論文の内容の要旨

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論文題目

Molecular studies of male-side factors essential for compatible pollination in *Arabidopsis thaliana*

(シロイヌナズナの和合受粉過程に必須の雄側因子の分子生物学的な解析)

Introduction

Understanding core mechanisms of compatible pollination allow researchers and crop breeders to control fertilization under fluctuating agronomic field conditions, as well as enabling the transfer of agronomic traits from more diverse genetic background to increase crop production. In dry stigma species such as Brassicaceae, including the model plant *Arabidopsis thaliana*, stigmas are coated by a cuticle layer. Pollen-stigma interaction is especially important for these species. Only compatible pollen grains gain access to the water from the stigma and germinate. In *A. thaliana*, pollen hydration starts within a few minutes after stigma contact. Approximately after 20 minutes, the pollen grain germinates a tube and then penetrates into the cell wall of the stigmatic papilla cell. Compatible pollen triggers various responses in the papilla cell upon cell contact. A large number of genes are induced; for example, Ca^{2+}-ATPase gene *ACA13* is upregulated after pollination and localized to pollen tube penetration site. Actin filament concentrates at the pollen attachment site when pollen hydration starts. Active polarized secretion in the stigma is also important for pollen acceptation.

On the pollen side, dormant pollen grain swiftly becomes metabolically active during the hydration on a stigma. Pollen polarizes its cellular content to prepare for directional germination and rapid pollen tube growth. This polarization involves cell wall material trafficking to the germination site. The tethering of vesicles to plasma membrane is facilitated by the exocyst complex. Actin filament organization also mediates pollen germination and tube growth. The directional supply of water from the stigma contact site may provide directional cue for pollen polarity. A cytoplasmic Ca^{2+} gradient is also observed in the pollen at the side facing stigma prior to germination. These phenomena clearly show that compatible pollination requires cooperative pollen-stigma interaction.

Although the accumulating knowledge has been advancing our understanding on the compatible pollination, important questions remain unanswered such as: what is the *bona fide* determinant for compatible pollination, and how quiescent pollen grains become active very rapidly? To dissect molecular components involved in these early post-pollination processes, I decided to perform a forward genetic screen. In this study, I established a novel luciferase-based assay to monitor early compatible pollination process in stigma. I searched for pollen mutants lacking ability to induce compatible response in the stigma. I focused my study on *VPS13a*, one of the identified genes, on its physiological and molecular functions regarding *Arabidopsis* biology of pollen after pollination.

Chapter 1. Identification of male genes involved in compatible pollination of *Arabidopsis thaliana*

I established the luciferase-based assay system based on a transcriptomic data of a previous study. The promoter of genes highly induced in the stigmas after compatible pollination (namely $At2g44840_{pro}$, $At4g29780_{pro}$ and $At4g24570_{pro}$) were fused to *Photinus pyralis* luciferase reporter gene. I introduced the constructs into the Col-0 strains to generate transgenic reporter plant lines. The stigmas of reporter lines were pollinated with compatible pollen to compare bioluminescence induction after pollination. The reporter line containing $At4g24570_{pro}$:*Luciferase* (*Luc*) construct showed the highest induction (mean±SD, 11.89±3.47 fold at 3 h after pollination) when compared with the other two lines ($At2g44840_{pro}$:*Luc*, 1.96±0.77 fold; and $At4g29780_{pro}$:*Luc*, 1.77±0.30 fold); thus, I selected this line as the reporter line in further analysis. To confirm that the luciferase induction is specific to pollen-stigma compatible communication, the reporter pistil was tested against three known incompatible pollen mutants, *cer1*, *cer6-2* and *fkp1*. These mutants are conditional male-sterile mutants which have affected pollen coat content and do not hydrate on a stigma under normal pollination condition. Pollination using pollen from these mutants did not induce luciferase activity of the reporter pistil. As a result, I succeeded in establishing the luciferase-based pollination assay specifically responds to compatible pollination.

I used the established assay in a forward genetic screen to search for mutants defective in inducing the bioluminescence in the reporter line. Pollen grains from about 2,400 M_2 mutant lines obtained from the EMS-mutagenesis were used to pollinate the reporter pistils. I isolated mutant lines which showed low or non-induction of stigma bioluminescence for causal gene identification. Among the 11 isolated mutants, seven independent lines causing non-induction (four lines) or low luciferase induction (three lines) phenotype were back-crossed to the wild-type Col-0 strain, and then self-fertilized to obtain the segregating BC_1F_2 generation. Pooled DNA from recessive mutant progenies that failed to induce the luciferase activity of the reporter line was subjected to the high-throughput sequence analysis, to identify the candidate causal gene by bulked-segregant analysis pipeline. Conserved SNPs in the pooled-genome of each mutant line suggested that candidate causal genes of two low induction mutants were At2g47240 (*CER8*, long-chain acyl-CoA synthase gene; missense mutation G382E) and At5g35700 (*FIMBRIN5*, actin bundling factor gene; nonsense mutation W491*). Previous reports suggested that *CER8* involves in pollen coat formation, and *FIMBRIN5* in pollen germination and tube growth. This indicated that my screening is able to find previously known mutants involved in the compatible pollination process. Causal mutations within the same gene At1g48090 were found from the four independent mutants (two nonsense mutations and two mRNA splice-site mutations), that belonged to the non-inducing phenotypic class. At1g48090 was annotated as a calcium-dependent lipid-binding family protein gene according to the Araport11 database, and is uncharacterized in *Arabidopsis*. I found that three independent T-DNA insertion lines for At1g48090 had similar phenotype to the isolated mutants, thus concluded that this was the causal gene controlling compatible pollination phenotype. Study of T-DNA insertion lines of the candidate causal genes for the remaining low induction mutant, failed to reproduce the mutant phenotype.

In this chapter, I successfully established new pollination assay and identified genes involved in compatible pollination through a genetic screen. The physiological importance and molecular characteristics of the novel gene At1g48090 are explained in the subsequent chapters.

Chapter 2. Physiological functions of *Arabidopsis VPS13a*

Based on the phylogenetic analysis of At1g48090, this gene was found as the homolog of the yeast *Vacuolar Protein Sorting 13* (*VPS13*). At1g48090 was renamed as *VPS13a* in this study. *Arabidopsis* carries two other

VPS13 domain-containing proteins, At4g17140 (named as *VPS13b* in this study) and At5g24740 (named as *SHRUBBY* in a previous study). *VSP13a* and *VPS13b* are the forms specifically evolved in the land plant species. Transcriptomic databases showed that *VPS13a*, *VPS13b* and *SHRUBBY* are ubiquitously expressed genes. I analyzed tissue specificity of *VPS13a* at higher resolution by generating *VPS13a*$_{pro}$:*GUS* transgenic lines. GUS activities were observed in vasculature of leaf and root, guard cell and root tip. In reproductive organs, I observed strong GUS activities in various stages of anther and mature pollen. Only half of the pollen grains from hemizygous GUS-reporter plant were positively stained suggesting that *VPS13a* expression in pollen starts after meiotic division of microsporocyte where haploid microspores are completely separated. From the genetic screen, only *VPS13a* but not *VPS13b* was found to be the gene required for compatible pollination. Therefore, I studied the function of VPS13a through the comparison with its closest phylogenetic homolog VPS13b.

Nuclei staining by DAPI showed that both *vps13a* and *vps13b* pollen developed into mature tricellular pollen stage, same as wild-type. The time needed for complete hydration of the *vps13a* and *vps13b* pollen (mean ± SE, 8.8 ± 0.29 min and 9.2±0.60 min, respectively) was similar to that of wild-type pollen (8.7 ± 0.38 min; ANOVA, p>0.05). However, none of the *vps13a* pollen grains germinated within the time observed (45 min), while 94.1% of the wild-type and 88.2% of *vps13b* pollen grains germinated at an average time of 21.4 ± 0.52 min and 20.1±0.82 min, respectively. In addition, the *vps13a* pollen grains germinated less efficiently under *in vitro* conditions. The mean (± SE) germination rates after 24 h of wild-type and *vps13b* pollen were, 53.2 ± 3.67% and 51.87 ± 1.73% respectively. On the contrary, germination rate of *vps13a* pollen was 15.5 ± 2.94%, significantly lower than the wild-type (ANOVA, p<0.001). These results suggested that while neither the *VPS13a* nor *VPS13b* played substantial role in pollen hydration on stigma, *VPS13a* is the main isoform important for efficient pollen germination. I also tested transmission efficiency of pollen by the reciprocal cross experiment between wild-type and *VPS13a* hemizygote. Although the hemizygote produced half *vps13a_Km*$_r$ and half *VPS13a_Km*$_s$ pollen, only 6.5% of progenies were resistant to kanamycin (Km_r:Km_s = 218:3,116). The ratio was distorted from Mendelian's law of segregation suggesting that VPS13a expressed in pollen is important for the pollen fertilization success under this condition (Chi-square test, X^2=1,553, p<0.001). The *vps13a* plant suffered reduced seed-set most likely from its compromised pollen germination abilities. The *vps13a/vps13b* double mutant pollen showed improper development as some grains only had unicellular or bicellular signals. This suggests genetic redundancy of *VPS13a* and *VPS13b* in male gametophyte development.

Chapter 3. Molecular functions of *Arabidopsis VPS13a*

VPS13a was predicted to have a Ca^{2+}-dependent lipid-binding (C2) domain in its sequence, while this domain was absent in VPS13b. I considered that the domain may explain why VPS13a but not VPS13b plays a notable role in pollen germination. To support this hypothesis, I used genome-editing system with two single guide RNAs targeting C2 domain in *VPS13a*. This CRISPR/Cas9 editing generated an in-frame *VPS13a* mutant that lacks the complete C2 domain (*VPS13aΔC2*) but the rest of protein remain intact as confirmed by cDNA sequencing. Similar to the *vps13a* mutant, 97.0% of pollen grains from *VPS13aΔC2* mutant failed to germinate within 45 min on the papilla cells. Although pollen grains of the *VPS13aΔC2* mutant retained small germination activity, the C2 domain was considered to be crucial for efficient and rapid pollen germination. This may largely explain why VPS13b which lacks the C2 domain cannot compensate for VPS13a during pollen germination. I used fusion protein Yellow cameleon 3.6 (YC3.6), a fluorescence resonance energy transfer-based Ca^{2+}

biosensor, to observe dynamics of cytoplasmic Ca^{2+} level in pollen grains after pollination. After pollination, wild-type background reporter pollen (*WT_VPS13a*/*YC3.6*) showed a rapid decrease of cytoplasmic Ca^{2+} level soon after pollen hydration initiation, reaching the lowest point at about 4-5 min, then gradually increased and maintained until germination. The *vps13a*/*YC3.6* pollen grains showed similar cytoplasmic Ca^{2+} pattern to the wild-type pollen (comparing YFP/CFP signal ratio at each timepoint; Student's t-test, $p>0.05$). This suggested that VPS13a does not influence Ca^{2+} concentration in pollen grains but might be involved in transducing the Ca^{2+} dynamics to the downstream cellular events.

N-terminus of VPS13 forms a hydrophobic cavity that is required for its lipid-transfer function between membranes. I found that N-terminal hydrophobic cavities are conserved in the three *Arabidopsis* VPS13 forms based on their homology-based 3D models by Phyre[2]. Human HsVPS13A is recently found to function as a tethering molecule between rough endoplasmic reticulum (rER) and lipid body (LB). It contains an LB-targeting amphipathic helix in the ATG_C domain near C-terminus. Helical wheel projection of corresponding sequences of the *Arabidopsis* VPS13 isoforms suggested that the amphipathic helix was also conserved in plants. These structural conservations suggested that *Arabidopsis* VPS13 potentially maintained the proposed rER-LB tethering function of their eukaryotic homologs. To observe possible effects of VPS13a in regulating these organelles dynamic during pollination, I used transmission electron microscopy to investigate pollinated pollen grains on stigma. In un-hydrated grains (immediately after pollination), no obvious difference was found between wild-type and mutant pollen. Coupled egg-cup-like structures of rER and LB were abundantly found in both wild-type and *vps13a* pollen grains. Numbers of LB visualized by Nile red was not different between the two genotypes (mean±SE: 138±11 particles in wild-type and 129±6.9 particles in *vps13a*; from 8.5 μm optical section covering whole grain area, around the center of pollen ellipsoid; Student's t-test, $p=0.501$). Interestingly, at 10 min after pollination, 47.0±5.9% (mean±SE) of LBs synchronously dissociated from the rER in hydrated wild-type pollen grains. Such dissociation was hardly observed in mutant pollen (1.0 ± 1.0%) suggesting that the phenomenon was dependent on the VPS13a activity.

To my knowledge, this is the first report on a synchronous release of LBs from rER. Many eukaryotic cells are known to use LB for various cellular activities. It is possible that this synchronous dissociation is a required process for pollen germination. LB forms at late stage during pollen development and its biogenesis mutants are compromised in pollen grain size and/or viability. It is possible that LB released from rER serving as a motile lipid-reservoir for forming organelle structures in pollen, such as vacuole that is rapidly enlarged in germinating grains, and plasma-membrane that are extended in the growing tubes. More functional tests are still needed to understand the contribution of this phenomenon in rapid pollen germination.

Conclusion

In this study, I identified *Arabidopsis* VPS13a as an essential factor for causing the release of LBs from the rER during pollen germination. The pollen grain is a very unique cell that can rapidly shift from a dry quiescent state to a rapidly growing phase within a short time after pollination, and loss-of-function of *VPS13a* disrupted this process. In flowering plants, like in most reproductive systems, sperm delivery for fertilization is a competitive process with natural selection for competent male gametophytes. Pollen may accommodate this remarkable shift from dormancy to elongation via a specific mechanism such as the rER-LB reorganization controlled by VPS13a.