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Arabidopsis LIM proteins PLIM2a and PLIM2b regulate actin configuration during pollen tube growth

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Abstract

The pollen tube grows rapidly, exclusively at its tip, to deliver its sperm for fertilization. The polarized tip growth of pollen tubes is dependent on the highly dynamic actin cytoskeleton. Plant LIM proteins (named after initials of containing proteins Lin11, Isl-1, and Mec-3) have been shown to regulate actin bundling in different cells, however, their roles in pollen tube growth have remained obscure. Here, we report the function of *Arabidopsis* LIM proteins PLIM2a and PLIM2b in pollen tube growth. The *PLIM2a* mutation resulted in short and swollen *Arabidopsis* pollen tube with defective actin bundles. The expression of the construct green fluorescent protein (GFP)-PLIM2b led to fluorescence of the actin bundles in germinating pollen and also the long actin bundles along the growing pollen tubes in *Arabidopsis*, but not of the short and sparse actin bundles that characterize the tip regions of the pollen tubes. There is a partially redundant function between PLIM2a and PLIM2b in the shank actin bundle organization during *Arabidopsis* pollen tube growth, as PLIM2b could rescue for the defective shank actin bundles in PLIM2a mutation pollen tubes. This report suggests critical roles of PLIM2a/PLIM2b in actin configuration during *Arabidopsis* pollen germination and tube growth.

Additional key words: actin bundle, cytoskeleton, fertilization, green fluorescent protein, pollen germination.

Introduction

Pollen germination and tube growth are critical for plant sexual reproduction, many molecules are involved in the regulation of this essential physiological process. These molecules form a large and complex signalling network that regulates, among other things, vesicle target and fusion, cytoskeletal dynamics, and cell wall assembly (Cheung *et al.* 2008). Actin dynamics plays an important role during pollen tube tip growth (Gu *et al.* 2005). In general, there are three kinds of actin networks in the growing pollen tube: *1*) long, parallel actin bundles along the shank; *2*) short actin bundles in the subapical region; and *3*) sparse actin filaments (AFs) in the apical region (Bartles 2000, Lovy-Wheeler *et al.* 2005). A large number of actin-binding proteins (ABPs) modulate both the structure and function of these actin networks. Actin bundlers represent an important subset of ABPs which directly bind adjacent AFs to assemble higher order structures (*i.e.*, bundles) and complex actin networks (Puius *et al.* 1998, Bartles 2000, Winder 2003). These ABPs typically contain two actin-binding domains (ABDs): a spacer domain and a regulation domain. The length of the spacer domain, which generally separates the two ABDs, is a critical determinant of the higher order actin network structures (Kovar *et al.* 2000).

Actin cytoskeleton is employed as molecular tracks for long distance transport and it is responsible for changing the cytoplasm in response to developmental and environmental factors. Mutations in actin regulatory proteins result in cell growth defects (Le *et al.* 2003, El-Assal *et al.* 2004). Actin bundlers are assumed to confer stability of transvacuolar strands and serve as tracks for cytoplasmic traffic (Shimmen *et al.* 1995, Tominaga *et al.* 2000, Shimmen and Yokota 2004). Recent research demonstrates that the *Schizosaccharo-*

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Abbreviations: ABPs - actin-binding proteins; ABD - actin-binding domain; ADF - actin depolymerizing factor; AFs - actin filaments; GFP - green fluorescent protein.

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myces pombe actin bundler Fim1 is important for yeast to assemble actin filaments into endocytic actin patches, polarizing actin bundles, and the contractile ring (Skau *et al*. 2011). Studies concerning actin bundlers in plants are limited to a few important protein families which include villin (Vidali *et al*. 1999, Yokota *et al*. 2003, Huang *et al*. 2005), fimbrin (Kovar *et al*. 2000), and formin (Cheung and Wu 2004, Ingouff *et al*. 2005, Ye *et al*. 2009). These proteins assemble actin filaments (AFs) cooperatively to establish large actin networks, which fulfill a wide variety of cellular requirements. Plant LIM proteins represent a novel family of actin-bundling protein that use LIM domains as actin-binding motifs (Thomas *et al*. 2006, 2007, Wang *et al*. 2008, Papuga *et al*. 2010). The cystein-rich protein (CRP) subfamily of LIM proteins usually has two conserved LIM domains separated by a spacer (40 - 50 residues). Most plant LIM proteins belong to this CRP LIM subfamily (Baltz *et al*. 1992, Eliasson *et al*. 2000, Kadrmas 2004). Studies concerning LIM domain-containing proteins, the tobacco NtPLIM2b and WLIM1, and lily LlLIM1, demonstrate the LIM domain functions as an autonomous actin-binding domain that involves in the regulation of actin cytoskeleton configuration (Thomas *et al*. 2007, Cheung *et al*. 2008, Wang *et al*. 2008).

There are six genes in *Arabidopsis* that encode LIM domain proteins, three of which are predominantly expressed in pollen (*PLIM2a*, *PILM2b*, and *PLIM2c*) (Eliasson *et al*. 2000, Arnaud *et al*. 2007). These proteins can bundle actin filaments *in vitro* and in a variety of vegetative cell types (Papuga *et al*. 2010). To date, however, there is no genetic evidence that these genes function in pollen development or tube growth. Recently, we have demonstrated a role of the PLIM2s in pollen development by RNAi and inferred the possible major role of PLIM2c in this development process (Ye and Xu 2012). In this study, we focused on the two *Arabidopsis* LIM proteins PLIM2a and PLIM2b. These analyses have revealed critical roles for these proteins in pollen germination and tube growth.

Materials and methods

Arabidopsis thaliana L. (ecotype Columbia; wild type, WT) seedlings were grown in a growth chamber at temperature of 22 °C, a 16-h photoperiod, irradiance of 100 μmol m^{-2} s^{-1} and relative humidity of 80 %. The *Arabidopsis* mutant *plim2a* (accession number SALK_066351) was obtained from the *Arabidopsis* Biological Resource Center (ABRC). Surface-sterilized seeds were plated onto Murashige and Skoog (1962) agar medium supplemented with 30 mg dm^{-3} hygromycin (*Roche*, Branford, USA) or 50 mg dm^{-3} kanamycin (*Invitrogen*, Beijing, China).

Plant transformation was performed according to the methods of Bechtold (1998). A pair of oligonucleotide primers, 2aS (5′-ATGCTTCATTGTTTAAAAG-3′) and 2aA (5′-AGACTC AACGACCGGCTCAG-3′), was used to amplify *PLIM2a* cDNA (At2g45800). A second pair of primers, 2bS (5′-ATGTCTTTCACAGGAACTCTCG-3′) and 2bA (5′- AGACTCTGAAACGCCATTCTC-3′), was used to amplify *PLIM2b* cDNA (At1g01780). PCR products were cloned into the *pGEM-T* vector (*Promega*, Madison, USA) and sequenced. After digestion with *Bgl*II/*Kpn*I, both *PLIM2* cDNA fragments were inserted into the *pLAT52::GFP* vector at the 3′-end of the green fluorescent protein (GFP) coding sequence, thereby generating *pLat52::GFP-PLIM2a* and *pLat52::GFP-PLIM2b*. In this way, the *GFP-PLIM2a* or *GFP-PLIM2b* gene products were driven by the pollen-specific Lat52 promoter (Twell *et al*. 1990, 1991, Gu *et al*. 2005). Later, GFP coding sequence in *pLat52::GFP-PLIM2a* was cut out by *Bam*HI/*Bgl*II to generate the *pLat52::PLIM2a.*

Further, a pair of primers, 2b*Sal*S (5′-GCGTCG ACCATCACTCTCCTGCTCCAT-3′) and 2b*Xba*A (5′-GCTCTAGAGACTTGTGGTAAGGCATTC-3′), was used to amplify the promoter region (~1.9 kb) of the *PLIM2b* gene. After digestion with *Sal*I/*Xba*I, the Lat52 promoter of the *pLat52::GFP-PLIM2b* vector was replaced with the *PLIM2b* promoter, thereby generating *pPro2b::GFP-PLIM2b*. These constructs were introduced into *Agrobacterium tumefaciens GV3101* by electroporation and then transformed into *Arabidopsis* (ecotype Columbia). Seeds from T1 plants were harvested and the selected T2 plants were analyzed for pollen tube growth and actin localization.

Characterization of pollen tube phenotypes and other analyses were performed on *Arabidopsis* flowers collected 2 weeks after bolting. Pollen was germinated on standard agar medium containing 2 mM Ca^{2+} [with an equal molar ratio of $CaCl_2$ and Ca $(NO_3)_2$], as described previously (Li *et al*. 1999). Approximately 3 or 4 h after germination, images of pollen tubes were recorded through a cooled CCD camera (model *C4742-95*, *Hamamatsu*, Japan) attached to an eclipse inverted microscope (model *TE300, Nikon*, Japan). Images were analyzed using the *MetaMorph v 4.5* software. Each experiment was performed more than three times and approximately 100 pollen tubes were chosen randomly for length and width measurements.

Pollen tubes were stained with *Alexa-488* phalloidin as described by Snowman *et al*. (2002). To quantify F-actin in pollen grains after 1 h of germination, images of pollen grains with short tubes from wild-type and the mutant *plim2a* were processed to determine the average pixel intensity with *LAS AF* software according to Ye *et al*. (2009). To visualize the actin cytoskeleton of pollen tubes, pollen was geminated for 3 h in pollen germination medium as described by Ye *et al*. (2009) and Lovy-

Wheeler *et al*. (2005). F-actin fluorescence was measured with a confocal laser-scanning microscope (*Zeiss*, Jena, Germany) using the argon laser (excitation wavelength of 488 nm). Optical sections of 0.5 µm were scanned and captured. Two Kalman-filtered scans were averaged for each optical section. Projections of 25 optical sections were used to generate final images for each pollen tube.

For semi-quantitative RT-PCR analysis, total RNA was isolated from inflorescence tissues of both WT and *plim2a* plants with *TRIzol* reagent (*Invitrogen*). RNA quantity and quality were determined *via* electrophoresis and spectrophotometry, respectively. For cDNA synthesis, 5 µg total RNA from different tissues was used for reverse transcription using *MMLV* reverse transcriptase (*Promega*). A 1 mm^3 aliquot of cDNA from each sample was used to amplify either: *1*) *PLIM2a* cDNA (296 bp) (primers 2aS, 5′-AAGACCGAGAAGCCC AAT-3′ and 2aA, 5′-TGGTTAGCAGCGGCTT GAT-3′); *2*) *PLIM2b* cDNA (~480 bp) (primers R2bS, 5′-CCCTTCAGATGAGCAACTATTC-3′ and R2bA, 5′-GAAACGCCATTCTCTTCCT-3′); or *3*) a *PLIM2c* fragment (~580 bp) (primers R2cS, 5′-GCGTTT ACAGGGACAACAG-3′ and R2cA, 5′-GCTTCATCT TCTTTGGGTTC-3′). The *actin 8* gene was used as an internal control using a pair of primers (5′-CACATG CTATCCTCCGTCTC-3′ and 5′-CAATGCCTGGAC CTGCTT-3′). PCR products after 25 cycles of amplification were subjected to 1.5 % (m/v) agarose gel electrophoresis.

All experiments were carried out with three biological replications. Differences in pollen tube length and width were considered significant when $P < 0.05$.

Results

In *Arabidopsis plim2a* mutant, a T-DNA is inserted into the third exon of the *PLIM2a* gene. Homozygous *plim2a* mutants were identified by examining pollen tube phenotype and genomic DNA of the T_4 progenies. All the

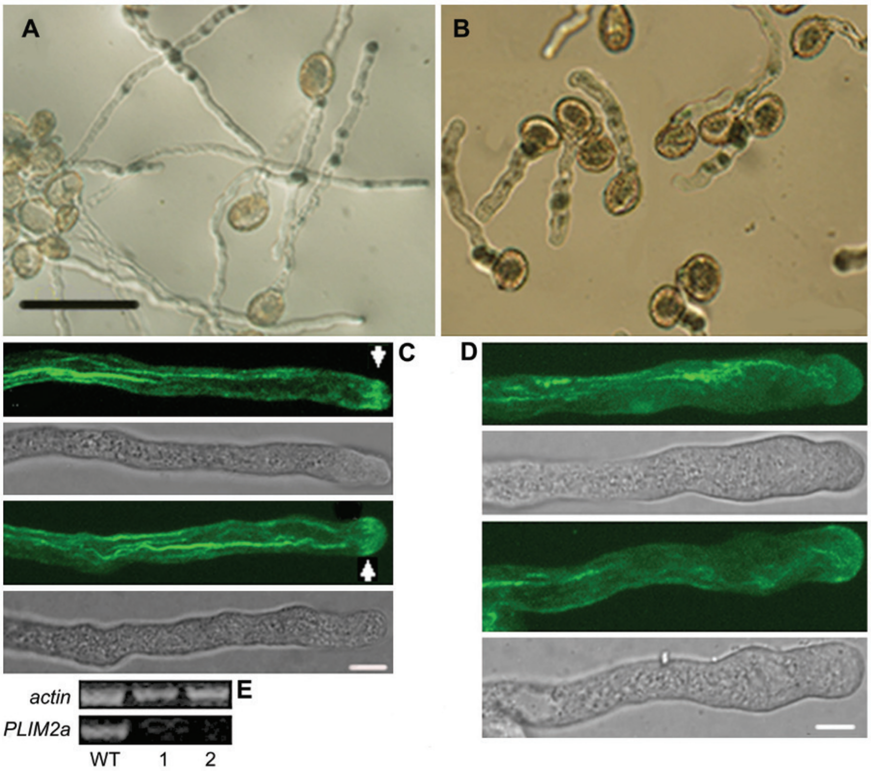

Fig. 1. The *PLIM2a* mutation induced aberrant actin configuration in *Arabidopsis* pollen tubes: *A* - long and thin pollen tubes of the control (WT) after 3 h germination; *B* - a T-DNA insertion into the *PLIM2a* gene (*plim2a*) caused short and broadened pollen tubes after 3 h germination, multiple lines were observed to display the same phenotype. Actin configuration in pollen tubes of the WT pollen tubes (*C*, *arrow* indicates sub-apical short actin bundles or the cortical fringe) or *plim2a* mutants (*D*) was stained by *Alexa-488* phalloidin. All micrographs are maximum projections of Z-stacks acquired with the confocal microscope. Actin filament in ~30 pollen tubes was examined for each sample. *E* - *PLIM2a* expression in inflorescence tissues of WT or two *plim2a* mutant plants (1 and 2) by semi-quantitative RT-PCR analysis. *Scale bars*: 100 µm in *A* and *B* and 10 µm in *C* and *D*.

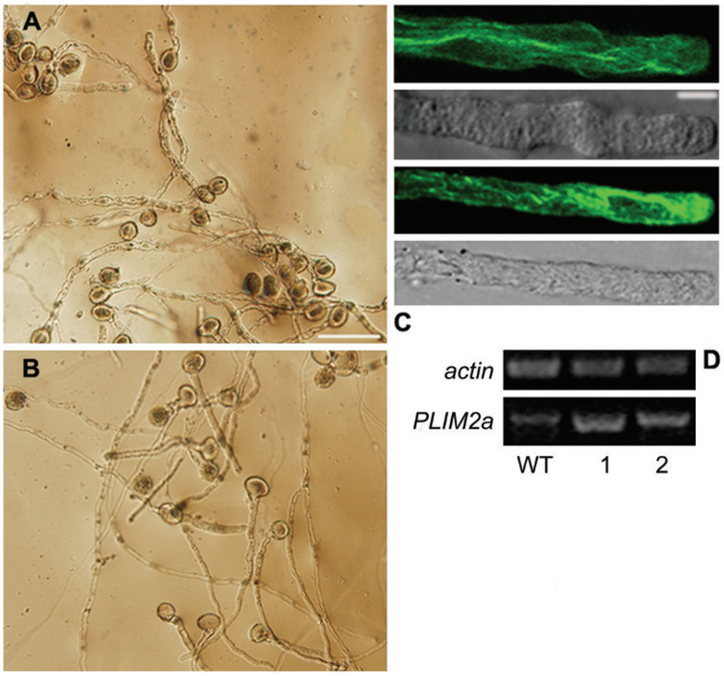

Fig. 2. Stable expression of *PLIM2a* in the *plim2a* mutant rescued its defect pollen tube growth. The *pLat52:PLIM2a* construct was transformed into the *plim2a* mutant by the *Agrobacterium*-mediated flower-dipping method. *A*, *B* - Pollen tube phenotypes of the transformed plants (more than 10 lines were observed to display similar phenotype) and WT (*scale bar* 50 µm). *C* - Actin configuration in pollen tubes of *plim2a* mutants complemented with *PLIM2a* expression by *Alexa-488* phalloidin staining (T_2 homozygous plants; AF in more than 10 pollen tubes was examined, *scale bar* 10 µm). *D* - Expression of *PLIM2a* in inflorescence tissues of WT or two complement plants (1 and 2) by semi-quantitative RT-PCR analysis.

plim2a pollen tubes had swollen tip and were shorter and broader when compared with pollen tubes of WT *Arabidopsis* plants (Fig. 1*A*, *B*). This homozygous *plim2a* mutant was selected for further analysis. When germinated for 3 h, the average length of WT pollen tubes was 395.6 ± 68.3 µm, whereas length of *plim2a* mutant tubes was only 128.9 ± 30.6 µm. The mutant pollen tubes, however, were wider than WT tubes (the average width was 11.9 ± 3.5 µm and 7.8 ± 2.3 µm, respectively). In addition, *plim2a* pollen tubes elongated more slowly than WT pollen tubes and a significant difference in pollen tube length was measured after 3 h of growth (Table 1). No other morphological defects were detected and the mutant plants exhibited normal fertility. This suggested that PLIM2a is required for normal pollen tube growth.

Table 1. The length [µm] of the *Arabidopsis* pollen tubes. At each time point after germination, about 100 pollen tubes were measured for the control WT and the mutant *plim2a* sample (Means ± SE, **, *** differences significant at $P < 0.001$ and 0.0001, respectively).

Genotype	1 d	2 d	3 d	4 d
plim2a	17.4±5.4	93.4±13.2	128.9±30.6**	168.2±21.4***
WT	21.9±5.6	164.2±57.5	395.6±68.3	551.8±116.2

Table 2. Length and width of pollen tubes of the *plim2a* mutant, WT, and mutant transformed with *pLat52::PLIM2a* (complement). Means ± SE, *n* = 100.

Genotype	Length [µm]	Width [µm]
plim2a	172.8±99.6	11.5±2.3
WT	434.5±236.3	7.5±2.1
Complement	393.1±196.8	8.9±2.4

Fluorescent dye *Alexa-488* phalloidin was used to visualize AF configuration. In WT pollen tubes, AFs were arranged in long parallel bundles along the shank, whereas short actin bundles were found in sub-apical regions. Very few AFs were found apically (Fig. 1*C*). Inside the *plim2a* tubes, however, long, longitudinally parallel actin bundles were almost absent in the shank as well as the short bundles in the sub-apical region. The mutant pollen tubes were characterized by a random distribution of irregular AFs (observed under a high background signal) (Fig. 1*D*). We confirmed a few extra nucleotides inserted into the *PLIM2a* coding sequence by amplification and sequencing the *PLIM2a* locus from both the mutant and WT samples. No *PLIM2a* expression could be detected in *plim2a* mutant by RT-PCR (Fig. 1*E*). In order to test the complementary effect of *PLIM2a* expression in the mutant, a *pLat52::PLIM2a* construct was inserted into the *plim2a* mutant. *PLIM2a* expression in the *plim2a* mutant induced the pollen tubes having similar grow rate and actin filament organization as the WT tubes (Fig. 2*A*,*B*). There was no obvious difference between the average length of the complement pollen tubes and that of the WT tubes whereas the average width of the complement pollen tubes was a little wider than that of the WT tubes (Table 2). The shank long actin bundles and the subapical short actin bundles were visualized by Alex-488 labeled phalloidin in the complement tubes (Fig. 2*C*). The expression of *AtPLIM2a* in the complement inflorescence was confirmed by RT-PCR (Fig 2*D*). These data suggest that *PLIM2a* expression could complement the growth defect of the mutant pollen tube.

To investigate the biological function of *PLIM2b* in *Arabidopsis* pollen germination and tube growth, we generated transgenic plants in which the native *PLIM2b* promoter was used to drive the expression of a GFP-fusion PLIM2b (*pPro2b::GFP-PLIM2b*). No other defects were observed in the transgenic plants contained *pPro2b::GFP-PLIM2b*, except the short pollen tubes (Fig 3*A*) with average length of 326.2 ± 33.7 μm whereas average length of WT tube was 489.6 ± 41.3 μm. The GFP-PLIM2b signal was evident in mature pollen, some of the germinated pollen, and also in the tube shank (Ye and Xu 2012) but it was absent in the tube tip (Fig. 3*A*). Expressed under its native promoter, GFP-PLIM2b was distributed evenly in mature pollen grains with the exception of a few concentrated dots. After germination, however, GFP-PLIM2b was concentrated near the germinating pore and formed a thick, round, filamentous mesh (Fig. 3*B*). This mesh persisted in the pollen grains and growing tubes. Long bundles extended from the thick mesh into the tube shank (Fig. 3*C*) but did not reach the tip of the tube (Fig. 3*D*). The most abundant distribution of GFP-PLIM2b was near the germinating pore of the pollen grain and decreased toward the tip. Then, rhodamine-phalloidin was used to visualize AF distribution within these *GFP-PLIM2b*-expressing pollen tubes. GFP-PLIM2b presence on the actin bundles was judged by the AF distribution depicted by rhodamine-phallodin signal (Fig. 3*E*,*F*). A similar pattern of co-localization of GFP-PLIM2b and AF was observed in

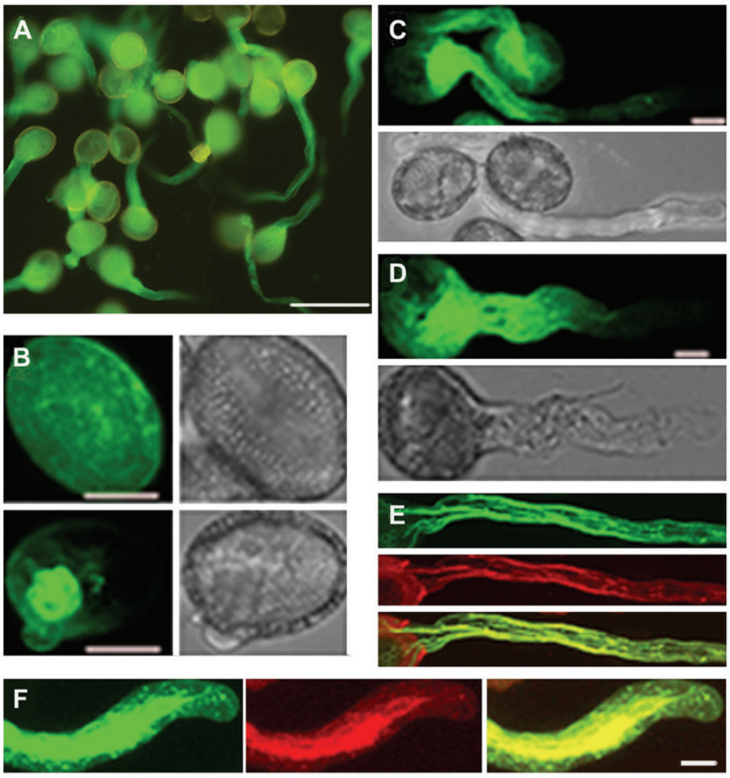

Fig. 3. GFP-PLIM2b decorated actin bundles in growing *Arabidopsis* pollen tubes. *A* - Pollen tubes expressing *GFP-PLIM2b* driven by the native *pPro2b* promoter from homozygous plants for 3 h germination. *B* - Cytoplasmic localization of GFP-PLIM2b in mature (*upper*) and germinating (*bottom*) pollen expressing *pPro2b::GFP-PLIM2b* from homozygous plants. *C*, *D* - Cytoplasmic localization of GFP-PLIM2b in growing pollen tubes expressing *pPro2b::GFP-PLIM2b.* GFP-PLIM2b was restricted to part of the pollen grain and the central part of the tubes but not in the tube tip. GFP-PLIM2b co-localized with rhodamine-phalloidin-labeled shank actin bundles in pollen tubes expressing *pPro2b:GFP-PLIM2b* from multiple transgenic lines (*E*) whereas both the GFP-PLIM2b and the rhodamine signal was devoid in the tip region of the tubes (*F*). Red and green images represent the rhodamine and GFP-PLIM2b signal, respectively. Actin filament in ~30 pollen tubes was examined. *Scale bars*: 100 μm in *A* and 10 μm in *B*-*F*.

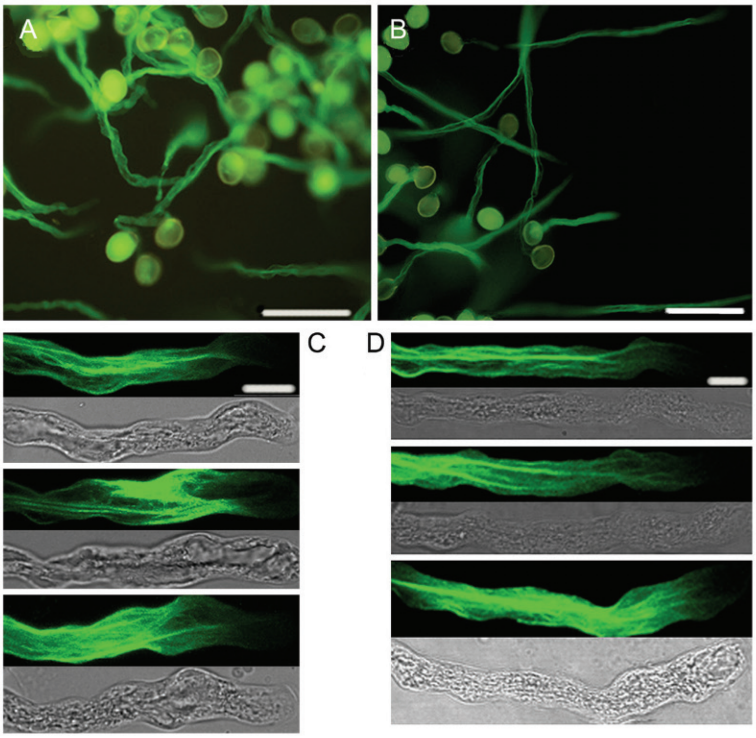

Fig. 4. *PLIM2b* expression rescued the defective shank actin bundle organization in *plim2a* mutant pollen tubes. The pollen tube phenotype (*A*, *B*) and actin filament organization visualized by GFP-PLM2b (*C*, *D*) of the T2 homozygous progeny pollen tube which was derived from crosses between *plim2a* and *pPro2b::GFP-PLIM2b* plants. *A* and *B,* each represents a T2 homozygous line where *pPro2b::GFP-PLIM2b* pollen was transferred to *plim2a* pistil. *C*, *D* - Higher magnification of the tips of the growing pollen tubes expressing *pPro2b::GFP-PLIM2b* to show that GFP-PLIM2b has a similar subcellular localization pattern in WT (*C*) and *plim2a* background (*D*) (especially to magnify and focus on the tip region). Actin filament in ~50 pollen tubes was examined for each sample. Multiple lines from each genotype were observed to display identical actin filament organization. *Scale bars*: 100 µm in *A* and *B*, 10 µm in *C* and *D*.

Table 3. The pollen tube length and width of the *plim2a* mutant, WT plants transformed with *pPro2b:GFP-PLIM2b* (TransWT), their cross progenies (Cross), and WT plants. About 100 pollen tubes germinated for 4 h were measured for each sample. Means ± SE.

Genotype	Length [µm]	Width [µm]
plim2a	232.8± 99.6	11.9±2.5
TransWT	336.9±105.3	11.4±2.2
Cross	465.7±146.1	11.3±2.3
WT	483.5±134.2	8.1±1.6

more than 35 tubes.

Both the stained AFs and the GFP-PLM2b signals were absent in the tip region (Fig. 3*F*). These indicate that the filaments labelled by GFP-PLIM2b in these *pPro2b::GFP-PLIM2b* pollen tubes were AFs. All these results suggest that *PLIM2b* is involved in actin bundle organization during *Arabidopsis* pollen germination and tube growth.

The *plim2a* mutant pollen tubes were typically short and swollen when compared to the WT tubes with defective actin bundles in both the shank and sub-apical regions (Fig. 1). Moderate expression of *PLIM2b* driven by its native promoter (*pPro2b::GFP-PLIM2b*) also resulted in a short pollen tube (compared to the WT tubes, Fig. 3) with defective actin configuration in the tip of these tubes (Fig. 3*D*). To test whether *PLIM2a* and *PLIM2b* were functionally redundant, the *plim2a* pistils were pollinated with *Arabidopsis* pollen from different *pPro2b::GFP-PLIM2b* transgenic lines. Moderate expression of *GFP-PLIM2b* in *plim2a* mutant pollen tubes resulted in significantly longer pollen tubes (Fig. 4*A*,*B*) with GFP-PLIM2b in actin bundles distributed from the germination pore to the subapical region but not in the apical region (Fig. 4*D*) similarly to the defective actin configuration in the tip of the WT (Fig. 4*C*). In contrast, there were no significant differences in tube width among these three genotypes (*plim2a*, WT containing *pPro2b::GFP-PLIM2b*, and *plim2a* containing *pPro2b::GFP-PLIM2b*) (Table 3). Although the average length of these growth rescued pollen tubes (*plim2a* containing *pPro2b:GFP-PLIM2b*) approached WT values, these tubes were wider than WT *Arabidopsis* pollen tubes (Table 3). The complement was observed in progenies from crosses between different WT containing *pPro2b:GFP-PLIM2b* line to *plim2a*. That is, *PLIM2b* partially rescued the growth defects of *plim2a* pollen tubes. This suggests that *PLIM2a* and *PLIM2b* are partially redundant in regulating *Arabidopsis* pollen tube growth.

Discussion

The dynamic of the actin cytoskeleton is critical for pollen tube growth, as growth is impaired when actin polymerization is either inhibited (by latrunculin) (Chen *et al*. 2007, Lovy-Wheeler *et al*. 2007) or promoted (by jasplakinolide) (Cardenas *et al*. 2005). Numerous ABPs have been identified that regulate actin cytoskeletal flexibility. These ABPs include plant LIM proteins which control actin bundle assembly in a variety of cell types (Thomas *et al*. 2006, Wang *et al*. 2008, Papuga *et al*. 2010). All six *Arabidopsis* LIM proteins have been shown to bind to AFs and cross-link to form actin bundles (Papuga *et al*. 2010). During the lost-of-function analysis of the PLIM2s, a possible role of PLIM2c in *Arabidopsis* pollen development was demonstrated. Their precise role in regulating actin bundling assembly in *Arabidopsis* pollen tube, however, remains unclear. In our present study, the other two *Arabidopsis* PLIM2 proteins, PLIM2a and PLIM2b, were shown to play pivotal roles in actin bundle organization during pollen tube growth.

GFP-PLIM2a is reported to be present on AFs of vegetative cells and bundles AFs *in vitro* (Papuga *et al*. 2010). *PLIM2a* lost-of-function resulted in short and swollen pollen tubes and in the loss of regular actin bundles in both the shank and sub-apical region of the *in vitro* grown pollen tubes (Fig. 1). Actin bundling is critical for cell elongation and morphogenesis (Le *et al*. 2003). The defective actin bundles that characterize the *plim2a* mutant therefore, only slowed the pollen tube growth rate and did not affect the pollination and fertility. The actin cytoskeleton is particularly dynamic in the sub-apical region of pollen tubes and many factors (such as actin depolymerization factor, ADF) are thought to regulate the continuous AF assembly/disassembly that characterizes this cellular compartment and is critical during pollen tube growth. When *PLIM2a* was over-expressed in tobacco pollen tubes, it randomly distributed in the tubes and induced the depolarized tube grow and excessive amount of short actin bundles in the sub-apical region of the tubes (data not shown). Similarly, over-expression of *AtPLM2a* in WT *Arabidopsis* induced the short and broad pollen tubes (data not shown). The growth defects of the pollen tube maybe due to the increased content of *AtPLIM2a*. These results suggest that PLIM2a is involved in actin bundle assembly during pollen tube growth. As the activity of both promoter *pLat52* and *proPLIM2a* are strong in the developing pollen and growing pollen tubes (Eady *et al*. 1995, Papuga *et al*. 2010), stable expression of *PLIM2a* in *plim2a* mutants was under the control of *pLat52* and induced the mutant pollen tube grow similar as that of WT (Fig 2). The molecular mechanisms by which PLIM2a regulates actin bundle assembly during pollen tube growth, however, remain unresolved.

The longitudinal actin bundles that run along the shank of the pollen tube were replaced by short bundles of actin in the sub-apical cellular region (also called the cortical actin fringe) in growing pollen tube (Lovy-Wheeler *et al*. 2005, Cheung and Wu 2008, Vidali *et al*. 2009). The gradients of Ca^{2+} and pH in the tip region of growing pollen tubes play a critical role in this actin configuration conversion through their regulation of some ABPs (Yokota *et al*. 2005, Lovy-Wheeler *et al*. 2006). In mature pollen, the PLIM2b protein generally was sparse with only a few spots of concentrated protein. Following germination, however, a thick, round actin mesh appeared near the germinating pore (Fig. 3*C*,*D*). This pattern is similar to PLIM1 in sunflower which also concentrates at the germinating pore of mature pollen grains (Baltz *et al*. 1999). The expression of *GFP-PLIM2b* in WT pollen tubes increased PLIM2b content due to summation of endogenous PLIM2b and expressed *GFP-PLIM2b*. This induced too many actin bundles to assemble near the germination pore and the base of the tubes (Fig. 3*C*). Maybe due to this disturbance, the elongation and the morphology of pollen tubes were affected and became aberrant. These might be the reasons for the moderate *GFP-PLIM2b* expression inducing the shorter tubes. GFP-PLIM2b depicted the long actin bundles along the shank of growing *Arabidopsis* pollen tubes, but it failed to depict the short actin bundles and sparse actin filament that characterize the sub-apical and apical cellular compartments, respectively (Fig. 3). An alkaline band is present in the sub-apical region of growing pollen tubes (Lovy-Wheeler *et al*. 2006). Within this band, the pH oscillates between 6.8 and 7.5 which profoundly affects actin remodeling. This alkaline band might help to explain why GFP-PLIM2b is present only on shank actin bundles. A pH value < 6.8 is required to activate PLIM2b (Papuga *et al*. 2010), so the relatively low pH of the tube shank may also help explain why the PLIM2b only bound to AFs in the shank.

As LIM proteins often have overlapping expression patterns in plant tissues (for example, PLIM2a, PLIM2b, and PLIM2c are all predominantly expressed in *Arabidopsis* pollen), it has been suggested that they have a high degree of functional redundancy (Papuga *et al*. 2010). Actin bundles in both the shank and tip regions were disrupted in *plim2a* mutant and PLIM2b was involved in actin bundle organization in the tube shank.

Therefore, *Arabidopsis* pollen containing *pPro2b::GFP-PLIM2b* was pollinated to *plim2a* pistils (actin can be visualized by GFP-PLIM2b signal to confirm the defect rescue of *plim2a*). GFP-PLIM2b has been shown to bind AFs in the shank of pollen tube but not in the tip (Fig. 3). It is not surprising that PLIM2b was only able to rescue actin bundle assembly in the shank of *plim2a* mutant, not in the tip region (Fig. 4) in multiple cross lines. The total amount of PLIM2s in the tubes and the configuration along the tubes are finely regulated by many factors, such as Ca^{2+} and pH. It is accessible to see the expression of GFP-PLIM2b in *plim2a* recovered tube length, both the *plim2a* mutant tubes (the endogenous PLIM2a decreased) and the *pPro2b::GFP-PLIM2b* tubes (PLIM2b elevated) were shorter than the WT tubes. In other words, the imbalance of actin distribution along the tubes induced the tubes to grow shorter which might be due to decrease or elevation of total amount of PLIM2s in the tubes. These results demonstrate that PLIM2a and PLIM2b are partially redundant as both are involved in long actin bundle assembly in the shank of *Arabidopsis* pollen tubes. PLIM2a is also likely involved in actin bundle organization in the tip region whereas PLIM2b may be involved in actin bundle organization during pollen germination.

As the finely regulated actin organization and actin dynamics in the tip region are critical for the pollen tube growth, any disturbance on these would affect the elongation and the morphology of pollen tubes and induce the tubes to be aberrant. The ectopic over-expression of the WLIM2a (At2g39900) or WLIM2b (At3g55770) under *pLat52* induced tobacco pollen tubes to grow aberrantly (our unpublished data). Although the expression pattern of the three PLIM2s overlapped in *Arabidopsis* pollen (Papuga *et al.* 2010), their regulation mechanism was different and they might play different roles during the pollen development, germination, and tube growth. Although we have *plim2a* mutant as well as *PLIM2b* and *2c* knockdown lines (Ye and Xu 2012), no single mutant of PLIM2b or PLIM2c has been obtained. Plants harbouring different combinations of double or triple defective PLIM2 showed different degree of sterility, with collapsed and empty pollen, non-germinated pollen, and/or short broadened pollen tubes (Ye and Xu 2012). Consequently, the specific function of these PLIM2 proteins in pollen germination and tube growth needs further investigation.

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