Planta (1997) 203: 495–505

Planta

Root hair growth in *Arabidopsis thaliana* is directed by calcium and an endogenous polarity

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Received: 22 April 1997 / Accepted: 14 May 1997

Abstract. Tip growth of plant cells has been suggested to be regulated by a tip-focused gradient in cytosolic calcium concentration ($[Ca^{2+}]_c$). However, whether this gradient orients apical growth or follows the driving force for this process remains unknown. Using localized photoactivation of the caged calcium ionophore Br-A23187 we have been able to artificially generate an asymmetrical calcium influx across the root hair tip. This led to a change in the direction of tip growth towards the high point of the new $[Ca^{2+}]_c$ gradient. Such reorientation of growth was transient and there was a return to the original direction within 15 min. Root hairs forced to change the direction of their growth by placing a mechanical obstacle in their path stopped, reoriented growth to the side, and grew past the mechanical blockage. However, as soon as the growing tip had cleared the obstacle, growth returned to the original direction. Confocal ratio imaging revealed that a tip-focused $[Ca^{2+}]_c$ gradient was always centered at the site of active growth. When the root hair changed direction the gradient also reoriented, and when growth returned to the original direction, so did the $[Ca^{2+}]_c$ gradient. This normal direction of apical growth of *Arabidopsis thaliana* (L.) Heynh. root hairs was found to be at a fixed angle from the root of 85 ± 6.7 degrees. In contrast, *Tradescantia virginiana* (L.) pollen tubes that were induced to reorient by touch or localized activation of the caged ionophore, did not return to the original growth direction, but continued to elongate in their new orientation. These results suggest that the tip-focused $[Ca^{2+}]_c$ gradient is an important factor in localizing growth of the elongating root hair and pollen tube to the apex. However, it is not the primary determinant of the direction of elongation in root hairs, suggesting that other information from the root is acting to continuously reset the growth direction away from the root surface.

Key words: *Arabidopsis* (root hair growth) – Cytosolic calcium – Pollen tube – Root hair – Tip growth – *Tradescantia* (pollen tube growth)

Introduction

Apically growing plant cells include root hairs, rhizoids, leaf hairs, and pollen tubes. These cells extend by synthesis and expansion of the cell wall at the extreme tip. Ultrastructural analysis of root hairs and other tip-growing cells has revealed that their cytoplasmic contents are highly organized and polarized (Rosen et al. 1964; Emons 1987). The tip of these cells predominantly contain secretory vesicles (Rosen et al. 1964) although microtubules and actin filaments may also be present (e.g. Lloyd and Wells 1985; Traas et al. 1985; Emons 1987). Further from the tip are found mitochondria, dictyosomes, vesicles, endoplasmic reticulum, and plastids (Rosen et al. 1964; reviewed in Sievers and Schnepf 1981). The high degree of spatial resolution with which this growth gradient is maintained has suggested that some factor, such as a gradient of a particular ion, maintains polarity of the apically growing cells.

Calcium plays an important role in the regulation of many cellular processes in higher plants, including cell division, cell expansion and cytoplasmic streaming (Bush 1995). Research on the tip growth of pollen tubes, algal rhizoids, fungal hyphae and root hairs suggests that a localized, highly focused gradient of Ca^{2+} is a strong candidate for the spatial information determining the direction of growth. Growing root hairs and pollen tubes posses a localized gradient of cytoplasmic free Ca^{2+} ($[Ca^{2+}]_c$) towards the growing apex and the intensity of this gradient correlates with the growth rate of these cells (Pierson et al. 1996; Felle and Hepler 1997; Wymer et al. 1997; and references therein). Further evidence for the role of Ca^{2+} arises from electrophysiological studies using the vibrating probe which suggest that Ca^{2+}-influx is higher at the tip than

Abbreviation: $[Ca^{2+}]_c$ = cytosolic calcium concentration

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at the base or sides of a root hair (Schiefelbein et al. 1992; Herrmann and Felle 1995; Jones et al. 1995) or pollen tube (Pierson et al. 1994). Also Ca^{2+}-ionophores and Ca^{2+}-channel blockers can inhibit tip growth of pollen tubes (e.g. Herth et al. 1990) and root hairs (e.g. Herrmann and Felle 1995; Wymer et al. 1997). Microinjection of Ca^{2+}-buffers that disrupt the $[Ca^{2+}]_c$ gradient also inhibits tip growth in these cells (Miller et al. 1992; Pierson et al. 1994; Herrmann and Felle 1995), further implicating a Ca^{2+} requirement in sustaining tip growth.

Malho and Trewavas (1996) tested the importance of the $[Ca^{2+}]_c$ gradient in determining the direction of apical growth in pollen tubes. They imposed intracellular $[Ca^{2+}]_c$ gradients and noted the effect on the direction of growth. Localized release of Ca^{2+} in the tip of the pollen tube was demonstrated to change the direction of apical growth towards the site of elevated $[Ca^{2+}]_c$. These results strongly suggest that a gradient in $[Ca^{2+}]_c$ is one of the factors that can direct the tip-growth process in pollen tubes. However, in vivo there may be many factors orienting the pollen tube growth that are not present in vitro. For example, Malho and Trewavas (1996) proposed that an apoplasmic Ca^{2+} gradient inside the stigma might direct tip growth. The difficulties of monitoring pollen growth and the dynamics of Ca^{2+} in situ, in the stigma have precluded direct testing of this theory to date.

Despite both pollen tubes and root hairs elongating via tip growth, root hairs possess many unique features. For example, their cell wall structure diverges from that of pollen tubes. The cell wall at the tip of the growing root hair consists of randomly arranged cellulose microfibrils (the α-layer of the cell wall). Basipetal to the growing tip a secondary cellulosic cell wall (β-layer) is synthesized. In contrast, the secondary pollen tube wall is formed mainly of callose (Belford and Preston 1961; Derksen and Emons 1990; Giddlings and Staehelin 1991). Also, root hair growth rate is approximately one-tenth that of pollen tubes (Jones et al. 1995; Wymer et al. 1997). Thus wall deposition and growth control in root hairs may differ from that in pollen tubes, and the role of the tip-focused $[Ca^{2+}]_c$ gradient in regulating root hair growth remains unproven. Root hairs growing from an intact root also offer a chance to monitor tip growth in vivo and in situ, under normal conditions of growth regulation. We were therefore able to investigate whether the $[Ca^{2+}]_c$ gradient is a primary determinant of the direction of root hair growth, as has been seen for pollen tubes grown in vitro (Malho and Trewavas 1996).

Data are presented showing that both an artificially generated gradient in $[Ca^{2+}]_c$ and a touch stimulus can elicit a transient reorientation of root hair growth. This change in direction is associated with a reorientation of the apically focused $[Ca^{2+}]_c$ gradient. However, the root hairs always return to their initial direction of growth, suggesting some factor other than the apical $[Ca^{2+}]_c$ gradient determines the direction of root hair growth in vivo.

Materials and methods

Plant material. Seeds of *Arabidopsis thaliana* (L.) Heynh. (Columbia ecotype) were surface-sterilized by immersing seeds in 95% ethanol and 10% (v/v) bleach for 10 min followed by five rinses in sterile distilled water. Sterile seeds were then embedded in a thin film of 1% (w/v) Phytagel layered on a 48×65 mm^2, No. 1, glass coverslip (Clay Adams, Lincoln Park, NJ, USA). The Phytagel (pH 5.7) was supplemented with the following macronutrients (mM): KNO_3, 3.0; $Ca(NO_3)_2$, 2.0; $MgSO_4$, 1.0; $(NH_4)_2PO_4$, 1.0; thiamine, 3.0; pyridoxine-HCl, 2.5; nicotinic acid, 4.0; *myo*-inositol, 0.56; 2-(N-morpholino)ethanesulfonic acid (Mes), 2.3; sucrose, 30; and micronutrients (μM); KCl, 25; H_3BO_3, 17.5; $MnSO_4$, 1.0; $ZnSO_4$, 1.0; $CuSO_4$, 0.25; $(NH_4)_6MoO_4$, 0.25; (ethylenedinitrilo)tetracetic acid (Fe-Na EDTA), 25. The cover glasses, with planted seeds were placed in 90-mm-diameter Petri dishes, wrapped with Parafilm, and placed in a growth chamber with constant irradiance of 36 μmol photons $\cdot$ m^{-2} $\cdot$ s^{-1} and temperature of 23 ± 1 °C. Plants were used 4–7 d after planting at which time the roots were 1–3 cm long. *Tradescantia virginiana* (L.) pollen was germinated and grown as previously described (Lewandowska and Charzynskaet 1977). Unless otherwise stated, chemicals were obtained from Sigma Chemical Co. (St Louis, Mo., USA). Caged compounds were obtained from Molecular Probes (Eugene, Ore., USA).

Video imaging and determination of growth rate. The growth of *A. thaliana* root hairs or *T. virginiana* pollen was monitored on a Nikon Diaphot 300 microscope (Nikon, Melville, N.Y., USA) using a 40× dry, Nikon, 0.7 N.A. objective and DIC optics. Images were recorded using a CH250A cooled CCD camera (Photometrics, Tucson, Ariz., USA) and a Quadra 800 computer (Apple Computer, Cupertino, Calif., USA). Images were captured every 30 s and growth rates were measured using IPLabs Spectrum image processing software (Signal Analytics, Vienna, Va., USA).

Ratiometric measurement of $[Ca^{2+}]_c$. For measurement of $[Ca^{2+}]_c$, root hairs or pollen tubes were simultaneously microinjected with the fluorescent calcium indicator calcium-green-2 linked to a 10-kDa dextran and rhodamine linked to a 10-kDa dextran (Molecular Probes). Pseudo-ratio images were then constructed by dividing the Ca^{2+}-dependent fluorescence from the calcium green with the calcium-independent fluorescence from the rhodamine signal, essentially as described previously for constructing ratio images from Indo-1 (Gilroy 1996). Indo-1 was not usable in these studies as its UV excitation is incompatible with the UV-activated caged probes outlined below. We have also been unable to obtain high quality ratiometric images using the true ratiometric approach of imaging a calcium green/texas red dextran (Haugland 1996). However, the pseudo-ratiometric approach used here was found to provide a reliable measure of the quantitative and spatial dynamics of cytoplasmic $[Ca^{2+}]$ in root hairs when compared with $[Ca^{2+}]$ monitored simultaneously using the truly ratiometric indicator Indo-1 (Wymer et al. 1997).

For root hair injection, the seedlings were first incubated for 15 min with 2 mL of fresh growth medium containing no Phytagel in order to fully hydrate the gel. Root hairs or pollen tubes were then impaled with micropipettes (10–20 MΩ resistance) pulled from filament electrode glass (World Precision Instruments, New Haven, Conn., USA) using a PC-84 puller (Sutter Instruments, Novato, Calif., USA) and containing 1 mM dextran-conjugated dyes. Dye was then pressure injected into the root hair base using a PV830 pneumatic picopump (World Precision Instruments, Sarasota, Fla., USA) using a series of 25-psi pressure pulses. Injected cells were allowed to recover from the microinjection for 20 min prior to ratio imaging. Root hairs that failed to regain normal growth rates at this time were excluded from further analysis. Using these criteria, the success rate of root hair injection was approximately 10%.

Coverslips with the microinjected seedlings were then placed on the stage of a Zeiss axiovert inverted microscope (Carl Zeiss, Melville, N.Y., USA) attached to an LSM410 laser scanning confocal microscope and imaged using a Zeiss 40×0.75 N.A., dry objective. Ratio images were collected using the Zeiss LSM 410 confocal microscope, using excitation from the 488 nm laser and emission collected at 515–540 nm (calcium green-2) and > 590 nm (rhodamine). Emission wavelengths were selected using a 488-nm primary dichroic mirror, 560-nm secondary dichroic and the appropriate Zeiss interference filters. Each frame represents a single 8-s scan of the laser. The calcium green/rhodamine signal was calibrated in vivo and in vitro as described previously for calcium green and Indo-1 (Gilroy 1996). Photobleaching represented $< 5\%$ per channel per scan for each ratio image. Pseudocolor ratio images of the $[Ca^{2+}]_c$ distribution were calculated as described in Gilroy (1996). Image processing was carried out on a PowerMac 8100 (Apple Computer) using IPLabs Spectrum image analysis software. Autofluorescence and dark current represented less than 5% of the calcium green or rhodamine signal. The dextran-conjugated indicators remained in the cytoplasm of root hair cells for > 8 h (Wymer et al. 1997).

For microinjection of pollen tubes, pollen was germinated in vitro in 0.5% (w/v) ultra low melting point agarose (Sigma; Type IX) and allowed to grow until the pollen tubes were between 50 and 100 μm long. These were injected with dextran-conjugated dyes as described above for root hairs except that the pollen tube was impaled at least 50 μm back from the tip. The pollen tube was then allowed to recover for 15 min and only those returning to growth rates of more than $5\ \mu m \cdot min^{-1}$ and showing no morphological disruption of the tip were studied further. Using these criteria, the success rate of pollen tube injection was approximately 5%.

Bright-field images were also taken at each experimental time point using the transmission detector of the confocal microscope and the 633 nm He/Ne laser attenuated to 10% with neutral density filters.

Application of touch stimulus. Glass microinjection micropipettes were filled with growth medium and positioned 10–20 μm away from the tip of growing root hairs or pollen tube using micromanipulators (M2; Narashige, Tokyo, Japan). The root hair or pollen tube was then allowed to grow into the micropipette such that the pipette touched the center of the cell tip. The electrode was maintained in a fixed position throughout each touch experiment.

Loading and photoactivation of the caged molecules. All procedures involving caged probes were performed in the dark or under dim safe light conditions. *Arabidopsis thaliana* root hairs and *T. virginiana* pollen tubes were treated for at least 20 min with 20 μM 1-(4,5-dimethoxy-2-nitrophenyl)ethyl ester (DMNPE-caged) Br-A23187 (Molecular Probes), diluted from 10 mM stock in dimethylsulfoxide (DMSO). The final DMSO concentration was 0.1% (v/v) and did not effect either root hair or pollen tube morphology or growth kinetics (data not shown). The caged compound was photoactivated by illumination with the UV laser (Enterprise, Coherent, Auburn, Calif., USA) of the LSM-410 confocal microscope. Uncaging efficiency was assessed by adding 10 μM caged fluorescein along with the caged ionophore (Gilroy 1996). Caged fluorescein is nonfluorescent until UV-illuminated and therefore provides a useful in-situ calibration of uncaging efficiency (Allan et al. 1994). The percentage of fluorescein photoactivated was monitored on the LSM 410 microscope; excitation 488 nm, 488 nm primary dichroic, emission 515–540 nm.

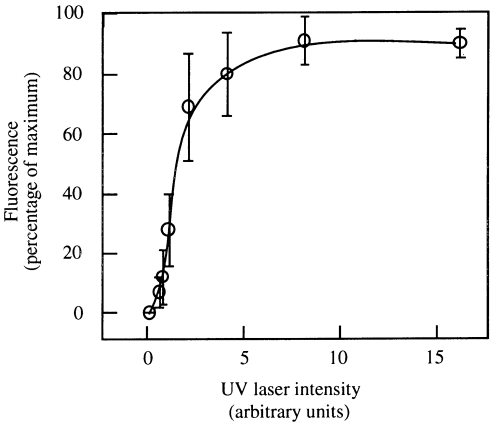


Fig. 1. Effect of UV illumination intensity on the uncaging of caged fluorescein. A gel, identical to that used to grow the *Arabidopsis* seedlings but supplemented with 10 μM caged fluorescein was placed on the stage of the Zeiss LSM 410 UV confocal microscope and illuminated using the UV laser of the confocal microscope set at a range of power levels. The amount of uncaged fluorescein was monitored by the appearance of fluorescence monitored at 515–540 nm, excitation 488 nm using the phototmulitplier detectors of the confocal microscope. 100% photoactivation was the fluorescence intensity produced when repeated UV illumination led to no further increase in fluorescence intensity. Results show mean ± SE, $n = 5$

Results

Effect of photoactivation of caged Br-A23187 on root hair cytoplasmic Ca^{2+} levels. To directly test whether the tip-focused $[Ca^{2+}]_c$ gradient could drive root hair growth we manipulated $[Ca^{2+}]_c$ using the caged calcium ionophore Br-A23187 and observed its effects on growth. Caged compounds are biologically inactive until photoactivated with UV light. Thus caged Br-A23187 is inactive as a Ca^{2+}-ionophore until illuminated with UV light. We reasoned that a localized UV irradiation would activate the ionophore, leading to a localized influx of Ca^{2+} either across the plasma membrane or from internal stores. Increasing the intensity of illumination should also activate more ionophore and consequently induce a larger Ca^{2+} influx. We were able to define the intensity of the UV scan as well as the area of illumination using the UV laser of the confocal microscope. Figure 1 shows a calibration of the system using caged fluorescein. Caged-fluorescein is non-fluorescent until photoactivated. Production of fluorescein therefore indicates the efficiency of UV photoactivation. Using this approach we were able to demonstrate that the single 0.7-s scan of the UV laser released 1–5% of the caged molecules, and that increasing the intensity of UV illumination led to increased photoactivation to saturation at approximately 4 times the intensity of the 0.7-s pulse (Figure 1). Although the caging group on the fluorescein molecule is not identical to that of caged Br-A23187, caged fluorescein has been successfully used to monitor the efficiency of uncaging of a variety of caged compounds (Allan et al. 1995; Gilroy 1996). The data in Fig. 1 indicate that the intensity of the UV irradiation can be used to establish a quantitative control over the amount of caged compound that is

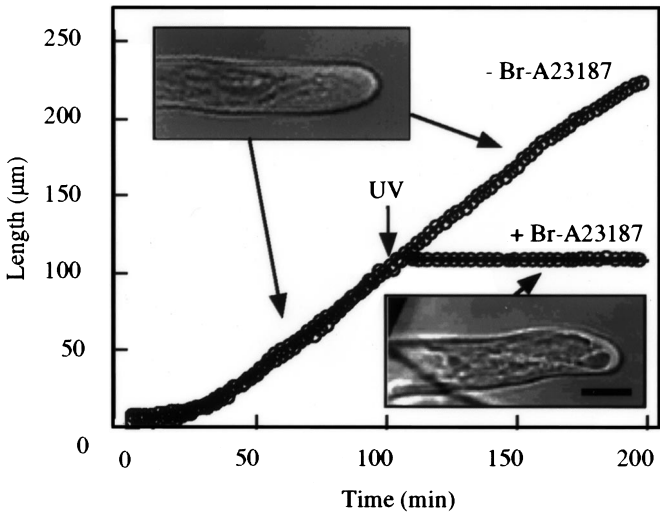


Fig. 2. Effect of photoactivation of caged Br-A23187 on tip growth of *Arabidopsis* root hairs. Roots were incubated in 20 μM caged Br-A23187 and maintained on the stage of the LSM 410 confocal microscope. Images were taken every 2.5 min and roots illuminated with 8 s of UV light at the indicated time. Control root hairs were treated identically but without caged ionophore (-Br-A23187). These are representative images of 14 independent experiments. Bar = 10 μm

photoactivated. Thus increased UV irradiation of caged Br-A23187 should lead to increased ionophore activation.

Having established the quantitative control of the uncaging of caged compounds by the UV laser of the confocal microscope, we applied this approach to manipulating root hair and pollen tube $[Ca^{2+}]_c$. Roots were treated with 20 μM caged Br-A23187 and subjected to a range of intensities and spatial patterns of UV illumination. Figure 2 shows that UV illumination of root hairs in the absence of the caged ionophore had no effect on root hair growth rate. Similarly, the loading of root hairs with caged ionophore but without UV illumination did not change their growth rate, direction or morphology (Fig. 2, Table 1). Figure 3A shows that a single 8-s pulse of UV light that illuminated the entire apex of the growing root hair led to an increase in the apical root hair $[Ca^{2+}]_c$ to more than 1 μM. This

Table 1. The effect of UV illumination on the growth rate of *Arabidopsis* root hairs loaded with, or without caged Br-A23187. Root hairs were treated with 20 μM caged Br-A23187 and subjected to 8 s of UV illumination. Growth rate was monitored over 50 min prior to UV illumination (not UV-illuminated) and 50 min after UV illumination (UV-illuminated). Note a single 8-s pulse of UV illumination applied to ionophore-treated root hairs led to complete inhibition of growth. Results represent mean ± SE

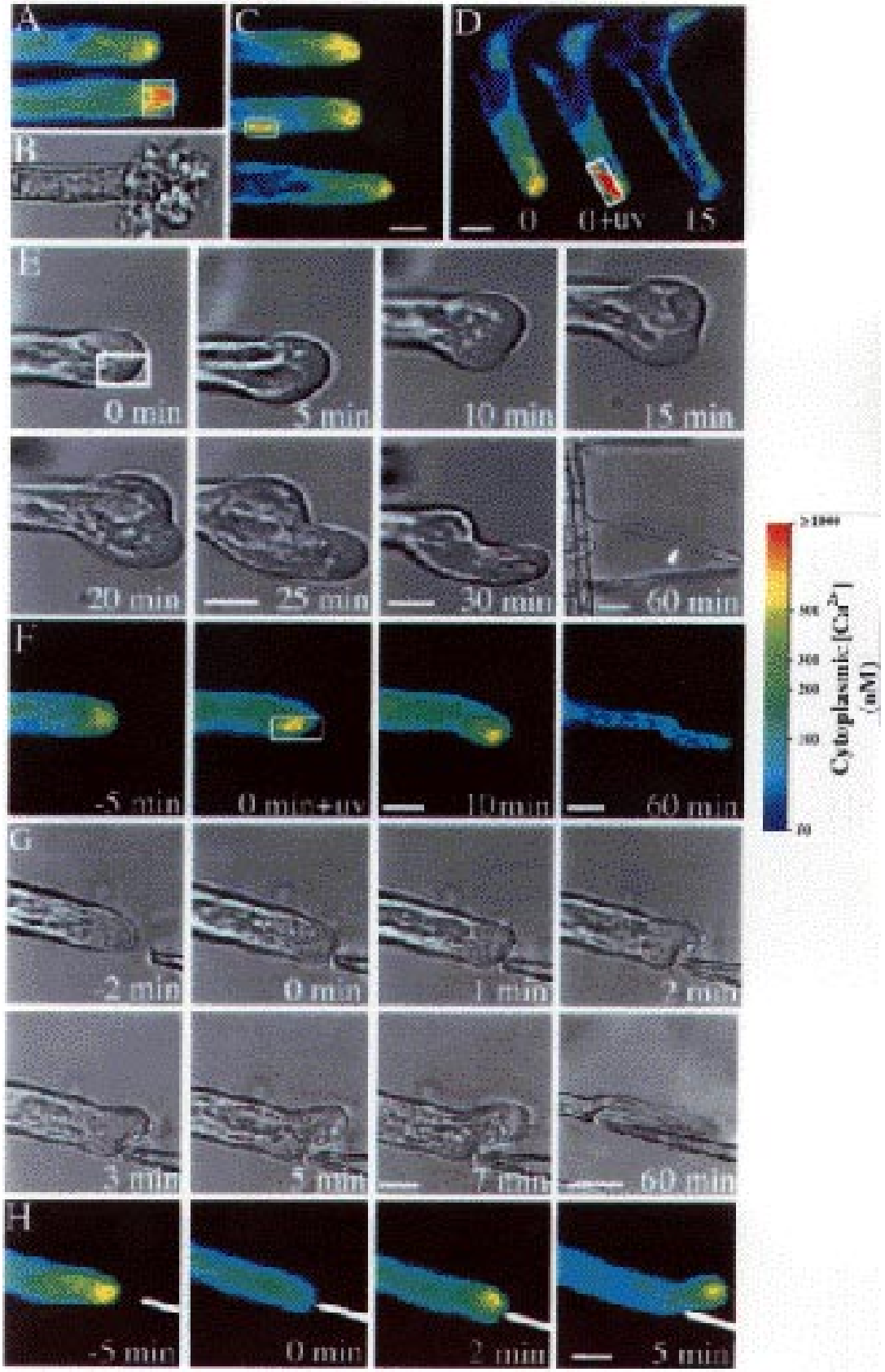
	Growth rate (μm · min^{-1}			
	Not UV-illuminated		UV-illuminated	
+Br-A23187	1.29 ± 0.19[a]	$n = 8$	0.00 ± 0.00	$n = 7$
−Br-A23187	1.3 ± 0.15[a]	$n = 10$	1.32 ± 0.18[a]	$n = 8$

[a]not significantly different. Student test, $P < 0.05$

increase in Ca^{2+} was associated either with inhibition of growth and vacuolation at the tip of growing root hairs (62.5%, $n = 32$; Fig. 2), or bursting of the tip within 1–2 min (Fig. 3B). Vacuolation and inhibition of apical growth were also observed when 1 μM non-caged Ca^{2+} ionophore Br-A23187 was added to root hairs (data not shown).

Although photoactivation of the caged Br-A23187 results in release of free Br-A23187, we were concerned that the observed effects of the UV illumination could have been related to the photolysis procedure rather than release of the ionophore. We therefore repeated the ionophore experiment outlined above but using caged fluorescein instead of caged Br-A23187. Photolysis of the caged-fluorescein did not lead to elevated levels of $[Ca^{2+}]_c$ (Fig. 4) or any observable effects on root hair growth, orientation or morphology (Fig. 5). Likewise, photoactivation of 20 μM caged ATP failed to cause changes in $[Ca^{2+}]_c$ or growth (data not shown). The 1-(4,5-dimethoxy-2-nitrophenyl)ethyl ester (DMNPE) caging group on the ATP molecule is identical to that of the caged Br-A23187 and should release the same byproducts during photoactivation. The lack of an effect of photoactivating caged-ATP suggests that any observed effects of caged ionophore on cytoplasmic $[Ca^{2+}]_c$ or growth are unlikely to be due to the side effects of the photolysis procedure.

Fig. 3A–H. The effect of UV activation of caged Br-A23187 and touch stimuli on growth and $[Ca^{2+}]_c$ in root hairs of *Arabidopsis*. **A** The effect of an 8-s UV pulse applied to the whole apical region of the growing root hair on apical $[Ca^{2+}]_c$. *Upper image*, 2 min before illumination; *lower image*, UV-illuminated within the region defined by box. Note that after ionophore photoactivation with UV light, $[Ca^{2+}]_c$ in the apical region is dramatically increased. Representative view of 7 root hairs from 6 individual roots. **B** Light image of tip bursting induced by photoactivation of caged Br-A23187 as in **A**. **C** The effect on $[Ca^{2+}]_c$ of localized asymmetrical UV illumination (*box*) for 0.7 s, 20 μm behind the growing apex of a root hair which was treated with 20 μM caged Br-A23187. Note that the tip-focused $[Ca^{2+}]_c$ gradient is present at all times, and that the root hair continues growth in the same direction as before illumination. Images represent 0 min (*upper*), 0 min + UV (*middle*), 5 min after illumination (*lower*). Representative view of 12 root hairs from 10 individual roots. **D** The effect of extensive (8 s) asymmetrical UV illumination at the tip (*box*) of a growing root hair loaded with caged Br-A23187. Time represents minutes after UV illumination. Representative view of 9 root hairs from 4 individual roots. **E** Response of a root hair that was loaded with caged Br-A23187 and illuminated with a single 0.7-s UV scan to the side of the apex (*box*). Time represents minutes after UV illumination. *Arrowhead* in the 60-min time point shows the site of UV irradiation. **F** Confocal ratio images of $[Ca^{2+}]_c$ at representative time points through the response shown in **E**. Time represents minutes after UV illumination. **G** The effect of touch stimulus applied by placing a micropipette in the path of the root hair's growth. Note the root hair transiently reoriented the direction of growth and then continued to grow in the original direction. Representative view of 19 root hairs from 11 individual roots. **H** Confocal ratio images of $[Ca^{2+}]_c$ at representative time points through the response shown in **G**. Time represents minutes after application of touch stimuli. $[Ca^{2+}]_c$ in the ratio images have been pseudo-color-coded according to the inset scale. Bars = 5 μm except for **E–G** at 60 min where the Bar = 15 μm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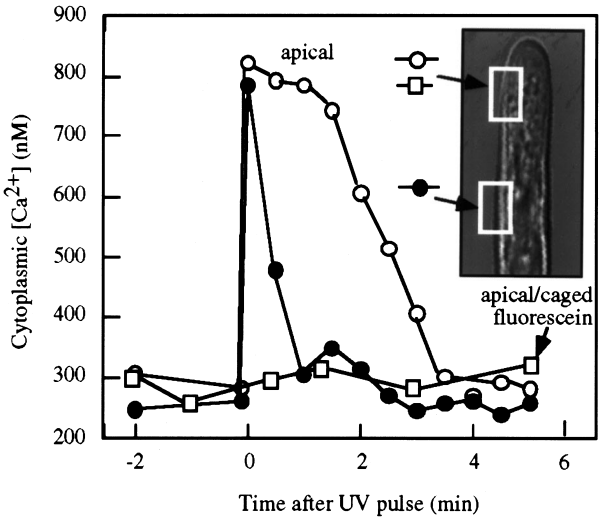


Fig. 4. Kinetics of the change in $[Ca^{2+}]_c$ induced by photoactivation of caged Br-A23187 and caged fluorescein in growing root hairs of *Arabidopsis*. Root hairs were microinjected with dextran-conjugated calcium green and rhodamine, and cytoplasmic $[Ca^{2+}]$ monitored by confocal ratio imaging. After treatment with 20 μM caged Br-A23187, the root hair was subjected to 0.7 s UV illumination localized to the site indicated (*box*) approximately 20 μm from the tip (●), or apical region (○) of the root hair, and the effect on $[Ca^{2+}]_c$ at this site monitored. Control root hairs were treated identically except that caged fluorescein was substituted for caged ionophore (□). Note that the photoactivation of caged fluorescein does not lead to change in $[Ca^{2+}]_c$. Representative traces of at least 8 root hairs per treatment

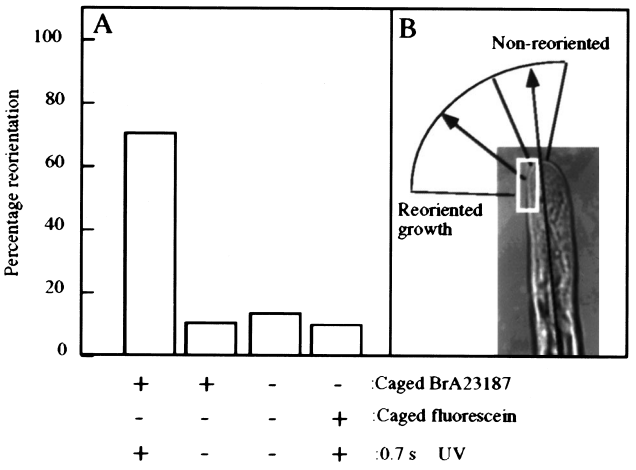


Fig. 5A,B. Reorientation of *Arabidopsis* root hairs by release of caged Br-A23187. Root hairs were treated with either 20 μM caged Br-A23187, or caged fluorescein and the caged compounds photoactivated with a single 0.7-s pulse of the UV laser on the confocal microscope limited to the side of the tip (*box* in **B**). The orientation of root hair growth was then assessed. Root hairs were classed as showing no reorientation if they grew within a 10 degree angle of the pre-UV photoactivation orientation of growth **(B)**. Reoriented root hairs showed at least 5 μm of new growth to within a 90 degree cone centered to the side of illumination

Localized photoactivation of caged Br-A23187 redirects root hair growth. Having established that the caged ionophore could lead to an increase in $[Ca^{2+}]_c$, we next limited the photoactivation of caged Br-A23187 to different regions of root hairs. Figure 3 (A, C, D, F) indicates that photoactivation of the caged ionophore was accompanied by an elevated $[Ca^{2+}]_c$ limited to the region of UV illumination.

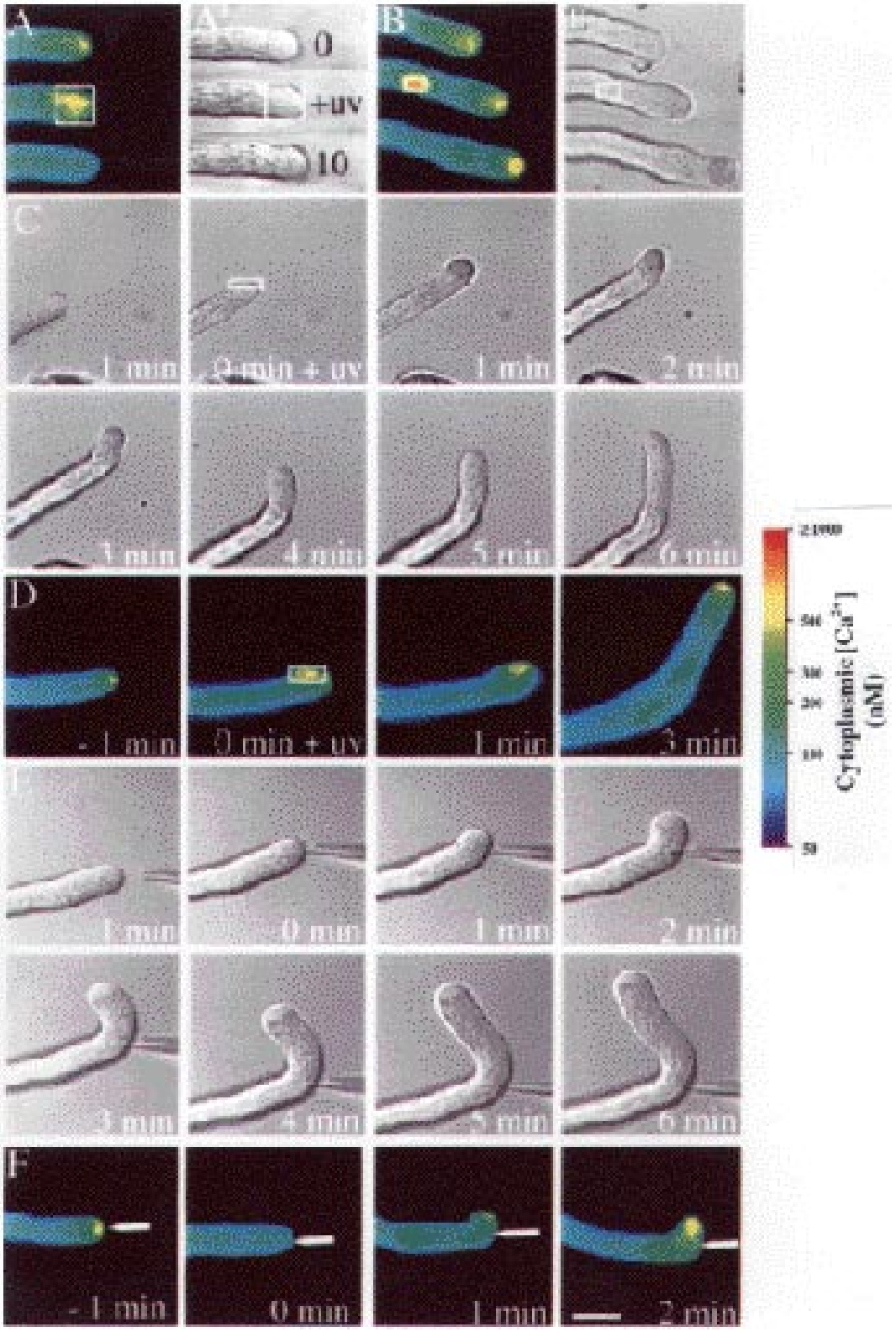
It was found that generating $[Ca^{2+}]_c$ gradients that did not closely mimic endogenous gradients was ineffective at reorienting growth. Thus, generation of localized lateral $[Ca^{2+}]_c$ gradients further than 5 μm from the tip did not affect the orientation of root hair elongation or growth rate (Fig. 3C, $n = 12$). The gradients generated greater than 5 μm behind the tip were also much shorter lived than those generated at the tip (see below), being sustained for only 1–2 min (Fig. 4). These differences may reflect the variations in cytoplasmic streaming patterns between the tip and the lateral cytoplasm of the root hair, and/or differential regulation of $[Ca^{2+}]_c$ at different places within the root hair cytoplasm.

Use of a UV scan of more than 0.7 s led to lateral $[Ca^{2+}]_c$ gradients larger than the endogenous gradients normally seen in growing root hairs, ranging from >1.5 μM at the site of illumination to 200–300 nM at the opposite side of the tip. Reorientation of growth was not observed under these conditions. Instead these large lateral gradients generally led to cessation of growth and vacuolation at the root hair tip, or bursting (Fig. 3D, $n = 9$).

However, when an asymmetrical gradient in $[Ca^{2+}]_c$ was applied by uncaging Br-A23187 on one side of root

Fig. 6A–F. The effect of UV activation of caged Br-A23187 and touch stimuli on growth and $[Ca^{2+}]_c$ in pollen tubes of *Tradescantia*. **A** The effect on $[Ca^{2+}]_c$ of an 8-s UV pulse applied to the whole apical region of the elongating pollen tube. The pollen tube was illuminated with 8 s of UV light in the region defined by the box. Note after ionophore photoactivation with UV light, $[Ca^{2+}]_c$ in the apical region is dramatically increased and pollen tube elongation ceases. Representative view of 6 pollen tubes. **A′** shows corresponding light images, which are representative of 27 pollen tubes. **B** The effect of localized asymmetrical UV illumination (*box*) of a pollen tube treated with 20 μM caged Br-A23187. The pollen tube was illuminated 30 μm behind the growing apex for 0.7 s. Note that a tip-focused $[Ca^{2+}]_c$ gradient is present at all times and that the pollen tube continues growth in the same direction as before illumination. **B′** shows corresponding light images, which are representative of 11 pollen tubes. **C** Response of a pollen tube that was loaded with caged Br-A23187 and illuminated with a single 0.7-s UV scan to the side of the apex (*box*). Time represents minutes after UV illumination. Note the sustained reorientation of growth to the side of illumination. **D** Confocal ratio images of $[Ca^{2+}]_c$ at representative time points through the response shown in **C**. Time represents minutes after UV illumination. Representative view of 7 pollen tubes. **E** The effect of touch stimulus applied by placing a micropipette in the path of the pollen tube growth. Representative view of 14 pollen tubes. **F** Confocal ratio images of $[Ca^{2+}]_c$ at representative time points through the response shown in **E**. Representative view of 7 pollen tubes. For confocal ratio imaging, pollen tubes were co-injected with calcium green-2 and rhodamine conjugated to 10 kDa dextrans. Calcium green and rhodamine fluorescence were simultaneously monitored using the confocal microscope. The cytoplasmic calcium concentrations in the ratio images have been pseudo-color-coded according to the inset scale. Bright-field images were taken using the transmission detector of the confocal microscope with 633 nm illumination. Bars = 10 μm

hair apex with a single 0.7-s scan of the laser, 70% ($n = 40$) of root hairs changed the direction of apical growth towards the illuminated side i.e. towards the site of elevated Ca^{2+} (Figs. 3E,F and 5). The $[Ca^{2+}]_c$ gradient ranged from approximately 700–1000 nM at the side of illumination to 200–300 nM at the opposite side of the root hair tip. This is similar in magnitude to the endogenous $[Ca^{2+}]_c$ gradients seen in root hair tips (e.g. compare Fig. 3F before and after UV illumination) and was sustained at the tip for 3–5 min before returning to the prephotoactivated $[Ca^{2+}]_c$ distribution (Fig. 4). Fig. 3E,F shows that although the generation of an



asymmetrical gradient in $[Ca^{2+}]_c$ did reorient growth, this was transient, with the growth returning to the original direction within 15 min.

Asymmetrical illumination of root hairs in the absence of caged ionophore or in the presence of 20 μM caged fluorescein did not have any effect on the $[Ca^{2+}]_c$ (Fig. 4) or the direction or rate of apical growth (Table 1, Fig. 5). These results suggest non-specific effects of the photolysis procedure were unlikely to account for the $[Ca^{2+}]_c$ gradient generated or its transient effects on the orientation of growth.

Touch leads to a transient root hair reorientation. Having established that the direction of root hair growth changes only transiently to imposed apical $[Ca^{2+}]_c$ gradients, we next tried to induce a sustained root hair reorientation using a touch stimulus. A micropipette was placed in the path of a root hair's growth and the subsequent effect observed. As shown in Fig. 3G, in all cases ($n = 19$) upon encountering the mechanical barrier of the micropipette, the root hairs showed a reorientation of their growth and grew sideways past the blockage. However, this lateral orientation of growth was not sustained and after clearing the obstacle growth reoriented and continued in the original direction (Fig. 3G). These results again suggest the direction of root hair growth is highly regulated to elongate away from the root.

Confocal ratio imaging revealed that reorientation in response to the touch stimulus was accompanied by a transient decrease in the tip-focused $[Ca^{2+}]_c$ gradient coincident with the tip encountering the blockage, and a subsequent re-establishment of a $[Ca^{2+}]_c$ gradient to the site of new lateral growth (Fig. 3H). Upon reinstatement of the original axis of growth, the $[Ca^{2+}]_c$ gradient was likewise reoriented to the original direction.

Characterizing the angle of root hair projection from the root surface. As root hairs seem to be continually reset to grow in their original direction of growth, we characterized how well regulated this initial growth angle was. Under our growth conditions, the angle at which root hairs projected from the epidermal surface was highly regular at 85 ± 6.7 degrees ($n = 84, 9$ separate roots). These root hairs also normally grew straight with deviations from the initial angle of growth being very rare. The mean deviation in growth angle along the length of the root hair from straight growth was 0 ± 0.6 degrees ($n = 41, 10$ separate roots).

Effect of localized photoactivation of Br-A23187 on pollen tube $[Ca^{2+}]_c$ gradient and orientation. The data presented above suggest that the $[Ca^{2+}]_c$ gradient in root hairs can only transiently reorient growth. Work on pollen tubes, however, has shown that the $[Ca^{2+}]_c$ gradient itself can lead to a sustained reorientation of growth (Malho and Trewavas 1996). We therefore repeated the experiments described above on in-vitro-germinated pollen tubes to determine whether this difference in behavior was due to the caged-ionophore approach we had used to generate Ca^{2+} gradients or to an inherent difference between the control of pollen tube and root hair elongation.

Tradescantia pollen tubes were microinjected with dextran-coupled calcium-green-2 and rhodamine, and $[Ca^{2+}]_c$ levels determined by confocal ratio imaging. Figure 6 (A,B,D,F) shows that, similar to previous reports (Rathore et al. 1991; Miller et al. 1992; Malho et al. 1994, 1995; Pierson et al. 1994, 1996; Malho and Trewavas 1996), we could observe a tip-focused Ca^{2+} gradient in growing pollen tubes. Treatment with caged ionophore, but without UV activation, did not affect pollen tube growth kinetics or morphology (data not shown).

Similar to our observations on root hairs, generation of lateral gradients in $[Ca^{2+}]_c$ that did not closely mimic this endogenous gradient in position or magnitude did not lead to reorientation of pollen tube growth. For example, illuminating the whole tip of pollen tubes treated with caged-ionophore led to an increased tip-focused $[Ca^{2+}]_c$ gradient which was larger than the endogenous gradients (Fig. 6A). This gradient subsided within 2–3 min leaving no detectable elevated tip-focused gradient, and no further growth for as long as we followed the pollen tube (at least 30 min, 63%, $n = 8$). In the remaining 37% of pollen tubes this unnaturally high tip-focused gradient led to rapid bursting at the tip within seconds.

When asymmetrical photolysis of caged Br-A23187 was performed more than 15 μm away from the tip, a lateral gradient in $[Ca^{2+}]_c$ was generated but did not affect the subsequent orientation of growth (Figs. 6B, 7). However, when a lateral gradient in $[Ca^{2+}]_c$ was generated across the apical part of growing *Tradescantia* pollen tubes, a sustained reorientation of the pollen tube was induced in the direction of ionophore uncaging in 91% of tubes examined, $n = 22$ (Figs. 6C,D and 8). Figure 7 shows that the asymmetrical $[Ca^{2+}]_c$ generated in the tip of the pollen tube was sustained. In contrast, the gradients generated further from the tip were transient in nature, lasting 2–3 min.

UV illumination of pollen tubes treated without caged ionophore or incubated with caged fluorescein did not affect cytoplasmic $[Ca^{2+}]_c$ or lead to the highly reproducible reoriented growth seen with the caged ionophore ($n = 13$, Figs. 6, 8).

Touch leads to sustained reorientation of pollen tubes. Touch also led to a sustained, predictable reorientation in the pollen tube (Fig. 6E). In all cases ($n = 25$), upon touching the micropipette, the pollen tubes reoriented their growth and grew sideways past the blockage. This reorientation was accompanied by a transient decrease in the apical $[Ca^{2+}]_c$ gradient as the pollen tube touched the mechanical blockage. The gradient was then reestablished focused to the site of new growth (Fig. 6F). This reorientation and $[Ca^{2+}]_c$ gradient was sustained and did not return to the pretouch direction of growth (Fig. 6E).

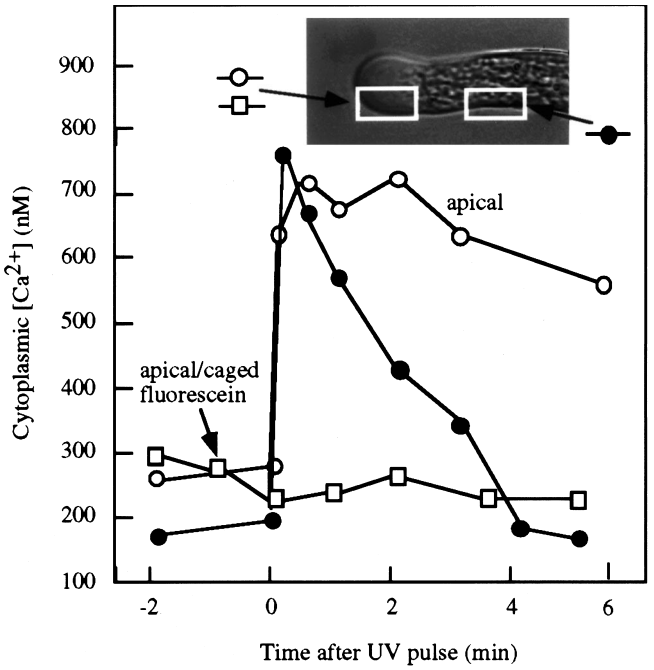


Fig. 7. Kinetics of the change in $[Ca^{2+}]_c$ induced by photoactivation of caged Br-A23187 on pollen tubes. Pollen tubes were microinjected with dextran-conjugated calcium green and rhodamine, and $[Ca^{2+}]_c$ monitored by confocal ratio imaging. After treatment with 20 μM caged Br-A23187, the pollen tube was subjected to 0.7 s UV illumination localized to the apex (○) or approximately 20 μm from the tip (●), and the effect on $[Ca^{2+}]_c$ at this site monitored. Control pollen tubes were treated identically except that caged fluorescein was substituted for caged ionophore (□). Representative traces of at least 9 pollen tubes per treatment

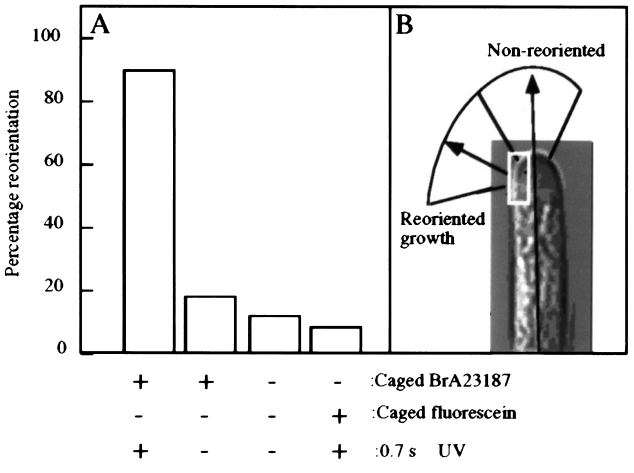


Fig. 8A,B. Reorientation of pollen tubes by release of caged Br-A23187. Pollen tubes were treated with either 20 μM caged Br-A23187, or caged fluorescein and the caged compounds photoactivated with a single 0.7-s pulse of the UV laser on the confocal microscope limited to the side of the tip (*box* in **B**). The orientation of pollen tube growth was then assessed. Pollen tubes were classed as showing no reorientation if they grew within a 20 degree angle of the pre-UV photoactivation orientation of growth **(B)**. Reoriented pollen tubes showed new growth to within a 90 degree cone centered to the side of illumination. Although pollen tubes grown in vitro do show spontaneous growth reorientations, the highly reproducible reorientation to the side induced by localized UV activation cannot be accounted for by such spontaneous reorientations. Results are the average of more than 20 pollen tubes per treatment

Discussion

Root hairs grow by extension at their tip, in which the region of growth is highly limited to the apex of the elongating cell. The nature of the positional information localizing this region of growth is unknown but the tip-focused gradient in $[Ca^{2+}]_c$ observed in apically growing cells (e.g. algal rhizoids: Brownlee and Pulsford 1988; pollen tubes: Reiss and Herth 1979; Miller et al. 1992; Pierson et al. 1994, 1996; Malho et al. 1995; root hairs: Herrmann and Felle 1995; Felle and Hepler 1997; Wymer et al. 1997) has received intensive study as a potential determinant of unidirectional growth. We therefore reoriented this tip-focused gradient in root hairs and pollen tubes to test whether the gradient could drive tip growth in vivo, and also whether it was a determinant of the direction of tip growth in situ in the plant. Using controlled release of the caged Ca^{2+}-ionophore Br-A23187, gradients of $[Ca^{2+}]_c$ were generated in the tips of growing root hairs and pollen tubes. Generation of such an asymmetrical gradient in $[Ca^{2+}]_c$ across the tip reoriented root hair and pollen tube growth towards the site of elevated $[Ca^{2+}]_c$. However, for root hairs the reorientation was transient, and growth returned to the original direction over 10–15 min (Fig. 3E). Reorientation was highly dependent on matching the spatial extent and magnitude of the endogenous gradients. Generating gradients away from the apex of the growing tip or gradients more extensive in magnitude than the tip-focused gradients normally observed in pollen tubes or root hairs, did not lead to reorientation of growth and usually inhibited elongation. These results suggest the $[Ca^{2+}]_c$ gradient localized to the tip of the growing pollen tube or root hair can provide the positional information to localize growth at the tip. However, for root hairs additional factors must be orienting growth.

The UV pulse used to uncage the Br-A23187 and generate the $[Ca^{2+}]_c$ gradient in root hairs was less than 1 s in duration, yet the $[Ca^{2+}]_c$ gradients so generated were sustained for up to 5 min (Fig. 4). This suggests that either diffusion of ionophore is very limited in the membrane at the tip, leading to a sustained localized influx, or that once the $[Ca^{2+}]_c$ gradient is established, it has some self-sustaining characteristics. For example, the elevated Ca^{2+} gradient may be regulating Ca^{2+} channels and pumps to maintain and reinforce this localized increase. Such a mechanism has been proposed in the self-electrophoretic model of polarity establishment in algal zygotes (Jaffe et al. 1974). Elevated $[Ca^{2+}]_c$ could then drive docking and fusion of exocytotic vesicles at the tip of the elongating cell. Elevated $[Ca^{2+}]_c$ has been shown to facilitate secretory activity in plants (Zorec and Tester 1992; Gilroy 1996) and by analogy with the elements of the mammalian and yeast secretory systems, Ca^{2+}-dependent events in such steps as secretory vesicle docking and fusion with the plasma membrane are likely to occur (reviewed in Battey and Blackbourn 1993). Calcium-binding proteins, e.g. annexins, are prime candidates for factors that could mediate these events, and annexins have been localized

to the tip of lily pollen tubes (Blackbourn et al. 1992). Interactions between the cytoskeleton and a $[Ca^{2+}]_c$ gradient may also mediate regulation of tip growth by Ca^{2+} (Kropf 1994). Thus a localized, tip-focused, self-sustaining $[Ca^{2+}]_c$ gradient could localize secretion and growth at the apex of tip-growing cells.

However, several lines of evidence suggest that the $[Ca^{2+}]_c$ gradient is not the primary determinant of the direction of root hair elongation. Root hairs grow at a precise angle from the root and continue at this angle until fully elongated. Artificially setting a new lateral gradient in $[Ca^{2+}]_c$, using caged ionophore, only led to a transient reorientation of growth before the normal root hair polarity was re-established (Fig. 3E,F). Similarly, the results of using touch to halt and reorient root hair growth show that although transient reorientation in growth direction can occur, the direction is continually being reset to the initial direction relative to the axis of the main root. The tip-focused $[Ca^{2+}]_c$ gradient is always focused to the point of elongation at the tip (Figs. 3F, 6D), implying its role in this process. However, the direction of growth is not simply directed by the site of this $[Ca^{2+}]_c$ gradient.

In contrast to root hairs, we found that pollen tubes showed a sustained reorientation in response to the same two treatments (touch and artificial Ca^{2+} gradient). When a $[Ca^{2+}]$ gradient was artificially formed, pollen tube growth was reoriented to the new site of elevated $[Ca^{2+}]_c$. In this case, once established in a new direction, the tip-focused $[Ca^{2+}]_c$ gradient and growth was sustained in this new orientation. Malho and Trewavas (1996) have similarly shown that creation of a lateral gradient in $[Ca^{2+}]_c$ across the tip of a pollen tube is sufficient to cause sustained reorientation of pollen tube growth to the side of elevated $[Ca^{2+}]_c$. Manipulating $[Ca^{2+}]_c$ elsewhere in the tube had lesser effects. As in our experiments, Malho and Trewavas (1996) induced lateral $[Ca^{2+}]_c$ gradients by caged-probe technology, but the caged probes used (caged Ca^{2+} and caged Ca^{2+}-chelator) were chemically different from the caged ionophore used here. Caged Ca^{2+} and caged Ca^{2+}-chelator induced a $[Ca^{2+}]_c$ gradient by directly changing cytoplasmic $[Ca^{2+}]_c$. In our experiments the increase in $[Ca^{2+}]_c$ was mediated through a localized influx into the cytoplasm, either across the plasma membrane and/or from organelles, caused by ionophore photoactivation. In addition, the pollen tubes studied were from a different species (*Agapanthus umbelliferus*) than the *Tradescantia virginiana* used here. Thus the sustained reorientation of pollen tubes induced by the $[Ca^{2+}]_c$ gradient seems to be a general phenomenon not linked to pollen source or the chemical nature of the probe used to produce the change in $[Ca^{2+}]_c$.

The guidance mechanisms of pollen tube growth in vivo in the stigma are unknown and it is likely that in the stigmatic tissue other factors will direct the $[Ca^{2+}]_c$ gradient and so control tube elongation. Pollen tube growth in vitro is likely to be divorced from these normal directional cues. Pollen tubes naturally grow towards the ovule but this directional growth is disrupted by addition of Ca^{2+} to the growth media (Mascarehnas and Machlis 1962), leading Malho and Trewavas (1996) to suggest that pollen tubes could possess sensors of extracellular $[Ca^{2+}]$, as seen in animal cells (Brown et al. 1995). These sensors could orient pollen tube elongation to an apoplasmic $[Ca^{2+}]$ gradient in the stigma. However, there is no direct evidence to date for the existence of either a $[Ca^{2+}]$ gradient inside the ovule, or molecular data identifying Ca^{2+} sensing receptors on the pollen tubes. Alternative positional information may be relayed through the pollen tube sensing cell surface features, as shown to be possible for fungal hyphae (Correa et al. 1996), or sensing electrical gradients in the stigma (Malho et al. 1994).

Clearly in root hairs, growth is being directed by factors other than the apical $[Ca^{2+}]_c$ gradient. These factors lead to a predefined orientation of root hair growth relative to the main root axis. The mechanism that allows the root hairs to sense their growth angle and orient themselves in relation to the root surface is unknown. Electrical fields around the root and root hairs are one candidate for this positional information. Electrical currents have been observed around growing roots and root hairs (Weisenseel et al. 1979) and could effect the distribution of open Ca^{2+} channels, as hypothesized for the self-sustaining Ca^{2+} gradient in algal zygote polarization (Kropf 1994). Tip-growing systems such as pollen tubes and fungi have been shown to exhibit both positive and negative galvanotropism (pollen tubes: Malho et al. 1994; fungi: Lever et al. 1994). In pollen tubes' galvanotropic growth was demonstrated to effect the direction of the tip-focused $[Ca^{2+}]_c$ gradient, and it has been proposed that the pollen tube in vivo may orient its growth in response to the small potential gradient down the stigma (Malho et al. 1994). Galvanotropic growth may be mediated through voltage-gated Ca^{2+} channels clustered at the growing tip (Garril et al. 1993). Circumstantial evidence for clustering of open Ca^{2+} channels at the apex of growing root hairs and pollen tubes arises from observations of enhanced Ca^{2+} influx at their tips, monitored by vibrating microprobe (e.g. pollen tubes: Pierson et al. 1994; root hairs: Schiefelbein et al. 1992; Herrmann and Felle 1995; Jones et al. 1995), and enhanced Mn^{2+}-quench kinetics focused at the tip of pollen tubes (Malho et al. 1995) and root hairs (Wymer et al. 1997). The distribution and regulation of Ca^{2+} channels and the galvanotropic behavior of root hairs remains unknown and certainly worthy of future investigation.

In summary, we have observed that the direction of root hair growth is continually reset relative to the axis of the root whereas pollen tubes grown in vitro may lack the normal external factors that direct their growth. The tip-focused $[Ca^{2+}]_c$ gradient of root hairs is closely correlated with the process of tip growth where it appears to act as part of the machinery localizing secretory activity to the apex of the cell. The bursting associated with the generation of an unnaturally high tip-focused Ca^{2+} gradient (Fig. 3B) may well reflect an induction of a sudden, excessive burst of secretion, leading to disruption of the tip. Even though the tip-focused $[Ca^{2+}]_c$ gradient can transiently orient the

direction of growth in root hairs, the gradient in itself is not the primary determinant of growth direction. As yet unknown factors direct this Ca^{2+}-dependent growth and maintain a fixed angle of growth away from the root surface. The angle of root hair emergence from the root surface is likely to be highly regulated and vary between species (Row and Reeder 1957). Such maintenance of an angle of approximately 90 degrees from the *Arabidopsis* root surface may well help ensure the root hairs explore the greatest volume of soil and extend the limits of the rhizosphere to their maximum.

The authors thank Sian Ritchie and Elison Blancaflor for critical reading of the manuscript. This work was supported by the USDA/DOE/NSF Interdisciplinary Research Training Group in Advanced Root Biology (NSF BIR-9220330) and grants to SG from DOE (93ER79239) and NSF (9513991).

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