Running Head:

PI staining of root hairs and pollen tubes

Corresponding Author:

Peter K. Hepler

Address:

Biology Department, University of Massachusetts, Amherst, MA 01003

Telephone:

413-545-2083

Email:

hepler@bio.umass.edu

Research Area:

Cell Biology
Title:

Propidium iodide competes with Ca$^{2+}$ to label pectin in pollen tubes and arabidopsis root hairs

Authors:

Caleb M. Rounds,*§ Eric Lubeck,†‡ Peter K. Hepler,* Lawrence J. Winship†

Institutions:

*Department of Biology, University of Massachusetts, Amherst, MA 01003
†School of Natural Science, Hampshire College, Amherst, MA 01002
Footnote: This work was supported by the National Science Foundation (http://www.nsf.gov/) (grant no. MCB-0847876 to P.K.H.). Corresponding author: Peter K. Hepler, hepler@bio.umass.edu. §Present Address, Department of Biology Mt. Holyoke College South Hadley, MA 01075 ‡Present Address, Department of Chemistry. California Institute of Technology Pasadena, CA 91106
Abstract:

We have used propidium iodide (PI) to investigate the dynamic properties of the primary cell wall at the apex of Arabidopsis thaliana root hairs and pollen tubes, and Lilium formosanum pollen tubes. Our results show that in root hairs as in pollen tubes, oscillatory peaks in PI fluorescence precede growth rate oscillations. Pectin forms the primary component of the cell wall at the tip of both root hairs and pollen tubes. Given the electronic structure of PI, we investigated whether PI binds to pectins in a manner analogous to Ca\textsuperscript{2+} binding. We first show that Ca\textsuperscript{2+} is able to abrogate PI growth inhibition in a dose dependent manner. PI fluorescence itself also relies directly on the amount of Ca\textsuperscript{2+} in the growth solution. Exogenous PME treatment of pollen tubes, which de-methoxylates pectins freeing more Ca\textsuperscript{2+} binding sites, leads to a dramatic increase in PI fluorescence. Treatment with pectinase leads to a corresponding decrease in fluorescence. These results are consistent with the hypothesis that PI binds to de-methoxylated pectins. Unlike other pectin stains, PI at low yet useful concentration is vital and specifically does not alter the tip-focused Ca\textsuperscript{2+} gradient or growth oscillations. These data suggest that pectin secretion at the apex of tip growing plant cells plays a critical role in regulating growth, and PI represents an excellent tool for examining the role of pectin and of Ca\textsuperscript{2+} in tip growth.
Introduction

The apical wall of tip-growing cells participates directly in the process of growth regulation (McKenna et al., 2009; Winship., et al 2010) yet few methods permit monitoring the wall properties of living cells. Despite this, several recent studies have enhanced our understanding of the apical cell wall. Chemical analyses of isolated pollen tube wall material have revealed a complex mixture of pectic polysaccharides with regions comprising long sequences of polygalacturonic acid. Important patterns of pectin methoxylation have been detected using immunocytochemical approaches, but these are limited to fixed cells (Dardelle, et al 2010). In a recent study, Parre and Geitmann used microindentation to show significant correlations between wall strength and growth rate (2005). None of these techniques allow for easy investigation of the cell wall during growth.

In an earlier study we found that PI vitally stains pollen tubes of lily and tobacco, and in particular reveals with great clarity the thickened apical cell wall (Fig. 1) (McKenna et al., 2009). In addition, the apical PI fluorescence oscillates and in lily pollen tubes correlates tightly with oscillations in wall thickness measured by differential interference contrast (DIC) optics. Finally these studies indicated that the PI fluorescence predicted cell growth rates with high confidence, suggesting that PI binding may provide useful information about the physical and chemical properties of the cell wall.

PI is commonly used to visualize plant cell walls by wide-field fluorescence and confocal microscopy (Tian et al., 2006; Fiers et al., 2005; Estevez et al., 2008) and to select viable cells during cell sorting (Deitch et al., 1982; Jones and Senft, 1985). A positively-charged phenanthridine derivative, the propidium ion stains cell walls but does not pass through the intact cell membranes of living cells. It readily diffuses into dead cells and forms highly fluorescent complexes by intercalation between base pairs of double stranded nucleic acids, thus acting as an excellent indicator for cell vitality (Hudson et al., 1969). Binding to cell walls presumably occurs by a different mechanism since it is not accompanied by the dramatic increase in fluorescence and shift in absorption and emission maxima observed when PI binds to nucleic acids. The mechanism of PI binding needs further exploration as does the potential for broader use in other tip growing plant cells.
In this report we test two hypotheses: first, that PI stains other tip growing cells with pectin-containing cell walls; and second that PI and Ca\(^{2+}\) bind to the same sites in these walls. This binding would occur through the interaction of partial positive charges caused by localized deficits in \(\pi\)-orbital electrons associated with 3 of the 4 nitrogen atoms of PI (Luedtke, et al., 2005) coordinating with negatively charged carboxyl and hydroxyl groups on homogalacturonans (HGAs) as has been suggested in *Oedogonium bharuchae* (Estevez et al., 2008). Our findings indicate that both hypotheses are satisfied. Notably, oscillatory changes in apical PI fluorescence occur, and are observed to anticipate oscillations in growth rate in arabidopsis root hairs and arabidopsis pollen tubes. In addition, competition studies indicate that PI and Ca\(^{2+}\) bind to the same sites in cell walls. Supporting these studies, we demonstrate that pectin methyl esterase (PME) creates more sites for PI binding, presumably by de-methoxylating HGAs as they are secreted, and that pectinase reduces PI fluorescence dramatically. However, unlike other pectin binding dyes, PI does not block Ca\(^{2+}\) channels at the concentration used in live cell studies, nor does it alter oscillatory growth characteristics. Our findings provide evidence that PI may be employed as a quantitative measure of Ca\(^{2+}\) binding sites, and thus may have use as an indicator of the degree of cross-linking of HGAs and of cell wall extensibility.

**Results**

**Apical PI labeling of arabidopsis root hairs and pollen tubes oscillates.**

Because PI vitally labels lily and tobacco pollen cell walls and predicts growth rate, we asked whether a similar phenomenon existed in two other well studied tip-growing plant cells: the arabidopsis root hair and pollen tube. Monshausen et al (2007) have shown that root hair growth rate oscillates and they have speculated that changes in cell wall properties lead to these oscillations. The specific composition of root hair cell walls remains unknown (Knox, 2008), although it is clear that HGAs are important to growth (Diet et al., 2006) and that arabinogalactan proteins, frequently associated with pectin deposits, localize to the tip of maize root hairs (Šamaj et al., 1999).

We germinated arabidopsis seeds on vertically oriented agar pads affixed to microscope slides. Before imaging, we added water with or without supplemental PI. Root hairs displayed a markedly different distribution of PI fluorescence from pollen tubes (compare Fig. 1A, C and D
Supplemental movies S1 and S2). In pollen tubes, the shanks are fluorescent, but the predominant labeling is at the cell apex. It is this apical fluorescence that has been shown to oscillate and predict growth (Fig. 1A, B and Video S1) (McKenna et al., 2009). In root hairs, in contrast, the predominant labeling occurs along the shanks. Although the tip is fluorescent, it is much dimmer than the rest of the cell wall (Fig 1 C, D and Video S2). Also in root hairs the DIC image shows no dramatic differences in wall thickness at the apex versus the shank. Nevertheless, ultrastructural studies of root hairs show a thick cell wall at the apex though it is unclear how different the thickness is from the wall of the shank (Galway et al., 1997).

Using time lapse recordings of PI fluorescence and DIC images of root hairs, we examined changes in the PI fluorescence at the root hair tip and compared this to the growth rate (Fig 1 D, E). Notably, we observed distinct rhythmic fluctuations in the apical fluorescence (Fig 1 D, E). Root hairs grow more slowly than lily pollen tubes and thus when collecting images at a rate of 1 per 3 sec, the changes in growth are less than the resolution of the microscope, resulting in growth rate data that are noisy. We averaged the growth rate over five successive frames to correct for this high frequency noise. The resulting plots reveal a clear oscillatory pattern to the growth rate (Fig. 1 E blue line), and an equally clear and dramatic oscillatory pattern in the apical PI fluorescence (Fig. 1 E red line). While the growth rate period and PI fluorescence period are similar, they are out of phase.

We next investigated the fluorescence pattern of PI in arabidopsis pollen tubes. Several lines of evidence demonstrate the presence of pectin at the arabidopsis pollen tube tip. PME mutants have been identified that show significant fertility or growth deficits (Jiang et al., 2005; Tian et al., 2006). Furthermore transcriptome analysis demonstrates that pectin related enzymes are up-regulated substantially in growing pollen tubes (Honys and Twell 2003). Arabidopsis pollen tubes exhibit more erratic growth rate changes than lily pollen tubes and arabidopsis root hairs. Roughly 33% of the pollen tubes we imaged sustained growth rate oscillations over several minutes (n=14) (Supp. Fig 1, Supp Video 3). The pattern of PI fluorescence in arabidopsis pollen tubes is remarkably similar to that of both tobacco (McKenna et al., 2009) and lily pollen tubes (compare Fig. 1A and F). All show higher signal at the tip and a more uniform signal along the shank. We examined the changes in this fluorescence over time and compared it to growth rate
changes (Fig. 1 G). Both the growth rate and the fluorescence oscillate, though both signals are more erratic than in root hairs and lily pollen tubes.

**PI fluorescence oscillations precede growth rate oscillations in arabidopsis root hairs and pollen tubes**

Given the oscillations in PI fluorescence and growth rate in arabidopsis root hairs and pollen tubes, we felt it was important to explore their phase relationship. For this we performed cross correlation analysis to evaluate whether these two observed oscillatory phenomena were correlated as they are in lily and tobacco pollen tubes (McKenna et al., 2009).

We collected the growth rate and corresponding PI fluorescence data form 10 growing root hairs. Growth rates varied from as slow as 17 nm·s⁻¹ to as fast as 28 nm·s⁻¹ with an average of 22 nm·s⁻¹ (+/- 1.3 nm·s⁻¹ SEM, n=10). Similarly, oscillatory period varied from 72 s to as much as 104 s or roughly 30% of the mean growth rate of 92 s (+/- 2.0 SEM) (Table I). Cross correlation analysis revealed that the PI fluorescence peaks precede the growth rate oscillation peaks by -112°. These data are remarkably similar to the offsets of -99° for lily and -124° for tobacco pollen tubes (McKenna et al., 2009).

For arabidopsis pollen tubes the data are less consistent. Growth rates (Supp. Fig 1, Table 1) ranged from 29.8 nm·s⁻¹ to as fast as 65 nm·s⁻¹ with a mean rate of 52 nm·s⁻¹ (+/- 7 nm·s⁻¹ SEM, n=5). The oscillation period for growth rate was surprisingly similar to that in both lily and tobacco pollen tubes with an average of 32 s (+/- 4s SEM). Cross correlation analysis showed that peaks in the PI signal preceded growth rate peaks by -47° (+/- 8°).

We express the phase shift in degrees or fractions of a single cycle, where one complete cycle is 360°, rather than in an absolute value in seconds (McKenna et al., 2009). Support for this decision appears in Supplemental Fig. 2, which shows comparisons between growth rate and the phase shift in seconds as well as between the growth rate and period in arabidopsis root hairs (Supp Fig 2 A and B). The changes in period do not correlate with changes in growth rate; neither does the phase shift in seconds. However, the longer the period the longer the phase shift in seconds (Supp Fig. 2C), indicating that maximum PI staining consistently occurs at a well-regulated fraction of a cycle before the peak in growth rate, regardless of the actual rate.
PI fluorescence is modulated by Ca\(^{2+}\) and Mg\(^{2+}\)

PI has long been used as a root cell wall stain, though the mechanism of action is unclear (Tian et al., 2006; Fiers et al., 2005; Estevez et al., 2008). Since the PI molecule carries two positive charges, we hypothesize that PI is binding to the same sites in the cell wall as Ca\(^{2+}\). While there are no published studies on the electronic structure of PI, we can look to investigations of ethidium bromide which contains an identical phenanthridine core. X-ray crystallography, UV/Vis and IR absorption, fluorescence emission, and NMR spectroscopy studies (Luedtke et al., 2005) show that the carbon and hydrogen atoms of the phenanthridium structure have relatively high electron densities while nitrogen atoms are electron deficient resulting in three positively-charged regions in the molecule spaced 6 to 7 Å apart. We reasoned that if PI were interacting with the negative carboxyl groups in HGs, as our pollen tube and root hair data suggested (McKenna et al., 2009), then Ca\(^{2+}\) and Mg\(^{2+}\), two ions that interact with negative charges in the cell wall, might affect PI fluorescence. In this scenario, if we increased the Ca\(^{2+}\) or Mg\(^{2+}\) in solution, we should see a corresponding decrease in PI fluorescence. Similarly an increase in PI concentration should have less effect on the observed fluorescence in a solution with higher Ca\(^{2+}\) than in one with relatively lower Ca\(^{2+}\).

As a first step in investigating this hypothesis, we transferred arabidopsis roots that had been grown on Hoagland’s medium in vertically oriented plates, to water supplemented with 6.4 µM PI and increasing concentrations of Ca\(^{2+}\) (Fig. 2A). The microscope and exposure settings were kept constant and the look up table is identical for each image, yet clearly as the Ca\(^{2+}\) concentration increased the PI fluorescence decreased markedly. These images were collected from above the expansion zone of the roots. The root hairs in the 100 mM image are too dim to visualize whereas in the 1mM image they are shorter. The images were taken within 20 min of immersion in PI, so a growth affect does not account for the difference in root hair density. These results suggested that Ca\(^{2+}\) and PI compete for the same sites in the cell walls.

To further characterize this interaction we chose to work with lily pollen because controlling growth conditions is more efficacious and larger numbers are easier to gather, compared to root hairs or to arabidopsis pollen. We grew pollen tubes in our standard medium then prepared them for imaging. We then supplemented the growth medium with 6 different concentrations of PI and 3 of Ca\(^{2+}\). To assess fluorescence, we measured a defined area at the tip of the pollen tube and
subtracted background fluorescence. Figure 2B shows that in a low Ca\(^{2+}\) growth media of 10 μM (triangles), PI fluorescence is strongly dependent on concentrations. In contrast, at a higher Ca\(^{2+}\) concentration (1 mM), the fluorescence increases only slightly as PI concentration is increased (Fig. 2B circles). The standard solution (100 μM Ca\(^{2+}\)) shows an intermediate response (Fig. 2B squares).

To investigate whether increasing concentrations of Mg\(^{2+}\) would alter PI fluorescence, we performed the same experiment outlined above with the following ion concentrations (Fig. 2C): 10 μM Ca\(^{2+}\) (triangles), 100 μM Ca\(^{2+}\) (squares), 10 μM Ca\(^{2+}\) + 90 μM Mg\(^{2+}\) (diamonds), or 100 μM Ca\(^{2+}\) + 1 mM Mg\(^{2+}\) (X’s). As expected, the medium supplemented with 10 μM Ca\(^{2+}\) showed the most dramatic change in PI fluorescence as PI concentration increased. In keeping with our hypothesis, if Ca\(^{2+}\) and Mg\(^{2+}\) were both competing with PI for the same sites in the cell wall, we saw that 100 μM Ca\(^{2+}\) and 10 μM Ca\(^{2+}\) + 90 μM Mg\(^{2+}\), which contain the same molarity of bivalent cation, showed the same response. When the Mg\(^{2+}\) concentration was increased to 1mM, the PI fluorescence shows the same response visible when 1 mM Ca\(^{2+}\) is added (Compare Fig 2B circles to 2C X’s).

Analysis of the dependence of fluorescence on Ca\(^{2+}\), Mg\(^{2+}\) and PI concentrations by non-linear least squares fitting showed that the best fit model was a Hill equation with a Hill coefficient between 1.8 and 2, consistent with previously reported cooperative binding of Ca\(^{2+}\) to pectins during gelation (Garnier et al, 1994). Although cooperative binding kinetics usually is evidence of a conformational change during sequential binding of ligands, in the case of linear association of HGs, we may be seeing the effect of the filling and un-filling of sequences of Ca\(^{2+}\) binding sites (Michel, 2007). Linear arrays of 8 to 15 galacturonic acid sub-units, as described in the modified egg-box model for pectin Ca\(^{2+}\) binding (Braccini and Perez, 2001; Vincent and Williams, 2009), are thought to result from the action of plant PME on esterified HGs and constitute the main binding regions for Ca\(^{2+}\) ions.

**PI growth inhibition can be ameliorated with Ca\(^{2+}\), but not Mg\(^{2+}\)**

High Ca\(^{2+}\) concentrations (>10 mM) cause pollen tube growth to arrest (Holdaway-Clarke et al., 2003). Though similar experiments have not been published with regards to Mg\(^{2+}\), we expected that high concentrations would interfere with growth through binding to carboxyls in the HGs.
We reasoned that this would also be true of PI. Furthermore, Ca\(^{2+}\) and Mg\(^{2+}\) might be able to partially offset growth inhibition at high PI concentrations.

We germinated *Lilium formosanum* pollen then transferred them to multi-well plates containing concentrations of PI from 0 to 120 µM. The cells were allowed to recover for ~10 min then imaged once. After 20 min we imaged them again. Average lengths for >40 pollen tubes were determined for each time point and concentration, and then the mean change in growth was determined (Fig. 3A). In 100 µM Ca\(^{2+}\), our standard lily growth medium (squares), pollen tubes grew very close to control growth rate up to 60 µM PI where they grew at 70% of control. At 120 µM PI the pollen tubes show almost no growth. When the Ca\(^{2+}\) in the medium is raised to 1 mM (circles in Fig. 3A) cells grow at 70% of control growth rate up to 120 µM PI. Conversely, when grown in 10 µM Ca\(^{2+}\) pollen ceased growing with 60 µM PI in solution. That is, the magnitude of the growth inhibition caused by PI depends on the Ca\(^{2+}\) concentration.

We do not see this effect in medium supplemented with Mg\(^{2+}\). At 100 µM Mg\(^{2+}\), 60 µM PI inhibits pollen tube growth ~50%, whereas pollen tubes in 30 µM PI grow as well as pollen tubes in standard medium without PI (Fig 3B). Increasing the concentration of Mg\(^{2+}\) in solution to 1 mM inhibits growth in 30 µM PI, suggesting that Mg\(^{2+}\) itself has an inhibitory effect. When the PI concentration is increased to 60 µM, no additive effect is seen (Fig 3B). The fluorescence data (Fig. 2) presented above suggest that all three ions are competing for the same binding sites on HGs. As Mg\(^{2+}\) or PI concentrations increase, there is a detrimental effect on growth as they bind to carboxyls, but do not cross-link the HGs. These data support our hypothesis that PI is binding to the same sites in the cell wall as both Ca\(^{2+}\) and Mg\(^{2+}\).

**PI does not alter oscillatory behavior**

The oscillatory growth of the lily pollen tube provides an excellent tool for ordering the processes that regulate tip growth (Holdaway-Clarke and Hepler, 2003; Moreno et al., 2007; McKenna et al., 2009; Rounds et al., 2010). Recent work has shown that during oscillatory growth in lily pollen tubes the apical cell wall thickness increases before growth rate peaks (McKenna et al., 2009). Given our data suggesting that PI at high concentrations inhibits pollen tube growth, we investigated whether PI altered growth oscillations.
We grew lily pollen tubes in our standard growth media supplemented with concentrations of PI ranging from 0 to 80 μM in 20 μM increments. Our bulk growth studies (Fig 4A) show that at 80 μM PI in 100 μM Ca²⁺ we would expect significant growth inhibition in the entire population. To compare the effect on oscillations, we chose pollen tubes that were growing at 50 nm·s⁻¹ or greater and had appreciable clear zones; we selected for healthy tubes, thus excluding those that were not oscillating. We collected the growth rate data for each pollen tube and performed a fast Fourier transformation to generate a periodogram using R (see Materials and Methods). This allowed us to examine the oscillatory components for each pollen tube and determine the predominant period. As shown above (Supp Fig 1) for root hairs, growth rate does not correlate with period (Fig. 4A) in lily pollen tubes. The growth rates vary between just over 50 nm·s⁻¹ to as fast as 250 nm·s⁻¹ and the periods vary between 20s and 100s. No trend is observed as an attempted linear model gives an R² value of 0.16. This suggests that the oscillatory period and growth rate are not related. Similarly, when PI concentration is plotted against average period, no trend is apparent (R² = 0.07) (Fig. 4B). PI affects average growth rate as concentrations increase in large pollen tube populations. If one examines growing pollen tubes within this population, PI does not directly affect growth rate oscillations.

**Pectin methyl-esterase increases while pectinase decreases binding sites for PI**

Pectin methyl-esterase (PME) is secreted by pollen tubes at the cell apex where it de-methoxylates HGs (Bosch et al., 2005; Bosch and Hepler, 2005; Parre and Geitmann, 2005). It has already been demonstrated that addition of PME causes a dramatic increase in the cell wall thickness at the apex (Bosch et al., 2005; Parre and Geitmann, 2005). As there is no evidence that exocytosis has ceased, the wall thickening also may be attributed at least in part to continued deposition of wall material. Parre and Geitmann (2005) showed that the stiffness of the pollen tube cell wall increased upon treatment with PME. In the same study it was shown that JIM5 (Knox, 2008), an antibody specific to acidic pectins, which usually occur only along the shank, labeled the tip of pollen tubes after PME treatment (Parre and Geitmann, 2005). We reasoned that if PI binds to the same sites in HGs that Ca²⁺ does, then PI fluorescence should increase upon treatment with PME. The source of the de-methoxylated HG would be a mixture of those created by the exogenous PME and those de-methoxylated by endogenous PME.
We labeled growing pollen tubes with PI then added PME to the slides. After the addition of PME cells grow normally for ~10 min then quickly stop. We never observed growth recovery (Fig. 5 and Video S4). Concomitantly the PI signal doubles at the tip (Fig. 5 A, B and Video S3). Note that the thickened cell wall at the apex and the PI signal overlay one another. This finding is consistent with the hypothesis that PI binds to newly de-methoxylated sites created by PME.

As a complement to this experiment, we investigated the effect of pectinase on the PI fluorescence of growing pollen tubes. Pectinase is widely used for enzymatic digestion of cell walls. In Solanum chaconense, pectinase has been shown to increase growth rates at concentrations up to ~5 mg·mL⁻¹ (Parre and Geitmann 2005). Parre and Geitmann (2005) showed that Aspergillus niger pectinase specifically digested both acidic and esterified HGs in the pollen tube. If PI is indeed interacting with carboxyl groups on the HGs, we would expect PI fluorescence to dissipate as the enzyme digested the cell wall. Disruption of the cell wall could lead to cytolysis especially in a hypotonic solution, so we added sucrose to a final concentration of 10.5% along with 1 mg·mL⁻¹ pectinase.

Images of PI labeled pollen tubes were collected during treatment with pectinase. 10.5% sucrose alone can induce incipient plasmolysis, but does not cause alteration in PI fluorescence (data not shown). Line scans down the center of the PI images were collected from before treatment and after treatment. The signals from six pollen tubes were normalized to the peak fluorescence prior to addition of pectinase. Before treatment, there is a clear PI signal at the tip that is several times brighter than background or the shank walls (Fig 6A and B left panels Video S5). As pectinase takes effect, the PI fluorescence declines markedly at the tip and along the shank of the pollen tube. Ultimately, the signals at the tip and along the shank are roughly equivalent (Fig 6 A and B right panels). The tip swells somewhat and growth has stopped, but cyclosis continues, until after several minutes the pollen tube explodes when turgor pressure overwhelms the weakened wall. Presumably exocytosis continues, but the PI signal still drops markedly suggesting that the pectinase is digesting possible sites for PI binding.

**PI does not interfere with tip focused Ca²⁺ gradient in pollen tubes**

The tip focused Ca²⁺ gradient in pollen tubes has been studied extensively for nearly two decades (Miller et al., 1992; Rathore et al., 1991). Inhibition of growth has usually been shown to block
the Ca\textsuperscript{2+} gradient (Cárdenas, 2009; Hepler et al., 2001; Cole and Fowler, 2006). The pectin dyes ruthenium red (Picton and Steer, 1985; Bednarska, 1989; Ischebeck et al., 2008; Szumlanski and Nielsen, 2009) and coriphosphine O (Weis et al., 1988) have been used on plant cells but inhibit growth and block Ca\textsuperscript{2+} channels; they are not useful as vital stains of pectin. We have already shown that at working concentrations of PI and Ca\textsuperscript{2+} cells grow at control rates. We wished to investigate whether there would be changes in the Ca\textsuperscript{2+} gradient.

We injected pollen tubes with the ratiometric Ca\textsuperscript{2+} sensitive dye fura-2 dextran. After removing the needle we added PI to the growth solution then imaged the cells (Fig. 7). The DIC image shows that the cell retains standard morphology with a rounded tip and an apical clear zone (Fig 7 A and Video S6). The PI fluorescence also appears normal for a growing pollen tube (compare to Fig 1) (McKenna et al., 2009). The Ca\textsuperscript{2+} gradient has a tip high peak and decreases to a basal level of \(~ 150\) nM. If PI were interfering with Ca\textsuperscript{2+} channels we would expect an alteration in the magnitude of the gradient. Furthermore, as shown in Fig. 5B the oscillatory profiles of growth rate, PI and Ca\textsuperscript{2+} correspond to published data. These data give us confidence that PI is not interfering with Ca\textsuperscript{2+} uptake (McKenna et al., 2009; Pierson et al., 1996).

**Discussion**

In the current study we provide evidence that PI, which vitally stains plant cell walls, competes with divalent cations, especially Ca\textsuperscript{2+}, and binds to carboxyl residues on HGs. With these properties, PI staining allows analysis of the link between wall deposition and cell growth. Notably, we expand our understanding of oscillatory growth and show that in root hairs, as well as pollen tubes, an increase in apical HG accumulation precedes peaks in growth rate.

As PI represents an excellent tool for understanding the physiology of wall extensibility in divergent tip growing plant cells, we invested considerable effort in determining the mechanism of cell wall binding. The competition studies, for example, indicate that Ca\textsuperscript{2+}, Mg\textsuperscript{2+} and PI interact with a common wall component, namely the HGs. Several previous studies indicate that Ca\textsuperscript{2+} cross-links galacturonic acid residues lending strength to the cell wall (Thibault and Rinaudo, 1985). Although Mg\textsuperscript{2+} also interacts with these negative charges it does not create links between the pectin chains (Malovíková et al., 2004; Thibault and Rinaudo 1985). Models of the electrostatic charge on the surface of the PI molecule show three partial positive charges
associated with tertiary nitrogen atoms that are 6 to 7 Å apart. The negative charges on the galacturonic acid residues occur at similar intervals suggesting that Ca\(^{2+}\) and PI bind to the same sites in cell walls.

Because Ca\(^{2+}\) itself inhibits growth at high concentrations, we explored the interactions of Ca\(^{2+}\), Mg\(^{2+}\) and PI on growth and PI fluorescence in lily pollen tubes. We show that in low Ca\(^{2+}\) media, PI dramatically increases in fluorescence as its concentration increases (Fig 2B). In a higher Ca\(^{2+}\) concentration the effect is much more gradual and in the range tested the fluorescence is never as bright. The effect of increasing Mg\(^{2+}\) concentration is quite similar, supporting the hypothesis that all three ions are interacting with the same charges. Initially this suggested competitive binding of Ca\(^{2+}\) and PI for HGs. However, the regression model that most closely fits the data suggests cooperative binding. In this scenario PI and Ca\(^{2+}\) interact with the same binding sites, but only Ca\(^{2+}\) effectively cross-links HGs, so that a higher proportion of binding sites occupied by Ca\(^{2+}\) limits the ability of PI to intercalate between HGs (for model see Fig. 8). At sufficiently high PI concentrations, cross-linking by Ca\(^{2+}\) is reduced, and the wall loses strength so that tubes burst at or near to the tip. Thus, increased cross-linking due to high [Ca\(^{2+}\)] simultaneously makes PI binding energetically less favorable and stiffens the cell wall. This model is supported by findings showing that Ca\(^{2+}\) binds to HGs in this manner in vitro (Malovíková et al., 2004).

The interplay between cell wall extensibility and the constant of turgor pressure (Winship et al., 2010) ultimately regulates tip growth. The current study shows that the amount of de-methoxylated HG available for Ca\(^{2+}\) binding oscillates at the tips of root hairs as it does in pollen tubes. These data suggest a fundamental mechanism for tip growth based upon two counterpoised processes. First, exocytosis of largely methoxylated HG replaces wall material carried away from the tip by expansion, locally reducing the relative concentration of binding sites for Ca\(^{2+}\), thus making the wall more extensible. Second, de-methoxylation by PME increases the relative concentration of Ca\(^{2+}\) binding sites leading to a progressively less extensible wall due to Ca\(^{2+}\) cross-linking (see proposed model, Fig. 8). A previous study showed that PME exocytosis itself oscillates (McKenna et al., 2009), suggesting that, along with the exocytosis of HGs this could account for changes in cell wall extensibility and oscillations in growth rate. PI, by sampling a small fraction of the total Ca\(^{2+}\) binding sites available, reports on the deposition of cell wall (Fig. 8).
These data focus our attention on the control of exocytosis in our effort to understand the mechanisms of both oscillations and growth. There has been much compelling work concerning the targeting of exocytosis (Preuss et al., 2004; Nielsen et al., 2008; Szumlanski and Nielsen, 2009; Cheung et al., 2010; Lee et al., 2008). For pollen tubes in particular external signals influence the directionality of growth (Okuda et al., 2009; Chae and Lord, 2011). How can these two factors be coordinated? It seems unlikely that wall extensibility itself changes first. Somehow external signals are linked through intracellular processes to changes in cell wall extensibility. It is unclear what the intracellular signals are that could lead to directional growth changes. In addition to the molecular tools of over expressed proteins, and the probes for assessing the ionic changes in the cytoplasm, with PI we have a reporter for changes within the cell wall, which should allow further understanding of the basic mechanism of tip growing plant cells as they respond to their environment.

Materials and Methods

Pollen Tube Growth Conditions

Pollen was grown as described previously (Rounds et al., 2010). Briefly, all pollen was from Lilium formosanum stocks stored at –80°C and germinated for 1 to 1.5 h on a rotator at room temperature in a standard growth medium (LPGM): 7% (w/v) sucrose, 1.6 mM H₃BO₃, 0.1 mM CaCl₂, and 15 mM MES buffer adjusted to pH 5.7 with KOH (a final concentration of KOH of ~6 mM); all reagents were from Fisher Scientific unless otherwise noted. For microscopic observations, a pollen suspension was spread on custom-made well slides with a growth medium solution containing a final concentration of 0.7% (w/v) low-melting agarose (Sigma-Aldrich). The immobilized pollen was then covered with fresh growth medium for imaging. Cells were allowed to recover for at least 0.5 h before imaging or further manipulations.

PI staining of pollen tubes

For PI staining of the primary cell wall, the growth medium covering the immobilized and recovered cells on microscope slides was replaced with LPGM supplemented with 20-40 μM PI (Sigma). Cells were imaged >10 min after addition of the stain.

Root Hair Growth Conditions
Pre-stratified, surface sterilized *Arabidopsis thaliana* (columbia ecotype) seeds were germinated on agar pads affixed to microscope slides. The agar medium consisted of 1% (w/v) Bacto-agar (Difco) and 1% (w/v) sucrose in a modified Hoagland’s medium (Bannigan et al., 2006). The slides were placed in standard petri dishes and oriented so that the roots would grow across the slide. Seeds were germinated and grown in constant yellow light (ca.100 μmol m-2 s-1) at 22° C. For root hair growth analysis, seedlings were analyzed between 2 and 4 d. Slides were removed and the preparation was covered in modified Hoagland’s medium supplemented with 10 μM PI. The preparation was then covered with a cover slip and sealed on three sides with a 1:1:1 mixture of petroleum jelly, lanolin and paraffin (Vidali et al., 2009). Roots were allowed to recover for at least 3 h before imaging. Newly emerging root hairs were selected for imaging.

**Growth Inhibition Experiments**

PI growth inhibition experiments were performed largely as described (Rounds et al., 2010). Briefly, pollen was germinated in control LPGM for ~1 h. The pollen suspension was then aliquoted into 12-well plates with the indicated amounts of Ca2+ and PI. The cells were allowed to recover for 20 min then imaged. After 20 min the cells were imaged again. All images were calibrated with a micrometer. Imaging was performed using a 1x lens on a stereomicroscope (Leica MZ16FA) using a CCD camera (Leica DF300FX). Images were analyzed using ImageJ (sbweb.nih.gov/ij) and analysis was performed in Excel. Regression curves were generated using the non-linear least squares regression analysis command in R (Ihaka and Gentleman, 1996).

**PI/Ca2+ Fluorescence competition**

The experimental set-up was performed largely as described for the inhibition experiments. Data acquisition was performed with the Nikon TE300 microscope (see below) using a 20X objective and the light path settings described in microscopy and imaging. PI fluorescence exposure time was 1 s. Data analysis was performed using ImageJ (Abramoff et al., 2004). The average fluorescence in a 3X3 pixel box at the pollen tube tip was measured. Background was calculated by taking the average fluorescence in a 20 pixel box. ~40 pollen tubes were measured for each treatment. Data were tabulated in Excel and regression curves were generated using the non-linear least squares regression analysis command in R (Ihaka and Gentleman, 1996).
Microscopy and Imaging

PI and DIC images were acquired using a CCD camera (Quantix Cool Snap HQ; Roper Scientific) attached to a Nikon TE300 inverted microscope (Nikon Instruments) with a 40x/1.3 numerical aperture (N.A.) oil immersion objective lens for pollen and a 60X/1.4 N.A. oil immersion lens for root hairs. All the equipment was operated with MetaMorph/MetaFluor (Molecular Devices) software. A filter wheel system (Lambda 10-2; Sutter Instruments), mounted immediately before the CCD camera, controlled the position of a polarizing filter for DIC or an emission filter for fluorescence imaging. Fluorescence excitation light was provided by a 175-W ozone-free xenon lamp in a DG-4 switching system (Sutter Instruments). Transmitted light was provided by a low-voltage halogen lamp. We used the following filter setup for PI imaging: emission 495/10X, a 565 DCLP dichroic and excitation 509 LP (all filters from Chroma). Exposure times varied, but were generally~25 ms for DIC and ~800 ms for PI unless otherwise stated. For imaging fura-2 dextran simultaneously with PI, we employed the following filters: for fura emission we used a 340 nm and 380 nm filter and for PI 490 nm. A triple band (UV/D/F/R) dichroic was employed. No emission filter was used for fura, but a 640/25 emission filter was employed for PI. Excitations were as follows 150 ms at 340 nm, 50 ms at 380 nm, 15 ms for DIC, and 850 ms at 490 nm. The DG-4 excitation was set to 25% for fura-2 dextran imaging as brighter intensities tended to harm the cell.

In Vitro Growth Rate Experiment with PME and pectinase

Exogenous orange peel PME (Sigma Aldrich) treatment was carried out largely as described (Bosch et al., 2005). Briefly, pollen tubes were germinated then plated for microscopy with PI staining as described above. Once the pollen tubes had recovered 1 well vol of a solution containing orange peel PME at 30 U mL⁻¹ was added for a final concentration of 15 U mL⁻¹. Images (both DIC and fluorescence) were collected at 3 sec intervals. Pectinase experiments were carried out in much the same way using Pectinase (USB) from Aspergillus niger. The enzyme was added along with LPGM with extra sucrose to bring the final concentration to 10.5% sucrose and 1 mg mL⁻¹ pectinase. Linescans were performed using the linescan feature of Metamorph and statistical analysis was performed using Excel. The images used for analysis were from the tenth frame of image collection, before the addition of pectinase, and the last frame of image collection, after incubation in pectinase.
Intracellular Ca$^{2+}$ measurement

Cells were pressure injected with fura-2 dextran as described (Cárdenas et al., 2008). After image collection, analysis was performed using ImageJ. Masks were made for each frame in entire movies then background was subtracted. The images were Gaussian filtered then the entire movies were ratioed using the ratioplus plugin for ImageJ at 32 bits so as to obtain fractional ratio values. These were then calibrated using standard methods described elsewhere (Roy et al., 1999).

Growth Rate Measurement and Cross Correlation Analysis

Growth rate was measured using the tip-tracking feature of the MetaMorph software package (Molecular Devices). For both PI and ratioed fura-2 dextran images, the intensity along line scans (20 pixels wide) down the center of the tube was collected for each image in entire movies. A custom R script (Ihaka and Gentleman, 1996) was then used to find the peak PI or Ca value near the X,Y position of the tip of the pollen tube (or root hair) based on the MetaMorph tracking file. The resulting traces were de-trended to remove bleaching artifacts and cross-correlation was performed using a custom R script (Supplemental Protocol S1) and as described previously (McKenna et al., 2009; Cárdenas et al., 2006; Lovy-Wheeler et al., 2006).

Supplemental Material

Supplementary Figure 1: Oscillatory profiles of Arabidopsis
Supplementary Figure 2: Root hair oscillation
Supplemental Movie S1: Lily pollen tube labeled with PI.
Supplemental Movie S2: Arabidopsis root hair labeled with PI.
Supplemental Movie S3: Arabidopsis pollen tube labeled with PI
Supplemental Movie S4: PME treatment of PI labeled lily pollen tube.
Supplemental Movie S5: Pectinase treatment of PI labeled lily pollen tube.
Supplemental Movie S4: Fura-2 dextran injected and PI labeled lily pollen tube.
Supplemental Protocol S1: R script for cross correlating PI, Ca$^{2+}$ and growth rate.
Supplemental Protocol S2: R script for determining period of an oscillatory pollen tube.
Acknowledgements:

We thank Dr. M. Bezanilla and Dr. T.I. Baskin and members of their respective labs for helpful discussions.

Literature Cited


Cárdenas L, McKenna ST, Kunkel JG, Hepler PK. (2006) NAD(P)H oscillates in pollen
tubes and is correlated with tip growth. Plant Physiol **142**: 1460-1468.


Jones KH, Senft JA. (1985) An improved method to determine cell viability by simultaneous
staining with fluorescein diacetate-propidium iodide. J Histochem Cytochem 33: 77 -79.


**Figure Legends**

**Figure 1.** PI fluorescence and growth rate oscillate in Lily pollen tubes (A, B) arabidopsis root hairs (C, D, E) and pollen tubes (F, G). A, The top panel shows a DIC image of a lily pollen tube, the bottom panel shows PI fluorescence of the same tube. The PI fluorescence is pseudocolored with white representing high signal and blue low. Scale bar represents 10 µm. B, Growth rate (blue) and PI fluorescence (red) are plotted on a line graph. Both oscillate with the same period but with different phases. C, A DIC image (top panel) and PI fluorescence image (bottom panel) of an arabidopsis root hair. The scale bar represents 10 µm. B. Two PI fluorescence images of the same root hair focused on the apex representing peak (top) and trough (bottom) PI signals. Scale bar represents 5 µm. E, A line graph showing the growth rate (blue) and peak PI fluorescence at the apex (red) for the same root hair shown (C, D). F, The top panel shows a DIC image of an arabidopsis pollen tube, the bottom panel shows PI fluorescence of the same tube. The PI fluorescence is pseudocolored with white representing high signal and blue low. Scale bar represents 5 µm. G, Growth rate (blue) and PI fluorescence (red) are plotted on a line graph. Both oscillate with the same period but with different phases. The growth rate between individual 3 s frames was smaller than the pixel size for our optics in both arabidopsis cell types; to remove the noise this generated, a four (pollen) or five (root hair) image running average is shown.

**Figure 2.** PI fluorescence decreases with increasing Ca$^{2+}$ concentration. A shows Arabidopsis roots incubated in 6.4 µM PI in water with increasing amounts of Ca$^{2+}$. B and C represent fluorescence of PI in different concentrations of PI and Ca$^{2+}$ (B) or PI, Mg$^{2+}$ and Ca$^{2+}$ (C). In B squares represent 100 µM Ca$^{2+}$(dashed curve), triangles represent 10 µM Ca$^{2+}$ (Dotted and
dashed curve) and circles 1 mM Ca\textsuperscript{2+} (filled curve). B and C, regression curves represent Hill plots. B, Fluorescence depends on the concentration of Ca\textsuperscript{2+}. Decreasing Ca\textsuperscript{2+} results in more fluorescence at a given PI concentration. Each point represents the average of at least 20 pollen tubes and error bars represent S.E.M. In C triangles represent 10 µM Ca\textsuperscript{2+}(dotted and dashed curve), squares represent 100 µM Ca\textsuperscript{2+}(dashed curve), diamonds represent 10 µM Ca\textsuperscript{2+} + 90 µM Mg\textsuperscript{2+} (dotted curve) and circles 10 µM Ca\textsuperscript{2+} plus 1 mM Mg\textsuperscript{2+} (filled curve).

**Figure 3.** PI growth inhibition depends on Ca\textsuperscript{2+} concentration (A), but not Mg\textsuperscript{2+}(B). Increasing concentrations of PI inhibit pollen tube growth. This can be mitigated by increasing the concentration of Ca\textsuperscript{2+} or exacerbated by decreasing the concentration. In A Squares represent 100 µM Ca\textsuperscript{2+} (dashed curve), triangles represent 10 µM Ca\textsuperscript{2+} (dotted and dashed curve) and circles 1 mM Ca\textsuperscript{2+} (filled curve). Regression curves in A represent sigmoidal plots. Dotted and dashed curve is 10 µM Ca\textsuperscript{2+}, dashed curve is 100 µM Ca\textsuperscript{2+}, and filled curve is 1 mM Ca\textsuperscript{2+}. Each point represents the average of at least 40 pollen tubes and error bars are S.E.M. B, A similar experiment performed with Mg\textsuperscript{2+}. An increase in Mg\textsuperscript{2+} concentration does not reverse PI inhibition, though a dramatic decrease in growth rate can be seen at the higher Mg\textsuperscript{2+} concentration.

**Figure 4:** Increasing PI does not alter growth oscillations. Pollen tubes were grown in LPGM then plated and PI was added. Time series from growing tubes with appreciable clear zones were collected and the growth rates and periods were collected. A. The entire data set at all concentrations shows no significant correlation between growth rate and period. Black circles represent no PI, empty squares are 10 µM PI, diamonds are 20 µM PI, triangles are 40 µM PI, and circles are 80 µM PI. R\textsuperscript{2} = 0.16. B. The average period is plotted against the increasing PI
concentrations. Error bars are SEM. n>8 for each concentration. No linear correlation is seen as the $R^2$ value is 0.07.

**Figure 5.** PME causes a dramatic increase in PI fluorescence at the pollen tube apex. A, DIC (top) and PI fluorescence (bottom) images of a pollen tube before (0 min) and after (20 min) treatment with PME. At 20 min the tip is clearly thicker and the fluorescence has increased dramatically. Scale bar represents 10 µm. B, A line graph representing the time course for the same pollen tube shown above (A). Blue represents the growth rate and red the PI fluorescence at the pollen tube apex. Note that both growth rate and PI apex fluorescence continue to oscillate until the pollen tube stops, and then growth rate plummets and PI fluorescence surges in tandem. Black arrow (B) shows where PME was added to the slide.

**Figure 6.** Pectinase decreases PI fluorescence and stops growth. A. Pectinase was added to growing lily pollen tubes. The top panel shows DIC images before and after pectinase addition. Bottom panel shows the PI fluorescence of the same pollen tube at the same time points with the same look up table. B shows the mean intensity of a line scan down the middle of 6 pollen tubes before treatment with pectinase on the left and after on the right. The gray lines represent +/- SEM.

**Figure 7.** PI does not interfere with tip focused $[Ca^{2+}]_i$ gradient. A, DIC (top) PI fluorescence (middle) and $[Ca^{2+}]_i$ (bottom) images of a growing pollen tube that has been injected with Fura-2 dextran and labeled with PI. Scale bar represents 10 µm and the $Ca^{2+}$ gradient legend is in nM. B, Line graphs showing the growth rate (top), apical PI signal (middle) and $[Ca^{2+}]_i$ at the apex of the pollen tube shown in (A). Growth rate, PI and $[Ca^{2+}]_i$ continue to oscillate in the same manner as each of the phenomena measured individually.
**Figure 8.** Model for binding of Ca$^{2+}$ and PI to HGs during tip growth. Disorganized chains of highly methoxylated HG leave the growing cell by exocytosis. In the cell wall PME demethoxylates the chains creating linear regions of negatively charged carboxyl groups. In the presence of Ca$^{2+}$, cooperative binding occurs between HG chains creating a stiff gel. In the presence of Ca$^{2+}$ and PI, both ions bind to carboxyls, though PI cannot crosslink.
Table I: Cross correlation analysis between growth rate and maximal PI fluorescence was performed for 10 arabidopsis root hairs and pollen tubes. The data show that maximal PI fluorescence leads growth by 112.3 degrees +/- 3.9.
Figure 1. PI and growth rate oscillate in Lily pollen tubes (A, B), Arabidopsis root hairs (D, E), and pollen tubes (F, G). A, The top panel shows a DIC image of a lily pollen tube, the bottom panel shows PI fluorescence of the same tube. The PI fluorescence is pseudocolored with white representing high signal and blue low. Scale bar represents 10 µm. B, Growth rate (blue) and PI fluorescence (red) are plotted on a line graph. Both oscillate with the same period but with different phases. C, A DIC image (top panel) and PI fluorescence image (bottom panel) of an Arabidopsis root hair. The scale bar represents 10 µm. D, Two PI fluorescence images of the same root hair focused on the apex representing peak (top) and trough (bottom) PI signals. Scale bar represents 5 µm. E, A line graph showing the growth rate (blue) and peak PI fluorescence at the apex (red) for the same root hair shown (A, B). D, The top panel shows a DIC image of an Arabidopsis pollen tube, the bottom panel shows PI fluorescence of the same tube. The PI fluorescence is pseudocolored with white representing high signal and blue low. Scale bar represents 5 µm. E, Growth rate (blue) and PI fluorescence (red) are plotted on a line graph. Both oscillate with the same period but with different phases. The growth rate between individual 3 s frames was smaller than the pixel size for our optics in both Arabidopsis cell types; to remove the noise this generated, a four (pollen) or five (root hair) image running average is shown.
Figure 2. PI fluorescence decreases with increasing Ca\textsuperscript{2+} concentration. A shows Arabidopsis roots incubated in 6.4 µM PI in water with increasing amounts of Ca\textsuperscript{2+}. B and C represent fluorescence of PI in different concentrations of PI and Ca\textsuperscript{2+} (B) or PI, Ca\textsuperscript{2+} and Mg\textsuperscript{2+} (C). In B squares represent 100 µM Ca\textsuperscript{2+}, triangles represent 10 µM Ca\textsuperscript{2+} and circles 1 mM Ca\textsuperscript{2+}. Dotted and dashed curve is 10 µM Ca\textsuperscript{2+}, dashed curve is 100 µM Ca\textsuperscript{2+}, and filled curve is 1 mM Ca\textsuperscript{2+}. B and C, regression curves represent Hill plots. B, Fluorescence depends on the concentration of Ca\textsuperscript{2+}. Decreasing Ca\textsuperscript{2+} results in more fluorescence at a given PI concentration. Each point represents the average of at least 20 pollen tubes and error bars represent S.E.M. In C triangles represent 10 µM Ca\textsuperscript{2+}, squares represent 100 µM Ca\textsuperscript{2+}, diamonds represent 10 µM Ca\textsuperscript{2+} + 90 µM Mg\textsuperscript{2+} and circles 10 µM Ca\textsuperscript{2+} plus 1 mM Mg\textsuperscript{2+}. Dotted and dashed curve is 10 µM Ca\textsuperscript{2+}, dotted curve is 10 µM Ca\textsuperscript{2+} + 90 µM Mg\textsuperscript{2+}, dashed curve is 100 µM Ca\textsuperscript{2+} and filled curve is 10 µM Ca\textsuperscript{2+} 90 µM Mg\textsuperscript{2+}. 
Figure 3. PI growth inhibition depends on Ca\(^{2+}\) concentration (A), but not Mg\(^{2+}\). Increasing concentrations of PI inhibit pollen tube growth. This can be mitigated by increasing the concentration of Ca\(^{2+}\) or exacerbated by decreasing the concentration. In A Squares represent 100 µM Ca\(^{2+}\), triangles represent 10 µM Ca\(^{2+}\) and circles 1 mM Ca\(^{2+}\). Regression curves in A represent sigmoidal plots. Dotted and dashed curve is 10 µM Ca\(^{2+}\), dashed curve is 100 µM Ca\(^{2+}\), and filled curve is 1 mM Ca\(^{2+}\). Each point represents the average of at least 40 pollen tubes and error bars are S.E.M. B, A similar experiment performed with Mg\(^{2+}\). An increase in Mg\(^{2+}\) concentration does not reverse PI inhibition, though a dramatic decrease in growth rate can be seen at the higher Mg\(^{2+}\) concentration.
Figure 4: Increasing PI does not alter growth oscillations. Pollen tubes were grown in LPGM then plated and PI was added. Time series from growing tubes with appreciable clear zones were collected and the growth rates and periods were collected. A. The entire data set at all concentrations shows no significant correlation between growth rate and period. Black circles represent no PI, empty squares are 10 μM PI, diamonds are 20 μM PI, triangles are 40 μM PI, and circles are 80 μM PI. R² = 0.16. B. The average period is plotted against the increasing PI concentrations. Error bars are SEM. n>8 for each concentration. No linear correlation is seen as the R² value is 0.07.
**Figure 5.** PME causes a dramatic increase in PI fluorescence at the pollen tube apex. A, DIC (top) and PI fluorescence (bottom) images of a pollen tube before (0 min) and after (20 min) treatment with PME. At 20 min the tip is clearly thicker and the fluorescence has increased dramatically. Scale bar represents 10 µm. B, A line graph representing the time course for the same pollen tube shown above (A). Blue represents the growth rate and red the PI fluorescence at the pollen tube apex. Note that both growth rate and PI apex fluorescence continue to oscillate until the pollen tube stops, and then growth rate plummets and PI fluorescence surges in tandem. Black arrow (B) shows where PME was added to the slide.
Figure 6. Pectinase decreases PI fluorescence and stops growth. A. Pectinase was added to growing lily pollen tubes. The top panel shows DIC images before and after pectinase addition. Bottom panel shows the PI fluorescence of the same pollen tube at the same time points with the same look up table. Scale bar represents 10 μm B shows the mean intensity of a linescan down the middle of 6 pollen tubes before treatment with pectinase on the left and after on the right. The gray lines represent +/- SEM.
Figure 7. PI does not interfere with tip focused \([\text{Ca}^{2+}]_i\) gradient. A, DIC (top) PI fluorescence (middle) and \([\text{Ca}^{2+}]_i\) (bottom) images of a growing pollen tube that has been injected with Fura-2 dextran and labeled with PI. Scale bar represents 10 µm and the calcium gradient legend is in nM. B, Line graphs showing the growth rate (top), apical PI signal (middle) and \([\text{Ca}^{2+}]_i\) at the apex of the pollen tube shown in (A). Growth rate, PI and \([\text{Ca}^{2+}]_i\) continue to oscillate in the same manner as each of the phenomena measured individually.
Figure 8. Model for binding of Ca\textsuperscript{2+} and PI to HGs during tip growth. Disorganized chains of highly methoxylated HG are exocytosed from the growing cell. In the cell wall PME de-methoxylates the chains creating negatively charged carboxyl groups. In the presence of Ca\textsuperscript{2+}, crosslinks are formed between HGs creating a stiff gel. In the presence of Ca\textsuperscript{2+} and PI, both ions bind to carboxyls, though PI cannot crosslink.