Demonstration *in vivo* of the role of *Arabidopsis* PLIM2 actin-binding proteins during pollination

Keisuke Sudo^{1†}, Jong-In Park^{1,2†}, Satomi Sakazono^{1†}, Hiromi Masuko-Suzuki¹, Masaaki Osaka¹, Mizuho Kawagishi^{1,3}, Kotomi Fujita^{1,3}, Mayumi Maruoka^{1,3}, Hikaru Nanjo^{1,3}, Go Suzuki⁴, Keita Suwabe⁵ and Masao Watanabe^{1,6*}

¹Laboratory of Plant Reproductive Genetics, Graduate School of Life Sciences, Tohoku University, 2-1-1, Katahira, Aoba-ku, Sendai, Miyagi 980-8577, Japan

²Department of Horticulture, Sunchon National University, Jeonnam 540-742, Korea

³Faculty of Agriculture, Tohoku University, 1-1, Tsutsumidori-Amamiya-machi, Aoba-ku, Sendai, Miyagi 981-8555, Japan

⁴Laboratory of Plant Molecular Genetics, Division of Natural Science, Osaka Kyoiku University, 4-698-1, Asahigaoka, Kashiwara, Osaka 582-8582, Japan

⁵Laboratory of Molecular Genetics and Breeding, Graduate School of Bioresources, Mie University, 1577, Kurima-machiya, Tsu, Mie 514-8507, Japan

⁶Faculty of Science, Tohoku University, Aramaki-Aoba, Aoba-ku Sendai, Miyagi 980-8578, Japan

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In plant reproduction, pollination is the initial key process in bringing together the male and female gametophytes. When a pollen grain lands on the surface of the stigma, information is exchanged between the pollen and stigmatic cell to determine whether the pollen grain will be accepted or rejected. If it is accepted, the stigmatic papilla cell supplies water and other resources to the pollen for germination and pollen tube elongation. Cellular processes involving actin are essential for pollen germination and tube growth, and actin-binding proteins regulate these processes by interacting with actin filaments to assemble cytoskeletal structures and actin networks. LIM proteins, which belong to a subfamily of cysteine-rich proteins, are a family of actin-binding proteins in plants, and are considered to be important for formation of the actin cytoskeleton and maintenance of its dynamics. Although the physiological and biochemical characteristics of LIMs have been elucidated *in vitro* in a variety of cell types, their exact role in pollen germination and pollen tube growth during pollination remained unclear. In this manuscript, we focus on the pollen-specific LIM proteins, AtPLIM2a and AtPLIM2c, and define their biological function during pollination in *Arabidopsis thaliana*. The *atplim2a/atplim2c* double knockdown RNAi plants showed a reduced pollen germination, approximately one-fifth of wild type, and slower pollen tube growth in the pistil, that is 80.4 $\mu\text{m/hr}$ compared to 140.8 $\mu\text{m/hr}$ in wild type. These defects led to an occasional unfertilized ovule at the bottom of the silique in RNAi plants. Our data provide direct evidence of the biological function of LIM proteins during pollination as actin-binding proteins, modulating cytoskeletal structures and actin networks, and their consequent importance in seed production.

Key words: *Arabidopsis thaliana*, *in vivo* function, pollen germination, pollen-specific LIM proteins, pollen tube growth

INTRODUCTION

Actin is an abundant protein in eukaryote cells. Actin monomers polymerize into actin filaments (AFs) to form the core element of the cytoskeleton and the tracks for intracellular signaling networks (Volkman and Baluska,

1999; Drobak et al., 2004; Hamada et al., 2012). It has a crucial role in many cellular processes during the life of a cell, and it brings about changes in cell structure in response to developmental and environmental factors (Hussey et al., 2006; Staiger et al., 2010; Mugnai et al., 2012). To establish and maintain such cellular functions, a highly sophisticated system is required to regulate the organization and dynamics of the actin cytoskeleton. Regulation is carried out by actin-binding

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* Corresponding author. E-mail: nabe@ige.tohoku.ac.jp

† These authors equally contributed to this work

proteins (ABPs) and important subsets of ABPs are actin bundlers, which bind directly AFs to assemble cytoskeletal structures (actin bundles) and actin networks. ABP activities are regulated in association with many cellular parameters, such as Ca^{2+} , pH and phosphorylation, to fulfill cellular requirements (Huang et al., 2005; Yokota et al., 2005; Papuga et al., 2010; Wen et al., 2012).

In higher plants, pollen grains germinate on the stigma and the emerging pollen tubes grow to the ovules for fertilization to complete sexual reproduction (Gutierrez-Marcos and Dickinson, 2012; Osaka et al., 2013). This process involves many actin-dependent cellular functions, such as dynamics of AFs, vesicle transport, endocytosis/exocytosis and formation of the cell wall (Cheung and Wu, 2008). In particular, the dynamics and organization of AFs are considered to be essential components of pollen germination and pollen tube growth, because suppression of AF organization by chemical treatment with latrunculin B or jasplakinolide results in disruption of pollen germination and pollen tube growth (Cárdenas et al., 2005; Chen et al., 2007; Lovy-Wheeler et al., 2007), suggesting that actin is essential for morphogenesis and cell elongation in plant reproduction (Kojo et al., 2013). ABPs regulate polymerization and depolymerization of AFs, and four ABP families, formin, villin, fimbrin, and LIM proteins, have been identified in plants (Thomas et al., 2009). These proteins are thought to regulate the organization, stabilization and dynamics of AFs collectively to assemble actin networks for cellular functions (Hussey et al., 2006; Staiger and Blanchoin, 2006). In *Arabidopsis*, Formin3 (Ye et al., 2009) and VILLIN5 (Zhang et al., 2010) are associated with the stabilization and organization of AFs in pollen. FIMBRIN1 in lily (Ll-FIM1) stabilizes the actin fringe (an actin structure in the subapex of the pollen tube) by cross-linking AFs *in vitro*, and injection of Ll-FIM1 antibody into pollen tubes resulted in slower tube growth compared to non-injected tubes (Su et al., 2012). Hence, it is clear that ABPs are important factors for assembly of the actin cytoskeleton during pollen germination and pollen tube growth.

LIM proteins (LIMs) function as ABPs in plants (Thomas et al., 2009), and are defined as relatively short proteins (approximately 200 amino acids) with two double zinc-finger LIM domains (40 to 50 amino acids), which function in protein-protein interaction. They belong to a subfamily of cysteine-rich proteins (CRPs) (Schmeichel and Beckerle, 1997; Weiskirchen and Gunther, 2003). LIMs are further classified into two subclasses, WLIMs and PLIMs. LIM genes belonging to the WLIM subclass are expressed throughout the sporophytic tissues in various organs, whereas LIM genes belonging to the PLIM subclass are expressed exclusively or preferentially in pollen (Baltz et al., 1999; Mundel et al., 2000). Tobacco WLIM1 has been shown to bind directly to AFs and triggers the regulation of actin cytoskeleton organization

(Thomas et al., 2006). HaPLIM1 in sunflower co-localizes with AFs in the germination cone of the pollen grain during germination (Baltz et al., 1999). In lily, the *in vitro* interaction between pollen-enriched LIM1 (LILIM1) and AFs is regulated by pH and Ca^{2+} , and it is suggested that this plays a central role in the oscillation of actin cytoskeleton remodeling during pollen tube elongation (Wang et al., 2008). In the *Arabidopsis* genome there are six LIM genes, three of which are classified as WLIMs (*WLIM1*, *WLIM2a* and *WLIM2b*) and the other three as PLIMs (*PLIM2a*, *PLIM2b* and *PLIM2c*). *AtWLIMs* are expressed widely throughout all sporophytic organs, including root, stem, leaves and flower. By contrast, *AtPLIM2a* and *AtPLIM2c* are expressed specifically in pollen and *AtPLIM2b* is expressed in the whole plant in addition to pollen (Arnaud et al., 2007; Ye and Xu, 2012). Expression levels of these *PLIM2s* in pollen increased at tri-cellular to maturation stages of pollen development (Papuga et al., 2010). PLIM2s co-localize with AFs in the pollen tube and direct actin-binding activity of PLIM2s has been demonstrated *in vitro*. Reduced pollen germination and shorter pollen tubes *in vitro* were observed in the triple mutants (*AtPLIM2c*: knockout, *AtPLIM2a* and *AtPLIM2b*: knockdown) (Ye and Xu, 2012). Because different distribution patterns of PLIM proteins were observed for PLIM2a and PLIM2c (that is longitudinally parallel actin bundles in the shank and short bundles in the sub-apical region for PLIM2a and long and short actin bundles in the germination pore of the pollen grain and in the tube shank but not in tube tip for PLIM2c) (Papuga et al., 2010; Ye et al., 2013), it is suggested that each PLIM protein has a distinct function in AF formation in pollen germination and pollen tube growth. Although biochemical analysis and *in vitro* pollen assays suggested a close relationship between PLIM2s and AFs in pollen germination and pollen tube growth (Ye and Xu, 2012), a direct confirmation of the biological function of PLIMs in pollen was not clear.

In this manuscript, we report the biological function of PLIM2s during pollination. Our results showed a reduction in pollen germination and slower pollen tube growth in the pistil in the double knockdown RNAi plants of *AtPLIM2a* and *AtPLIM2c*, leading to an occasional unfertilized seed at the bottom of the silique and a decreased total seed number per silique. Our data provide direct evidence of the function of PLIMs in pollen germination and tube growth during pollination and reveal the importance of their role in seed production in *Arabidopsis*.

MATERIALS AND METHODS

Plant materials *Arabidopsis thaliana* ecotype Columbia (Col-0) plants were grown in a chamber (Panasonic-Sanyo, Tokyo, Japan) at 22°C under 16 hr light/8 hr dark. Genomic DNA was extracted from fully expanded leaves of 6 to 8-week-old plants. Mature leaves, stems,

roots, flower buds, and opened flowers were also collected for RT-PCR analysis.

RT-PCR analysis Semiquantitative RT-PCR was performed according to Park et al. (2010) with slight modifications. Briefly, mRNA was isolated from the tissues described in Plant Materials using a Fast Track 2.0 mRNA isolation kit (Invitrogen, San Diego, CA, USA). First-strand cDNA was synthesized from 150 ng of isolated mRNA with a cDNA synthesis kit (First-Strand cDNA Synthesis kit, GE Healthcare, Piscataway, NJ, USA). The reverse-transcribed cDNA was used as a template for RT-PCR amplification with a specific primer set as listed in Supplementary Table S1. PCR was performed with *ExTaq* DNA polymerase (TaKaRa Bio, Shiga, Japan) for 35 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 58°C and extension for 1 min at 72°C, followed by a final extension for 4 min. As a positive control, a gene encoding *actin4* (accession no. NM001085300) was used, as described in Hakozi et al. (2008).

Construction of RNAi binary vectors The 5' upstream region containing putative promoter of *AtPLIM2c* was identified from the genomic sequence (At3g61230) of the *Arabidopsis* Database (TAIR: <http://www.arabidopsis.org>). A 1.10-kb DNA fragment from position -1 to -1,097 bp (the first ATG was marked as +1) was amplified by PCR using a primer set (*AtPLIM2c*-promoter-Forward and *AtPLIM2c*-promoter-Reverse), as described in Supplementary Table S1. A PCR fragment from the 5' upstream region was subcloned into pCR2.1 plasmid vector (Invitrogen, San Diego, CA, USA). The nucleotide sequence was confirmed by sequencing of the plasmid using a ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The *AtPLIM2c* promoter was excised as a *Kpn* I-*Sal* I fragment from the plasmid and inserted into the *Kpn* I-*Sal* I site of the multicloning site in the binary vector pBI-GSH (Park et al., 2006), which contains a gene encoding β -glucuronidase (*GUS*; Ohta et al., 1990). This construct was named *AtPLIM2c::GUS* (Supplementary Fig. S1).

A binary vector *AtPLIM2c::AtPLIM2c* RNAi, having *AtPLIM2c* dsRNA interference (*AtPLIM2c* RNAi) governed by the *AtPLIM2c* promoter, was constructed. Sense and antisense 462-bp fragments of the 3'-region of *AtPLIM2c* from position 363 to 824 (with the first ATG marked as +1), containing the coding region and 3'-UTR, were amplified by PCR with a specific primer sets of *AtPLIM2c*-Sense-Forward and *AtPLIM2c*-Sense-Reverse, and *AtPLIM2c*-Antisense-Forward and *AtPLIM2c*-Antisense-Reverse, respectively (Supplementary Table S1). For the construction of a loop linker of RNAi vector (Chuang and Meyerowitz, 2000), the *GUS* coding region of 1,161 to 1,590 bp was amplified by the specific primer set of

AtPLIM2c-*GUS*-Forward and *AtPLIM2c*-*GUS*-Reverse (Supplementary Table S1). To connect the above three PCR products, the following sequential PCRs were performed: PCR products of sense orientation of *AtPLIM2c* and *GUS* loop were connected by PCR with the primer set of *AtPLIM2c*-*GUS*-Forward and *AtPLIM2c*-sense-Reverse for *GUS* loop and *AtPLIM2c* sense fragment (*GUS*-sense fragment), and PCR products of antisense orientation of *AtPLIM2c* and *GUS* loop were connected by PCR with the primer set of *AtPLIM2c*-antisense-Forward and *AtPLIM2c*-*GUS*-Reverse for *AtPLIM2c* antisense and *GUS* loop fragment (antisense-*GUS*) (Supplementary Table S1). Then, the *GUS*-sense and antisense-*GUS* fragments were connected to make the antisense-*GUS*-sense fragment by PCR with primers of *AtPLIM2c*-Antisense-Forward and *AtPLIM2c*-Sense-Reverse (Supplementary Table S1). The antisense-*GUS*-sense fragment was subcloned into pCR2.1 vector and the nucleotide sequence was confirmed by sequencing, as described above. The antisense-*GUS*-sense plasmid was digested with *Sal* I and *Sac* I and subcloned downstream of the *AtPLIM2c* promoter in the *AtPLIM2c::GUS* binary vector, by replacing the *GUS* coding region. This RNAi construct was named *AtPLIM2c::antisense-GUS-sense* (Supplementary Fig. S1). The *AtPLIM2c::antisense-GUS-sense* was introduced into *Agrobacterium tumefaciens* strain EHA105 (Hood et al., 1993) by the electroporation method (Mersereau et al., 1990).

Plant transformation Plant transformation was performed according to Park et al. (2006) and Park et al. (2010). Briefly, transgenic *Arabidopsis* plants were generated with the *Agrobacterium* binary vector by the floral dip method (Clough and Bent, 1998). Transgenic plants were selected on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing 0.8% agar, 1% sucrose and 50 mg L⁻¹ kanamycin. Transformants were grown in a growth chamber with conditions as described above.

Observation of pollen viability, pollen germination, and pollen tube growth For observations of pollen viability at flowering stage 13 (Smyth et al., 1990), the Alexander staining method was performed (Alexander, 1969). Flowers at flowering stage 15 (Smyth et al., 1990) were collected from *Arabidopsis* plants and applied to an *in vitro* pollen germination assay. Pollen grains were placed on solid medium (pH7.5) containing 10% sucrose, 0.01% boric acid (w/v), 1 mM MgSO₄, 5 mM CaCl₂, 5 mM KCl, and 0.5% agar, at 22°C for 6 hr (Boavida and McCormick, 2007). Germinated pollen grains and elongated pollen tubes were observed under a light microscope (Axio Imager A2, Carl Zeiss, Jena, Germany). For observations of pollen tube growth *in vivo*, pollinated pistils were collected every 2 hr, from 0 hr to 24 hr after

Fig. 1. Gene expression and morphology of the *atplim2a/atplim2c* RNAi plants. (A) RT-PCR analysis of *AtPLIM2a*, *AtPLIM2b*, and *AtPLIM2c* of wild type and RNAi lines 2c-5 and 2c-39. (B) floral morphology in wild type and RNAi lines. Upper photographs show whole flowers. Bar = 500 μ m. Lower photographs show the mature anther and pollen grains, with Alexander staining. Bar = 100 μ m. (C) silique formation in bolting inflorescences. Arrowheads indicate shorter siliques. Bar = 1 cm.

pollination. Pistils were fixed in a 9:1 mixture of ethanol and acetic acid, stained with 0.1% aniline blue in 0.1% K_3PO_4 for 6 hr (Shimizu and Okada, 2000), and observed by UV fluorescence microscopy (Nikon, Eclipse E800 microscope system).

RESULTS

Construction of RNA interference plants of *AtPLIM2a* and *AtPLIM2c* Of the three *Arabidopsis* *PLIMs*, *AtPLIM2a* and *AtPLIM2c* are expressed specifically in the pollen and *AtPLIM2b* is expressed in the whole plant in addition to pollen (Arnaud et al., 2007; Ye and Xu, 2012). Therefore in this study, we concentrated on elucidation of the role of pollen-specific *AtPLIM2a* and *AtPLIM2c* during pollination. RNA interference (RNAi) was used to investigate the biological functions of *AtPLIM2s* in pollination. In a previous report, an RNAi vector with the 5' region of the *AtPLIM2c* (from 1 to 348 bp downstream of the ATG codon) targeted all three *PLIM2* genes in transgenic plants, due to their high homology (Ye and Xu, 2012). Thus, to avoid this, RNAi plants specific for *AtPLIM2a* and *AtPLIM2c* were constructed using the 3' region of the *AtPLIM2c* cDNA as an RNAi vector. As expected, transgenic RNAi plants showed decreased transcript levels of *AtPLIM2a* and *AtPLIM2c* compared to wild type *Arabidopsis* plants, while the transcript level of *AtPLIM2b* was unaffected. Two levels of repression were identified: severe (line 2c-5) and moderate (line 2c-39) (Fig. 1A). In both lines,

there was no difference in plant growth and floral morphology compared to wild type (Fig. 1, B and C). However, some shorter siliques were found in line 2c-5 but not in line 2c-39 or wild type (Fig. 1C).

Repression of *AtPLIM2a* and *AtPLIM2c* affects pollen germination and pollen tube growth In order to investigate the shorter silique observed in line 2c-5, we evaluated pollen viability and silique content. Both wild-type and line 2c-5 showed normal pollen viability (Fig. 1B). However, the number of seeds per silique was different between wild type and line 2c-5 (Fig. 2 and Table 1): the average seeds per silique were 36.5 ± 7.3 in line 2c-5, compared to 47 ± 6.7 in wild type. This difference was significant in a *t*-test at 5% level. Furthermore, defective of seed development was frequently observed in the lower part of the silique in line 2c-5 (Fig. 2B). In reciprocal crosses between wild type and line 2c-5, defective seed development was observed (27.3 ± 5.3 seeds) when line 2c-5 was used as the pollen parent, but not as the pistil parent (41.5 ± 4.9 seeds) (Fig. 2A). The defect in seed development, in terms of number of seeds per silique and silique length, was commonly observed without respect to position of flowers in the inflorescence (Fig. 3). This result indicates that a repression of *AtPLIM2a* and *AtPLIM2c* affects pollen function in pollination.

To characterize the defect in pollen function in line 2c-5, pollen germination and pollen tube growth were analyzed *in vitro* and *in vivo*, respectively. In line 2c-5 only 16.2% of pollen grains germinated, compared to 80.9% of

pollen grains from wild type (Table 1). In addition, the reduction in percentage of pollen germinated was more severe in line 2c-5 than in line 2c-39. To observe pollen tube growth, pollinated pistils were fixed at 0, 2, 4, 6, 8, 10, 12, and 24 hr after pollination and observed by microscopy after aniline blue staining (Fig. 4). In the case of wild type, at 2 hr after pollination, pollen tubes were observed around the top part of the style, and at 12 hr after pollination pollen tubes had arrived at the basal part of the pistil, indicating that most pollen tubes reached the ovule for fertilization within 12 hr after pollination. In contrast, in line 2c-5, most pollen tubes were observed to be still in the stigma at 2 hr after pollination. At 6 hr after pollination, pollen tubes were in the upper quarter of the style, and at 12 hr after pollination pollen tubes were still around the middle part of the style, in contrast to the majority of pollen tubes in wild type, which had already arrived at the basal part of the style at this time. At > 24 hr after pollination, pollen tubes finally reached the basal part of style in line 2c-5. Based on these data, the rate of pollen tube growth was estimated at 140.8 $\mu\text{m/hr}$ in wild type and 80.4 $\mu\text{m/hr}$ in line 2c-5 (Fig. 5), and thus the rate of growth of pollen

tubes in line 2c-5 was approximately 60% that of wild type. Taken together, these results indicate that reduced expression of *AtPLIM2a* and *AtPLIM2c* affects both pollen germination and pollen tube growth, resulting in shorter siliques with unfertilized ovules. The results also indicate that the level of reduction in expression of *AtPLIM2a* and *AtPLIM2c* directly correlated with the reduction in pollen germination and pollen tube growth.

Table 1. Silique length, number of seeds per silique and pollen germination in wild type and RNAi plants

	silique length (mm)	No. of seeds*	pollen germination (%)**
Wild type	13.1 $\pm$ 1.80	47.0 $\pm$ 6.72	80.9 $\pm$ 3.56
line 2c-5	12.1 $\pm$ 1.18	36.5 $\pm$ 7.26	16.2 $\pm$ 3.71
line 2c-39	11.8 $\pm$ 0.87	41.8 $\pm$ 5.38	53.8 $\pm$ 5.81

Each value indicates mean $\pm$ SD. n = 30.

*Number of seeds per silique was significantly different between wild type and line 2c-5, and line 2c-5 and line 2c-39, respectively ($p < 0.05$).

**Pollen germination was significantly different between wild type and line 2c-5, wild type and line 2c-39, and line 2c-5 and line 2c-39, respectively ($p < 0.05$).

Fig. 2. Seed formation from self and cross pollination between wild type and the *atplim2alatplim2c* RNAi line, 2c-5. (A) number of seeds per silique in each pollination. Asterisks indicate the significant difference by *t*-test. (B) representative photographs of resulting seeds in each pollination. Arrowheads indicate the non-fertilized ovules. Bar = 2 mm.

Fig. 3. Characteristics of siliques in each pollination: (A) number of seeds per silique; (B) silique length. Vertical axis indicates position of flowers in the bolting inflorescence. Each value indicates mean $\pm$ SD.

Fig. 4. Pollen tube growth in the pistil of wild type and line 2c-5. Pollen tubes in the pistil are shown at 2-hr intervals, from 2 to 12 hr, and at 24 hr after pollination of (A–G) wild type and (H–N) line 2c-5. Arrowheads indicate the longest pollen tube in each panel. Bar = 200 μ m.

Fig. 5. Pollen tube growth along the time course after pollination in wild type and line 2c-5. Solid line indicates pollen tube length of wild type and dotted line indicates that of line 2c-5. Asterisks indicate the significant difference by *t*-test.

DISCUSSION

Pollen tube growth is amazingly rapid compared to other growth processes in plants and involves extensive endo/exocytosis to bring about tip growth and rapid remodeling of the cellular cytoskeleton to support extension of the pollen tube. Among these biological functions, control of actin cytoskeleton dynamics is fundamental for the regulation of organelle movement, cytoplasmic

streaming and vesicle transport (Hussey et al., 2006; Cárdenas et al., 2005; Suetsugu et al., 2012; Sugita et al., 2012; Kong et al., 2013). Four families of plant ABPs are thought to regulate formation of the actin cytoskeletal and maintenance of its flexibility (Thomas et al., 2009) and their physiological and biochemical characteristics have been elucidated *in vitro* in a variety of cell types (Yokota et al., 2005; Ye et al., 2009; Su et al., 2012). However, their exact contribution to pollen germination and pollen tube growth during pollination remained unclear, due to their functional complexity and gene redundancy (Ye and Xu, 2012).

LIM proteins are a family of ABPs in plants and their biochemical characteristics have been examined in tobacco, lily and *Arabidopsis* (Thomas et al., 2006; Wang et al., 2008; Papuga et al., 2010). WLIM1 in tobacco and LIM1 in lily directly bind to AFs and have been shown to modulate actin bundle assembly in cultured cells and *in vitro* pollen tubes. In *Arabidopsis*, the six *LIM* genes are categorized into two major groups by their expression pattern. Their expression patterns, however, are not specific but show considerable overlapped, and thus it is suggested that there is a high degree of functional redundancy. All *Arabidopsis* LIMs have been biochemically demonstrated to bind to AFs directly with different efficiencies (Papuga et al., 2010). LIM binding capabili-

ties are differently regulated by pH and Ca^{2+} : activities of all WLIMs are pH and Ca^{2+} independent while those of the three PLIM subclass members are pH dependent, being inhibited by high pH value (Papuga et al., 2010). In addition, activity of PLIM2c protein is down-regulated by high Ca^{2+} , indicating that its activity is regulated by both pH and Ca^{2+} . These characteristics suggest that *Arabidopsis* LIMs function in the same manner as other ABP proteins but also have specific functions in pollen.

In this study, pollen germination in *plim2a/plim2c* double knockdown RNAi plants was reduced significantly to $16.2 \pm 3.7\%$ in 2c-5 and $53.8 \pm 5.8\%$ in 2c-39, and correlated with the degree of reduction in expression of *PLIM2a* and *PLIM2c* (Fig. 1A and Table 1). Pollen tube growth was markedly delayed in *plim2a/plim2c* double knockdown plants and its speed was estimated to be $80.4 \mu\text{m/hr}$, approximately 60% of that in wild type (Figs. 4 and 5). These reductions in pollen germination and pollen tube growth resulted in a failure of pollen tubes to access ovules in the lower part of pistils, leading to unfertilized ovules (Fig. 2B). Consequently, there was a reduction in the number of seeds set per silique and silique length (Figs. 2A, and 3, Table 1), which is as expected for a mutant defective in pollen tube growth (Jiang et al., 2005; Chae et al., 2009; Tanaka et al., 2013). In addition, these defects were observed only when the *plim2a/plim2c* double knockdown plants were used as the male parent, i.e. when pollens of the *plim2a/plim2c* double knockdown plants were pollinated onto stigmas of wild type or *plim2a/plim2c* double knockdown plants but not *vice versa*. Taken together, these results provide clear and direct evidence for PLIM function in pollen germination and pollen tube growth during pollination and our data support a central role of LIM proteins as ABPs in the regulation of AF organization during pollen germination and pollen tube growth.

In growing pollen tubes, gradients of pH and Ca^{2+} in the tip region are critical for correct elongation, and these factors are thought to be closely associated with actin cytoskeleton dynamics through the activation of ABPs. ABP activities themselves are also tightly regulated by many cellular parameters, including pH, Ca^{2+} , phosphorylation, and protein-protein interactions (Yokota et al., 2005). For *Arabidopsis* pollen germination, a Ca^{2+} gradient in the pollen grain is required: thus, before pollen germination, Ca^{2+} increases at the potential germination site, presumably the germination pore, and pollen failing to establish a Ca^{2+} gradient does not germinate (Iwano et al., 2004). We observed defective pollen germination in *plim2a/plim2c* double knockdown RNAi plants (Table 1). Of the three PLIMs, only PLIM2c is responsive to Ca^{2+} concentration and is inactivated by high Ca^{2+} (Papuga et al., 2010), suggesting that modulation of actin organization by PLIM2c is involved in successful pollen germination. Our data support the proposition that each

PLIM has a distinct role in pollen germination and pollen tube growth, although the three PLIMs are expressed together in *Arabidopsis* pollen grains. Papuga et al. (2010) reported that, in addition to expression in pollen grains, *PLIM2a* was observed in pollen tubes and sometimes in leaves, and *PLIM2b* was regularly observed in roots and leaf vascular tissues, but not in pollen tubes, while *PLIM2c* was restricted to pollen grains. In pollen tubes of the *plim2a* knockdown plants, AFs were irregular and distributed at random, and actin bundles in the subapical region of the tube shank were almost absent, leading to slower pollen tube elongation compared to that of wild type (Ye et al., 2013), while Ye and Xu (2012), suggested that reduced *PLIM2a* expression does not affect pollen germination. In the *plim2a/plim2c* double knockdown plants, phenotypic defects were observed in both pollen germination and pollen tube growth. From these results, taken all together, it is suggested that PLIM2a is the exclusive regulator of AF modulation during pollen tube growth, whereas actin dynamics for pollen germination is regulated by a cooperation of PLIM2a and PLIM2c (and possibly PLIM2b), indicating different roles for each PLIM during pollen germination and pollen tube growth.

This study shows the importance of PLIM2a and PLIM2c as ABPs and of their cooperative functions in pollination. However, the contribution of other ABPs in actin bundle assembly during pollination remains unclear. Therefore, their specific and individual functions in pollination, together with PLIMs, would be attractive candidates for further investigations to increase our understanding of pollination biology.

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