



Pectate lyase-like lubricates the male gametophyte's path toward its mating partner

Youssef Chebli^{1,2} and Anja Geitmann^{1,*}

¹ Department of Plant Science, McGill University, Ste-Anne-de-Bellevue, Québec H9X 3V9, Canada

² ECP3-Multi-Scale Imaging Facility, McGill University, Ste-Anne-de-Bellevue, Québec H9X 3V9, Canada

*Author for correspondence: geitmann.aes@mcgill.ca

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (<https://academic.oup.com/plphys/pages/General-Instructions>) is Anja Geitmann.

Abstract

The pollen tube is an extension of the male gametophyte in plants and mediates sexual reproduction by delivering the sperm cells to the female gametophyte. To accomplish this task, the elongating pollen tube must break through the thick wall of the pollen grain and penetrate multiple pistillar tissues. Both processes require the loosening of cell wall material—that of the pollen intine and that of the apoplast of the transmitting tract. The enzymatic toolbox for these cell wall modifying processes employed by the invading male gametophyte is elusive. We investigated the role of the pectin-digesting pectate lyase-like (PLL) by combining mutant analysis with microscopy observations, fluorescence recovery after photo-bleaching experiments, and immuno-detection. We show that in *Arabidopsis* (*Arabidopsis thaliana*), PLLs are required for intine loosening during the first steps of pollen tube germination. We provide evidence that during pollen tube elongation, PLLs are released by the pollen tube into the extracellular space, suggesting that they may be employed to soften the apoplast of the transmitting tissue. The synergistic enzymatic action of PLLs in the pollen grain, the pollen tube, and the transmitting track contribute to an effective fertilization process.

Introduction

The pollen tube, or male gametophyte, is responsible for delivering the sperm cells to the ovary to perform double fertilization leading to fruit formation and seed set. It is a rapidly growing, cylindrical protuberance that is formed by the pollen grain upon landing on a receptive stigma. To perform its task, the pollen tube has to invade the tissues that separate the pollen grain from the female gametophyte: stigma, style, placenta, and ovule. Depending on the species, long distances (up to tens of centimeters) must be covered, especially while traversing the style. To navigate the style, depending on the species, the tube has to proceed either through a tissue-lined canal or by invading the solid transmitting tissue filling the central cavity of the style. Particularly in the case of a solid style, pollen tube growth is challenged mechanically by the impedance presented by the apoplast of the densely packed transmitting tissue (Reimann et al. 2020). In *Arabidopsis*

(*Arabidopsis thaliana*), the transmitting tract is made of cylindrical cells surrounded by a fibrous extracellular matrix (Lennon et al. 1998). Depending on the species, the pollen tube is thought to employ various methods to facilitate invasive growth by adapting its mechano-sensitive growth behavior to the anatomy of the pistil (Reimann et al. 2020) and by softening the transmitting tissue, for example by inducing programmed cell death and/or secreting digestive enzymes presumed to target the apoplast (Ori et al. 1990; Cordeiro et al. 1994; Sessa and Fluhr 1995; Ramalho-Santos et al. 1997; Vieira et al. 2001; Chen et al. 2002). However, although numerous cell wall-digesting enzymes are known to be produced by the pollen tube (Bosch and Hepler 2005), no direct experimental proof has been brought forward that these are actually released to the outside, which would be a requirement for their activity in digesting the apoplast of the transmitting tract. The reason for this lack of information is the

fact that pollen tube growth proper also requires the action of cell wall modifying enzymes for its own morphogenesis (Chebli and Geitmann 2017). Teasing apart the enzymes' morphogenetic role from the penetration-facilitating function has been a challenge.

The pollen tube grows by the constant addition of cell wall material at the apical plasma membrane. The apical cell wall continuously yields under the internal pressure of the tube (Geitmann and Steer 2006) and morphogenesis of the elongating cylindrical cell relies on the spatial and temporal control of the stiffening and maturation processes in the cell wall material (Fayant et al. 2010; Chebli et al. 2012). This maturation is reflected in the polar distribution of the cell wall components along the longitudinal axis of the tube (Fayant et al. 2010; Geitmann 2010). Many biochemical pathways involving numerous enzymes were shown to be implicated in regulating pollen tube cell wall expansion and pollen tube growth (Bosch and Hepler 2005; Geitmann and Steer 2006; Chebli and Geitmann 2007; Wolf et al. 2012). Among the crucial biochemical processes during pollen tube morphogenesis is the transformation of pectin during the maturation of the wall material (Chebli and Geitmann 2007; Chebli et al. 2012). Pectins are secreted in highly methyl-esterified form at the apex, and their de-esterification allows gelation and stiffening by calcium ions. Various pectin-modifying enzymes such as pectin-methyl-esterase, polygalacturonase, and pectate lyase (PL) are known to be implicated in the regulation of the pectin biochemistry in plant cell walls in general and pollen tubes in particular (Bosch and Hepler 2005). These biochemical reactions contribute to the regulation of cell wall mechanics by balancing between the required degrees of stiffness, elasticity, and plasticity of the wall, thus ensuring the morphogenesis of a perfectly cylindrical cell.

One of the cell wall-modifying enzyme families produced by the pollen tube is the PL family. These hydrolytic enzymes have been shown to be implicated in pectin hydrolysis during cell wall expansion, cell adhesion, and differentiation, and during the maturation and senescence of fruits. PLs are also major allergens for humans (Radauer et al. 2008; Shahali et al. 2012; Suzuki et al. 2012). In tomato (*Solanum lycopersicum*) (Marin-Rodriguez et al. 2002), banana (*Musa acuminata*) (Pua et al. 2001), and strawberries (*Fragaria xananassa*, Duch., cv Chandler) (Jimenez-Bermudez et al. 2002), PL activity enhances fruit maturation and their absence delays senescence. The enzyme acts on the pectic chains by cleaving the α -1,4-polygalacturonic acid chain by a β -elimination mechanism (Herron et al. 2000). A number of studies have shown that PL and PL-like (PLL) genes are highly expressed in pollen grains from alfalfa (*Medicago sativa*) (Wu et al. 1996), Japanese cedar (*Cryptomeria japonica*) (Taniguchi et al. 1995), tobacco (*Nicotiana tabacum*) (Wing et al. 1990; Kulikauskas and McCormick 1997), and Arabidopsis (Kulikauskas and McCormick 1997). More than 20 PL and PLL genes are expressed in pollen grains of Arabidopsis (Pina et al. 2005) prompting the speculation that they might be implicated in the early stages of pollen

germination or in the digestion of the transmitting tract. However, no experimental proof has been provided hitherto to show that the enzyme has the function to act on the surrounding growth substrate and that it is not simply implicated in the pollen tube morphogenesis by acting on its own wall.

Here we used a *semi-in vivo* experimental design (Supplemental Fig. S1) to investigate the role of the 3 genes coding for PLL enzymes: At1g14420 (PLL1), At2g02720 (PLL2), and At3g01270 (PLL3) during Arabidopsis pollen tube germination and elongation. Through fluorescence microscopy analysis we show that PLLs are important for the initiation of pollen tube germination and for pollen tube elongation. We also demonstrate that PLLs are released by the pollen tube, suggesting that they may be employed to loosen the transmitting tissue apoplast in its path to facilitate its elongation.

Results

PLL expression in pollen grains

The 3 PLL genes investigated here (At1g14420 [PLL1], At2g02720 [PLL2], and At3g01270 [PLL3]) were selected because of their high expression levels in pollen grains (Pina et al. 2005) and their very low or absence of expression in other mature organs such as the pistil. In immature growing organs such as 1 to 3-d old flowers or in siliques, the level of expression of the 3 PLLs is higher than in other organs. In mature Arabidopsis flowers, the 3 investigated PLLs are only expressed in anthers (Supplemental Fig. S2; Pina et al. 2005; Winter et al. 2007; Klepikova et al. 2016). RT-PCR performed on pollen grains imbibed for 3 h in a liquid culture medium (at which time >75% of the pollen grains had germinated and displayed a length of 200 μ m) showed that PLL1, PLL2, and PLL3 are expressed in pollen tubes (Fig. 1A).

To examine the effect of the lack of a given PLL on pollen tube development, T-DNA insertion lines were used. RT-PCR confirmed that, in hydrated pollen grains and pollen tubes, PLL1 and PLL2 were not expressed in the respective T-DNA insertion lines, whereas PLL3 was expressed, but at lower levels compared within the wild type (WT) (Fig. 1B).

To assess how the absence or reduction of PLL expression affects pollen tube growth, pollen tubes were grown both *in vitro* on a solidified agar medium and in *semi-in vivo* conditions (Supplemental Fig. S1). Only pollen grains from homozygous knock-out lines were used. The germination percentage of pollen from the 3 knock-out lines was less than half of that observed in the WT pollen (Fig. 2A). In *in vitro* conditions, WT pollen grains started to germinate 30 min after imbibition whereas germination in *pll2* pollen grains was delayed, occurring 1 h after imbibition, and *pll1* and *pll3* pollen grains germinated only 2 h after imbibition. Pollen grains were considered germinated when the pollen tube length was greater than the diameter of the pollen grain (Tuinstra and Wedel 2000). In both *in vitro* and *semi-in vivo*

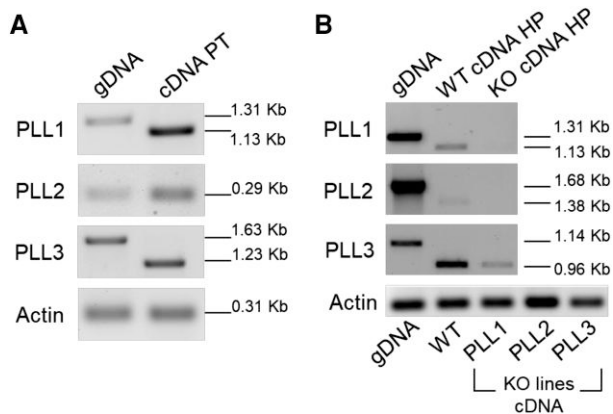


Figure 1. Expression of *PLLs* in WT and knock-out lines. PCR was performed on cDNA from WT pollen tubes (cDNA PT) **A** and on hydrated pollen grain cDNA from WT (WT cDNA HP) and knock-out lines (KO cDNA HP) using a different set of primers **B**. As a positive control, PCR on genomic DNA was also performed with the same sets of primers. Each gel represents the amplification of one *PLL* gene or *ACTIN*. Values of the molecular weight markers are indicated at the right of each gel.

conditions, the pollen tube length at 6 h after imbibition was reduced by 47% in the knock-out lines compared with WT pollen tubes (Fig. 2B). However, after 24 h of *semi-in vivo* growth, the length of the WT and knock-out pollen tubes was similar. To assert that the observed phenotypes were due to the lack of *PLL* expression, we complemented the *pll1*, *pll2*, and *pll3* knock-out lines with their respective fluorescently tagged *PLL* genes expressed under their respective native promoters. Pollen grains from homozygous complemented lines started to germinate 20 min after imbibition and although their germination rates were 18% to 25% lower than that of the WT, their germination rates were 30% to 40% higher than those of the corresponding knock-out lines. Pollen tube length after 6 h of imbibition was not significantly different from that of the WT, but significantly different from those observed in the corresponding knock-out lines (Fig. 2B).

Attempts were made to obtain double knock-out lines by crossing 2 different homozygous knock-out lines and then selfing. Crossing the resulting double heterozygous lines never yielded a double homozygous mutant. For all crossings, segregation in T2 was WT ($8.1\% \pm 3\%$), double T-DNA heterozygous ($33\% \pm 3\%$), T-DNA homozygous for one gene and T-DNA heterozygous for the other ($48.7\% \pm 3\%$) and WT for one gene and T-DNA homozygous for the other ($10.2\% \pm 3\%$).

PLLs are enriched at the collar region of the pollen grain aperture and the tip of the pollen tube

To determine the subcellular localization and to monitor the intracellular dynamics of *PLL1*, *PLL2*, and *PLL3*, we transformed *Arabidopsis* WT plants with the genes for their fluorescently tagged versions, controlled either by the respective native promoter or by the strong, anther specific protein

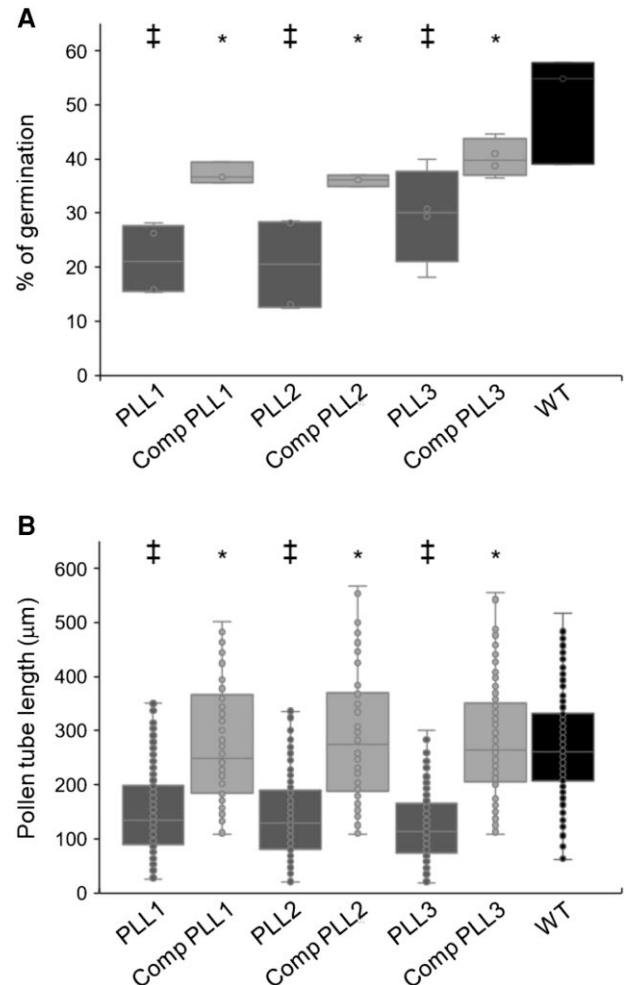


Figure 2. Germination phenotypes of WT, *PLL* knock-out, and complemented lines. Percentage of germination **A** and pollen tube length at 6 h **B** in knock-out lines (dark gray), complemented lines (light gray), and WT (black) grown in *in vitro* conditions. Gray bars represent the standard error from 5 repeats. The central lines represent the median values. The whisker lines indicate variability outside the upper and lower quartiles. The lower quartile represents the values under which 25% of data points are found. The upper quartile represents the value under which 75% of data points are found. Individual points represent the values of individual experiments. For each experiment, >50 pollen grains or tubes were analyzed. Crosses (‡) indicate that the corresponding sample is statistically different from the WT. Asterisks (*) indicate that the corresponding sample is statistically different from the knock-out mutant. (2-way t-test, $P < 0.001$ for all samples).

LAT52 promoter (Twell et al. 1991). The rationale for using 2 different approaches was (i) to understand the effect of *PLL* over-expression on pollen tube development, (ii) to be able to localize the fluorescently tagged *PLLs* in case the native promoter was weak, and (iii) to compare the subcellular localization in both cases, because the expression of a gene controlled by different promoters is known to affect its localization (Röckel et al. 2008).

Confocal laser scanning micrographs of pollen with the fluorescently tagged proteins showed that *PLLs* were

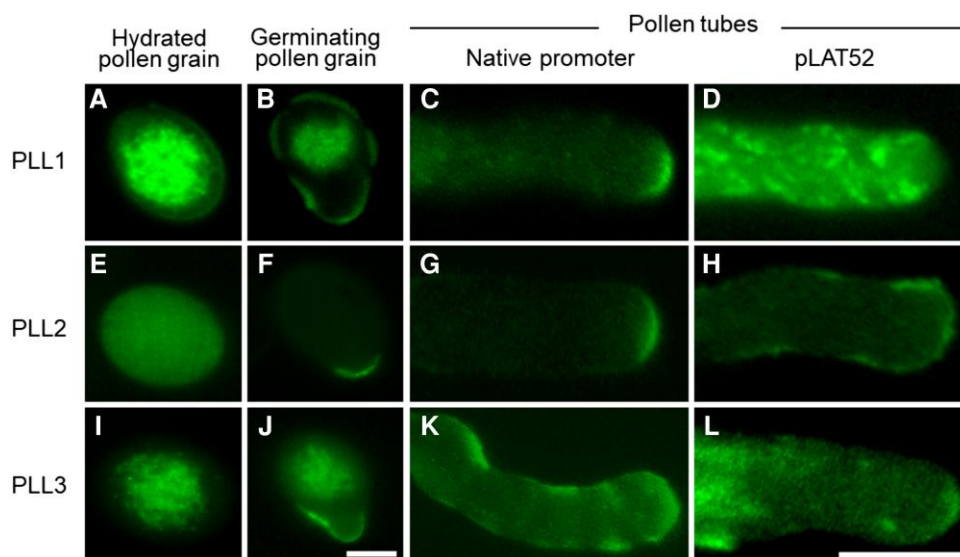


Figure 3. Localization of PLLs in pollen grains and tubes. Single confocal midsections of hydrated pollen grains **A, E, and I**), germinating pollen grains **B, F, and J**), and pollen tubes longer than 45 μm **C, D, G, H, K, and L**) expressing fluorescently tagged PLLs PLL1 **A to D**), PLL2 **E to H**) and PLL3 **I to L**) under the native promoter **A, B, C, E, F, G, I, J, and K**) or pLAT52 **D, H, and L**). Scale bar in **J** applies to all pollen grains **A, B, E, F, I, and J**) and scale bar in **L** applies to all pollen tubes **C, D, G, H, K, and L**). Scale bars = 10 μm .

abundant in the cytoplasm of hydrated pollen grains (Fig. 3, **A, E, and I**). Immediately before germination, PLLs were specifically located at the collar region delineating the site of outgrowth of the future pollen tube (Fig. 3, **B, F, and J**). Under their respective native promoters, PLL1 and PLL2 were located specifically at a narrow region on the apex of pollen tubes longer than 45 μm (Fig. 3, **C and G** and Supplemental Movie 1), whereas they could not be detected in the distal regions of the same tubes (Fig. 3 and Supplemental Movies S1 and S2). In tubes shorter than 45 μm , PLL1 and PLL2 could be seen at the very tip and in the distal region close to the aperture but they were largely absent from the subapical region. Just as for PLL1 and PLL2, PLL3 was predominantly localized at the apex of the pollen tube, but unlike the 2 others, PLL3 was quite abundant in the subapical and distal parts of the tube, raising the question of putative specific roles of the different PLLs during pollen tube elongation (Fig. 3K and Supplemental Movie 3).

Remarkably, while prolonged observation in the confocal laser scanning microscope caused tagged PLL fluorescence in the shank region to gradually bleach, the label in the continuously elongating apex did not bleach during observation (Supplemental Movie 2). This suggests that newly transported, unbleached protein was continuously delivered to the growing apex, consistent with the high exocytosis rate occurring in this region.

To prove that PLLs were not associated with the plasma membrane but with the apoplastic space, growing pollen tubes were treated with a 50% sucrose solution. In plasmolyzed pollen tubes, tagged PLLs were not localized with the plasma membrane but were mainly enriched in the periplasmic space between the retracted plasma membrane and the

apical cell wall (Fig. 4). Weaker label was also visible at the apical cell wall.

When *PLL1-CFP* and *PLL3-YFP* were expressed under pLAT52, the proteins were detected at the apical cell wall and were highly concentrated in cytoplasmic organelles, possibly Golgi, endosomes, and/or endoplasmic reticulum (Fig. 3, **D and L**). As this localization appeared to be an artifact induced by over-expression due to the strong promoter, further characterization of these organelles was not pursued. The pattern of subcellular localization of PLL2 when *PLL2-YFP* was expressed under pLAT52 was virtually identical to that observed when it was expressed under its native promoter (Fig. 3, **G and H**).

To assert that the chimeric genes expressed under their respective native promoters were not substantially overexpressed in the mutants, we assessed their relative expression levels in the inflorescence. The presence of the chimeric genes caused a decrease in the expression of the respective native genes. Expression of *PLL1-CFP* was substantially higher than the endogene expression, *PLL2-YFP* expression was statistically lower than the endogene expression and *PLL3-YFP* expression was similar to the corresponding endogene expression (Supplemental Fig. S3).

PLLs are released by the pollen tube at the apex

The absence of PLLs in the distal region of the pollen tube suggests that the enzymes either exert their function in the apical cell wall of the tube proper or that they are released into the surrounding growth substrate which would enable them to exert their role in the apoplast of the transmitting tissue. If the target of the PLLs was the pollen tube cell wall proper, an explanation for the spatially highly confined localization at the apex is warranted. Other enzymes inserted into

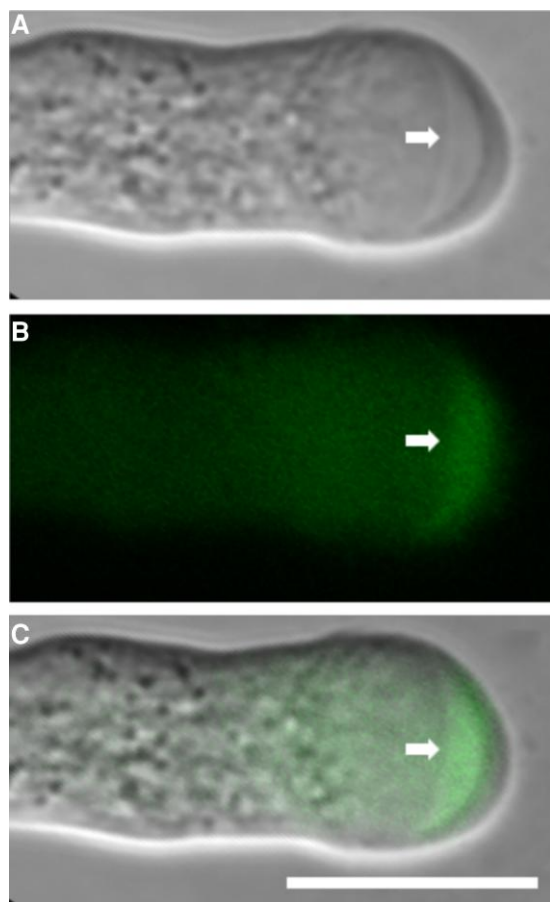


Figure 4. Periplasmic localization of PLL2-YFP in plasmolyzed pollen tubes. **A)** Differential interference contrast micrograph, **B)** confocal laser scanning micrograph of PLL2-YFP, and **C)** merged image. The arrow indicates the localization of the retracted plasma membrane. Bar = 10 μ m.

the pollen tube cell wall such as pectin methyl esterase can be found in the entire pollen tube cell wall (Röckel et al. 2008). However, a protein inhibitor of this enzyme, the pectin methyl esterase inhibitor is located only at the pollen tube apex and the spatial confinement of its localization has been hypothesized to be ensured by removal from the wall through endocytosis in the flanks of the tube (Röckel et al. 2008). Such a reincorporation mechanism could also be envisaged for the highly pole-focused localization of PLLs. It could then be hypothesized that the enzyme removed from the wall in the subapical region would be recycled immediately to the pole of the cell thus minimizing the amount of new PLLs to be synthesized and delivered to the apex. To test this hypothesis, we photobleached an area in the shank region enriched in secretory vesicles on their way to be delivered to the apex. If PLLs at the pole were predominantly obtained from the putative PLL pool reincorporated at the subapex, this bleaching would not be expected to have a dramatic effect on the fluorescence intensity of the PLL label at the pole. The results did not support this hypothesis. When shank-located vesicles containing fluorescently tagged PLLs were bleached, the fluorescence at the nonbleached pole disappeared almost completely for a period of 1 to 3 s (reduction of 80% and 75% of the fluorescence intensity of PLL1 and PLL2, respectively). This suggests that the majority of the tip-localized PLLs is not recycled in the subapical flanks but derive from the shank localized sites of synthesis, likely the Golgi. FRAP analysis showed that the half-time recovery for PLL1 and PLL2 was between 30 and 40 s and that the mobile fraction was 80% and 60%, respectively (Fig. 5). The findings were further confirmed by the fact that no internalization of fluorescence was visible at the apical plasma membrane. Interestingly, for PLL3, although the mobile fraction (75%) was within the same range as PLL1 and

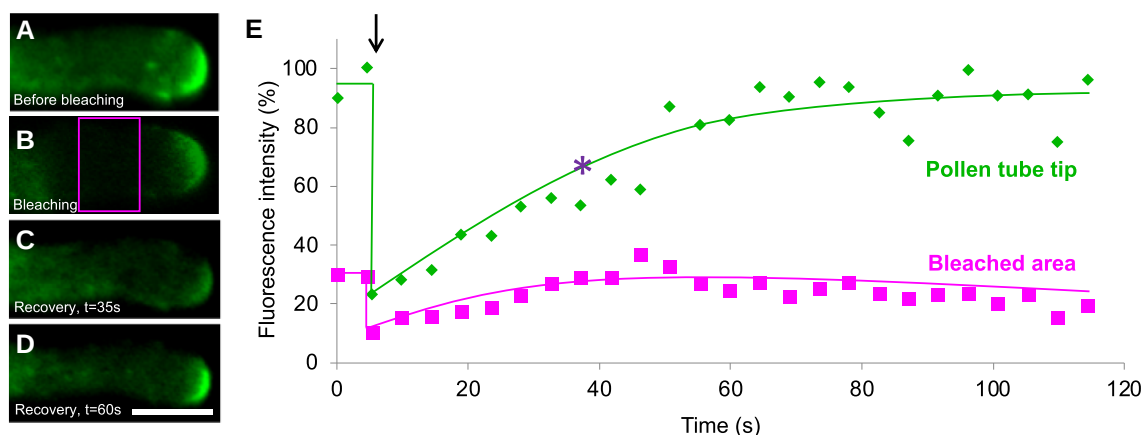


Figure 5. FRAP analysis of PLL1-CFP dynamics in the apex of a growing pollen tube. Single optical sections of a bleached pollen tube **A to D)** taken before bleaching **A)**, immediately after bleaching **B)**, 15 s **C)**, and 50 s **D)** after bleaching. **E)** Normalized fluorescence intensity in the bleached area (squares) and at the tip of tube (diamonds). Frame in **B)** identifies the bleached area. Arrow in **E)** indicates the time of bleaching. Purple star (*) indicates approximate half-time recovery of the pollen tube tip fluorescence. Bar = 10 μ m.

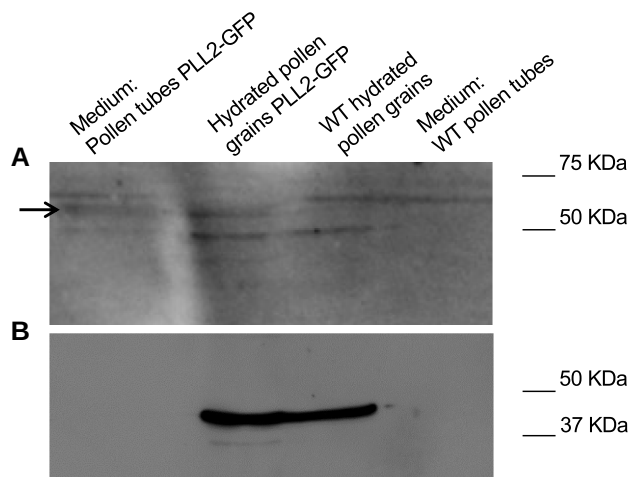


Figure 6. Immuno-detection of PLL2-GFP in the pollen tube growth medium by protein gel blot. **A)** PLL2-GFP detection and **B)** actin detection in PLL2-GFP pollen tube growth medium, in whole protein extract from PLL2-GFP hydrated pollen grains, in WT hydrated pollen grains, and in WT pollen tube growth medium. The black arrow indicates the band of PLL2-GFP at the expected ~60 kDa.

PLL2, the recovery rate calculated from the 15 s halftime recovery point was twice that determined for PLL1 and PLL2 (Supplemental Fig. S4). These findings indicate that PLL3 might be produced in higher quantities than PLL1 and PLL2, explaining also the fact that PLL3, contrary to PLL1 and PLL2, is highly enriched in the subapical and distal regions of the cell wall (Fig. 4K and Supplemental Movie 3).

Newly biosynthesized PLLs are constantly delivered to the apex (as suggested by the absence of bleaching under continuous laser illumination during imaging), but not reincorporated by endocytosis (as indicated by the FRAP approach). However, if confocal scanning is used intermittently only (i.e. no bleaching expected), the fluorescence intensity of PLLs at the pole or the meridional width of the PLL-rich domain does not increase over time. This suggests that the continuously delivered PLLs are released into the surrounding medium. This would be consistent with the periplasmic localization of PLLs upon plasmolysis which suggests that PLLs are highly soluble. To further corroborate that PLLs are truly released from the tube, we grew pollen tubes expressing *PLL2-GFP* in the *semi-in vivo* setup until they emerged from the cut end of the stigma into the liquid medium. The stigma and pollen tubes were then removed, and the medium was processed to test for secreted proteins. Salts and sugar were removed from the liquid medium by purification and a protein gel blot was performed to detect GFP. The blot showed that GFP-labeled PLL2 was present in the liquid medium, indicating that the chimeric enzyme had been released by the pollen tubes (Fig. 6). Label with anti-actin antibody was used as a negative control to demonstrate that the GFP-PLLs on the blot were not released as a result of pollen tube bursting.

Discussion

PLs-like are required during the initiation of pollen grain germination

The pollen grain wall is made of 2 thick and robust layers, the exine formed from sporopollenin, tryphine, and wax and the intine composed largely of cellulose and pectins (Van Aelst and Van Went 1992; Rowland and Domergue 2012). Together these wall layers form a very stiff envelope that protects the pollen grain from environmental stresses. To be able to emerge during germination, the pollen tube has to pass through the intine either by softening the different components through digestion or breaking of polymer linkages or by exerting sufficient force to push outwards or a combination of both (Geitmann 2010). A multitude of cell wall hydrolyzing enzymes and agents is produced by plants, fungi, and bacteria (Willats et al. 2001; Vincken et al. 2003; Cosgrove 2005) and among these many are accumulated in pollen. Proteomic and genomic data have indicated the accumulation of xylanase, xyloglucan endotransglycosylase/hydrolase, expansins, polygalacturonases, pectin methyl-esterases, and PLLs in pollen grains of *Arabidopsis* (Pua et al. 2001; Pina et al. 2005). Some of these enzymes have been shown and others have been stipulated to play important roles in the first stages of pollen tube initiation by hydrolyzing and loosening the grain wall at the aperture before germination (Bosch and Hepler 2005; Dai et al. 2007; Suen and Huang 2007; Valdivia et al. 2009). Other components such as reactive oxygen species appear during pollen hydration and during the lag phase before germination and were also shown to be important for pollen tube emergence (Speranza et al. 2012).

PLLs in particular, were shown to be accumulated in pollen grains (Pua et al. 2001; Pina et al. 2005). PLLs were stipulated to be involved in pollen wall softening but their temporal activation and subcellular localization have remained elusive (Kulikauskas and McCormick 1997; Pua et al. 2001; Bosch and Hepler 2005; Pina et al. 2005). It was hypothesized that their role during pollen germination is to cleave the pectin matrix of the intine. This would result in locally loosening the intine cover in the aperture, thus allowing the turgor pressure inside the hydrated pollen grain to initiate germination by pushing the cell wall outwards. Vesicles filled with cell wall precursors (mainly pectins) would then fuse at the collar region and elongation of the pollen would begin (Taylor and Hepler 1997). Evidence for the respective roles of enzymatic actions during the pollen grain germination had been elusive, but our results confirm that PLLs may play a role in modifying the cell wall composition of the pollen grain during the first steps of protrusion and germination.

Using RT-PCR and fluorescence localization we showed that PLLs are produced in hydrated pollen grains and that PLL1, PLL2, and PLL3 are abundant and specifically localized at the aperture of the pollen grain before germination. Pollen performance of knock-out lines showed that the germination percentage was reduced by up to 60% as a result of the suppression of a single PLL. Importantly, those grains that did

germinate required more time to do so, consistent with a role of PLL in intine loosening. Taken together these results suggest that PLLs are implicated in the initiation of pollen germination, likely by locally softening the intine wall, thus providing a specifically localized surface region that is conducive to the formation of a protuberance.

The pollen tubes that were able to emerge from the grains of knock-out lines were shorter than their WT counterparts at 6 h of *semi-in vivo* culture but not after 24 h. This suggests that individual PLLs may enhance pollen performance but are not essential for the overall capacity of the pollen tube to elongate *in vitro*. Their action seems to be more critical during the first stages of pollen germination since the percentage of germinated pollen grains was markedly reduced in the knock-out lines. Delayed germination due to a missing PLL would also explain why obtaining double homozygous mutants was not possible. According to normal Mendelian segregation, the percentages resulting from the self crossing of a double heterozygous t-DNA insertion line would be 6.25% WT for both genes, 50% homozygous for one gene, and heterozygous for the other, 25% double heterozygous, 12.5% homozygous for one gene and WT for the other and 6.25% double homozygous. This was not the case in the observed crossings, where no double homozygous individuals were obtained and the number of double heterozygous individuals increased drastically to 33%. One explanation for this would be that the double knock-out pollen grains (25% of the population of pollen grains formed by a heterozygous plant) were disadvantaged by delayed germination in comparison to the pollen grains containing only a single knock-out (50% of the population). Assuming that only WT and single knock-out pollen tubes would succeed in fertilizing the ovules, the expected theoretical ratios would be very close to those that were obtained: 8.3% WT (8.1% obtained), 25% double heterozygous (33% obtained), 50% homozygous for one gene and heterozygous for the other (48.7% obtained), 16.7% homozygous for one gene and WT for the other (10.2% obtained) and 0% double homozygous (0% obtained). Although the double knock-out pollen grains might eventually germinate, all available ovules would be fertilized by the time their tubes reach the ovary.

PLs-like are released by the pollen tube and may digest the transmitting tissue

When growing inside the pistil, the pollen tube has to invade the apoplastic space of the transmitting tract. The expansive growth activity of the pollen tube is located in the shoulder of its hemisphere-shaped apex (Geitmann and Dumais 2009). Therefore, this region is most exposed to friction forces created by the contact of the elongating pollen tube with the surrounding cells of the transmitting tract (Sanati Nezhad and Geitmann 2013). To minimize these friction forces and to facilitate pollen tube elongation within the pistil environment, plants have developed 2 major strategies: (i) the activation of programmed cell death of the transmitting tissue

and/or (ii) the induction of secretion of auto-digesting enzymes by the style. Both of these strategies allow the loosening of the transmitting tract which facilitates pollen tube elongation. In tobacco styles, the programmed cell death of the transmitting tissue is triggered by the landing of compatible pollen on the pistil. This leads to a change in the RNA stability and to substantial morphological changes in the styler cells ultimately leading to the death of the transmitting tissue (Wang et al. 1996; Wu and Cheung 2000). Cell death has been hypothesized to be necessary to provide nutrients to growing pollen tubes but also to facilitate pollen tube penetration. Auto-digestion of the styler tissues by hydrolytic enzymes is involved in the degradation and remodeling of the apoplast of the transmitting tract shortly before and during pollen tube growth. The style-specific β -glucanase SP41 in tobacco reaches its peak 2 d before anthesis and remains high until style senescence (Ori et al. 1990; Sessa and Fluhr 1995). Aspartic proteinases in Arabidopsis transmitting tissue (Chen et al. 2002) and in cardoon (*Cynara cardunculus*) transmitting tissue, stigmatic papilla and epidermal cells of the style degrade the transmitting tract and facilitate pollen tube penetration (Cordeiro et al. 1994; Ramalho-Santos et al. 1997; Vieira et al. 2001).

The auto-digestive activity of the transmitting tract is likely to considerably aid pollen tube invasion, but it has long been hypothesized that the pollen tube ensures successful passage by adding its own enzymes to the mix. The pollen tube is known to produce and therefore thought to secrete wall-digesting enzymes and agents such as xylanase (Suen and Huang 2007), β -expansins (Valdivia et al. 2009), or polygalacturonases (Marin-Rodriguez et al. 2002), but the actual release of these agents from the cell into the surrounding substrate has not been proven experimentally. Our findings show clearly that the Arabidopsis pollen tube releases PLLs into the *in vitro* growth medium. This is consistent with bio-informatics studies using several prediction models (Supplemental Results and Supplemental Fig. S5) showing that once exocytosed, the PLL's N-terminal domain is cleaved. This suggests that PLLs are not attached to the plasma membrane but rather relocated into the cell wall or, as suggested by our findings, into the extracellular space. It is therefore reasonable to presume that *in planta* these enzymes are used to soften of the cell walls and/or middle lamellae of the transmitting tissue. We propose that this process may decrease the friction forces between the advancing tube and the style and may facilitate invasion similar to a lubricant.

During pollen tube elongation, PLL localization in the cell wall is mostly confined to the tip of the pollen tube. No accumulation was visible in the distal maturing cell wall, except for PLL3. Confocal laser scanning microscopy and FRAP experiments showed that newly biosynthesized PLLs are continuously delivered to the tip of the pollen tube without accumulating in the wall, suggesting that PLLs are secreted into the extracellular environment. This was corroborated by evidence from protein gel blot analysis revealing the presence of GFP-labelled PLL2 in the liquid *in vitro* growth

medium. If PLLs were recycled in the subapical flanks or into the cytoplasm, an inward fluorescent movement would have been detected, as is the case with bilipidic membranes stained with styryl dyes and GFP-labelled membrane proteins that are internalized at the shank of the tube (Bove et al. 2008; van Oostende-Triplet et al. 2017; Kaneda et al. 2019). Moreover, upon plasmolysis, the PLLs accumulated in the periplasmic region, indicating that the enzymes are highly soluble and are likely to be continuously released during pollen tube growth. When expressing the fluorescently tagged PLLs under the strong *LAT52* promoter, PLL1, and PLL3 were not restricted to the apical cell wall but were also localized inside cytoplasmic organelles. The mislocalization of a protein when its gene is expressed under a strong promoter is a common phenomenon that was observed for example for the pectin methyl esterase inhibitor expressed under *LAT52* instead of its native promoter (Röckel et al. 2008). Given that PLLs are pectin degrading enzymes and highly soluble, high amounts may be toxic to the pollen tube if secreted in excess because it might damage its own cell wall. The relocalization of excess PLLs into cytoplasmic organelles (possibly endosomes) may therefore be a strategy to prevent the auto-destruction of the pollen tube's own wall.

PLL3 might be implicated in the regulation of the pollen tube cell wall properties

PLL3 is localized at the apex of the pollen tube like PLL1 and PLL2 but is also enriched in the subapical and distal parts of the pollen tube cell wall. Moreover, FRAP experiments showed that PLL3 might be produced more abundantly than PLL1 and PLL2 during pollen tube elongation. These findings suggest that, in addition to being implicated in the loosening of the pollen grain cell wall and the digestion of the transmitting track, PLL3 might also play a role in regulating the biochemistry of the pollen tube cell wall during its elongation. The localization of PLL3 in the pollen tube shoulder cell wall, where pectins are demethyl-esterified but not yet fully cross-linked with Ca^{2+} raises the possibility that PLL3 might, at this location, digest the demethylated pectins, contributing to the delicate equilibrium of strain and stress required at this location for the proper formation of the cylindrical shape of the pollen tube (Fayant et al. 2010; Chebli et al. 2012). PLL3 pollen tube knock-out mutants did not display any characteristic phenotype distinct from the WT or its PLL1 and PLL2 counterparts, suggesting that the role of PLL3 in regulating the cell wall properties is either non-essential or that, in this single mutant, mechanisms involving other pectin digesting enzymes might compensate for the absence of PLL3. Further investigation is warranted to understand if and how PLL3 activity affects the pectin organization and the mechanical properties of the pollen tube cell wall.

Digested pectins could act as signaling molecules

The potential role of PLL-mediated digestion of the apoplast of the transmitting tract may not be limited to a mechanical role

of lubrication but could be further reaching. PLLs cleave long pectin chains into oligogalacturonides. Oligogalacturonides play the role of signaling molecules; they are known to induce plant defense mechanisms, to regulate plant growth and development, and be implicated in the molecular communication between rhizobia and legumes (Ridley et al. 2001; Field 2009; Hématy et al. 2009; Moscatiello et al. 2012; Wolf et al. 2012). One could, therefore, argue that oligogalacturonides resulting from PLL activity may serve as signals that lead the pollen tube through the most “liquefied” path of the style. Though further investigation is required to reveal whether and how the oligogalacturonides resulting from pectin digestion may be involved in pollen tube guidance and in pollen tube-sensing by the synergid cell (Duan et al. 2020), the notion that sugar-rich components (such as arabinogalactan proteins) contribute to guiding the pollen tube throughout the style has been brought forward earlier (Coimbra and Pereira 2012). It is possible that the pollen tube itself actually causes these signaling molecules to be generated and might play a pivotal role in its own guidance. How these signals are integrated with other external cues provided by the ovary and ovule tissues remains to be investigated. Only a few guiding molecules have been identified so far, these include chemocyanin in lily (*Lilium* sp.), calcium, *Egg Apparatus* (EA1) in maize, and the defensin-like cysteine-rich LURE peptides in the wishbone flower (*Torenia fournieri*) (Cheung and Wu 2001; Higashiyama et al. 2003; Geitmann and Palanivelu 2007; Higashiyama and Hamamura 2008; Dresselhaus and Márton 2009; Higashiyama 2010; Palanivelu 2011; Palanivelu and Tsukamoto 2012). PLLs are essential for the initiation of pollen germination and they are released by the elongating tube, likely to facilitate its invasive growth by digesting the pectins composing the apoplast of the transmitting tissue. During the pollen tube growth in the pistil, the change in the mechanical environment properties (such as a softening of the transmitting tissue) alters the growth rate behavior of the pollen tube, suggesting that the pollen responds to environmental mechanical cues with a specific feedback-based control mechanism that is yet to be identified (Ghanbari et al. 2018; Reimann et al. 2020; Kapoor and Geitmann 2022). Figure 7 summarizes our conceptual model for PLL localization and dynamics during pollen tube development. Our findings open the door to a better understanding of the interaction between the male gametophyte and the gynecium.

Materials and methods

Plant material

Arabidopsis (*A. thaliana*) ecotype Columbia 0 plants were grown in trays in a greenhouse at 22°C day temperature and 20°C night temperature, 50% humidity under 16 h day-light, and 300 $\mu\text{E m}^{-2} \text{s}^{-1}$ light intensity. Plants were irrigated every day and fertilized every second day with Plant-Prod 20-20-20 fertilizer at 200 ppm. Pollen was collected every day during anthesis using a modified vacuum cleaner as

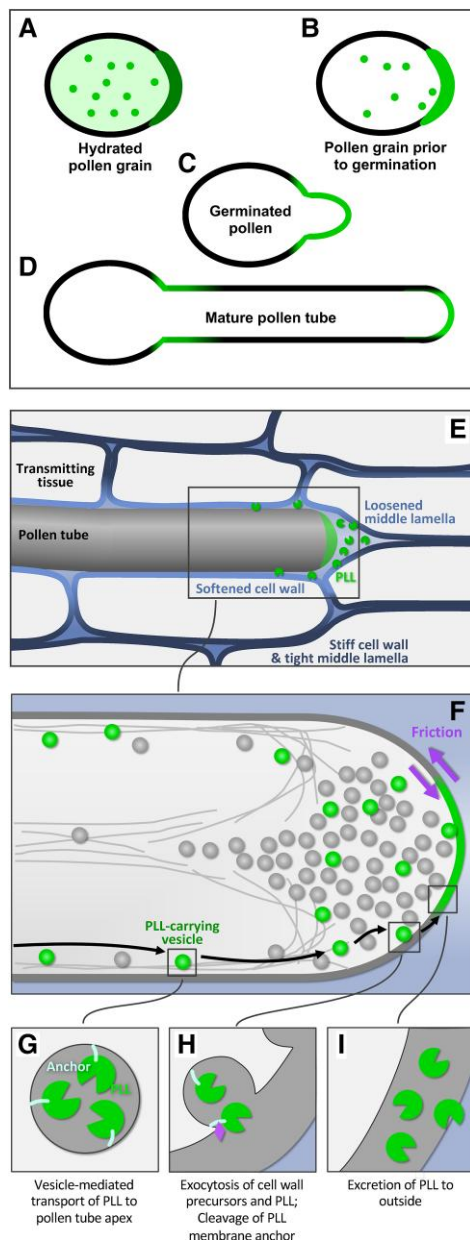


Figure 7. Overview of the localization and dynamics of PLLs during pollen tube development. Schematic of the localization **A to D**) of PLLs (green) in hydrated pollen grains **A**), in pollen grains few seconds before germination **B**), during the first minutes of pollen tube elongation **C**) and during sustained pollen tube growth. **E to I**) Intracellular and extracellular dynamics of PLLs. **E**) The pollen tube (dark gray, centre) invades the transmitting tract and is in physical contact with the neighboring walls (blue) of the transmitting tissue cells. PLLs (green pacman symbols) are released at the pollen tube tip and may contribute to softening the transmitting tract cell walls and loosening the middle lamella. The relative stiffness of the walls is color-coded with dark blue representing stiffer walls and light blue softened walls. **F**) Intracellular transport of PLLs to the pollen tube apex occurs in secretory vesicles containing cell wall precursors (gray) or enzymes (green). **G**) PLLs are anchored to the vesicle membrane by their N-terminal (light green line symbol) is cleaved upon exocytosis **H**). **I**) PLLs are released into the periplasmic space and cell wall before their release into the extracellular medium (green pacman symbols in **E**). Objects are not drawn to scale.

described by Johnson-Brousseau and McCormick (2004). The T-DNA mutants used for At1g14420 (GK-074A12-011892) and At2g02720 (GK-557E02-023923), were generated in the context of the GABI-Kat program and provided by Bernd Weisshaar (MPI for Plant Breeding Research; Cologne, Germany) (Rosso et al. 2003). The T-DNA mutant used for At3g01270 (GK-703E10-025186) was obtained from the National Arabidopsis Stock Center (NSAC) (Scholl et al. 2000). T-DNA insertion lines and lines expressing fluorescent tagged PLL genes were grown on a MS medium with the appropriate antibiotic ($50 \mu\text{g mL}^{-1}$ kanamycin, $50 \mu\text{g mL}^{-1}$ hygromycin, and/or $5.25 \mu\text{g mL}^{-1}$ sulfadiazine) before being transferred to the soil. Male sterile Arabidopsis Landsberg erecta plants were obtained from Ravishankar Palanivelu (University of Arizona).

In vitro and semi-in vivo pollen tube growth

In vitro pollen germination was conducted on solidified Arabidopsis pollen tube growth medium (Bou Daher et al. 2009) for 3 to 6 h depending on the experiment. Pollen grains were considered germinated when the length of the pollen tube exceeded the diameter of the pollen grain. Pollen tube length was measured after 6 h of imbibition. For semi-in vivo germination, stigmas of male sterile Arabidopsis plants were cut and placed on a solidified germination medium (Supplemental Fig S1). Hydrated pollen grains were placed on the stigma and left to germinate for 3 to 24 h depending on the experiment. Two-way *t*-test was performed to assess the statistical differences in pollen germination rates and pollen tube length.

Fluorescent tagging of PLLs

For native expression, the constructs included the promoter sequence 5'-UTR (about 1,000 bp), the 3'-UTR, and the terminator region (about 200 bp). The location of the fluorescent tag relative to the target gene was determined based on computer-assisted predictions of protein folding, cleavage sites, and functional domain probabilities. Arabidopsis plants expressing PLL1 (At1g14420), PLL2 (At2g02720), and PLL3 (At3g01270) were generated using the fluorescent tagging of full-length protein technique (Tian et al. 2004). Briefly, 2 sets of primers P1-P2 and P3-P4 were designed to amplify each of the target genes in 2 fragments. P1 and P4 contained attB1 and attB2 recombination sites respectively in addition to gene-specific sequences. P2 and P3 contained gene-specific sequences and fluorescent tag specific sequences. PLL1 was labeled with the cyan variant of the yellow fluorescent protein (CFP) while PLL2 and PLL3 were labeled with yellow fluorescent protein (YFP). Primer sequences are listed in Supplemental Table S1.

The fluorescent tag was combined with the 2 PCR fragments using a triple template PCR with a forward primer containing the attB sites. Individual PCR fragments were amplified using Phusion (Finnzymes) DNA polymerase and triple template PCR was performed using ExTaq (TaKaRa) DNA polymerase. DNA was extracted from gel using

QIAquick gel extraction kit (Qiagen). Final PCR fragments were introduced into pDONR Zeo (Invitrogen) entry vector using a BP recombination reaction according to the manufacturer's instructions (Invitrogen). Positive clones were verified by sequencing. The chimeric gene was introduced in pBIN-GW (Tian et al. 2004) and pMDC107 (obtained from the Arabidopsis Biological Resource Center) (Curtis and Grossniklaus 2003) destination vectors using LR recombination reaction according to the manufacturer manual (Invitrogen). Plasmid extraction from bacteria was done with QIAprep spin miniprep kit (Qiagen).

For *PLLs* expressed under the control of pLAT52, total RNA from leaves was extracted with Trizol (Invitrogen) according to the manufacturer manual. 5 µg was used to synthesize cDNA using the SuperScript III First-Strand Synthesis SuperMix (Invitrogen) in an Eppendorf AG 22331 thermocycler. *PLL* corresponding cDNA was amplified using the primer sequence shown in Supplemental Table S1. PCR fragments were amplified using ExTaq (TaKaRa) DNA polymerase. Final PCR fragments were introduced into pENTR TOPO vector (Invitrogen) using a TOPO cloning reaction according to the manufacturer manual (Invitrogen). Positive clones were verified by sequencing. The chimeric gene was introduced between the 3'-end of pLAT52 and the 5'-end of a GFP coding sequence in a modified pMDC107 vector.

Agrobacterium-mediated transformation

PLL1, *PLL2*, and *PLL3* knock-out lines and their corresponding WT of Arabidopsis were transformed using *Agrobacterium* mediated transformation. *PLL1*, *PLL2*, and *PLL3* knock-out plants were transformed with their respective fluorescent tagged *PLLs* expressed under the native promoter or under the LAT52 promoter. *Agrobacterium tumefaciens* cells (strain GV3101) were grown overnight in 500 mL of LB-medium supplemented with kanamycin (50 µg mL⁻¹), rifampicin (100 µg mL⁻¹), and gentamycin (50 µg mL⁻¹) until an OD of 1.7. After centrifugation at 4,000 × *g* for 15 min, the cells were suspended in 400 mL of a 4% (w/v) sucrose solution and 0.02% (v/v) Silwet 77 (v/v). Arabidopsis plants were submerged upside-down in the *Agrobacterium* solution for 15 s, then gently shaken for 5 s, wrapped in plastic foil, and kept on their side overnight. On the second day, plants were unwrapped and left to grow in the conditions stated above. Seeds were left to dry before collection.

Genotyping

Knock-out lines were verified by a triple primer PCR with a specific T-DNA primer and 2 specific gene primers (Supplemental Table S1). Arabidopsis lines expressing fluorescent-tagged *PLLs* were first verified by confocal laser scanning microscopy as described below and then by PCR using a primer specific to the gene and a primer specific to the fluorescent protein. Only homozygous lines were used in this study.

RT-PCR

RT-PCR was used to determine whether *PLLs* were expressed in whole flowers, hydrated pollen grains, and pollen tubes of WT and knock-out lines. Total RNA was extracted, and cDNA was synthesized as described above. Supplemental Table S2 summarizes the different PCR conditions. Primer sequences are listed in Supplemental Table S1. A total of 7 µL of each reaction mix was loaded on a 1% (w/v) agarose gel stained with ethidium bromide and band intensity on the photographs was quantified using ImageJ software (Schneider et al. 2012). The endogenous expression levels in the transgenic lines expressing fluorescent *PLLs* under their respective native promoters were used as a reference value for expression after normalization based on the actin expression profile. In order to amplify only the transgene, the corresponding reverse primer was chosen on the fluorescent protein.

Microscopy

In vitro and semi-in vivo grown pollen tubes were observed with a Zeiss Imager-Z1 microscope equipped with a Zeiss AxioCam MRm Rev.2 camera and AxioVision Release 4.5 software. Pollen tubes expressing fluorescent *PLLs* were observed with a Zeiss LSM 510 META/LSM 5 LIVE/Axiovert 200M system. In *Live* observation mode, GFP and YFP were excited with the 488 nm argon laser (4%) and an LP 505 emission filter, the detector gain was set at 600, the pinhole was set to 1 AU. In *Meta* observation mode GFP and YFP were excited with a 488 nm (2%) laser and respective emission filters of LP 530 and BP 505 to 550, the detector gain was set at 700. CFP was excited with a 458 nm (2%) laser and a BP 470 to 500 nm emission filter, the detector gain was set at 800, the pinhole was set to 1 AU. For FRAP experiments, the 488 nm line of the argon laser was used at maximal power, output set to 12% to 16% for image acquisition and 100% for photobleaching, and emission filter LP 505, the detector gain was set at 600, the pinhole was set to 1 AU. Quantification of FRAP was performed with the Zeiss LSM 5 Duo and Fiji software (Schindelin et al. 2012). Quantification of fluorescence intensity was performed at the tip of the pollen tube, the bleached region, and in an area of the image that was not bleached. Values were corrected to compensate for bleaching caused in the imaging mode by dividing fluorescence intensity in the bleached region of interest by that in an unbleached region. Fluorescence intensity values were normalized to the highest fluorescence intensity of the tube apex before the bleach. The mobile fraction of the *PLLs* at the tip of the tubes was calculated using the following formula: $M_f = (I_E - I_0) / (I_1 - I_0) \times 100$. The half-time recovery of *PLL* fluorescence at the tip of the tubes was determined as the time required for the tube apex to recover halfway between the initial bleaching of the vesicles in the sup-apex and the steady state of fluorescence intensity. The halfway recovery fluorescence was calculated using the following formula: $F_{1/2} = I_0 + (I_1 - I_0)/2$ where: I_E is the mean end value of the recovered

fluorescence intensity; I_0 is the first postbleach fluorescence intensity and I_1 is the initial (prebleach) fluorescence intensity. The results presented in this manuscript are averages of FRAP experiments conducted on at least 6 different pollen tubes. Images were adjusted for brightness and contrast.

Detection of PLL2-GFP in the growth medium

WT pollen grains and pollen grains expressing *PLL2-GFP* were grown on *Arabidopsis* male-sterile stigmas that had been cut and placed on top of a 35 μ m filter mesh soaking in a growth medium. Pollen tubes were grown until they passed through the cut stigma and proceeded to grow in the liquid medium for 3 h. The liquid growth medium was then collected and filtered with a 30 kDa Centricon regenerated cellulose membrane (Millipore) to remove sugar, salts, and small molecular weight proteins (under 30 kDa). For the detection of PLL2-GFP with an expected band at 60 kDa, 10 μ L of sample were mixed with 10 μ L of 4 $\times$ loading dye (100 mM Tris-base, 40% (v/v) glycerol, 5% (w/v) SDS, 0.005% (v/v) bromophenol blue, 10% (v/v) β -mercaptoethanol, pH 6.8), heated at 95°C for 10 min and separated by SDS-PAGE. Separated proteins in the gel were electrophoretically transferred onto a PVDF membrane at 35 V overnight at 4°C. The blotted membrane was rinsed in TBS buffer (140 mM NaCl, 2.7 mM KCl, 19 mM Tris-HCl, pH 7.4) and then blocked with 3.5% (w/v) skim milk in TBS for 2 h. The membrane was washed a second time with 3.5% milk. TBS and anti-GFP (Sigma) or anti-actin (Sigma) were added to a final concentration of 1:1,000 and 1:20,000, respectively, and incubated overnight at 4°C. The membrane was then washed 3 times with TBS containing 0.05% (v/v) Tween 20 for 10 min. A peroxidase conjugated IgG antimouse (BioShop) at a final concentration of 1:20,000 was used as a secondary antibody. The bound antibodies were detected with a chemiluminescent HRP substrate (Millipore) according to the manufacturer's instructions. Detection was done with an ImageQuant Las4000 biomolecular imager (GE). Brightness and contrast were adjusted in the images to reveal faint bands. A gamma correction of 1.2 was also applied.

Accession numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers 838004 (At1g14420), 814801 (At2g02720), and 821142 (At3g01270).

Acknowledgments

We would like to thank Jonathan Soulard and Mario Cappadocia (Université de Montréal, Montréal, QC, Canada) for kindly providing the pMDC107 and the modified pMDC107 with pLAT52 and GFP. We thank Natasha Raikhel (University of California, Riverside, CA, USA) for generously providing the pBIN-GW, p-Citrine3, and p-CFP plasmids and Ravishankar Palanivelu (University of Arizona, Tucson, AZ, USA) for providing the *Arabidopsis* male sterile line.

We acknowledge Firas Bou Daher for providing the images shown in [Supplemental Fig. S1, A and C](#).

Author contributions

Y.C. and A.G. designed the research and wrote the paper. Y.C. performed the experiments and analyzed the data.

Supplemental data

The following materials are available in the online version of this article.

Supplemental Figure S1. Semi-in vivo germination setup.

Supplemental Figure S2. Schematic representation of the expression profiles of *PLL1*, *PLL2*, and *PPL3* in all organs of *A. thaliana*.

Supplemental Figure S3. Relative expression levels of endogenes and transgenes.

Supplemental Figure S4. Examples of FRAP analysis of PLL1-CFP, PLL2-YFP, and PLL3-YFP.

Supplemental Figure S5. PLL1, PLL2, and PPL3 protein sequences and structure predictions.

Supplemental Table S1. Sequence of the primers used in this study.

Supplemental Table S2. Experimental details of the RT-PCRs performed in an Eppendorf AG 22331 thermocycler.

Supplemental Movie 1. Localization of PLL2-YFP in *Arabidopsis* pollen tube.

Supplemental Movie 2. Localization of PLL2-YFP in a pollen grain.

Supplemental Movie 3. Localization of PLL3-YFP in *Arabidopsis* pollen grain and tube.

Funding

Work in the Geitmann Lab is supported by a Discovery grant from the Natural Sciences and Engineering Research Council of Canada (NSERC) and by the Canada Research Chairs Program.

Conflict of interest statement. None declared.

Data availability

Data are available upon request to the corresponding author.

References

- Bosch M, Hepler PK. Pectin methylesterases and pectin dynamics in pollen tubes. *Plant Cell*. 2005;17(12):3219–3226. <https://doi.org/10.1105/tpc.105.037473>
- Bou Daher F, Chebli Y, Geitmann A. Optimization of conditions for germination of cold-stored *Arabidopsis thaliana* pollen. *Plant Cell Rep*. 2009;28(3):347–357. <https://doi.org/10.1007/s00299-008-0647-1>
- Bove JRM, Vaillancourt B, Kroeger J, Hepler PK, Wiseman PW, Geitmann A. Magnitude and direction of vesicle dynamics in growing pollen tubes using spatiotemporal image correlation spectroscopy and fluorescence recovery after photobleaching. *Plant Physiol*. 2008;147(4):1646–1658. <https://doi.org/10.1104/pp.108.120212>

- Chebli Y, Geitmann A.** Mechanical principles governing pollen tube growth. *Funct Plant Sci Biotechnol.* 2007;**1**(2):232–245.
- Chebli Y, Geitmann A.** Cellular growth in plants requires regulation of cell wall biochemistry. *Curr Opin Cell Biol.* 2017;**44**:28–35. <https://doi.org/10.1016/j.cceb.2017.01.002>
- Chebli Y, Kaneda M, Zerkour R, Geitmann A.** The cell wall of the *Arabidopsis* pollen tube—spatial distribution, recycling, and network formation of polysaccharides. *Plant Physiol.* 2012;**160**(4):1940–1955. <https://doi.org/10.1104/pp.112.199729>
- Chen X, Pfeil JE, Gal S.** The three typical aspartic proteinase genes of *Arabidopsis thaliana* are differentially expressed. *Eur J Biochem.* 2002;**269**(18):4675–4684. <https://doi.org/10.1046/j.1432-1033.2002.03168.x>
- Cheung AY, Wu H-M.** Pollen tube guidance—right on target. *Science.* 2001;**293**(5534):1441–1442. <https://doi.org/10.1126/science.1065051>
- Coimbra S, Pereira LG.** Arabinogalactan proteins in *Arabidopsis thaliana* pollen development. In: Çiftçi YÖ, editor. *Transgenic plants—advances and limitations.* Rijeka, Croatia: In Tech; 2012. p. 329–352.
- Cordeiro MC, Pais MS, Brodelius PE.** Tissue-specific expression of multiple forms of cyprosin (aspartic proteinase) in flowers of *Cynara cardunculus*. *Physiol Plant.* 1994;**92**(4):645–653. <https://doi.org/10.1111/j.1399-3054.1994.tb03035.x>
- Cosgrove DJ.** Growth of the plant cell wall. *Nat Rev Mol Cell Biol.* 2005;**6**(11):850–861. <https://doi.org/10.1038/nrm1746>
- Curtis MD, Grossniklaus U.** A gateway cloning vector set for high-throughput functional analysis of genes in planta. *Plant Physiol.* 2003;**133**(2):462–469. <https://doi.org/10.1104/pp.103.027979>
- Dai S, Chen T, Chong K, Xue Y, Liu S, Wang T.** Proteomics identification of differentially expressed proteins associated with pollen germination and tube growth reveals characteristics of germinated *Oryza sativa* pollen. *Mol Cell Proteomics.* 2007;**6**(2):207–230. <https://doi.org/10.1074/mcp.M600146-MCP200>
- Dresselhaus T, Márton ML.** Micropylar pollen tube guidance and burst: adapted from defense mechanisms? *Curr Opin Plant Biol.* 2009;**12**(6):773–780. <https://doi.org/10.1016/j.pbi.2009.09.015>
- Duan Q, Liu M-CJ, Kita D, Jordan SS, Yeh F-LJ, Yvon R, Carpenter H, Federico AN, Garcia-Valencia LE, Eyles SJ, et al.** FERONIA controls pectin- and nitric oxide-mediated male–female interaction. *Nature.* 2020;**579**(7800):561–566. <https://doi.org/10.1038/s41586-020-2106-2>
- Fayant P, Girlanda O, Chebli Y, Aubin C-E, Villemure I, Geitmann A.** Finite element model of polar growth in pollen tubes. *Plant Cell.* 2010;**110**(8):075754. <https://doi.org/10.1105/tpc.110.075754>
- Field RA.** Oligosaccharide signalling molecules. In: **Osbourn AE, Lanzotti V**, editors. *Plant-derived natural products.* New York: Springer; 2009. p. 349–359.
- Geitmann A.** How to shape a cylinder: pollen tube as a model system for the generation of complex cellular geometry. *Sex Plant Reprod.* 2010;**23**(1):63–71. <https://doi.org/10.1007/s00497-009-0121-4>
- Geitmann A, Dumais J.** Not-so-tip-growth. *Plant Signal Behav.* 2009;**4**(2):136–138. <https://doi.org/10.4161/psb.4.2.7633>
- Geitmann A, Palanivelu R.** Fertilization requires communication: signal generation and perception during pollen tube guidance. *Florim Ornament Biotechnol.* 2007;**1**(2):77–89.
- Geitmann A, Steer M.** The architecture and properties of the pollen tube cell wall. In: Malhó R, editor. *The pollen tube.* Plant cell monographs; 2006; vol. 3. Berlin, Heidelberg: Springer. p. 177–200.
- Ghanbari M, Packirisamy M, Geitmann A.** Measuring the growth force of invasive plant cells using Flexure integrated Lab-on-a-Chip (FiLoC). *Technology.* 2018;**6**(03–04):101–109. <https://doi.org/10.1142/S2339547818500061>
- Hématy K, Cherk K, Somerville S.** Host–pathogen warfare at the plant cell wall. *Curr Opin Plant Biol.* 2009;**12**(4):406–413. <https://doi.org/10.1016/j.pbi.2009.06.007>
- Herron SR, Benen JAE, Scavetta RD, Visser J, Jurnak F.** Structure and function of pectic enzymes: virulence factors of plant pathogens. *Proc Natl Acad Sci USA.* 2000;**97**(16):8762–8769. <https://doi.org/10.1073/pnas.97.16.8762>
- Higashiyama T.** Peptide signaling in pollen–pistil interactions. *Plant Cell Physiol.* 2010;**51**(2):177–189. <https://doi.org/10.1093/pcp/pcq008>
- Higashiyama T, Hamamura Y.** Gametophytic pollen tube guidance. *Sex Plant Reprod.* 2008;**21**(1):17–26. <https://doi.org/10.1007/s00497-007-0064-6>
- Higashiyama T, Kuroiwa H, Kuroiwa T.** Pollen-tube guidance: beacons from the female gametophyte. *Curr Opin Plant Biol.* 2003;**6**(1):36–41. [https://doi.org/10.1016/S1369-5266\(02\)00010-9](https://doi.org/10.1016/S1369-5266(02)00010-9)
- Jimenez-Bermudez S, Redondo-Nevedo J, Munoz-Blanco J, Caballero JL, Lopez-Aranda JM, Valpuesta V, Pliego-Alfaro F, Quesada MA, Mercado JA.** Manipulation of strawberry fruit softening by antisense expression of a pectate lyase gene. *Plant Physiol.* 2002;**128**(2):751–759. <https://doi.org/10.1104/pp.010671>
- Johnson-Brousseau SA, McCormick S.** A compendium of methods useful for characterizing *Arabidopsis* pollen mutants and gametophytically-expressed genes. *Plant J.* 2004;**39**(5):761–775. <https://doi.org/10.1111/j.1365-3113X.2004.02147.x>
- Kaneda M, van Oostende-Triplett C, Chebli Y, Testerink C, Bednarek SY, Geitmann A.** Plant AP180 N-terminal homolog proteins are involved in clathrin-dependent endocytosis during pollen tube growth in *Arabidopsis thaliana*. *Plant Cell Physiol.* 2019;**60**(6):1316–1330. <https://doi.org/10.1093/pcp/pcz036>
- Kapoor K, Geitmann A.** Invasive processes in the life cycle of plants and fungi. In: Jensen KH, Forterre Y, editors. *Soft matter plants: from biophysics to biomimetics*; 2022; The Royal Society of Chemistry. p. 203–226.
- Klepikova AV, Kasianov AS, Gerasimov ES, Logacheva MD, Penin AA.** A high resolution map of the *Arabidopsis thaliana* developmental transcriptome based on RNA-seq profiling. *Plant J.* 2016;**88**(6):1058–1070. <https://doi.org/10.1111/tpj.13312>
- Kulikauskas R, McCormick S.** Identification of the tobacco and *Arabidopsis* homologues of the pollen-expressed LAT59 gene of tomato. *Plant Mol Biol.* 1997;**34**(5):809–814. <https://doi.org/10.1023/A:1005856531693>
- Lennon KA, Roy S, Hepler PK, Lord EM.** The structure of the transmitting tissue of *Arabidopsis thaliana* (L.) and the path of pollen tube growth. *Sex Plant Reprod.* 1998;**11**(1):49–59. <https://doi.org/10.1007/s004970050120>
- Marin-Rodriguez MC, Orchard J, Seymour GB.** Pectate lyases, cell wall degradation and fruit softening. *J Exp Bot.* 2002;**53**(377):2115–2119. <https://doi.org/10.1093/jxb/erf089>
- Moscattello R, Baldan B, Squartini A, Mariani P, Navazio L.** Oligogalacturonides: novel signalling molecules in rhizobium-legume communications. *Mol Plant Microbe Interact.* 2012;**25**(11):1387–1395. <https://doi.org/10.1094/MPMI-03-12-0066-R>
- Ori N, Sessa G, Lotan T, Himmelhoch S, Fluhr R.** A major stylar matrix polypeptide (sp41) is a member of the pathogenesis-related proteins superfamily. *EMBO J.* 1990;**9**(11):3429–3436. <https://doi.org/10.1002/j.1460-2075.1990.tb07550.x>
- Palanivelu R.** Targeted growth of pollen tubes to ovules prior to completing fertilization. *Mol Reprod Dev.* 2011;**78**(12):893–893. <https://doi.org/10.1002/mrd.21403>
- Palanivelu R, Tsukamoto T.** Pathfinding in angiosperm reproduction: pollen tube guidance by pistils ensures successful double fertilization. *Wiley Interdiscip Rev Dev Biol.* 2012;**1**(1):96–113. <https://doi.org/10.1002/wdev.6>
- Pina C, Pinto F, Feijo JA, Becker JD.** Gene family analysis of the *Arabidopsis* pollen transcriptome reveals biological implications for cell growth, division control, and gene expression regulation. *Plant Physiol.* 2005;**138**(2):744–756. <https://doi.org/10.1104/pp.104.057935>
- Pua E-C, Ong C-K, Liu P, Liu J-Z.** Isolation and expression of two pectate lyase genes during fruit ripening of banana (*Musa acuminata*). *Physiol Plant.* 2001;**113**(1):92–99. <https://doi.org/10.1034/j.1399-3054.2001.1130113.x>

- Radauer C, Bublin M, Wagner S, Mari A, Breiteneder H.** Allergens are distributed into few protein families and possess a restricted number of biochemical functions. *J Allergy Clin Immunol*. 2008;**121**(4): 847–852.e847. <https://doi.org/10.1016/j.jaci.2008.01.025>
- Ramalho-Santos M, Pissarra J, Verissimo P, Pereira S, Salema R, Pires E, Faro CJ.** Cardosin A, an abundant aspartic proteinase, accumulates in protein storage vacuoles in the stigmatic papillae of *Cynara cardunculus* L. *Planta*. 1997;**203**(2):204–212. <https://doi.org/10.1007/s004250050183>
- Reimann R, Kah D, Mark C, Dettmer J, Reimann TM, Gerum RC, Geitmann A, Fabry B, Dietrich P, Kost B.** Durotropic growth of pollen tubes. *Plant Physiol*. 2020;**183**(2):558–569. <https://doi.org/10.1104/pp.19.01505>
- Ridley BL, O'Neill MA, Mohnen D.** Pectins: structure, biosynthesis, and oligogalacturonide-related signaling. *Phytochemistry*. 2001;**57**(6): 929–967. [https://doi.org/10.1016/S0031-9422\(01\)00113-3](https://doi.org/10.1016/S0031-9422(01)00113-3)
- Röckel N, Wolf S, Kost B, Rausch T, Greiner S.** Elaborate spatial patterning of cell-wall PME and PME1 at the pollen tube tip involves PME1 endocytosis, and reflects the distribution of esterified and de-esterified pectins. *Plant J*. 2008;**53**(1):133–143. <https://doi.org/10.1111/j.1365-3113X.2007.03325.x>
- Rosso MG, Li Y, Strizhov N, Reiss B, Dekker K, Weisshaar B.** An *Arabidopsis thaliana*; T-DNA mutagenized population (GABI-Kat) for flanking sequence tag-based reverse genetics. *Plant Mol Biol*. 2003;**53**(1/2):247–259. <https://doi.org/10.1023/B:PLAN.0000009297.37235.4a>
- Rowland O, Domergue F.** Plant fatty acyl reductases: enzymes generating fatty alcohols for protective layers with potential for industrial applications. *Plant Sci*. 2012;**193–194**:28–38. <https://doi.org/10.1016/j.plantsci.2012.05.002>
- Sanati Nezhad A, Geitmann A.** The cellular mechanics of an invasive lifestyle. *J Exp Bot*. 2013;**64**(15):4709–4728. <https://doi.org/10.1093/jxb/ert254>
- Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B.** Fiji: an open-source platform for biological-image analysis. *Nat Methods*. 2012;**9**(7):676–682. <https://doi.org/10.1038/nmeth.2019>
- Schneider CA, Rasband WS, Eliceiri KW.** NIH Image to ImageJ: 25 years of image analysis. *Nat Methods*. 2012;**9**(7):671–675. <https://doi.org/10.1038/nmeth.2089>
- Scholl RL, May ST, Ware DH.** Seed and molecular resources for *Arabidopsis*. *Plant Physiol*. 2000;**124**(4):1477–1480. <https://doi.org/10.1104/pp.124.4.1477>
- Sessa G, Fluhr R.** The expression of an abundant transmitting tract-specific endoglucanase (Sp41) is promoter-dependent and not essential for the reproductive physiology of tobacco. *Plant Mol Biol*. 1995;**29**(5):969–982. <https://doi.org/10.1007/BF00014970>
- Shahali Y, Sutra J-P, Haddad I, Vinh J, Guilloux L, Peltre G, Sénéchal H, Poncet P.** Proteomics of cypress pollen allergens using double and triple one-dimensional electrophoresis. *Electrophoresis*. 2012;**33**(3): 462–469. <https://doi.org/10.1002/elps.201100324>
- Speranza A, Crinelli R, Scoccianti V, Geitmann A.** Reactive oxygen species are involved in pollen tube initiation in kiwifruit. *Plant Biol*. 2012;**14**(1):64–76. <https://doi.org/10.1111/j.1438-8677.2011.00479.x>
- Suen DF, Huang AH.** Maize pollen coat xylanase facilitates pollen tube penetration into silk during sexual reproduction. *J Biol Chem*. 2007;**282**(1):625–636. <https://doi.org/10.1074/jbc.M608567200>
- Suzuki K, Yang L, Takaiwa F.** Transgenic rice accumulating modified cedar pollen allergen Cry j 2 derivatives. *J Biosci Bioeng*. 2012;**113**(2):249–251. <https://doi.org/10.1016/j.jbiosc.2011.10.005>
- Taniguchi Y, Ono A, Sawatani M, Nanba M, Kohno K, Usui M, Kurimoto M, Matuhashi T.** Cry j I, a major allergen of Japanese cedar pollen, has pectate lyase enzyme activity. *Allergy*. 1995;**50**(1):90–93. <https://doi.org/10.1111/j.1398-9995.1995.tb02489.x>
- Taylor LP, Hepler PK.** Pollen germination and tube growth. *Annu Rev Plant Physiol Plant Mol Biol*. 1997;**48**(1):461–491. <https://doi.org/10.1146/annurev.arplant.48.1.461>
- Tian G-W, Mohanty A, Chary SN, Li S, Paap B, Drakakaki G, Kopec CD, Li J, Ehrhardt D, Jackson D, et al.** High-throughput fluorescent tagging of full-length Arabidopsis gene products in planta. *Plant Physiol*. 2004;**135**(1):25–38. <https://doi.org/10.1104/pp.104.040139>
- Tuinstra MR, Wedel J.** Estimation of pollen viability in grain *Sorghum*. *Crop Sci*. 2000;**40**(4):968–970. <https://doi.org/10.2135/cropsci2000.404968x>
- Twell D, Yamaguchi J, Wing RA, Ushiba J, McCormick S.** Promoter analysis of genes that are coordinately expressed during pollen development reveals pollen-specific enhancer sequences and shared regulatory elements. *Genes Dev*. 1991;**5**(3):496–507. <https://doi.org/10.1101/gad.5.3.496>
- Valdivia E, Stephenson A, Durachko D, Cosgrove D.** Class B β -expansins are needed for pollen separation and stigma penetration. *Sex Plant Reprod*. 2009;**22**(3):141–152. <https://doi.org/10.1007/s00497-009-0099-y>
- Van Aelst AC, Van Went JL.** Ultrastructural immuno-localization of pectins and glycoproteins in *Arabidopsis thaliana* pollen grains. *Protoplasma*. 1992;**168**(1–2):14–19. <https://doi.org/10.1007/BF01332646>
- van Oostende-Triplet C, Guillet D, Triplet T, Pandzic E, Wiseman PW, Geitmann A.** Vesicle dynamics during plant cell cytokinesis reveals distinct developmental phases. *Plant Physiol*. 2017;**174**(3): 1544–1558. <https://doi.org/10.1104/pp.17.00343>
- Vieira M, Pissarra J, Verissimo P, Castanheira P, Costa Y, Pires E, Faro C.** Molecular cloning and characterization of cDNA encoding cardosin B, an aspartic proteinase accumulating extracellularly in the transmitting tissue of *Cynara cardunculus* L. *Plant Mol Biol*. 2001;**45**(5):529–539. <https://doi.org/10.1023/A:1010675015318>
- Vincken J-P, Schols HA, Oomen RfJ, McCann MC, Ulvskov P, Voragen AGJ, Visser RGF.** If homogalacturonan were a side chain of rhamnogalacturonan I. Implications for cell wall architecture. *Plant Physiol*. 2003;**132**(4):1781–1789. <https://doi.org/10.1104/pp.103.022350>
- Wang H, Wu H-M, Cheung AY.** Pollination induces mRNA poly(A) tail-shortening and cell deterioration in flower transmitting tissue. *Plant J*. 1996;**9**(5):715–727. <https://doi.org/10.1046/j.1365-3113X.1996.9050715.x>
- Willats WGT, McCartney L, Mackie W, Knox JP.** Pectin: cell biology and prospects for functional analysis. *Plant Mol Biol*. 2001;**47**(1/2): 9–27. <https://doi.org/10.1023/A:1010662911148>
- Wing R, Yamaguchi J, Larabell S, Ursin V, McCormick S.** Molecular and genetic characterization of two pollen-expressed genes that have sequence similarity to pectate lyases of the plant pathogen *Erwinia*. *Plant Mol Biol*. 1990;**14**(1):17–28. <https://doi.org/10.1007/BF00015651>
- Winter D, Vinegar B, Nahal H, Ammar R, Wilson GV, Provart NJ.** An “electronic fluorescent pictograph” browser for exploring and analyzing large-scale biological data sets. *PLoS One*. 2007;**2**(8):e718. <https://doi.org/10.1371/journal.pone.0000718>
- Wolf S, Hématy K, Höfte H.** Growth control and cell wall signaling in plants. *Annu Rev Plant Biol*. 2012;**63**(1):381–407. <https://doi.org/10.1146/annurev-arplant-042811-105449>
- Wu H-M, Cheung AY.** Programmed cell death in plant reproduction. *Plant Mol Biol*. 2000;**44**(3):267–281. <https://doi.org/10.1023/A:1026536324081>
- Wu Y, Qiu X, Du S, Erickson L.** PO149, a new member of pollen pectate lyase-like gene family from alfalfa. *Plant Mol Biol*. 1996;**32**(6): 1037–1042. <https://doi.org/10.1007/BF00041387>