Running Head: Ca$^{2+}$ and Root Hair Growth

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Imaging of the Yellow Cameleon 3.6 Indicator Reveals that Elevations in Cytosolic Ca$^{2+}$ Follow Oscillating Increases in Growth in Root Hairs of *Arabidopsis thaliana*

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ABSTRACT

In tip growing cells, the tip-high Ca²⁺ gradient is thought to regulate the activity of components of the growth machinery including the cytoskeleton, Ca²⁺-dependent regulatory proteins and the secretory apparatus. In pollen tubes, both the Ca²⁺ gradient and cell elongation show oscillatory behavior reinforcing the link between the two. We report that in growing root hairs of Arabidopsis thaliana, an oscillating tip-focused Ca²⁺ gradient can be resolved through imaging of a cytosolically expressed Yellow Cameleon (YC) 3.6 FRET-based Ca²⁺ sensor. Both elongation of the root hairs and the associated tip-focused Ca²⁺ gradient show a similar dynamic character, oscillating with a frequency of 2-4 min⁻¹. Cross-correlation analysis indicates that the Ca²⁺ oscillations lag the growth oscillations by 5.3 ± 0.3 s. However, growth never completely stops, even during the slow cycle of an oscillation, and the concomitant tip Ca²⁺ level is always slightly elevated compared to the resting Ca²⁺ concentration along the distal shaft, behind the growing tip. Artificially increasing Ca²⁺ using the Ca²⁺ ionophore A23187 leads to immediate cessation of elongation and thickening of the apical cell wall. In contrast, dissipating the Ca²⁺ gradient using either the Ca²⁺ channel blocker La³⁺ or the Ca²⁺ chelator EGTA is accompanied by an increase in the rate of cell expansion and eventual bursting of the root hair tip. These observations are consistent with a model where the maximal oscillatory increase in cytosolic Ca²⁺ is triggered by cell expansion associated with tip growth and plays a role in the subsequent restriction of growth.
Introduction

Tip growth of cells such as fungal hyphae, algal rhizoids, pollen tubes and root hairs is sustained by targeted secretion of new membrane and wall material to the apical few micrometers of their elongating tips. Turgor is then thought to drive expansion at the cell apex, with the subapical wall resisting these expansive forces. The combination of localized secretion coupled to regulation of wall properties would then lead to the elongated cylindrical morphology of these cells (for review, see Gilroy and Jones, 2000).

The tip-focused Ca\textsuperscript{2+} gradient, characteristic of tip-growing cells, seems to play an important role in the spatial control of these systems. The cytosolic free Ca\textsuperscript{2+} concentration is approximately 100 nM at the base of the polarized cell but rises up to micromolar levels over the apical few micrometers of the expanding tip (reviewed in Bibikova and Gilroy, 2002). This elevated apical Ca\textsuperscript{2+} is proposed to provide a spatial determinant for growth by facilitating membrane fusion at the tip and regulating a host of Ca\textsuperscript{2+}-dependent proteins required for tip growth. Dissipating the Ca\textsuperscript{2+} gradient in pollen tubes, fungal hyphae and root hairs has been shown to disrupt growth (e.g., Clarkson et al., 1988; Miller et al., 1992; Herrmann and Felle, 1995; Wymer et al., 1997), whereas experimentally altering the direction of the gradient leads to redirected growth, with the site of new expansion following where the new Ca\textsuperscript{2+} gradient is imposed (e.g. Malho and Trewavas, 1996; Bibikova et al., 1997). Thus, there is strong evidence supporting a regulatory role for the Ca\textsuperscript{2+} gradient through imposing spatial control on the site of cell expansion in these tip-growing systems. Indeed, the structure of the apical actin cytoskeleton and its regulatory proteins (villins, gelsolins and actin depolymerizing factors; Smertenko et al., 1998; Tominaga et al., 2000; Allwood et al., 2001; Ketelaar et al., 2003; Fan et al., 2004) are thought to be regulated by the tip high Ca\textsuperscript{2+} (Yokota
et al., 2005). Similarly, there is evidence that annexins (Blackbourn et al., 1992; Clark et al., 1992; Carroll et al., 1998), phosphoinositide metabolism (Preuss et al., 2006), calmodulin and protein kinases (Moutinho et al., 1998; Yoon et al., 2006) also play roles in sustaining tip growth and are regulated via the cytosolic Ca\textsuperscript{2+} gradient.

Tip growth in some systems has been shown to oscillate with periods of rapid expansion alternating with slower growth rates. While this has been confirmed for different species of pollen tubes by independent groups (Pierson et al., 1995; Pierson et al., 1996; Messerli and Robinson, 1997; Watahiki et al., 2004; Hwang et al., 2005), it has not been repeated for tip growth in fungal hyphae (Lopez-Franco et al., 1994; Sampson et al., 2003). Oscillating growth in root hairs has only recently been reported (Monshausen et al., 2007) and is under further investigation in this report.

One of the most extensively studied tip growing system has been lily pollen tubes. In this system growth oscillations were accompanied by oscillations in the tip focused Ca\textsuperscript{2+} gradient (e.g. Holdaway-Clarke et al., 1997; Messerli and Robinson, 1997; Messerli et al., 2000; Watahiki et al., 2004). Interestingly, the periodic increases in the Ca\textsuperscript{2+} gradient actually lagged the periodic increases in growth by about 4 s (Messerli et al., 2000). These observations have led to a model for pollen tubes where a mechanically sensitive Ca\textsuperscript{2+} channel may be gated via membrane tension during elongation growth. This stretch-activated channel would then support a Ca\textsuperscript{2+} increase that follows rather than coincides with maximal cell elongation (Messerli and Robinson, 2007). It has even been suggested that a burst of secretion may precede elongation in Agapanthus pollen (Coelho and Malho, 2006). Lily pollen tubes in culture (reviewed in Messerli et al., 2000) grow 5-7 times faster than Arabidopsis root hairs in culture (Monshausen et al., 2007) and it is unclear whether an
equivalent relationship between oscillatory growth and Ca\textsuperscript{2+} changes exists for the root hair. Although a stretch-activated Ca\textsuperscript{2+} influx channel has been identified in pollen tubes (Dutta and Robinson, 2004), the Ca\textsuperscript{2+} channel sustaining the tip focused Ca\textsuperscript{2+} gradient in root hairs is thought to be gated by membrane voltage and reactive oxygen species (ROS; Foreman et al., 2003). Thus, whether fluctuations in the root hair apical Ca\textsuperscript{2+} gradient occur and are functionally important for regulating cell elongation remain unknown.

In this paper we report that root hairs of \textit{Arabidopsis} exhibit both oscillatory growth and oscillations in the tip-focused Ca\textsuperscript{2+} gradient. In addition, we show that the maximum of the Ca\textsuperscript{2+} gradient lags the growth maxima by approximately 5 s. Treatments that dissipate the Ca\textsuperscript{2+} gradient promote tip expansion and eventual bursting whereas artificially elevating cytosolic Ca\textsuperscript{2+} leads to rapid growth arrest. These results indicate that one role for the maximal Ca\textsuperscript{2+} levels attained during the oscillatory increase in cytosolic Ca\textsuperscript{2+} that accompany root hair growth is actually to limit turgor-driven expansion after each burst of elongation.

RESULTS

Oscillations in Cytosolic Ca\textsuperscript{2+} Accompany Root Hair Tip Growth

To determine the kinetics of the tip focused Ca\textsuperscript{2+} gradient in growing root hairs, we used \textit{Arabidopsis} plants stably transformed with a soluble version of the green fluorescent protein-based Ca\textsuperscript{2+} sensor, yellow cameleon (YC) 3.6 (Nagai et al., 2004), driven by the CaMV 35S promoter. Expression of this protein had no detectable effect on root hair growth rates, density or general morphology (Figure 1, and data not shown) indicating it provided an appropriate approach for analysis of root hair growth. We imaged the YC3.6 signal using a Zeiss LSM 510 confocal microscope and found that root hair elongation was sensitive to laser irradiation
intensity, with higher levels of irradiation leading to a minor but significant inhibition of elongation rates (reduction from 2.01±0.29 to 1.34±0.26 µm min⁻¹ at 60% laser attenuation n=6, student t-test P<0.001, see Materials and Methods for specifics of the imaging protocol). However, by using lower laser power (90% attenuation) we were able to monitor root hairs for extended periods (>10 min) with frequent sampling (images every 2 s) without significant alteration of root hair growth rate (1.85±0.33 µm min⁻¹, n=6, student t-test P=0.38) or morphology (Figure 1), allowing us to make measurements of root hair Ca²⁺ dynamics during growth.

As previously reported (reviewed in Bibikova and Gilroy, 2002), growing root hairs were characterized by a tip-focused Ca²⁺ gradient. Invariably, Ca²⁺ levels were highest within 1-2 µm of the extreme apex and then rapidly declined with increasing distance from the tip until reaching resting Ca²⁺ concentrations approximately 20 µm behind the apex (Figure 1A-C). While this gradient persisted as long as root hairs continued to grow, our high temporal resolution measurements showed that the magnitude of the gradient oscillated with a frequency of approximately 2-4 peaks per min (Figure 1B, C). The largest changes in cytoplasmic Ca²⁺ occurred at the extreme tip of the root hair whereas Ca²⁺ levels in more subapical regions oscillated in phase but with smaller amplitudes (Figure 1A, B).

We were interested in whether these changes in Ca²⁺ were associated with alterations in growth rate. Applying high resolution tip tracking software previously used to measure growth of Arabidopsis root hairs (Messerli et al., 1999; Monshausen et al 2007), we were able to confirm that growth rates of YC3.6 expressing root hairs oscillated at the same frequency of 2-4 peaks per min as apical Ca²⁺ levels (Figure 1D) and as in untransformed wild-type root hairs (Monshausen et al 2007). The magnitude of both the oscillations in Ca²⁺ and growth rate
were variable even within a single root hair (Figure 1B, supplemental figure 1). While we could not observe a clear relationship between the amplitude of growth peaks vs amplitude of Ca\textsuperscript{2+} peaks using linear regression analysis (supplemental Figure 1) our measurements indicated a close temporal relationship between cytoplasmic Ca\textsuperscript{2+} and growth where each burst of growth appeared to be followed by a rapid elevation in Ca\textsuperscript{2+} (Figure 1D). Cross correlation analysis comparing the temporal kinetics of Ca\textsuperscript{2+} and growth oscillations (Messerli et al., 2000) confirmed that growth peaks most likely preceded Ca\textsuperscript{2+} increases by 5.3 ± 0.3 s (Figure 1E).

In agreement with previous observations (e.g. Wymer et al., 1997), non-growing root hairs showed no sustained tip-focused Ca\textsuperscript{2+} gradient and no oscillations in Ca\textsuperscript{2+} levels could be detected (supplemental Figure 2).

**Blocking Ca\textsuperscript{2+} Entry Leads to Uncontrolled Expansion**

Our observation that Ca\textsuperscript{2+} concentrations increased after growth, parallels results obtained with pollen tubes where it has even been suggested that pulsatile expansion may actually be divorced from the oscillating Ca\textsuperscript{2+} gradient (Messerli et al., 2000). To further assess the relationship between Ca\textsuperscript{2+} and tip growth, we therefore attempted to manipulate cytoplasmic Ca\textsuperscript{2+} levels while simultaneously monitoring cell expansion.

Published data indicates that Ca\textsuperscript{2+} enters the cytoplasm of tip growing root hairs from the extracellular environment (Herrmann and Felle, 1995; Wymer et al., 1997). To attenuate this influx, we incubated roots with La\textsuperscript{3+}, a blocker of Ca\textsuperscript{2+} permeable channels. Monitoring Ca\textsuperscript{2+} levels during La\textsuperscript{3+} treatment showed that 200 µM La\textsuperscript{3+} rapidly caused the dissipation of the tip-focused Ca\textsuperscript{2+} gradient. Thus, within less than 10 s of treatment, either no difference
between apical Ca\(^{2+}\) levels and those 20 µm from the tip could be observed or a slight decline to below these subapical Ca\(^{2+}\) levels immediately after La\(^{3+}\) treatment was observed (Figure 2A). These observation suggest that the tip focused gradient is largely supported by influx into the cytosol by La\(^{3+}\)-sensitive channels and collapses very rapidly upon their inhibition whereas, basal Ca\(^{2+}\) levels are largely maintained under these conditions. Interestingly, however, although inhibited relative to its rate before addition of La\(^{3+}\), expansion of the cell apex continued for a few minutes following La\(^{3+}\) treatment. This expansion accelerated until the root hairs eventually burst at their tips (Table 1, Figure 2B). At higher concentrations of La\(^{3+}\) (1 mM), almost all growing root hairs ruptured within 10 min of treatment, whereas at lower concentrations of the inhibitor (200 µM), only the vigorously growing root hairs closer to the apex of the root consistently burst; older, more basal root hairs ceased to elongate but continued to swell at the apex for some time (Table 1, data not shown). To ascertain that the growth effects of La\(^{3+}\) were indeed due to inhibition of Ca\(^{2+}\) influx rather than unspecific effects, we sought to attenuate Ca\(^{2+}\) influx by the alternative means of chelating extracellular Ca\(^{2+}\). Treatment with 4 mM EGTA also led to rapid bursting of almost all growing root hairs within 10 min of treatment (Table 1).

These results indicate that expansion of the root hair tip can be sustained by processes that are not strictly dependent on the high cytoplasmic Ca\(^{2+}\) concentrations normally found in the root hair apex. It is important here to distinguish between such experimentally-induced expansion/swelling that leads to eventual bursting and the highly controlled cell elongation that is sustained to allow normal growth to occur. The expansion observed in the absence of a clear tip-focused Ca\(^{2+}\) gradient seemed to occur in an uncontrolled manner leading to cell rupture. Endogenous oscillatory increases in Ca\(^{2+}\) levels at the growing root hair apex may
thus play a role in restricting expansion after each burst of growth to maintain control of the sustained elongation characteristic of tip growth.

**Increasing the Tip-high Ca\(^{2+}\) Gradient Arrests Growth**

To investigate this potential regulatory role of Ca\(^{2+}\) in restricting growth, we artificially increased cytoplasmic Ca\(^{2+}\) by treating root hairs with the Ca\(^{2+}\) ionophore A23187. Figure 3 shows that prior to application of 10 µM A23187, both the tip-focused Ca\(^{2+}\) gradient and the growth rate oscillated as described in Figure 1. Immediately after treatment, however, Ca\(^{2+}\) levels rapidly increased (Figure 3A). The elevated Ca\(^{2+}\) levels subsequently declined over 30-60s likely due to activation of Ca\(^{2+}\) homeostatic systems compensating for the increased influx elicited by the ionophore. Interestingly, upon A23187 treatment elongation was arrested within a few seconds of treatment (Figure 3B). In many root hairs, this high Ca\(^{2+}\)-induced cessation of cell expansion was accompanied by a thickening of the apical cell wall. This thickening was visible in bright field images as a cap-like structure with altered refractory properties (compare Figure 3C and E). To confirm the nature of this structure, we loaded root hairs with fluorescein diacetate, supplemented the external medium with fluorescein-dextran (10 kDa) and imaged the cell apex using confocal optical sections of less than 1 µm thickness. We found that if image acquisition was performed soon after addition of fluorescein-dextran to the medium, very little of the dye had yet permeated the cell wall. Thus, as both cytosolic fluorescein and extracellular fluorescein were excluded from the wall space, the thickness of the wall became apparent by the absence of fluorescence (Figure 3F). Although the extent of wall thickening was variable in ionophore-treated cells, it was most evident in younger root hairs and equivalent thickening was never found in untreated control
root hairs (Figure 3C-F). These observations indicate that while Ca\(^{2+}\) ionophore treatment rapidly arrested cell expansion of root hairs, secretion of wall material was sustained, leading to a thickening where tip growth-related secretion normally occurs. YFP::RabA4b is thought to mark the apical secretory vesicle machinery in growing Arabidopsis root hairs, dissipating once growth ceases (Preuss et al., 2004, 2006). Monitoring the distribution of this YFP marker in root hairs revealed that, as previously reported, RabA4b decorated an apical accumulation of vesicles (Figure 3H) and this accumulation was maintained despite A23187-arrested elongation (Figure 3J), consistent with the idea that secretion is sustained in these ionophore-treated non-growing cells.

**DISCUSSION**

The oscillations in elongation of pollen tubes are thought to reflect either the rapid usage of growth components, that must be re-accumulated to support the next phase of growth, or feedback in either the regulatory or metabolic machinery supporting tip growth (Feijo et al., 2001). Until recently, whether such oscillatory patterns represent an element in the process of root hair tip growth has been unclear. Figure 1 shows that when made with sufficient resolution, oscillations in growth rate could be monitored in root hairs of Arabidopsis with a frequency of 2-4 peaks per min (see also Monshausen et al., 2007). During these oscillations, elongation decelerated and accelerated, with growth rates rising to up to 3 times basal levels. It is important to note that elongation never completely paused during the slowest phase of the oscillation. Thus, the regulatory mechanisms behind the oscillatory growth most likely represent the effects of cellular machinery fine-tuning the rate of expansion. In lily pollen tubes, growth oscillates at approximately 1-3 peaks per min with peak growth rates reaching
2-5 times the basal level (Pierson et al., 1996; Messerli and Robinson, 1997). Thus, despite the slower growth rate of the root hair, the kinetics of its growth oscillations closely parallel those of pollen tubes suggesting a possibly conserved oscillatory mechanism.

Although genetically encoded Ca$^{2+}$ reporters such as the yellow cameleon 2.1 have been used as a non-invasive method to monitor Ca$^{2+}$ changes during pollen tube growth (e.g. Watahiki et al., 2004), until recently we have found that the small dynamic range of these probes has limited their usefulness in resolving the dynamics of the Ca$^{2+}$ gradient in root hairs. However, the enhanced dynamic range of the YC3.6 reporter (Nagai et al., 2004) has allowed us to monitor oscillatory changes in the tip-focused Ca$^{2+}$ gradient during oscillating growth (Figure 1). Cross-correlation analysis indicates that the oscillations of the Ca$^{2+}$ gradient lagged oscillations in growth by 5 s. A similar phenomenon has been observed in pollen tubes where cytosolic the Ca$^{2+}$ increase also lagged growth by 4 s (Messerli et al., 2000; Messerli and Robinson, 2007).

It is important to note that while the Ca$^{2+}$ gradient underwent regular oscillations, there was a statistically significant elevated Ca$^{2+}$ level at the tip throughout the oscillatory cycle (Figure 1). This consistently elevated Ca$^{2+}$ concentration is likely to support basal levels of exocytosis during apical growth. Indeed, Figure 3 shows that when Ca$^{2+}$ levels were artificially elevated by ionophore treatment, there was an increase in apical wall thickness, consistent with a Ca$^{2+}$-promoted fusion of secretory vesicles leading to exocytosis of wall material. A similar phenomenon has been reported in pollen tubes where Reiss and Herth (1978) observed an apical thickening as growth was arrested. Importantly, our analysis of the organization of the apical secretory membrane system using YFP::RabA4b indicates that even after growth arrest by application of Ca$^{2+}$ ionophore and Ca$^{2+}$ elevation, the apical secretory
machinery remains in place, consistent with the idea that the elevated Ca\(^{2+}\) level could drive this apparatus to high levels of secretion and so to the wall thickening we observed.

However, during normal tip growth of the root hair, oscillatory Ca\(^{2+}\) increases are superimposed on the stable tip-focused Ca\(^{2+}\) gradient. The maximum of each Ca\(^{2+}\) oscillation occurred after an increase in growth, suggesting that while the basal gradient may be supporting sustained growth, the Ca\(^{2+}\) peaks may play additional role(s) in organizing spatial and temporal aspects of root hair elongation. One possibility is that the increase in Ca\(^{2+}\) is acting to ‘prime’ the hair for the subsequent pulse of growth, i.e. acting to prepare the secretory apparatus for the next round of cell expansion. However, the strong cross-correlation of the Ca\(^{2+}\) increase to following the period of maximal growth rate, rather than preceding it, suggests a role in processes following the burst of increased growth. Alternatively, elevated Ca\(^{2+}\) levels could be acting on enzymes that regulate wall structure to rigidify the wall and so help limit turgor-driven expansion. Such dual Ca\(^{2+}\)-dependent regulation is well characterized for many mammalian regulatory processes where the spatial and temporal dynamics of a change in Ca\(^{2+}\) can trigger different responses in the same cell. For example, in B lymphocytes, the transcriptional regulators NF\(_{\kappa}\)-B and c-Jun N-terminal kinase (JNK) are selectively activated by a large transient increase in Ca\(^{2+}\), whereas, in the same cells, up-regulation of the NFAT transcription factor requires a sustained, low level increase in the same ion (Dolmetsch et al., 1997). It is also important to note that our cytosolic Ca\(^{2+}\) measurements are monitoring bulk cytosolic Ca\(^{2+}\) levels and could reflect Ca\(^{2+}\) influx from both across the plasma membrane and from internal sites that may well help support different aspects of the tip-focused Ca\(^{2+}\) gradient (i.e maintaining the basal gradient versus generating the oscillatory component) and have specific targets and actions within the cell.
Our observations that (1) blocking Ca$^{2+}$ influx into the root hair leads to uncontrolled expansion/bursting, (2) artificially increasing Ca$^{2+}$ causes growth arrest, and that (3) the peak of the Ca$^{2+}$ oscillation occurs after a burst in growth are all consistent with a role for the maximal component of the oscillatory cytosolic Ca$^{2+}$ increase in limiting rather than facilitating expansion. Similar effects have been seen in fungi where the hyphae of *Saprolegnia ferax* show abnormal growth with enlarged hyphal diameter when they are transferred to nominally zero Ca$^{2+}$ media (Jackson and Heath, 1989), suggesting that the restraints on growth may also have changed. Previous analyses of treatments that alter the Ca$^{2+}$ gradient in the root hair often reported a cessation of root hair growth (Clarkson et al., 1988; Miller et al., 1992; Herrmann and Felle, 1995; Wymer et al., 1997). For the channel blockers used in these previous experiments, the cessation of growth likely reflects growth conditions, such as much higher Ca$^{2+}$ levels in the medium, where the bursting we observe is suppressed.

During oscillating growth we have also measured dynamic increases in extracellular ROS and pH that oscillate with a similar frequency as growth but lag growth oscillations by 7-8 s (Monshausen et al., 2007). These extracellular changes are thought to play a role in restricting growth at the tip (pH) and along the shank immediately behind the tip (ROS). Intriguingly, these oscillations in extracellular ROS and pH lag the oscillating increases in the intracellular Ca$^{2+}$ gradient we show here. Therefore it is possible that the oscillatory nature of the cytosolic Ca$^{2+}$ gradient, and of the extracellular ROS and pH changes may be linked as part of a system to limit growth once an initial burst of elongation has occurred with elevation in Ca$^{2+}$ being driven by each growth pulse and itself triggering subsequent ROS and pH response systems to limit further expansion. Such a model fits well with the likely Ca$^{2+}$
dependency of the NADPH oxidases, which contain an EF-hand-like Ca\textsuperscript{2+} binding domain that appears critical for supporting tip growth and recent data suggesting that ROS and Ca\textsuperscript{2+} regulation of growth form a feedback loop to sustain tip growth (Takeda et al., 2008). The spatial and temporal aspects of these three oscillatory parameters in relation to growth are depicted in the model shown in Figure 4.

Our observation that La\textsuperscript{3+} and EGTA buffering of the medium prevents the formation of the oscillations in tip focused Ca\textsuperscript{2+} (Figure 2) supports the idea that influx across the plasma membrane is a key element regulating the dynamics of the gradient, possibly acting as a primer to trigger Ca\textsuperscript{2+} release from internal sites, as proposed for pollen (Messerli and Robinson, 1997; Messerli et al., 1999; Messerli and Robinson, 2003). One possible influx mechanism is through Ca\textsuperscript{2+} permeable channels directly gated by tension in the plasma membrane, as seen in pollen tubes (Dutta and Robinson, 2004). These pollen tube channels are known to be Gd\textsuperscript{3+}-sensitive (Dutta and Robinson, 2004) and the Ca\textsuperscript{2+} influx into root hairs is likewise Gd\textsuperscript{3+} sensitive (supplemental Figure 3). Alternatively, cytoskeletal elements may play a role in regulating mechanosensitive channel activity, as suggested for pollen tubes (Wang et al., 2004). The relationship between the ROS/hyperpolarization-activated Ca\textsuperscript{2+} channel thought to support the gradient in root hairs (e.g. Foreman et al., 2003) and such a mechanical response remains to be defined. It is probable that more than one Ca\textsuperscript{2+}-permeable channel exists at the tips of root hairs similar to the tip-growing rhizoids of *Fucus* that contain two Ca\textsuperscript{2+}-permeable channels, one with and one without mechanosensitivity (Taylor et al., 1996). Integration of the activity of these channels may well lie at the heart of the system that must precisely balance the promotion and restriction of turgor-driven expansion to permit root hair elongation without runaway expansion and the associated catastrophic failure of the
apical wall. The molecular identity of these channels, how they relate to enhanced exocytosis of wall material and their relationships to the extracellular ROS production and proton transport systems linked to growth restriction (Monshausen et al. 2007) is a major challenge for future research.

MATERIALS AND METHODS

Plant Material

Seeds of *Arabidopsis thaliana* Columbia were surface sterilized and germinated on Murashige & Skoog medium (Sigma) supplemented with 1% (w/v) sucrose and 1% (w/v) agar at 21°C under continuous light conditions. Four-day-old seedlings were chosen for experiments.

Imaging of Cytosolic Ca$^{2+}$ Levels

*Arabidopsis* seedlings expressing the FRET-based Ca$^{2+}$ sensor yellow cameleon YC3.6 (Nagai et al., 1999) were transferred to purpose-built cuvettes and mounted as described previously (Monshausen et al. 2007). After several hours of growth in agar containing 0.1 mM KCl, 1 mM NaCl and 0.1 mM CaCl$_2$, pH ~6, supplemented with 1% (w/v) sucrose, root hairs were ratio imaged with the Zeiss LSM 510 laser scanning confocal microscope (Carl Zeiss Inc., Thornwood, NY) using a 40x water immersion, 1.2 numerical aperture, C-Apochromat objective. The YC3.6 Ca$^{2+}$ sensor was excited with the 458 nm line of the argon laser. The CFP (473-505nm) and FRET-dependent Venus (526-536 nm) emission were collected using a 458 nm primary dichroic mirror and the Meta detector of the microscope. Bright-field images were acquired simultaneously using the transmission detector
of the microscope. For time-lapse analysis, images were collected every 2 or 3 s, with each individual image scan lasting 1.57 s.

*In situ* calibration was performed by raising Ca\(^{2+}\) to saturating levels for YC3.6. This was attempted by treatment with 1 M CaCl\(_2\), or 50% EtOH or mechanical perturbation just below the threshold for causing cell rupture. The maximum FRET/CFP ratio was attained in response to mechanical perturbation (\(R_{\text{max}} = 2.5\)). The minimum FRET/CFP ratio (\(R_{\text{min}} = 0.65\)) was recorded by treating the plants with 1 mM BAPTA-AM (Molecular Probes). Ca\(^{2+}\) levels were then calculated according to the equation \(\text{Ca}^{2+} = K_d \frac{(R-R_{\text{min}})}{(R_{\text{max}}-R)^{1/n}}\) where \(R\) represents the FRET/CFP ratio measured during the experiment (Miyawaki et al., 1999), \(n\) represents the Hill coefficient which has been determined as 1 for YC3.6, and the \(K_d\) for Ca\(^{2+}\) of 250 nM (Nagai et al., 2004). Due to the inherent uncertainties of the precise *in vivo* \(K_d\) in such *in situ* calibrations, the raw FRET/CFP ratio data is included in each figure.

We used two different lines of transgenic *Arabidopsis* Columbia expressing 35S-driven YC3.6: line 1 was transformed with YC3.6 in the binary vector pGreenII (generous gift of Jeffrey Harper, University of Nevada, Reno). To generate line 2, pGreenII was restriction-digested with NcoI and EcoRI to obtain YC3.6 with NOS terminator. This fragment was ligated into the Gateway entry vector pENTR11 (Invitrogen) and subsequently recombined into the binary Gateway-compatible destination vector pEarleyGate100 (Earley et al. 2006) according to published protocol (Invitrogen). Because pENTR11 and pEarleyGate100 both contain kanamycin resistance as a selection marker, the backbone of the entry clone was cleaved with PvuII and SspI prior to recombination. Recombined plasmids were transformed into *E. coli* Mach1 cells (Invitrogen) and clones were selected on LB/kanamycin plates. Recombinant plasmids were then transformed into *Agrobacterium tumefaciens* via
electroporation, followed by transformation of *Arabidopsis* by floral dip (Clough and Bent, 1998). No differences in the Ca$^{2+}$ oscillations were observed between these two independent *Arabiopsis* lines and neither showed discernable alterations in root hair morphology or growth rate relative to wild type plants.

**Measurement of Root Hair Growth**

Bright-field images were collected every 2 s simultaneously with fluorescence images using 458 nm excitation. High resolution growth measurements were made using the computer vision tracking software as previously described (Messerli et al., 1999) providing 1/10 pixel resolution. For high-resolution analysis of root hair tip growth, plants were normally imaged with the root growing through a gel matrix because this approach restricted movement of the main root axis and allowed us to determine minute changes in root hair tip position caused by apical growth. To observe the effect of the Ca$^{2+}$-modulating reagents EGTA, Ca$^{2+}$ channel blocker La$^{3+}$ and Ca$^{2+}$ ionophore A23187 on root hair growth, growth measurements were performed on root hairs immersed in liquid medium. After observing growth of a root hair for several minutes before treatment, the reagent (at 2x concentration) was gently mixed into the medium and growth measurements were continued on the same cell. The use of liquid medium allowed noticeable shifting of the root axis and made measurements of root hair tip growth more difficult. However, this approach afforded the necessary rapid access of the reagents to the root hairs without delays due to diffusion through an agar medium.

To study the effect of irradiation intensity on root hair growth, elongation rates were monitored under three different imaging conditions: (i) using the 458 nm line of the argon
laser (at 4.7 A tube current, 90% attenuation), a root hair was imaged once at time 0 and again after 5 min; (ii) a root hair was imaged every 2s for 5 min (458 nm at 4.7 A, 90% attenuation); (iii) a root hair was imaged every 2s for 5 min (458 nm at 4.7 A, 60% attenuation). The average growth rate was calculated on the basis of the increase in root hair length during these 5 min.

Cross-correlation analysis was performed to determine the temporal relationship between tip-restricted Ca\(^{2+}\) oscillations and growth oscillations. The correlation coefficient 

\[
   r = \frac{SP_{XY}}{\sqrt{SS_{X}SS_{Y}}}
\]

was determined as the measurements of the growth oscillations were shifted in time with respect to the Ca\(^{2+}\) oscillations. \(SS_{X}\) and \(SS_{Y}\) are the sum of the squares for corresponding Ca\(^{2+}\) and growth recordings while \(SP_{XY}\) is the sum of the products of the two corresponding recordings. A perfect sine wave produces a correlation coefficient of 1 at \(t = 0\) when compared to itself and a value of \(-1\) at \(t=0\) when compared to itself 180 degrees out of phase. As data sets were shifted in time with respect to each other the data points at the tail ends no longer overlapped. These points were removed from the analysis. The temporal resolution of the analysis is the same as that used to acquire the images but there is an offset of one-half of the temporal resolution due to the fact that the growth rate measurements were plotted at the half-way point in between the corresponding images used to determine the growth rate.

**Monitoring Root Hair Cell Wall Thickening**

*Arabidopsis* roots were treated with 5 µM fluorescein diacetate for 5 min. After washing, fluorescein-dextran (10 kDa) was added to the medium until intracellular and
extracellular fluorescein fluorescence intensities were approximately equal. Root hair apices were imaged with the Zeiss LSM 510 microscope using the 40x water immersion objective described above. Fluorescein was excited with the 488 nm line of the argon laser. Emission was collected using a 488 nm primary dichroic mirror and a 505 nm long pass filter. Optical sections of less than 1 µm thickness were acquired. Bright-field images were acquired simultaneously using the transmission detector of the microscope.

**Monitoring YFP::RabA4b localization in *Arabidopsis* root hairs**

Root hairs of *Arabidopsis* expressing YFP-RabA4b (Preuss et al., 2004) were imaged using the Zeiss LSM 510 and the same imaging parameters as described above for fluorescein.

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FIGURE LEGENDS

**Figure 1.** *Arabidopsis* root hairs show an oscillating tip-focused Ca$^{2+}$ gradient that peaks after maximal growth. A, Root hairs undergoing tip growth in *Arabidopsis* plants expressing the Ca$^{2+}$ sensor YC3.6 targeted to the cytosol were imaged every 3 s. Cytosolic Ca$^{2+}$ levels have been calibrated as described in the Materials and Methods and pseudo-color coded according to the inset scale. Numbers represent time in seconds. Scale bar=10 µm. Representative of n>40 measurements. B, Quantitative analysis of cytosolic Ca$^{2+}$ oscillations in a representative growing root hair. Ca$^{2+}$ levels were measured in 5 µm$^2$ regions of interest (ROI) along the root hair length outlined in the inset. Increase in the ratio of FRET/CFP reflects an increase in cytoplasmic Ca$^{2+}$ level. C, Average Ca$^{2+}$ levels during peaks and troughs of Ca$^{2+}$ oscillations. Values selected for calculation of averages are depicted as *asterisks* (peaks) and *arrowheads* (troughs) in (B). D, Quantitative analysis of root hair growth rates and cytosolic Ca$^{2+}$ levels at the root hair apex. Ca$^{2+}$ was measured in a ~30 µm$^2$ ROI outlined in (A). Representative of n>10 measurements. E, Cross correlation analysis of Ca$^{2+}$ oscillations with growth oscillations indicate that the increases in cytosolic Ca$^{2+}$ lag increases in growth rate by approximately 5 s. Cross correlation was performed on data from eight separate root hairs.

**Figure 2.** Effect of La$^{3+}$ on cytosolic Ca$^{2+}$ and growth of *Arabidopsis* root hairs. A, Treatment with 200 µM La$^{3+}$ triggers rapid dissipation of the tip-focused Ca$^{2+}$ gradient in a growing root hair. Ca$^{2+}$ levels were measured in a ~30 µm$^2$ ROI at the root hair apex as outlined in Figure 1A. The rapid increase in Ca$^{2+}$ at the end of this recording is due to Ca$^{2+}$ entry during bursting of the root hair. B, Treatment with 200 µM La$^{3+}$ causes acceleration of elongation and
eventual bursting of growing root hairs. Arrow denotes Ca\(^{2+}\) increase due to bursting. Representative of \(n = 7\) (Ca\(^{2+}\)) and \(n = 10\) (growth) measurements, respectively.

**Figure 3.** Effect of Ca\(^{2+}\) ionophore A23187 on cytosolic Ca\(^{2+}\) and growth of *Arabidopsis* root hairs. A, Treatment with 10 \(\mu\text{M}\) A23187 triggers a rapid increase of cytosolic Ca\(^{2+}\) in a growing root hair. Ca\(^{2+}\) levels were measured in a \(<30\ \mu\text{m}^2\) ROI at the root hair apex as outlined in Figure 1A. Representative of \(n = 10\) measurements. B, Treatment with 10 \(\mu\text{M}\) A23187 arrests root hair tip growth. All detectable growth of the root hair had ceased by 14 s when measurements resumed. The, non-growing root hair did not remain in the same plane as the root axis continued to shift after treatment, necessitating constant refocusing. At or below the limits of resolution for the tracker (0.2 \(\mu\text{m}/\text{min}\)), this combination of changes in focal plane and root expansion appear as very slow growth for this example. Representative of \(n = 10\) measurements. C-F, Bright-field and fluorescence images of root hairs loaded with FDA and immersed in medium containing fluorescein-dextran. C and D, Untreated, growing control root hair. Cell wall (arrowhead) thickness is indicated by the exclusion of cytosolic and extracellular fluorescein fluorescence. E and F, A23187-induced Ca\(^{2+}\) increase and growth inhibition is accompanied by a thickening of the apical root hair cell wall (arrowhead). Images were acquired 1 h after start of ionophore treatment. Representative of \(n = 11\) measurements. G-J, Bright-field and fluorescence images of root hairs expressing YFP-RabA4b. G and H, Untreated, growing control root hair. Note that YFP-RabA4b accumulates at the apex of the growing root hair. I and J, A23187-induced apical cell-wall thickening is accompanied by an accumulation of YFP-RabA4b at the cell apex. The root hair was immersed in medium containing fluorescein-dextran to help visualize cell wall thickness.
(arrowhead). Representative of $n = 10$ measurements. Sale bar = 10 µm.

**Figure 4.** Temporal and spatial relationships between growth, the tip-focused $\text{Ca}^{2+}$ gradient, surface (wall) pH and surface (wall) ROS. Relative timings of growth, pH and ROS were taken from Monshausen et al., 2007.
Table 1. Frequency of root hair bursting in response to EGTA and La$^{3+}$. Root hairs were treated as indicated and % burst hairs in the apical 400-µm region of the root hair zone were scored after 10 min. Data are from at least six different roots for each treatment.

| Treatment        | Number of root hairs analyzed | % burst root |
|------------------|-------------------------------|--------------|
| 4 mM EGTA        | 119                           | 98.5         |
| 1 mM La$^{3+}$   | 87                            | 96.0         |
| $^{a}$0.2 mM La$^{3+}$ apical 200 µm | 54                            | 86.2         |
| $^{a}$0.2 mM La$^{3+}$ apical 400 µm | 117                           | 65.4         |

$^{a}$Root hairs were monitored in either the apical 200 or 400 µm region of the root hair zone.
Supplementary data

Supplementary movie 1. Cytosolic Ca\textsuperscript{2+} oscillations during tip growth of an *Arabidopsis* root hair. Cytosolic Ca\textsuperscript{2+} was monitored in plants expressing the soluble Ca\textsuperscript{2+} sensor YC3.6 as described in the Materials and Methods. Images were taken every 3 s. Movie duration 3.5 min. Ca\textsuperscript{2+} levels have been pseudocolor-coded according to the scale in Figure 1.

Supplementary Figure 1. Regression analysis of the relationship between peak Ca\textsuperscript{2+} levels during each oscillation and the preceding or following peak in growth rate. Peak Ca\textsuperscript{2+} level and preceding and following growth rate were plotted for 8 separate root hairs over 4-5 min of observation each equating to between 12 and 18 growth and Ca\textsuperscript{2+} peaks. Regression analysis was performed using Excel. Note that when plotting amplitudes of growth peaks versus subsequent Ca\textsuperscript{2+} peaks, the slopes of all trend lines are positive, whereas slopes are more variable for Ca\textsuperscript{2+} peaks versus subsequent growth peaks. However, in all cases, the regression coefficients are low, suggesting that either a poor relationship exists between these factors, or that the relationship between them is not a simple linear one. Alternatively, variability in the data likely related to making such measurements in Ca\textsuperscript{2+} levels and especially growth rate at the limits of resolution of current technology may also be obscuring the relationship.

Supplementary Figure 2. Ca\textsuperscript{2+} levels at the apex of a non-growing root hair. Root hairs that had ceased undergoing tip growth in *Arabidopsis* plants expressing the Ca\textsuperscript{2+} sensor YC3.6 targeted to the cytosol were imaged every 3 s. Note the lack of detectable Ca\textsuperscript{2+} oscillations at the apex.
Supplementary Figure 3. Effect of Gd$^{3+}$ treatment on cytosolic Ca$^{2+}$. Root hairs undergoing tip growth in *Arabidopsis* plants expressing the Ca$^{2+}$ sensor YC3.6 targeted to the cytosol were imaged every 3 s. 1 mM GdCl$_3$ was added and the effect on growth and Ca$^{2+}$ dynamics monitored. Note the reduction in tip Ca$^{2+}$ levels induced by addition of Gd$^{3+}$. The sudden rapid increase in cytosolic Ca$^{2+}$ at the end of the recording (arrow) was caused by Ca$^{2+}$ entry as the cell ruptured. These effects mimic those seen upon La$^{3+}$ treatment.
Figure 1. Arabidopsis root hairs show an oscillating tip-focused Ca\textsuperscript{2+} gradient that peaks after maximal growth. A, Root hairs undergoing tip growth in Arabidopsis plants expressing the Ca\textsuperscript{2+} sensor YC3.6 targeted to the cytosol were imaged every 3 s. Cytosolic Ca\textsuperscript{2+} levels have been calibrated as described in the Materials and Methods and pseudo-color coded according to the inset scale. Numbers represent time in seconds. Scale bar=10 μm. Representative of n>40 measurements. B, Quantitative analysis of cytosolic Ca\textsuperscript{2+} oscillations in a representative growing root hair. Ca\textsuperscript{2+} levels were measured in 5 μm\textsuperscript{2} regions of interest (ROI) along the root hair length outlined in the inset. Increase in the ratio of FRET/CFP reflects an increase in cytoplasmic Ca\textsuperscript{2+} level. C, Average Ca\textsuperscript{2+} levels during peaks and troughs of Ca\textsuperscript{2+} oscillations. Values selected for calculation of averages are depicted as asterisks (peaks) and arrowheads (troughs) in (B). D, Quantitative analysis of root hair growth rates and cytosolic Ca\textsuperscript{2+} levels at the root hair apex. Ca2+ was measured in a <30 μm\textsuperscript{2} ROI outlined in (A). Representative of n>10 measurements. E, Cross correlation analysis of Ca\textsuperscript{2+} oscillations with growth oscillations indicate that the increases in cytosolic Ca\textsuperscript{2+} lag increases in growth rate by approximately 5 s. Cross correlation was performed on data from eight separate root hairs.
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