown to bind S proteins *in vitro* (Hearn *et al.*, 1996). We hypothesize that SBP either acts as an accessory receptor in conjunction with the *S*-specific receptor in pollen, or that it is the *S*-receptor, and has both high and low-affinity binding sites (Jordan *et al.*, 1999). Since the present study demonstrates that when incompatible stigmatic S proteins interact with the pollen *S*-receptor, SI-induced increases in Ca^{2+} influx are stimulated, it appears likely that the pollen *S*-receptor is in some way associated, or interacts with, channels that conduct Ca^{2+}. In this way, the S-specific influx of Ca^{2+} could be achieved. Our data also indicate that Ca^{2+} influx stimulates increases in $[Ca^{2+}]_i$, which suggests some contribution from intracellular stores as well as extracellular sources. These increases in $[Ca^{2+}]_i$ trigger the SI-induced signalling cascade (Figure 9b) described earlier.

In summary, we have demonstrated that normally growing poppy pollen tubes exhibit relatively large

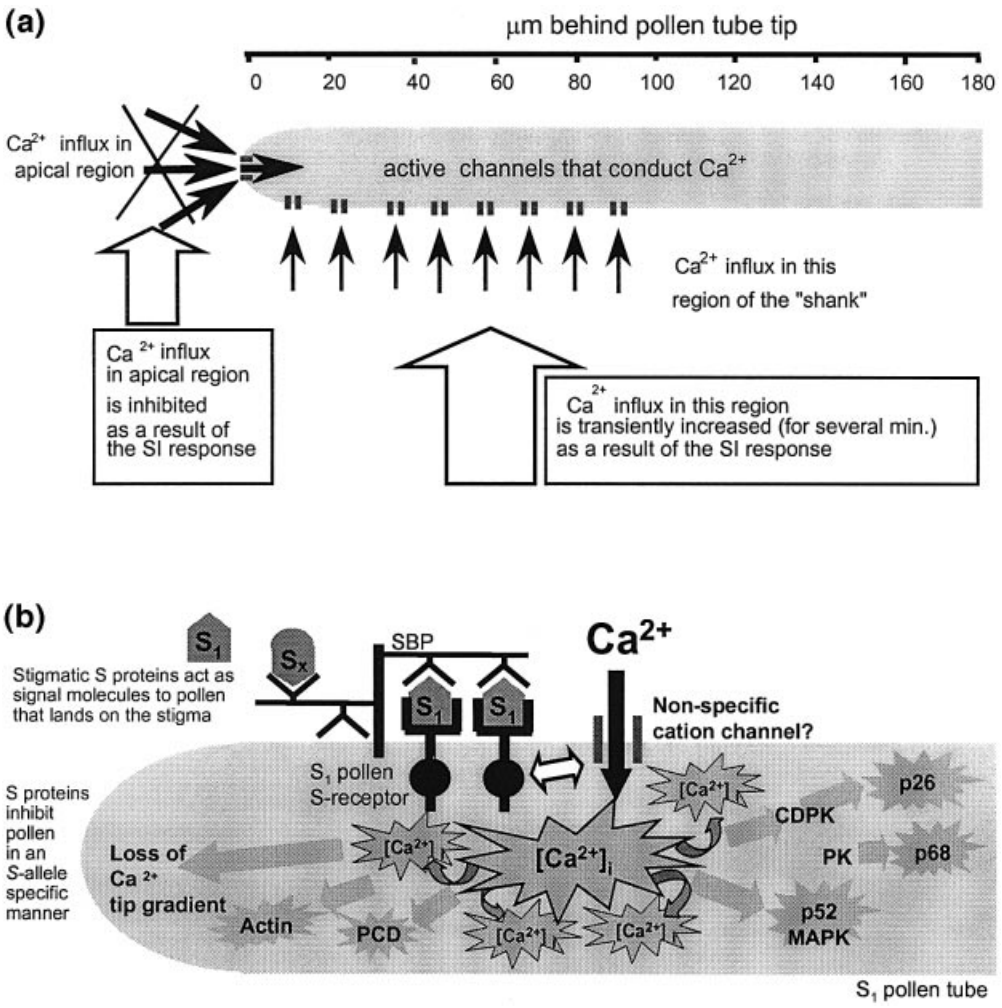


Figure 9. Models for Ca^{2+} fluxes in a poppy pollen tubes and alterations induced by the SI response.
(a) This model is based upon the Ca^{2+} influx data presented. In normally growing poppy pollen tubes there is evidence of Ca^{2+} influx (indicated by black arrows) at the tip and the shank. This implies there are Ca^{2+}-conducting channels in the pollen tube (grey boxes). The effects of an incompatible SI response on Ca^{2+} influx (indicated by open arrows) are Ca^{2+} influx at the tip is inhibited and significant influx is detected in the shank of the pollen tube.
(b) Model indicating some of the events identified as being SI-stimulated, how they might interrelate, and how alterations in $[Ca^{2+}]_i$ may affect them. Stigmatic S proteins (S_1, S_x) interact with assumed *S*-specific pollen receptor via SBP (all bind SBP, but only S_1 protein can interact with S_1 receptors thereby generating incompatible *S*-specificity). Binding the *S*-receptor stimulates increases in Ca^{2+} influx (perhaps through a non-specific cation channel), which is postulated to interact (indicated by the double-headed arrow) in some way with the pollen *S*-receptor to achieve S-specific influx of Ca^{2+}. Evidence suggests that Ca^{2+} influx stimulates increases in $[Ca^{2+}]_i$, with some contribution from intracellular stores as well as extracellular sources. This activates several protein kinase cascades. Other events known to be triggered downstream of increases in $[Ca^{2+}]_i$ are indicated.

influxes of Ca^{2+} in the shank region. Of particular importance are the findings that these shank influxes are amplified during the SI response. It seems plausible therefore that the SI receptor is in some way associated with Ca^{2+}-conducting channels along the shank of the tube. These results further emphasize the role of extracellular Ca^{2+} currents as part of the complex signalling cascade leading to the inhibition of pollen tube tip growth.

Experimental methods

Plant material

Plants of *P. rhoeas* L. (var. Shirley) segregated for known incompatibility genotypes (S_1S_3 and S_2S_4) were used for these experiments (Franklin-Tong *et al.*, 1988). Pollen was stored at –20°C, over silica gel, until required.

Expression of recombinant S-proteins and assessment of S-protein activity

Recombinant S proteins for alleles S_1 and S_3 (S_1e and S_3e, respectively) were used to challenge pollen. Nucleotide sequences specifying the mature peptide of the S gene for S_1 and S_3 were cloned into the expression vector pMS119 to produce pPRS100 and pPRS300, respectively (Foote *et al.*, 1994). High-level expression of recombinant S proteins were induced in these *E. coli* cells containing pPRS100 or pPRS300 with 1 mM isopropyl β-D-thiogalactoside. The resulting inclusion bodies were isolated, purified and refolded as described in Kakeda *et al.* (1998). Protein concentration was estimated using the Coomassie Brilliant Blue R-250 dye-binding method (Bradford, 1976). The recombinant S proteins S_1e and S_3e were assayed for their S-specific pollen inhibitory activity prior to each experiment. They were dialyzed against liquid germination medium (see *In vitro* system for growing pollen) and tested against incompatible (S_1S_3) pollen and compatible (S_4S_5 or S_2S_4) pollen genotypes for S-specific inhibitory activity; see Kakeda *et al.* (1998) for full details.

In vitro *system for growing pollen for vibrating probe work*

Pollen was grown using an *in vitro* system, adapted from Franklin-Tong *et al.* (1993) so that the vibrating probe technique could be used to make measurements from growing pollen tubes. The concentration of Ca^{2+} in the germination medium (GM) was adjusted to 50 μM, which was the lowest concentration that would support good growth. This GM comprised 7.5% sucrose, 5% polyethylene glycol 6000, 0.01% KNO_3, 0.01% H_3BO_3, 0.01% $Mg(NO_3)_2{\cdot}6H_2O$, 50 μM $CaCl_2{\cdot}2H_2O$. Slides were prepared as described in Franklin-Tong *et al.*, 1997. Briefly, pollen was prehydrated and a pre-warmed chamber-slide was prepared with GM and 1.5% low-temperature gelling agarose (type VII; Sigma-Aldrich Co. Ltd, Poole, UK). Pollen was sown on this thin layer of germination medium, left to cool and solidify for approximately 30 sec. This was then covered with 300 μl liquid germination medium and incubated at room temperature in a moist chamber until pollen tubes of approximately 100 μm had grown.

Vibrating probe work

Measurements of Ca^{2+} fluxes were made using Ca^{2+} selective vibrating probes (Smith *et al.*, 1994). These were made at various points at the extreme apex and along the shank region of growing pollen tubes. For the SI challenges, we chose a region of the pollen tube approximately 50 μm behind the pollen tube tip to position the probe, and monitored Ca^{2+} fluxes over time.

Some 2-dimensional scans were made of the shanks of growing pollen tubes, which enabled the mapping of both the magnitude and vector direction of the Ca^{2+} fluxes. The Ix and Iy were made sequentially, not simultaneously or interleaved. As a result, saying the Ix is stable for the time the measurements were made does not necessarily mean that the Iy, measured next, shares the same stability. Matlab scripts were used to place vectors on grey scale images with appropriate scale bars. A sample script is available for download at the following URL: http://marlin.bio.umass.edu/biology/kunkel/pub/matlab/. Data matrices produced by the ion selective probe software, as described in Holdaway-Clarke *et al.* (1997), were analyzed using an MS Excel (Excel 97, Microsoft) spreadsheet to convert data from the background-mV estimation of concentration and microvolt-difference estimation of the local gradient into specific ion flux (pmole cm^{-2} sec^{-1}). A model MS Excel spreadsheet is available at http://marlin.bio.umass.edu/biology/kunkel/mage/uv2flux.html

A Nernst slope and intercept was determined for each electrode used. Calibration of the efficiency of the probe in a dynamic state was performed using point sources of Ca^{2+} as previously described (Smith *et al.*, 1994). The background mV response was measured prior to each series of 1D measurements and prior to each 2D measurement. Background mV values away from and close-by the tube were routinely determined to establish if the local environment of the pollen tubes was being modified by evaporation or local cellular metabolism. Mean data on the Ca^{2+} fluxes was generated from various data sets. Means of fluxes for data where no detectable changes were elicited (e.g. at a certain point behind the pollen tube tip, or before challenge) were taken from a complete data set where that measurement was made. 'Mean maximal' fluxes for comparisons of data where changes were detected (e.g. oscillations at pollen tube tips and fluxes triggered by the SI response) were taken from 10 adjacent data points in time where the largest influx was detected. For those experiments examining challenges, means were calculated only from the pollen tubes subsequently challenged with compatible or incompatible S-proteins (i.e. 'before' and 'after' challenge comparisons).

Use of Gd^{3+} and La^{3+} to block calcium influx and growth in pollen tubes

A range of concentrations between 10 μM and 500 μM of Gd^{3+} and La^{3+} was used to establish concentrations required to block Ca^{2+} influx. These were added to pollen tubes prior to measurement with the ion-selective vibrating probe. Pollen tubes were also treated with addition of La^{3+} and Gd^{3+} prior to addition of incompatible S proteins. To establish the concentrations of La^{3+} and Gd^{3+} required to inhibit pollen tube growth, pollen tubes were grown on germination medium solidified with 0.5% Agarose MP (Boehringer-Ingelheim, Bracknell, Berkshire, UK). After 30 min addition of $LaCl_3$ or $GdCl_3$ were made by adding liquid germination medium supplemented with 0, 10, 25, 50, 75, 100, 250 or 500 μM $LaCl_3$ or $GdCl_3$. At this point, a control was fixed in 2% glutaraldehyde for t = 0. The pollen tubes were grown for 30 min

after treatment, fixed in 2% glutaraldehyde and the length of 15 tubes for every treatment was measured. Independent experiments were repeated (n = 7), and the data analyzed using Minitab software.

Microinjection, treatment and $[Ca^{2+}]_i$ imaging of fura-2 dextran-loaded pollen tubes

Fura-2 dextran was used to visualize the $[Ca^{2+}]_i$ in individual living pollen tubes in order to determine if La^{3+} was effective in blocking intracellular $[Ca^{2+}]_i$, as in our vibrating probe studies it was found to block Ca^{2+} influx stimulated by the SI response. Pollen tubes grown *in vitro* were microinjected with fura-2 dextran (10 000 Mr) (Molecular Probes Inc., Eugene, OR, USA) as described in detail by Franklin-Tong *et al*. (1997). $[Ca^{2+}]_i$ was measured in pollen tubes pre-treated with 500 μM La^{3+} using ratiometric imaging before and after SI induction using biologically active S proteins, as described in Franklin-Tong *et al*. (1997). A series of pairs of images imaged at 10-sec intervals, at the wavelengths 340 nm and 360 nm was captured in a timed sequence. Pollen tube $[Ca^{2+}]_i$ was analyzed through a series of images captured by fluorescence ratio-imaging on a Nikon Diaphot microscope (Nikon UK Ltd., Kingston, Surrey, UK) outfitted with a highly regulated mercury vapor lamp and a thermoelectrically cooled CCD (Pierson *et al*., 1994, 1996). Images were obtained and processed using the PMIS imaging software package (Photometrics, Tucson, AZ, USA) and the ratio data were plotted using excel.

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