Doctoriate Dissertation

博士論文

# Studies on functions of ANTH domain proteins in *Arabidopsis thaliana*

# （シロイヌナズナ ANTH ドメインタンパク質の機能に関する研究）

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Department of Biological Sciences, Graduate School of Science,

The University of Tokyo

東京大学大学院理学系研究科生物科学専攻

Keita Muro

室　啓太

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I appreciate all the laboratory members for their encouragement and supports. Especially I thank Dr. Kazuo Ebine, Dr. Junpei Takagi and Dr Tomohiro Uemura for technical advises and valuable comments.

**Abbreviations**

| | |
|---|---|
| ANTH | AP180 N-terminal homology |
| ANX | ANXUR |
| AP-2 | adaptor protein complex 2 |
| CAP | putative clathrin assembly protein |
| CLC | clathrin light chain |
| CME | clathrin-mediated endocytosis |
| DAPI | 4',6-diamidino-2-phenylindole |
| DMSO | dimethyl sulfoxide |
| ECA | epsin-like clathrin adaptor |
| ENTH | epsin N-terminal homology |
| FM4-64 | (N-(3-Triethylammoniumpropyl)-4-(6-(4-(Diethylamino) Phenyl) Hexatrienyl) Pyridinium Dibromide) |
| GFP | green fluorescent protein |
| GUS | β-glucuronidase |
| mRFP | monomeric red fluorescent protein |
| PICALM | phosphatidylinositol binding clathrin assembly protein |
| PM | plasma membrane |
| SNARE | soluble N-ethylmaleimide-sensitive factor attachment protein receptor |
| SYP | syntaxin of plants |

**Abstract**

Eukaryotic cells internalize nutrients, proteins, and membrane materials via multiple endocytic pathways. Clathrin-mediated endocytosis (CME) is the best-characterized pathway and is responsible for a number of major endocytic activities in plant cells (Chen *et al.* 2011). In mammals, epsin N-terminal homology (ENTH) and AP180 N-terminal homology (ANTH) domain-containing proteins are proposed to function in initiation of clathrin-coated pit formation, bridging coat components and cargo proteins to donor membranes (Legendre-Guillemin *et al.* 2004). The *Arabidopsis thaliana* genome encodes eighteen ANTH domain proteins whereas metazoa and fungi have much fewer ANTH domain proteins, which implies that plant cell ANTH proteins have more divergent and significant functions than non-plant systems (De Craene *et al.* 2012; Zouhar and Sauer 2014). However, despite their assumed importance, little is known about the physiological significance of this family in plants. Here I report the roles of redundantly functioning ANTH domain proteins, PHOSPHATIDYLINOSITOL BINDING CLATHRIN ASSEMBLY PROTEIN 5a (PICALM5a, aka ECA2, At1g03050) and PICALM5b (At4g02650). The *picalm5a picalm5b* double mutant exhibits reduced fertility. The double mutant is defective in pollen tube growth both *in vivo* and *in vitro*, with premature bursts and knotted pollen tube morphology, which is

similar to that of the double mutant of ANX1 and ANX2 receptor-like kinases. Although subcellular localization of tip-localized SNARE proteins (Enami *et al.* 2009) was not affected by the double mutation, the tip localization of ANX1 and ANX2 was severely impaired in *picalm5a picalm5b* pollen tubes, which indicated compromised recycling of ANX1 and ANX2. I propose that PICALM5a and PICALM5b developed as adaptor proteins during plant evolution to recycle ANX1 and ANX2, and whose activities are essential for sustained pollen tube growth.

## Chapter 1: Introduction

Eukaryotic cells comprise membrane-bound structures called organelles, such as endoplasmic reticulum, Golgi apparatus, plasma membrane. Each organelle contains specific set of proteins and carries out its distinct function in the survival of the cell. Therefore, highly regulated molecular traffickings between organelles are essential. Endocytosis is one of such trafficking pathway by which cells internalize membrane materials, extracellular materials and membrane proteins from plasma membrane. Among multiple types of endocytosis, clathrin-mediated endocytosis (CME) is one of the most notable one characterized by polyhedral lattice surrounding vesicles and is responsible for a number of major endocytic activities in plant cells (Chen *et al.* 2011).

There are five major steps in CME: nucleation, cargo selection, clathrin coat assembly, membrane scission, and uncoating (McMahon and Boucrot 2011). The AP-2 adaptor complex is well-known heterotetrameric protein complex that works in the initiation of CME, bridging coat components and cargo proteins to donor membranes (McMahon and Boucrot 2011). Besides this, epsin N-terminal homology (ENTH) and AP180 N-terminal homology (ANTH) domain-containing proteins are also proposed to function in initiation of clathrin-coated

pit formation in mammals (Legendre-Guillemin *et al.* 2004).

ENTH domain-containing proteins (ENTH proteins) and ANTH domain-containing proteins (ANTH proteins) are protein families that contain highly conserved ENTH/ANTH domains at their N-terminus. The ENTH domain of epsin 1 and the ANTH domain of AP180, mammalian ENTH and ANTH domain-containing proteins, respectively, are reported to bind inositol phospholipids, with preference for phosphatidylinositol-4,5-bisphosphate (Ford *et al.* 2001; Itoh *et al.* 2001). Their C-terminal, relatively unstructured regions contain motifs for interaction with clathrin and AP-2, indicating their functions in bridging coat components to membrane phospholipids (Figure 1, Legendre-Guillemin *et al.* 2004). Moreover, disruption of ENTH/ANTH proteins results in mislocalization of specific membrane proteins, suggesting their function as cargo-specific adaptors (Nonet *et al.* 1999; Miller *et al.* 2007; Miller *et al.* 2011; Messa *et al.* 2014).

ANTH protein family members are highly diversified in Arabidopsis (Figure 2, Table 1). While human and mouse have only four members, the *Arabidopsis thaliana* genome encodes eighteen ANTH proteins (De Craene *et al.* 2012; Zouhar and Sauer 2014). This implies that plant cell ANTH proteins have more divergent and significant functions than non-plant systems. However, despite their assumed importance, little is

known about the physiological significance of this family in plants.

Some of ANTH proteins in *Arabidopsis* have been characterized. These include PICALM1a/ECA1, PICALM5a/ECA2, and PICALM4a/ECA4, which are localized on the plasma membrane (PM), endosomes, and cell plates (Song *et al.* 2012). PICALM4a and PICALM4b/CAP1 have been shown to directly interact with TML, one of the core components of the TPLATE complex (Gadeyne *et al.* 2014). These results suggest that ANTH proteins have roles in plant endocytic processes. However, the molecular and physiological functions of ANTH proteins remain unclear. The expansion of family members in Arabidopsis could result in overlapping functions, which might explain why the loss of function effects of these genes have not been reported to date. Therefore, I started my study by generating multiple mutants of ANTH proteins by crossing T-DNA insertion mutants of ANTH proteins, which provided very interesting information on ANTH protein functions in plant reproduction, specifically tip-localization of ANXUR receptor kinases, which play pivotal roles in pollen tube growth through an autocrine signaling pathway.

| Gene ID | Name | Other Name |
|---|---|---|
| At2g01600 | PICALM1a | ECA1 |
| At1g14910 | PICALM1b | |
| At5g57200 | PICALM2a | |
| At4g25940 | PICALM2b | |
| At5g35200 | PICALM3 | |
| At2g25430 | PICALM4a | ECA4 |
| At4g32285 | PICALM4b | CAP1 |
| At1g03050 | PICALM5a | ECA2 |
| At4g02650 | PICALM5b | |
| At1g05020 | PICALM6 | AP180 |
| At1g33340 | PICALM8 | |
| At1g25240 | PICALM9a | |
| At1g68110 | PICALM9b | |
| At2g01920 | PICALM9c | |
| At1g14686 | PICALM9d | |
| At4g40080 | PICALM10a | |
| At5g10410 | PICALM10b | |
| At5g65370 | PICALM10c | |

Table 1. List of ANTH domain-containing proteins in Arabidopsis

ANTH domain-containing proteins in Arabidopsis were renamed. Their names follow those established before (De Craene *et al.* 2012; Zouhar and Sauer 2014).

## Chapter 2: Results

### The *picalm5a picalm5b* double mutation leads to reduced male fertility

To identify the physiological processes in which ANTH proteins are involved, I examined the phenotypes of multiple mutants of ANTH proteins. Among the higher-ordered mutants generated, I found that the *picalm5a picalm5b* double mutant had the most marked phenotypic abnormality (Figure 4). Although the vegetative growth of the *picalm5a picalm5b* plants was indistinguishable from the wild type and the single mutant plants, their siliques were significantly shorter than those produced by the wild-type plants. I then cleared these siliques to observe the seeds inside, and found that the seed numbers contained in the mutant siliques were significantly reduced compared to the wild type (Figure 5). I also noticed that seeds were formed only on the apical side of mutant siliques, which suggested that the basal part of the mutant siliques had not been successfully fertilized.

Next, I performed reciprocal cross-pollination between the wild-type and *picalm5a picalm5b* double mutant plants to investigate whether this phenotype resulted from a defect in the male or female organs (Figure 6). When pistils of the *picalm5a picalm5b* mutant were pollinated with wild-type pollen grains, the silique length and the number of seeds were comparable to those of the self-pollinated wild type. Conversely,

when pistils of the wild-type plants were pollinated with pollen grains from the *picalm5a picalm5b* mutant, the siliques were shorter, fewer seeds were generated, and they were only produced on the apical part of the siliques. These results indicate that the *picalm5a picalm5b* mutation leads to defective pollen function, which reduces fertility.

**PICALM5a and PICALM5b are essential for sustained pollen tube growth**

I then examined the defect in the male gametes of the *picalm5a picalm5b* double mutant. First, I tested the possibility of abnormal development of pollen grains. The mature double mutant pollen grains were not morphologically distinguishable from the wild-type pollen, and two sperm and one vegetative nuclei were observed when the pollen grains produced on *picalm5a picalm5b* and the wild-type plants were stained with DAPI (Figure 7). This suggested that the pollen successfully developed in the *picalm5a picalm5b* mutant.

The seed distribution observed in the double mutant silique suggested that fertilization succeeded only on the apical part of the pistil, which could be due to limited pollen tube growth in pistils. To verify this possibility, I examined pollen tube elongation *in vivo*. Hand-pollinated wild-type and *picalm5a picalm5b* pistils were stained using methyl blue at 12 hours after pollination. As a result, the *picalm5a*

*picalm5b* double mutant pollen tubes were significantly shorter than the wild-type and single mutant pollen tubes. Furthermore, they were unable to reach the ovules in the basal part of the pistils (Figure 8).

I also examined pollen tube elongation *in vitro.* The pollen grains were spread on cellulose cellophane sheets placed on solidified pollen germination medium and incubated at 23°C for 5 hours. The *picalm5a picalm5b* double mutant pollen tubes were significantly shorter than the wild type pollen tubes, and knotted pollen tubes were frequently observed, whereas lengths and the morphology of pollen tubes of the *picalm5a* and *picalm5b* single mutants were similar to the wild type pollen tubes (Figure 9). Furthermore, I found that many pollen tube tips of the *picalm5a picalm5b* mutant burst and released their cytoplasm during incubation. These results indicate that PICALM5a and PICALM5b are required for sustained pollen tube growth, which is essential for fertilization in the basal part of the pistil.

### ***PICALM5a*** **and** ***PICALM5b*** **are expressed in mature pollen grains and pollen tubes**

According to the Arabidopsis eFP Browser microarray database (Schmid *et al.* 2005; Winter *et al.* 2007), *PICALM5a* and *PICALM5b* are mainly expressed in mature pollen grains and germinated pollen tubes (Figure 10). This expression pattern was

confirmed by the promoter-reporter assay. I generated transgenic plants expressing β-glucuronidase under the regulation of *PICALM5a* or *PICALM5b* promoters in wild-type Arabidopsis (*PICALM5a*$_{pro}$*:GUS* and *PICALM5b*$_{pro}$*:GUS*). GUS signals were mainly observed in the mature pollen grains and pollen tubes growing in the pistils of both transgenic plants (Figure 11), which was consistent with the microarray data.

**PICALM5a and PICALM5b are localized to the subapical region PM in pollen tubes**

I then expressed GFP-tagged PICALM5a and PICALM5b in the *picalm5a picalm5b* double mutant. These constructs rescued the defective pollen tube growth of the double mutant *in vitro* and *in vivo,* and increased fertilization efficiency and silique growth (Figures 4, 8—9). The results confirmed that these phenotypes were caused by *PICALM5a* and *PICALM5b* loss of function, and also demonstrated the functionality of the GFP-tagged PICALM5 proteins.

I then observed subcellular localizations of PICALM5a-GFP and PICALM5b-GFP expressed in their respective single mutants. Both fluorescently tagged PICALM5 proteins were localized to the subapical region PM of the growing pollen tubes (Figure 12). It has been suggested that ANTH proteins play a role in

clathrin-dependent endocytosis (Legendre-Guillemin *et al.* 2004). Therefore, I examined whether these PICALM5 proteins were co-localized with clathrin. I generated a transgenic plant that co-expressed GFP-fused Clathrin Light Chain 1 (CLC1-GFP) driven by the LAT52 promoter and mRFP-tagged PICALM5a under the regulation of its own promoter. The CLC1-GFP was localized to the punctate cytoplasmic structures and the subapical PM in the growing pollen tubes. It was also co-localized with PICALM5a-mRFP on the plasma membrane (Figure 13). The co-localization was especially evident at the punctate foci in the subapical PM, which was further demonstrated by the quantified fluorescence intensity in the membrane region. These results suggest that the PICALM5 proteins are involved in CME on the subapical PM of the growing pollen tubes.

**PICALM5a and PICALM5b are required for the correct localization of ANXUR receptor-like kinases**

I then observed CLC1-GFP in the pollen tubes of the *picalm5a picalm5b* mutant, but found no marked effect on its distribution (Figure 14). Therefore the effect of PICALM5a and PICALM5b absence on general CME would be subtle, if any. Thus, I speculated that the *picalm5a picalm5b* pollen tube elongation defect may result from

the disordered transport of specific proteins necessary for proper pollen tube elongation. To identify such proteins, whose transport is mediated by the PICALM5 proteins, I looked for PM proteins, impairments of which result in a similar pollen tube defects to the *picalm5a picalm5b* mutant. A literature survey suggested that pollen tubes with defective ANXUR (ANX) 1 and ANX2 receptor-like kinases, which redundantly regulate pollen tube growth via an autocrine signaling pathway (Ge *et al.* 2017; Mecchia *et al.* 2017), result in short and knotted pollen tubes that are associated with premature rupture (Miyazaki *et al.* 2009; Mizuta and Higashiyama 2014). The similar pollen tube phenotypes for the *anx* and *picalm5* mutations prompted me to localize the ANX proteins in the *picalm5a picalm5b* double mutant. Intriguingly, ANX1-GFP and ANX2-GFP exhibited remarkably different localizations between in the wild type and *picalm5a picalm5b* plants (Figure 15). In the wild-type pollen tubes, tip-focused cytoplasmic and PM localizations were observed for ANX1-GFP and ANX2-GFP. However, in the *picalm5a picalm5b* mutant, these proteins were localized to a broader region of the pollen tube without tip-enriched cytoplasmic signal. This localization suggested that the *picalm5a picalm5b* mutant failed to retrieve ANX1 and ANX2 from the subapical PM of the pollen tubes, which led to broadened ANX localization on the PM. When expressed in the *picalm5a* and *picalm5b* single mutants, ANX1-GFP and

ANX2-GFP showed similar subcellular localization to the wild type plants, which indicated the redundant functions of PICALM5a and PICALM5b in the endocytosis of ANX proteins. Mislocalization of ANX2-GFP in *picalm5a picalm5b* pollen tubes was rescued by the expression of PICALM5a-mRFP, which further indicated that PICALM5 proteins are responsible for the proper localization of ANX receptor-like kinases as well as the functional redundancy between PICALM5 proteins (Figure 16).

**PICALM5a and PICALM5b are not required for the correct localization of the pollen-specific SYP1 proteins.**

I then examined whether the effect of the *picalm5* mutations is specific to ANX proteins. It has been reported that the triple mutation in *SYP124*, *SYP125*, and *SYP131*, which are pollen-specific *SYP1* members that mediate membrane fusion at the PM, results in severe pollen tube growth defects (Slane *et al.* 2017). The GFP-tagged SYP124, SYP125, and GFP-SYP131 were localized to distinct regions of the PM in the growing pollen tubes of wild-type plants (Figure 17), which was consistent with previous studies (Enami *et al.* 2009; Slane *et al.* 2017). No obvious difference in localizations of these proteins between in the wild-type and in the *picalm5a picalm5b* double mutant, indicating that the *picalm5a picalm5b* mutation did not affect the

distribution of these proteins in pollen tubes (Figure 17). This result indicated that PICALM5a and PICALM5b were specifically required for the correct localization of ANX proteins (Figure 18).

## Chapter 3: Discussion

During fertilization, pollen tubes reach ovules by growing very rapidly in one direction (Qin and Yang 2011). In growing pollen tubes, secretory vesicles are delivered to an inverted cone-shaped region in the tip region to supply cell wall and PM materials (Rosen and Gawlik 1966; Tiwari and Polito 1988; Lancelle and Hepler 1992; Derksen *et al.* 1995). Meanwhile, excessive PM materials are sequestered via endocytosis (Picton and Steer 1983; Steer and Steer 1989; Derksen *et al.* 1995; Ketelaar *et al.* 2008). Studies on endocytosis in growing pollen tubes using the endocytic tracer FM4-64 suggested that most of the endocytosed materials were rapidly recycled to the secretory pathway (Parton *et al.* 2001). Pollen tube endocytosis occurs mainly at the lateral region adjacent to the apex (Derksen *et al.* 1995; Blackbourn and Jackson 1996). Although clathrin-independent endocytosis has been reported, CME is a major pathway for internalizing membrane materials in plants (Moscatelli *et al.* 2007). Several proteins that have a role in CME, such as clathrin light chain, clathrin heavy chain, and PICALM6/AP180 proteins, are reported to accumulate in this region (Zhao *et al.* 2010; Feng *et al.* 2016). However, the molecular mechanisms that sort specific cargos and their physiological significance have not been elucidated thus far.

In this study, PICALM5a and PICALM5b were localized to the PM of the

lateral subapical region in pollen tubes, which, together with co-localization with the clathrin light chain, strongly suggested that they had roles in the CME process. The subcellular localizations of clathrin light chain and pollen-specific SYP1 members in the pollen tubes were not affected by the *picalm5a picalm5b* mutation. This suggests that PICALM5a and PICALM5b may act as adaptors that are specific to a subpopulation of PM proteins, including ANX proteins. Pollen tubes produced by the *anx1 anx2* double mutant rupture shortly after germination (Miyazaki *et al.* 2009). Conversely, pollen tubes produced by *picalm5a picalm5b* burst after growing to some extent, which indicates that these adaptors are required for sustained pollen tube growth. This phenotypic difference is most likely due to the fact that the ANX proteins remained in the subapical PM region and could fulfill their functions during the early period of pollen tube growth, whereas during later stages, not enough ANX proteins to sustain pollen tube growth could be recycled back from the lateral region because of the absence of these specific adaptors. The ANX protein levels at the tip must be tightly regulated because over-accumulation of ANX proteins also results in defective pollen tube growth (Boisson-Dernier *et al.* 2013). This is caused by excessive exocytosis and abnormal deposition of cell wall materials in the tip region of the pollen tube. Therefore, exocytic and endocytic activities must be properly balanced during tip growth, which

would also hold true for PM proteins that are not ANX proteins.

My finding indicates that a specific set of ANTH proteins is responsible for the regulation of specific cargos during pollen tube growth. Fertilization mediated by pollen tubes is a relatively late-acquired trait during land plant evolution, and close homologs of PICALM5a and PICALM5b do not exist in basal land plants, such as lycophytes and bryophytes, which do not show pollen tube-mediated delivery of male gametes (Zouhar and Sauer 2014). Therefore, the rapid diversification of the ANTH protein group during land plant evolution could be associated with the acquisition of new plant functions, including pollen tube growth. Future studies need to identify the molecular and physiological functions of other ANTH protein members so that the relationship between the diversification of endocytic mechanisms and the evolution of plant physiology can be elucidated.

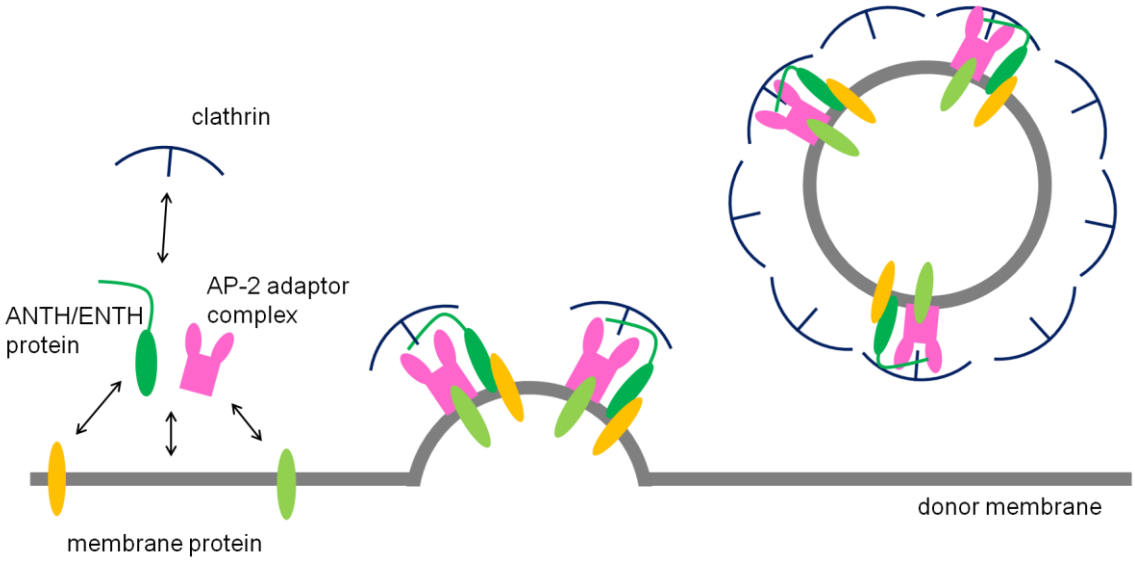



Figure 1. Model of adaptor proteins in clathrin mediated endocytosis.

In clathrin-coated vesicle formation, AP-2 complex and ANTH/ENTH domain containing proteins serve as bridges interacting with donor membrane, clathrin and cargo membrane proteins.

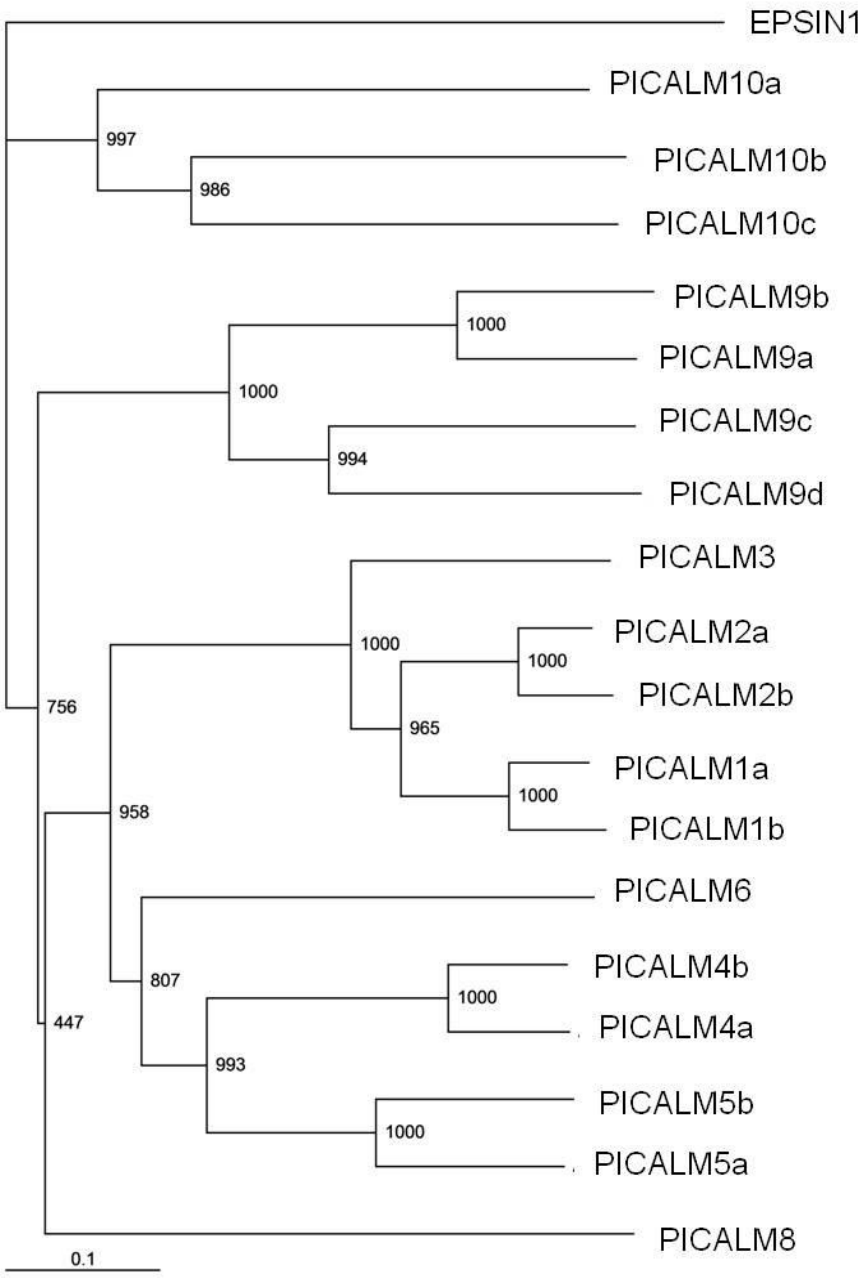



Figure 2. Phylogenetic tree of Arabidopsis ANTH Domain-containing proteins

First 200 amino acids, comprising the ENTH/ANTH domains, from 18 ANTH proteins and a ENTH protein EPSIN1 as an outgroup were aligned using ClustalW version 2.1 (http://clustalw.ddbj.nig.ac.jp) and a phylogenetic tree was drawn using TreeView software. Bootstrap values are indicated on the nodes. Bar represents substitutions per site.

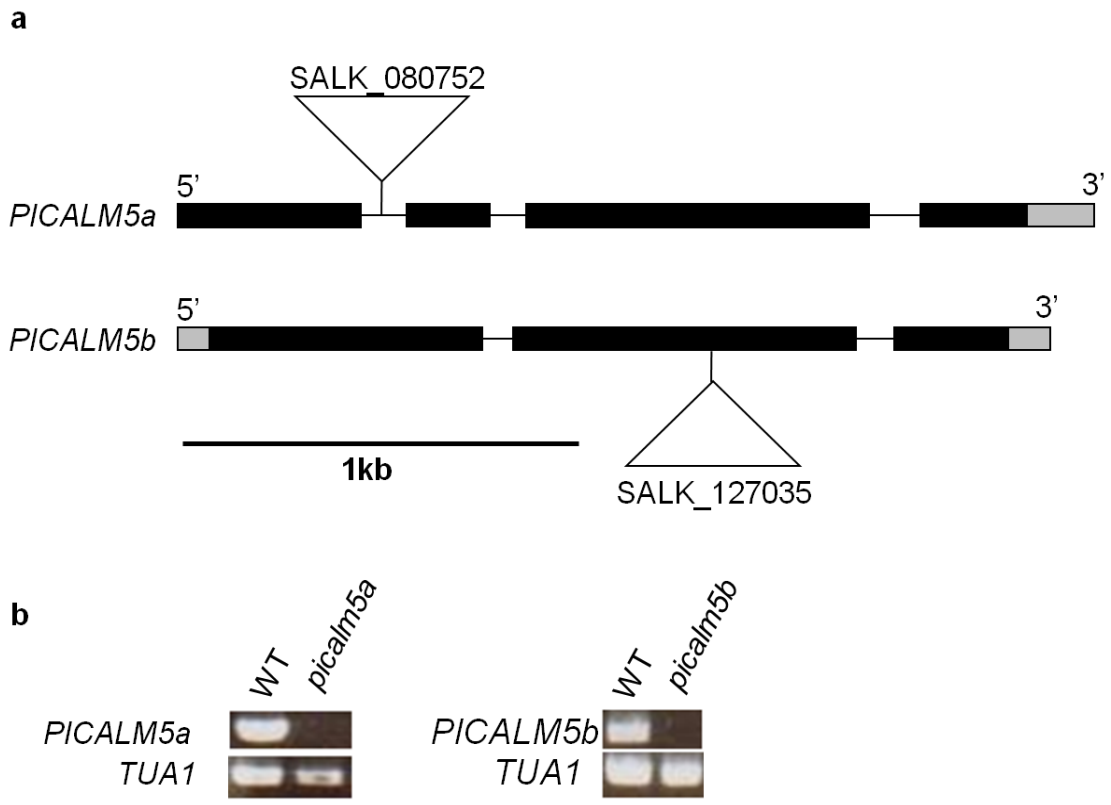



Figure 3. Mutant lines inserted with T-DNA used in this study

a, Locations of the T-DNA insertions in *picalm5a* and *picalm5b*. Exons and untranslated regions are indicated with black and gray boxes, respectively. b, RT-PCR analysis of the full-length transcripts of *PICALM5a* and *PICALM5b* in the flowers of the wild type (WT) and respective mutant plants. *TUA1* was used as a control.


Figure 4. Shoots (top) and inflorescences (bottom) of six-week-old wild-type, *picalm5a*, *picalm5b*, *picalm5a picalm5b*, *PICALM5a-GFP* in *picalm5a picalm5b*, and *PICALM5b-GFP* in *picalm5a picalm5b* plants. Scale bars = 10 cm (top) and 1 cm (bottom).

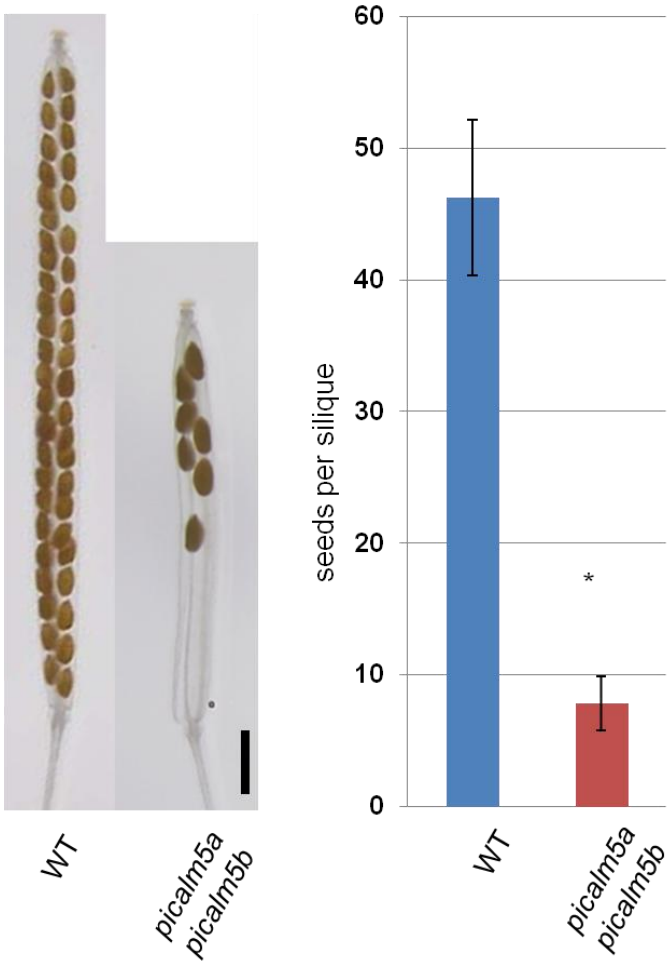



Figure 5. (left) Representative cleared siliques of self-pollinated wild type and *picalm5a picalm5b* plants. Scale bar = 1 mm. (right) Quantification of the seed number per silique for wild type and *picalm5a picalm5b* plants. Values are presented as means ± S.D. ($n$ = 28). Asterisk indicates a significant difference from the wild-type according to Welch's $t$ test at $p < 0.01$.

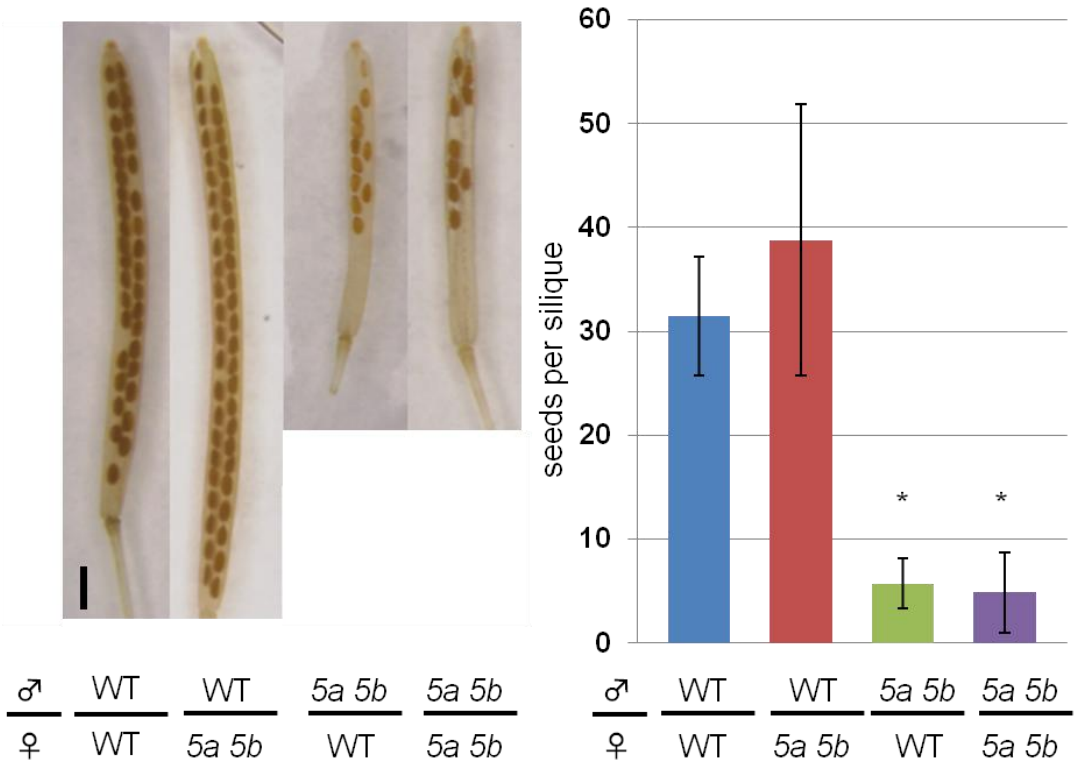



Figure 6. (left) Representative cleared siliques obtained by cross pollination between parents with the indicated genotypes. 5a 5b represents the picalm5a picalm5b double mutant. Scale bar = 1 mm. (right) Quantification of the seed number per silique for cross-pollinated siliques. 5a 5b represents the picalm5a picalm5b double mutant. Values are presented as means ± S.D. (n = 10). Asterisks indicate significant differences from the value for the crossing between male and female wild-type plants according to a Welch's t test at $p < 0.01$.

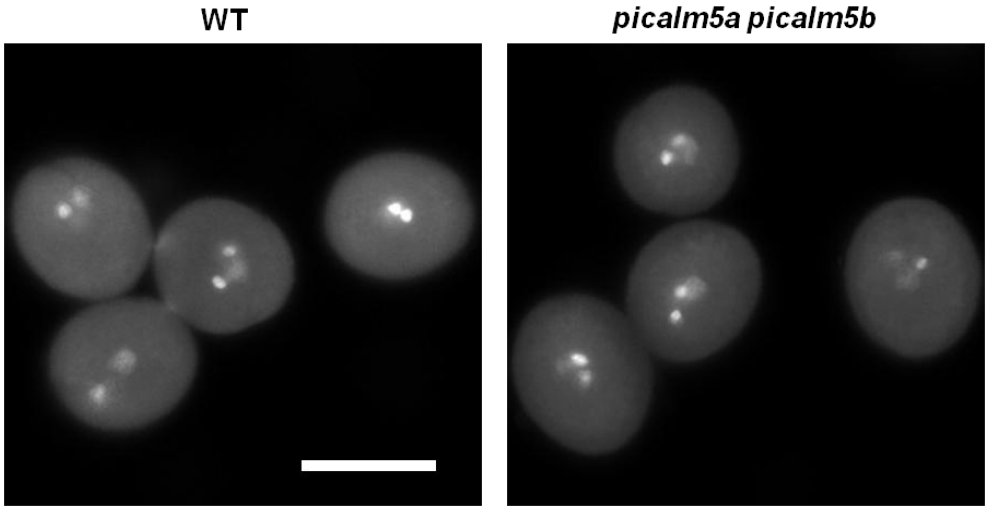



Figure 7. DAPI-staining of wild-type and *picalm5a picalm5b* pollen. Scale bars = 20 µm.

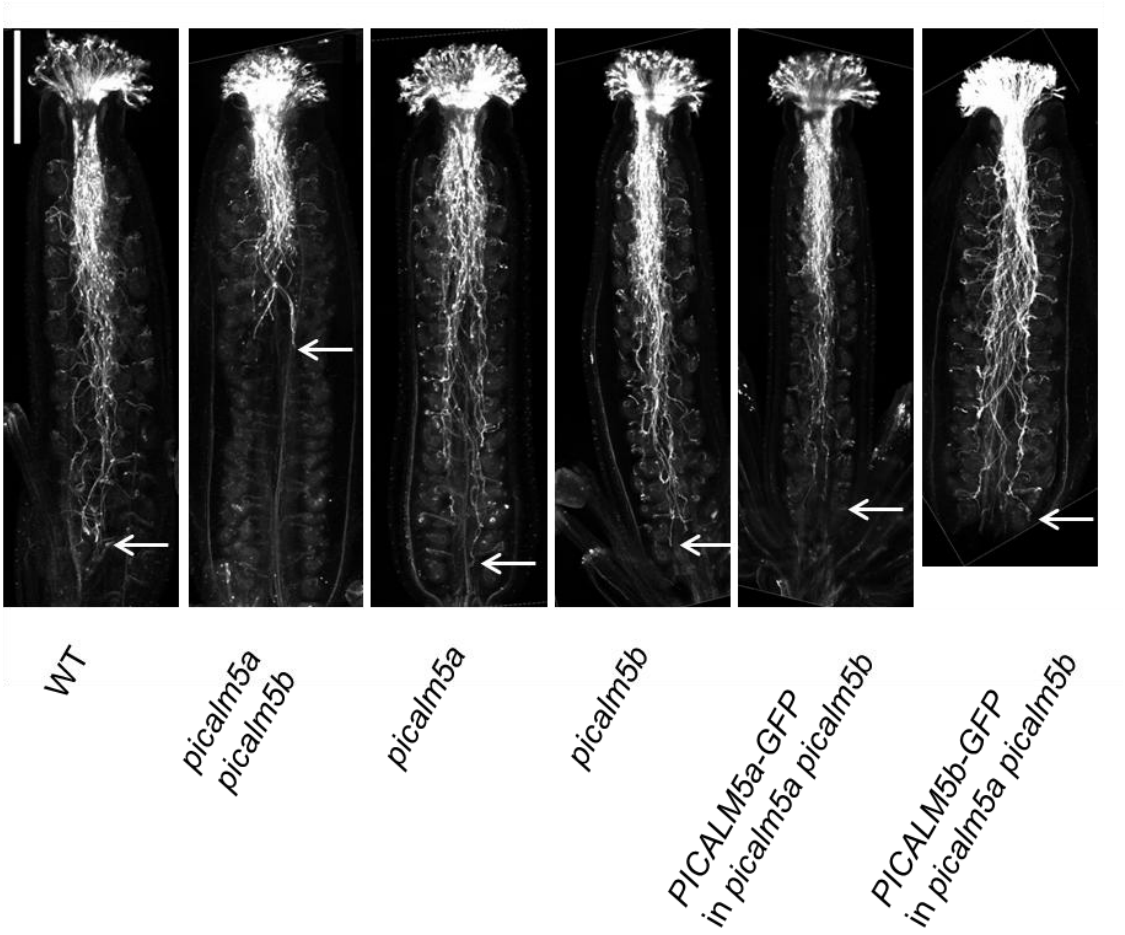



Figure 8. Methyl blue staining of pistils from wild-type, *picalm5a, picalm5b, picalm5a picalm5b, PICALM5a-GFP* in *picalm5a picalm5b,* and *PICALM5b-GFP* in *picalm5a picalm5b* plants. Siliques were harvested at twelve hours after hand-pollination. White arrows indicate the tips of the longest pollen tubes. Scale bar = 500 μm.

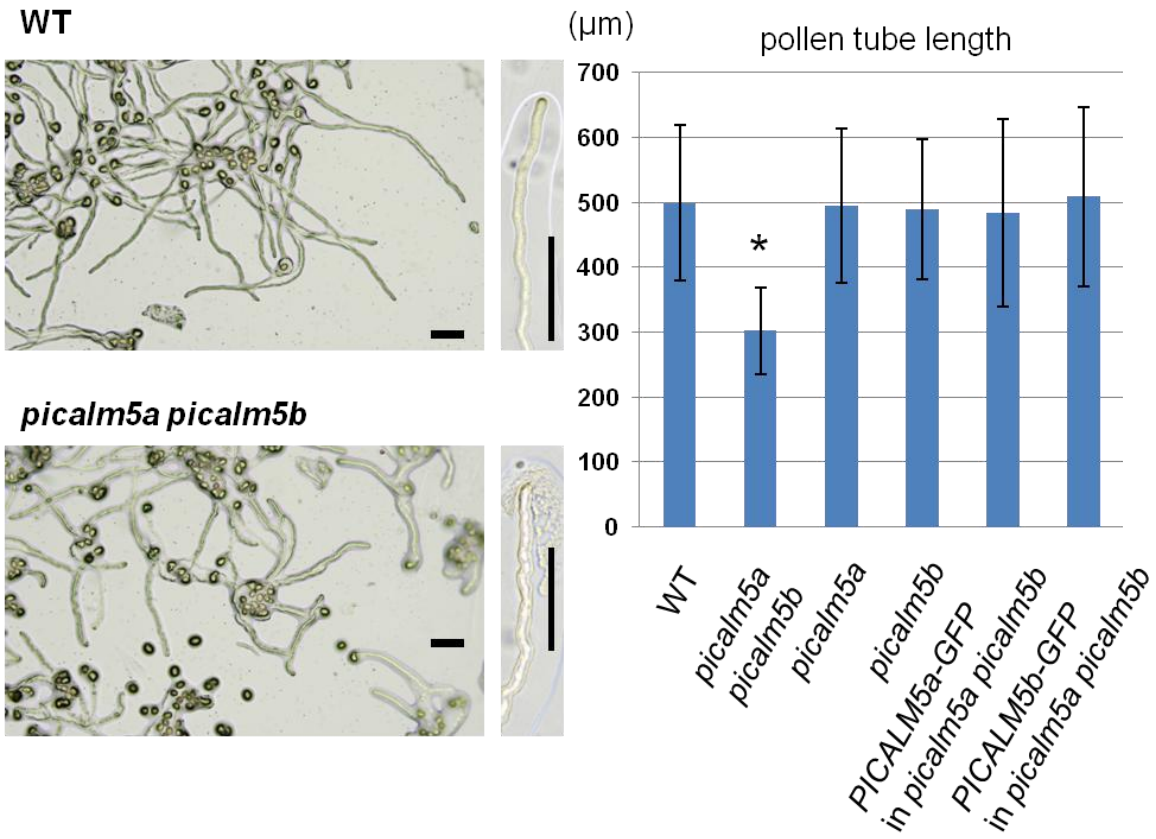
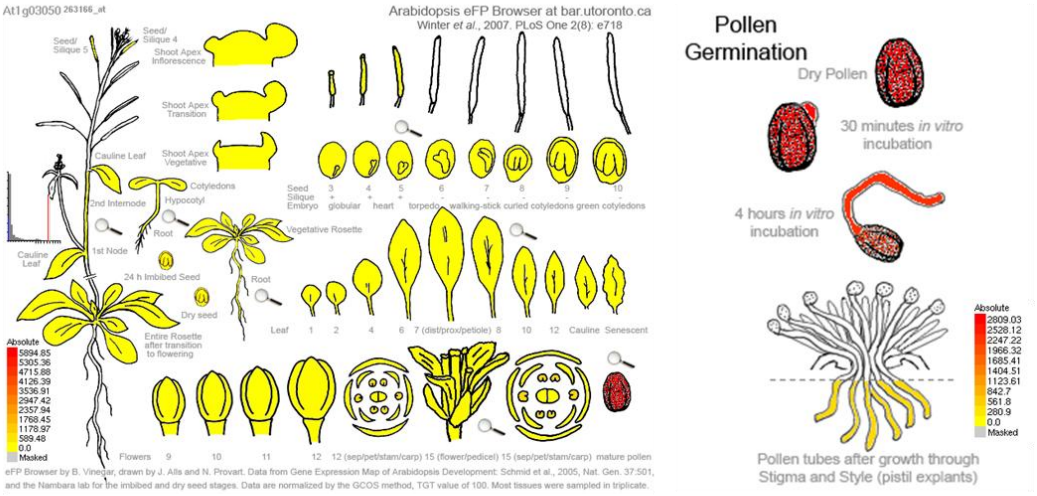



Figure 9. (left) Wild-type and *picalm5a picalm5b* pollen tubes grown in vitro. Scale bars = 100 µm. (right) Quantification of wild-type, *picalm5a*, *picalm5b*, *picalm5a picalm5b*, *PICALM5a-GFP* in *picalm5a picalm5b*, and *PICALM5b-GFP* in *picalm5a picalm5b* pollen tube lengths after 5 hours incubation. Asterisk indicates a significant difference from the wild type according to a Welch's t test at $p < 0.01$.

*PICALM5a*




*PICALM5b*

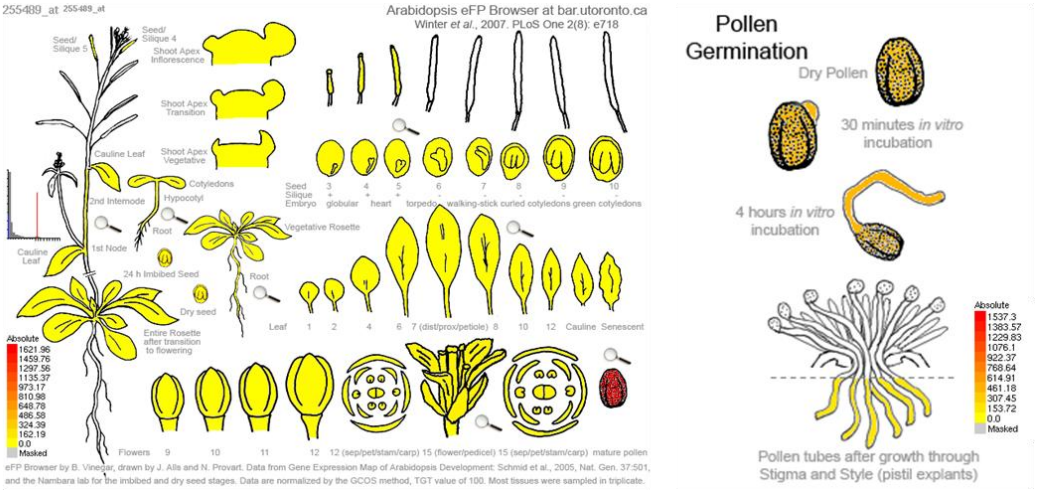



Figure 10. Expression patterns of *PICALM5a* and *PICALM5b*. Data was collected from the Arabidopsis Electronic Fluorescent Pictograph (eFP) Browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi).

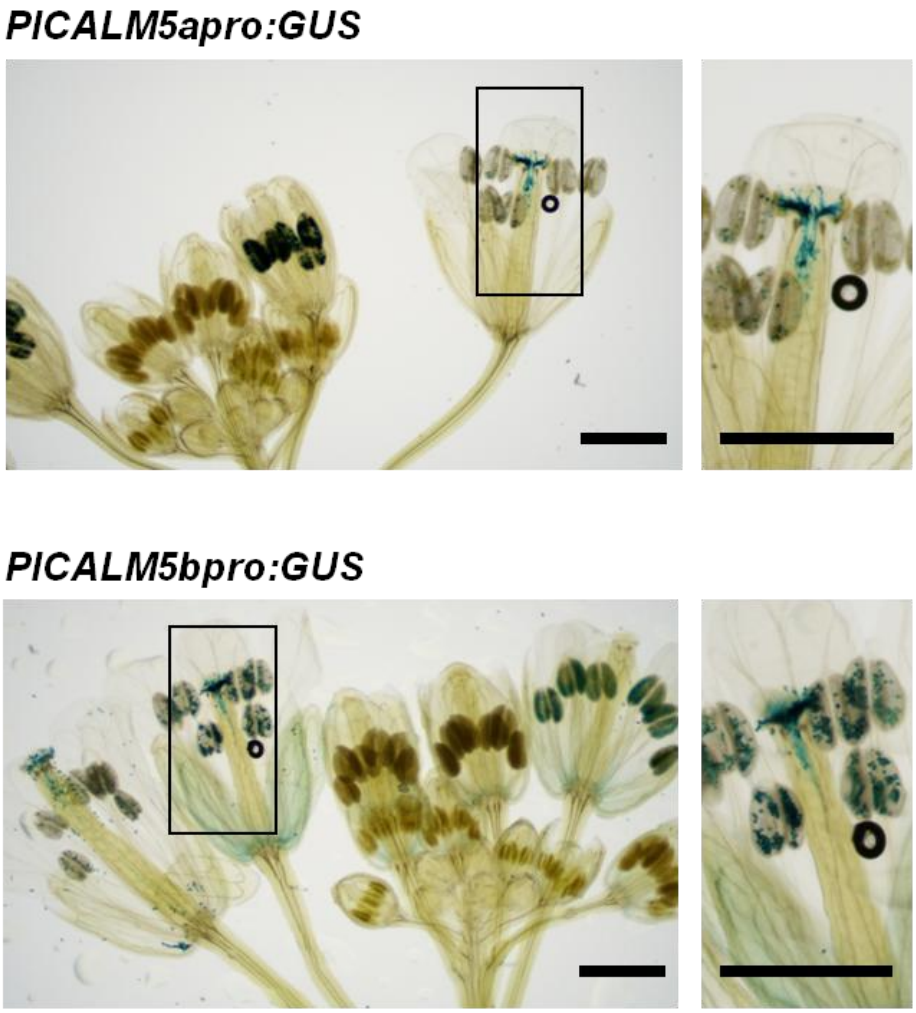



Figure 11. GUS staining of the inflorescences and pistils from *PICALM5apro:GUS* and *PICALM5bpro:GUS* plants. Right panels show the magnified views of the squared area in the left panels. Scale bars = 1 mm.

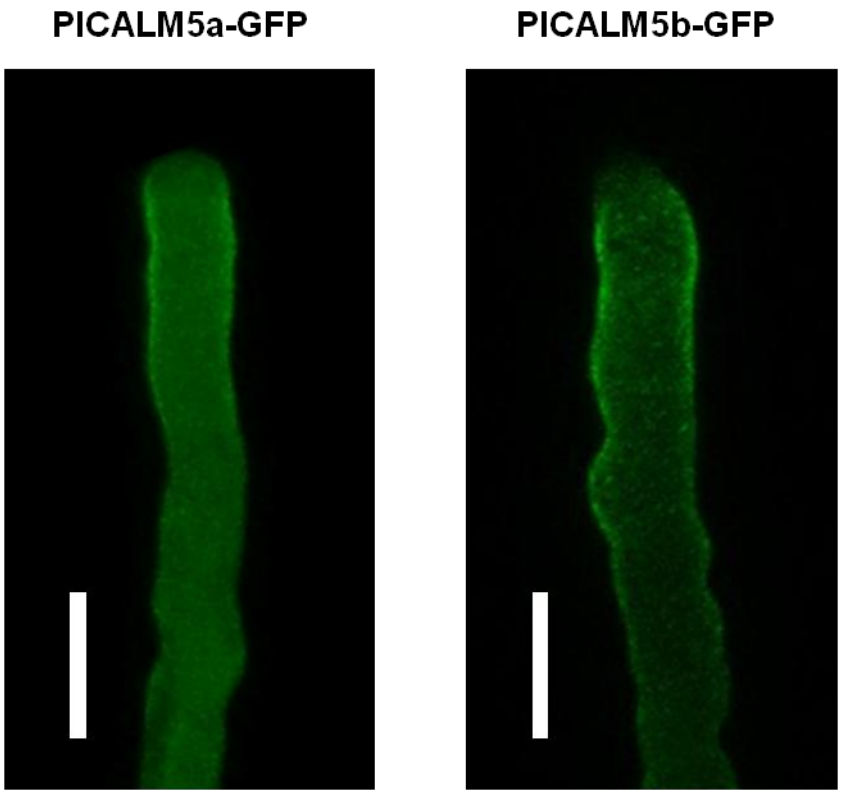



Figure 12. Subcellular localization of PICALM5a-GFP (left) and PICALM5b-GFP (right) in pollen tubes from the picalm5a and the picalm5b mutants, respectively. Scale bars = 10 μm.

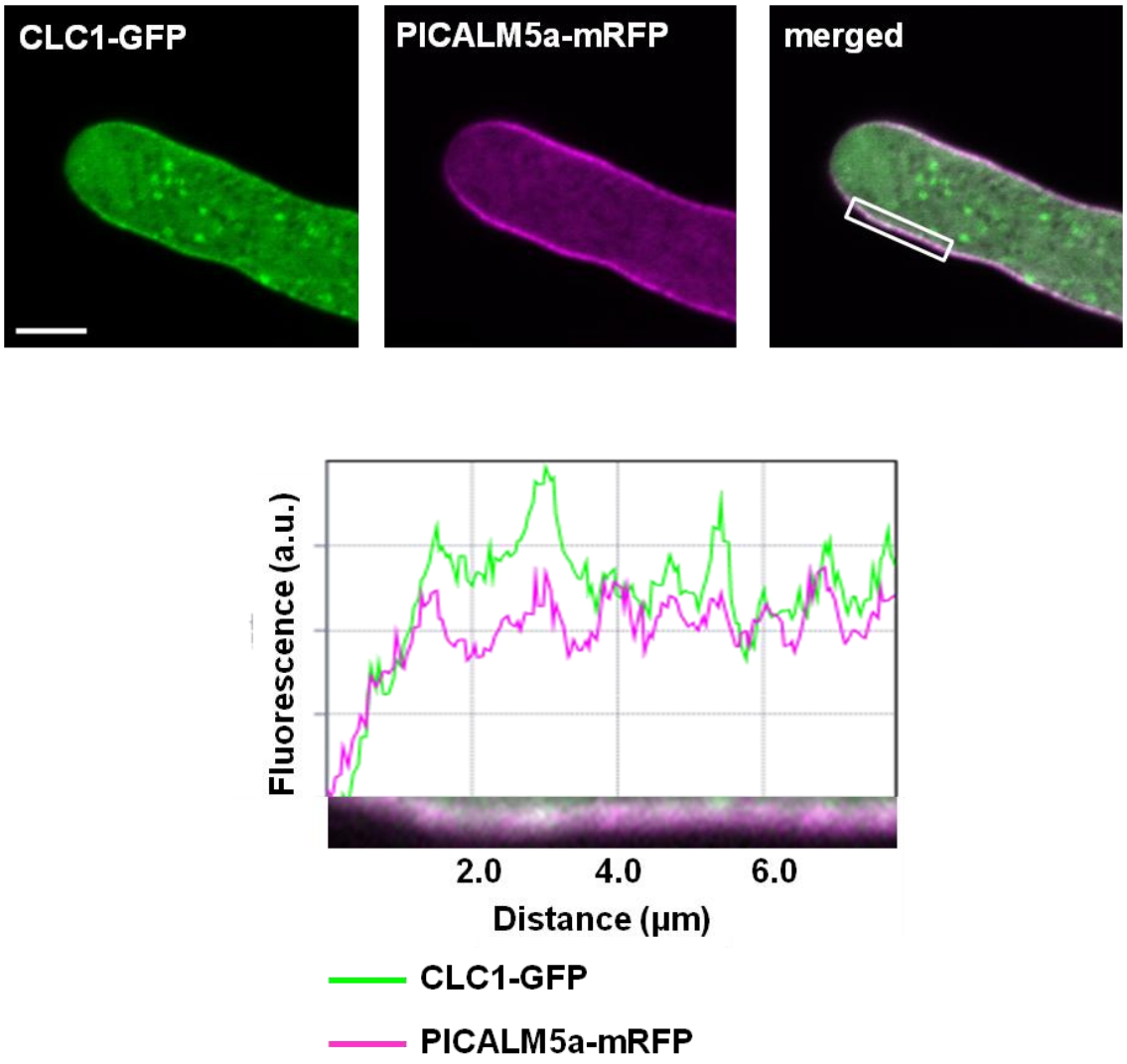



Figure 13. (top) Subcellular localizations of CLC1-GFP and PICALM5a-mRFP in a wild-type pollen tube. Scale bar = 5 μm. (bottom) Fluorescence intensity profiles for the white box shown in top panels.

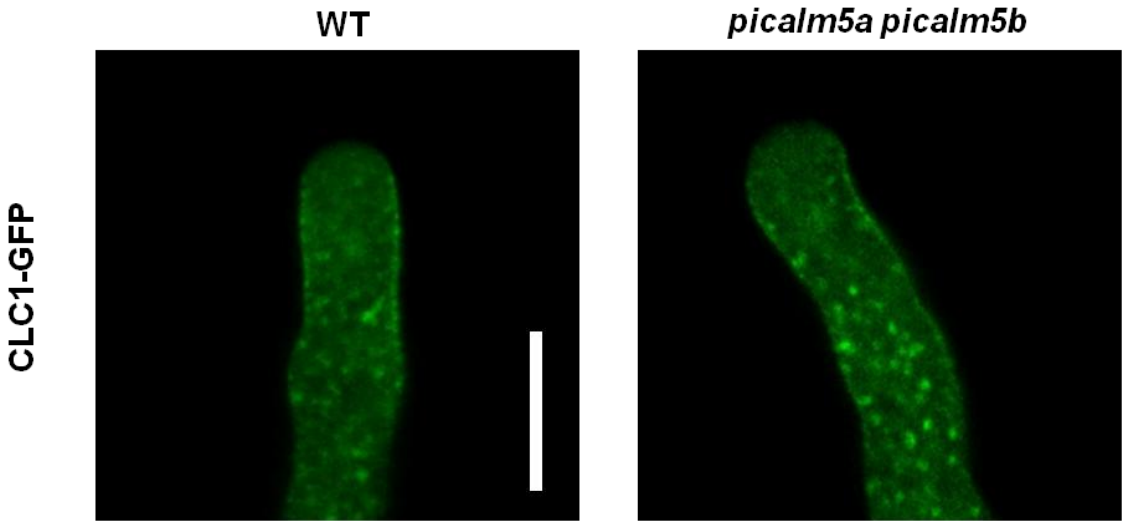



Figure 14. Subcellular localizations of CLC1-GFP in a growing pollen tube from a wild type (left) and a *picalm5a picalm5b* (right) plant. Scale bar = 10 μm.

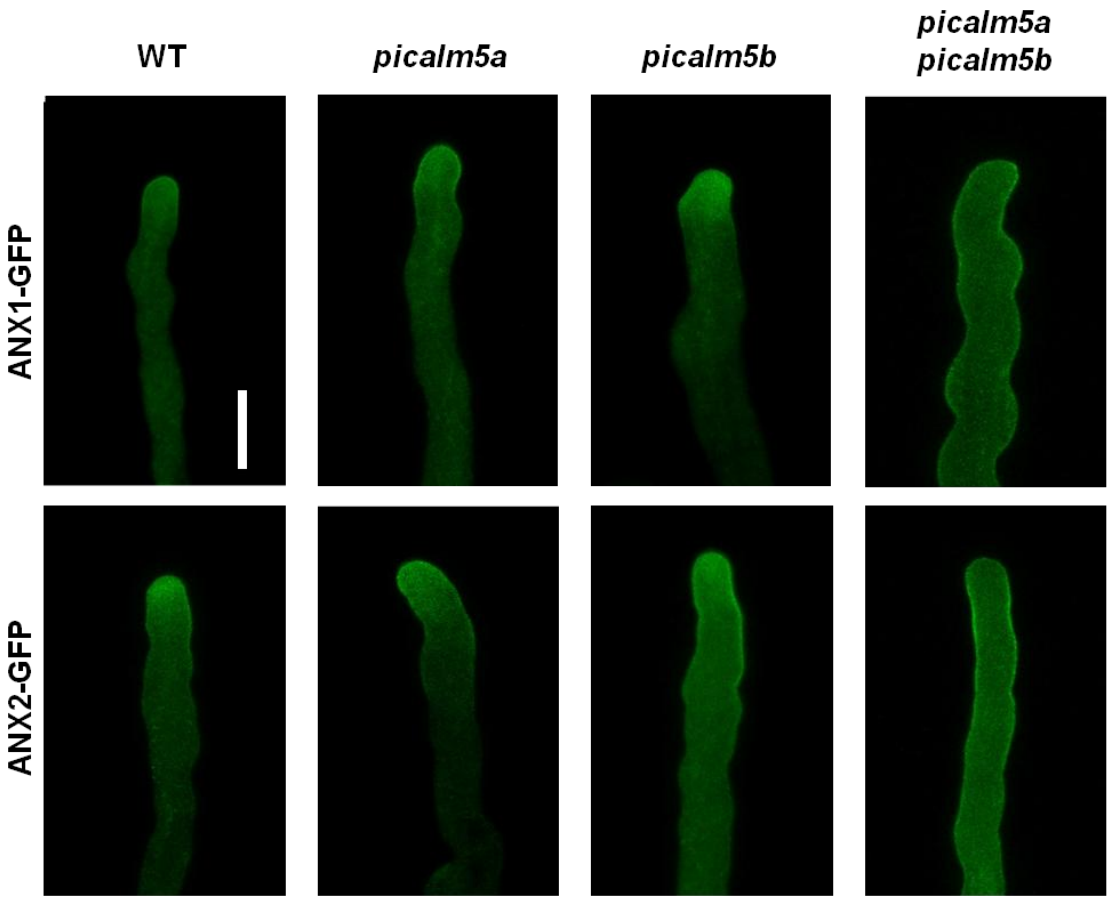



Figure 15. Subcellular localizations of ANX1-GFP and ANX2-GFP in the growing pollen tubes of wild type, *picalm5a*, *picalm5b*, and *picalm5a picalm5b* plants. Scale bar = 10 μm.

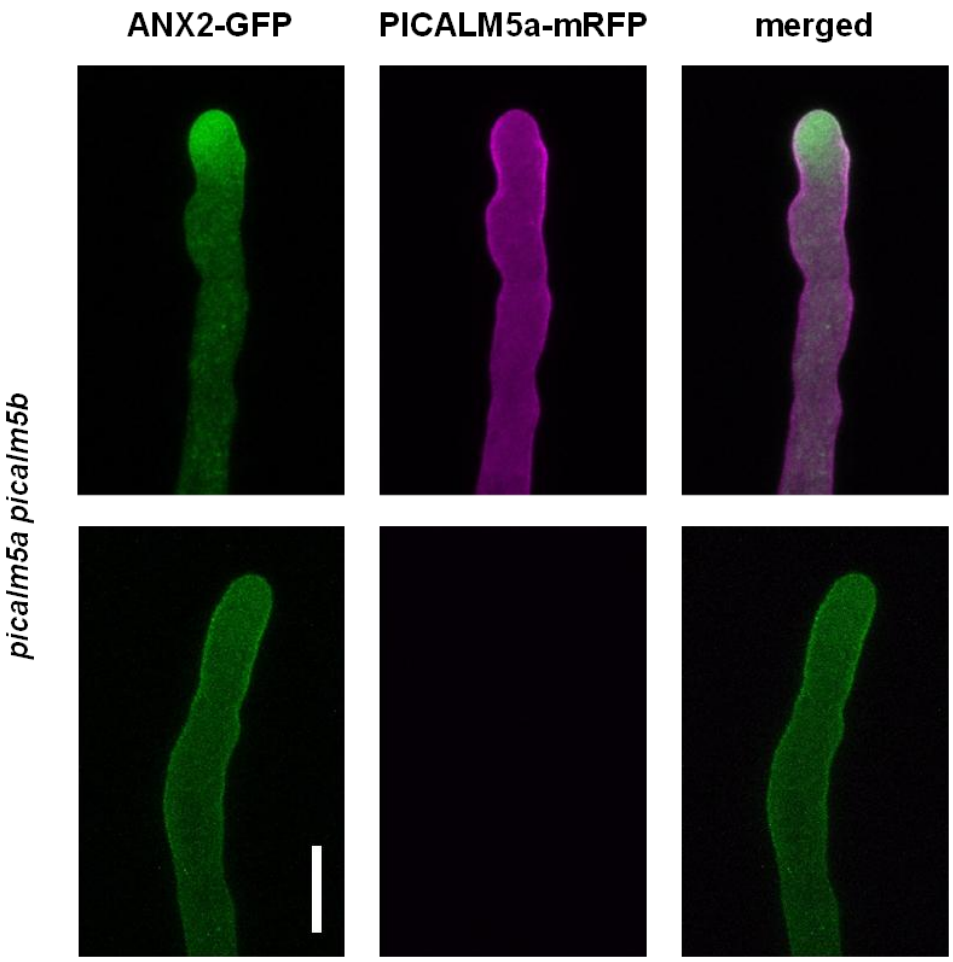



Figure 16. Subcellular localizations of ANX2-GFP in growing pollen tubes of transgenic plants harboring a single copy of *ANX2-GFP* expressed with (top panels) or without (bottom panels) *PICALM5a-mRFP* on the *picalm5a picalm5b* background. Scale bar = 10 μm.

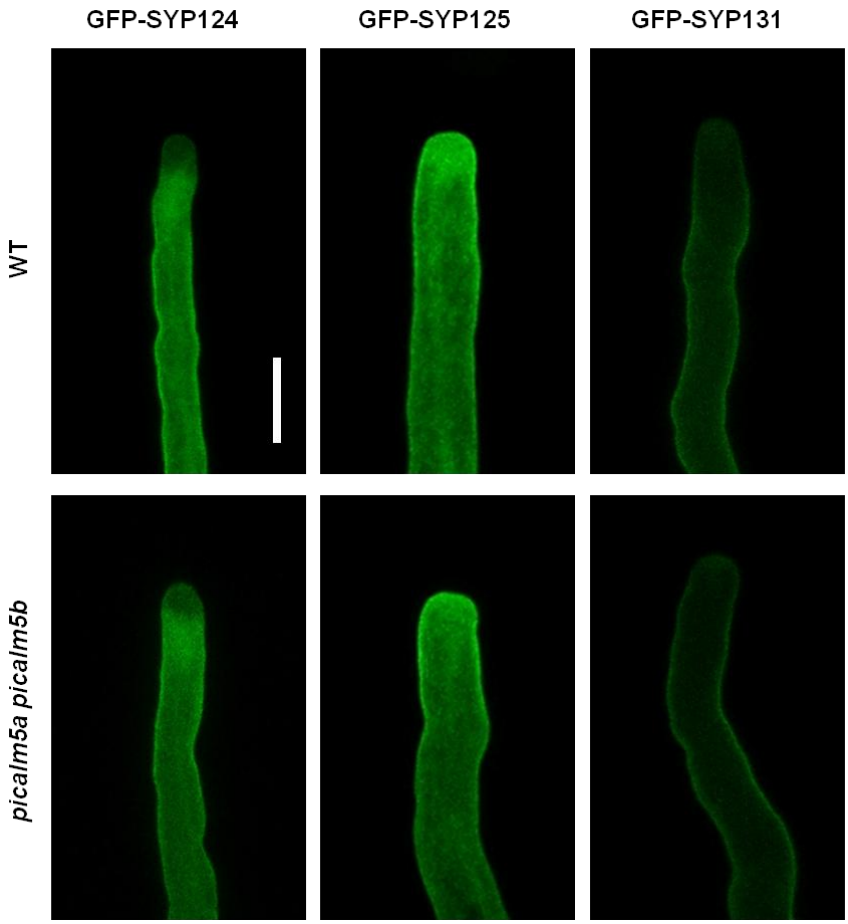



Figure 17. Subcellular localizations of GFP-SYP124, GFP-SYP125, and GFP-SYP131 in growing pollen tubes of wild-type plants and *picalm5a picalm5b* plants. Scale bar = 10 μm.

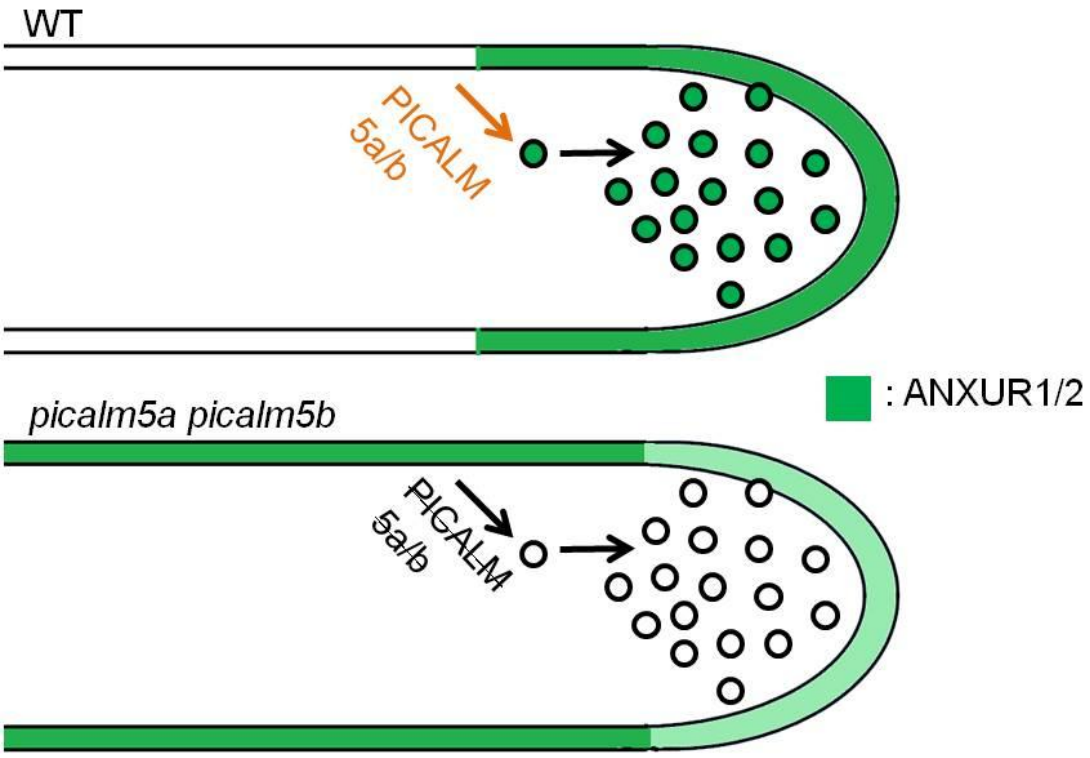



Figure 18. Model of the PICALM5a/b functions on the tip localization of ANXUR receptor-like kinases.

The results from this study suggested that PICALM5a/b have overlapping function as cargo-specific adaptors loading ANXUR1/2 into endocytic vesicles, which subsequently recycled into secretory pathway.

## Materials and Methods

### Plant materials and growth conditions

All *Arabidopsis thaliana* plants used in this study are a Col-0 accession background. Seeds were grown on Murashige and Skoog (MS) agar medium containing 0.2% sucrose at 23°C under continuous light. Two-week-old plants were transplanted into soil and grown at 23°C under long-day light-dark cycles (16 hours light and 8 hours dark).

### Plasmid construction

To generate *PICALM5a-GFP*, *PICALM5a-mRFP*, *PICALM5b-GFP*, *GFP-SYP124*, *GFP-SYP125*, and *GFP-SYP131*, approximately 2 kb of upstream and 1 kb downstream sequences for the coding regions of each gene were PCR-amplified with the primers listed in Table S2. The amplified fragments were then cloned into the pENTR/D-TOPO entry vector (Thermo Scientific). The clones were amplified by inverted PCR and combined with cDNA for GFP or mRFP using an In-Fusion HD Cloning Kit (Clontech). To generate *ANX1-GFP* and *ANX2-GFP*, PCR-amplified genomic fragments containing approximately 2 kb promoter sequences and coding regions for *ANX1* and *ANX2* were cloned into the pENTR/D-TOPO entry vector. The clones were recombined with the pGWB4 vector (Nakagawa *et al.* 2007) using an LR Clonase II enzyme mix (Thermo

Scientific).

To generate *LAT52p:CLC1-GFP*, the genomic fragment containing the coding region for *CLC1* without the stop codon was PCR-amplified and subcloned into the SpeI site of the *YMv036* vector (Mizuta *et al.* 2015) using an In-Fusion HD Cloning Kit (Clontech).

Pollination and silique clearing

Buds that were about to open were emasculated one day before pollination. Cross-pollinated siliques were harvested 10–14 days after hand pollination and fixed/decolorized overnight in ethanol:acetic acid solution (6:1). Before observation, the siliques were cleared with chloral hydrate solution (8 g chloral hydrate, 1 mL glycerol, 2 mL water). Images were obtained using a Leica MZ16 FA fluorescence stereomicroscope.

DAPI staining

DAPI staining was performed as described before (Park *et al.* 1998). Five to six open flowers were soaked in 300 µL of DAPI staining solution (0.4 µg/mL DAPI, 0.1% Triton X-100, 1 mM EDTA, 0.1 M $NaPO_4$, pH 7.0) in microtubes. After briefly vortexing the samples, the flower debris was removed with a pair of tweezers and the

microtubes were centrifuged to spin-down pollen grains. Pollen grains were transferred to a microscope slide and observed under a Zeiss LSM780 confocal microscope excited with UV.

Methyl blue staining

Methyl blue staining was performed as described before. (Kaya *et al.* 2014). The pistils were harvested 12 hours after hand-pollination and fixed overnight in acetic acid:ethanol solution (1:3) at room temperature. The fixed pistils were softened in 1 N NaOH for 30 min at 60°C. After three rinses with 2% $K_3PO_4$, the pistils were stained with 0.01% methyl blue in 2% $K_3PO_4$ for 2 to 4 hours in the dark. Fluorescence images were obtained using a Zeiss LSM780 inverted confocal microscope.

GUS staining

The inflorescences were fixed in 90% acetone on ice for 15 minutes. The fixed inflorescences were briefly washed twice with 100 mM $NaPO_4$ (pH 7.0) and placed into GUS staining solution (0.5 mg/mL X-Gluc, 1 mM potassium ferricyanide/ferrocyanide, 0.1% Triton X-100, 10 mM EDTA, and 100 mM $NaPO_4$, pH 7.0). The samples in the GUS staining solution were vacuum-infiltrated for 15 min and incubated for 3 to 5

hours at 37°C. The stained samples were washed with 70% ethanol and decolorized with ethanol:acetic acid solution (6:1).

*In vitro* pollen germination

*In vitro* pollen germination was carried out essentially as described before (Boavida and McCormick 2007) with some modification. Pollen grains were applied to a cellulose cellophane sheet (Futamura Chemical) (Rodriguez-Enriquez *et al.* 2013) that was placed on a thin layer of pollen tube germination medium (0.01% $H_3BO_3$, 5 mM $CaCl_2$, 5 mM KCl, 1 mM $MgSO_4$, 10% sucrose (pH 7.5), 1.5% low-melting agarose) supplemented with 10 μM epibrassinolide (Sigma-Aldrich) (Vogler *et al.* 2014) on coverslips and incubated at 23°C in a humid chamber. Images were obtained using an Olympus CKX53 inverted microscope, and pollen tube lengths were measured using ImageJ software (National Institutes of Health).

Fluorescent protein imaging

Transgenic pollen grains were germinated in liquid pollen tube germination medium (0.01% $H_3BO_3$, 5 mM $CaCl_2$, 5 mM KCl, 1 mM $MgSO_4$, 10% sucrose, pH 7.5) supplemented with 10 μM epibrassinolide. After incubation for 5 to 7 hours at 23°C,

fluorescent images were captured using a Zeiss LSM780 inverted confocal microscope.

## Quantification and statistical analysis

To compare the length of pollen tube germinated *in vitro*, I performed three independent experiments. The lengths of twenty pollen tubes were measured for each experiment and genotype. Statistical comparison was performed using Welch's *t* test, and statistically significant differences ($p < 0.01$) are indicated with an asterisk.

In the fertility analysis, the number of seeds per silique was counted in 28 self-pollinated siliques or 10 hand-pollinated siliques for each genotype. Statistical comparisons were performed using Welch's *t* test, and statistically significant differences ($p < 0.01$) are indicated with an asterisk.

## Accession numbers

The Arabidopsis Genome Initiative locus identifiers for the genes mentioned in this article are At1g03050 (*PICALM5a*), At4g02650 (*PICALM5b*), At2g20760 (*CLC1*), At3g04690 (*ANX1*), At5g28680 (*ANX2*), At1g61290 (*SYP124*), At1g11250 (*SYP125*), and At3g03800 (*SYP131*).

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