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SUMMARY OF DOCTORAL THESIS

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Title:

Analysis of pollen development and pollen wall formation in *Arabidopsis thaliana* (L.) Heynh.
（シロイヌナズナにおける花粉発達と花粉表層形成に関する研究）

The research described in this thesis aimed to improve our understandings of the important processes of pollen development and exine wall formation and to isolate and characterize new genes involved in such complicated processes. Vast numbers of genes work cooperatively to regulate various biological processes including pollen development and exine wall formation. In order to understand the functions and molecular mechanisms underlying these processes, analyses of transgenic plants that concomitantly express two protein-coding genes are often required.

In **Chapter 1**, the author presents a general introduction to the work. **Chapter 2** describes a high-magnification level screening aimed to isolate new genes affecting anther and pollen development in *Arabidopsis thaliana*. Unlike most of the previous screens which were mainly based on isolating male sterile and semi-sterile plants neglecting a large number of mutants with severe to mild defects in pollen structure, however, still fertile; the screen described here using scanning electron microscopy could isolate such mutants. Several mutants with a range of defects in anther/pollen development and exine wall formation were recovered; some of them exhibit novel surface-structure phenotypes. A total of one hundred and one mutant plants were isolated (some of them were found to be allelic) and classified according to their phenotypic characters into three classes including multiple subclasses and types. The identification of the mutation location was performed mainly by the bulked-segregant analysis using next generation sequencing rather than using the traditional mapping methods. Twenty-three mutations were successfully mapped to specific regions of particular chromosomes of the Arabidopsis genome; thirteen of them were linked to specific genes. This work illustrates that high magnification level screens (i.e., using scanning electron microscopy) are highly beneficial and may even be required to isolate mutants with novel phenotypes, (i.e., most mutants with severe defects that can be found by naked-eye visual screen or dissecting microscope-level screen most likely will be alleles of previously identified genes). Characterization of the genes responsible for mutants isolated in this screen will expand our knowledge of the molecular mechanism underlying several vital processes in pollen development, including pollen wall formation, sporopollenin synthesis, polymerization and transport, and exine patterning. This screen provides an additional resource for plant researchers to analyze functions of genes involved in anther/pollen development and exine wall formation processes and will help unrevealing previously unknown players in such processes.

COPII proteins are involved in lipid and protein transport events from the endoplasmic reticulum to the Golgi in the early secretory pathway. **Chapter 3** provides phenotypic characterization of two components of the Arabidopsis coat protein complex II (COPII) proteins, *AtSEC23A* and *AtSEC23D*, and shows how these genes are involved in regulating plant growth and development. The study revealed that both *AtSEC23A* and *AtSEC23D* are essential for normal pollen and tapetum development. Single *atsec23a and atsec23d* mutant plants, although normally fertile,

showed a compromised pollen wall formation with less sporopollenin deposition in the outer exine layer. Double *atsec23ad* mutant plants were semi-sterile and exhibited a wide range of developmental defects in pollen and tapetal cells. Pollen grains of *atsec23ad* plants had defective exine and intine layers and their cytoplasm have shown signs of degeneration or leakage. Moreover, development of tapetal cells in *atsec23ad* plants was altered, with structural abnormalities in inner organelles (i.e., ER, Golgi, elaioplasts, and tapetosomes). Both AtSEC23A and AtSEC23D exhibited the characteristic localization pattern of COPII proteins, indicating that they are functional COPII components. The results indicated that *AtSEC23A* and *AtSEC23D* may only share partial redundant functions and may have differing substrate preferences. Thus, this work suggests that *AtSEC23A* and *AtSEC23D* organize pollen wall development and exine patterning by regulating ER export of lipid and proteins necessary for pollen wall formation in tapetal cells. This work provides a direct link between the tapetum early secretory pathway and the processes of sporopollenin deposition and exine patterning. In addition, the results presented in this Chapter shed light on the functional heterogeneity of SEC23 homologs in plants.

To better understand the functions and molecular mechanisms underlying various biological processes including pollen development and exine wall formation, developing new systems to deliver two genes simultaneously are often required. For example, exploring the expression patterns and co-localizations of the two SEC23 proteins described in Chapter 3 required utilizing vector systems that enable simultaneous cloning of two expression cassettes. For these reasons, two vector systems allowing simple cloning of two expression cassettes simultaneously in plant cells were developed and presented in **Chapter 4** and **Chapter 5**. **Chapter 4** describes a vector system that enables the simultaneous cloning of two desired sets of promoters and open reading frames, named the R4 dual-site gateway cloning system. **Chapter 5** presents a vector system allows the simultaneous cloning of two genes under control of the moderate nopaline synthase promoter, named the dual-site gateway cloning system. These vectors have been used successfully not only in exploring the expression patterns and co-localizations of the two SEC23 proteins but also in the co-localization of each of AtSEC23A and AtSEC23D with other organelle markers (e.g., endoplasmic reticulum, Golgi, and plastid) as described in Chapter 3. These vectors also facilitate the protein-protein interaction analysis by bimolecular fluorescence complementation (BiFC) assay and co-immunoprecipitation. Investigating protein-protein interactions is required in many cases to dissect the functions and molecular mechanisms underlying multiple biological processes including pollen development and exine wall formation. In addition, the two developed vector series are versatile cloning tools with multiple flexibility features, including multiple types of tags and 4 kinds of plant selection markers. Both cloning systems will be invaluable experimental tools with multiple applications in plant research, e.g., analysis of co-localization patterns of transgenes and investigation of protein-protein interactions by BiFC or co-immunoprecipitation.

In the **final Chapter**, the author provides proposed conclusion remarks and discusses the findings presented in this study.

In sum, the large scale screening (~ 10,000) using scanning electron microscopy at a high-ma gnification level was successful to isolate mutants with mild defects in the exine surface struc ture that would be neglected with using other screening strategies. Multiple mutants with nov el phenotypes were recovered. Two genes, *AtSEC23A* and *AtSEC23D*, were characterized in d etail; both were found to be required for proper pollen wall development and exine patterning and they may function by regulating ER export of lipid and proteins necessary for pollen w all formation in the tapetal cells. Two novel vector systems were developed for analysis of th e genes revealed in this study. These systems are beneficial tools in exploring the functions a nd molecular mechanisms underlying various biological processes including pollen developmen t and exine wall formation.