DIVISION OF EVOLUTIONARY BIOLOGY

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All living organisms evolved from a common ancestor that lived more than 3.5 billion years ago, and the accumulation of mutations in their genomes has resulted in the present biodiversity. Traces of the evolutionary process are found in the genomes of extant organisms. By comparing the gene sequences and gene networks of different organisms, we can infer (1) the phylogenetic relationships of extant organisms and (2) the genetic changes that caused the evolution of morphology and development. The inferred phylogenetic relationships provide important insights into problems in various fields of evolutionary biology. Our group focuses on biogeography, the evolution of morphological traits, and systematics in a wide range of taxa. Concerning the evolution of morphology and development, we hope to explore the genetic changes that led to the evolution of the plant body plan. We have selected several land plants and some green algae as models to compare the functions of genes involved in the development of both reproductive and vegetative organs in land plants.

I. Evolution from unicellular to multicellular organisms

The first evolutionary step from unicellular to multicellular organisms is to form two different cells from a single cell via asymmetric cell division. The first cell division of a protoplast isolated from the protonemata of the moss *Physcomitrella patens* is asymmetric regarding its shape and nature, and gives rise to an apical meristematic cell and a differentiated non-meristematic cell. A systematic overexpression screening for genes involved in asymmetric cell division of protoplasts in *P. patens* was performed. After eliminating genes that are not directly involved in asymmetric cell divisions, such as photosynthesis genes, we used 3000 clones as materials for the overexpression screening. Individual cDNAs were subcloned under a constitutive promoter and introduced into the protoplasts of *P. patens* for transient expression. We observed and categorized phenotypes of the regenerating protoplasts. We identified 58 cDNAs whose overexpression caused the defects in asymmetric cell divisions in two repeated experiments. We knocked in a cytrin gene just before the stop codon of each candidate gene and examined the cellular localization of a fused protein under its native promoter. Thus far, we have examined 32 of 58 candidates and nine fused proteins were detected to be specifically localized in an apical meristematic cell. Further characterization of these genes by the overexpression of the genes in protoplasts with GFP-tubulin or GFP-talin and the loss-of-function experiments using RNAi are now in progress. Functional analyses of these genes should help us to understand the molecular mechanisms of how plants generate distinct meristematic cell lineages to build their multicellular bodies. This work was performed as a collaborative work with Dr. Tomomichi Fujita (Hokkaido University).

II. Evolution from cells to tissues

The most prominent difference between plant and animal cells is that plant cells have a cell wall and do not move during development. Therefore, the plane of cell division and the direction of cell elongation determine the morphology of differentiated tissues and organs.

2-1 Mechanism of cytokinesis

The cells of land plants and their sister group charophycean green algae divide by the insertion of cell plates at cytokinesis. This is in contrast to other green algae, in which the invagination of plasma membrane separates daughter cells at cytokinesis. The cell plate appears in the middle of daughter nuclei, expands centrifugally towards a cell periphery, and finally fuses to a parental cell wall. Cell wall materials are transported to the expanding cell plate with a phragmoplast, which is mainly composed of microtubules. A centrifugal expansion of the phragmoplast is a driving force for that of the cell plate, although the molecular mechanism for the centrifugal expansion of the phragmoplast was a challenge. We observed tobacco BY-2 cells expressing GFP-α-tubulin with live imaging technique and found that a new microtubule at the margin of the phragmoplast appeared at the side of an existing phragmoplast microtubule. We further confirmed the microtubule appearance site using a live imaging of tobacco BY-2 cells expressing GFP-AtEB1b, a marker of growing microtubule plus ends. γ-tubulin, an essential component of a protein complex for microtubule nucleation, was concentrated at the base of newly formed microtubules. We propose a hypothesis that cytosolic γ-tubulin complexes are recruited onto existing phragmoplast microtubules and nucleate new microtubules as branches by a similar mechanism to the cortical microtubule formation at interphase (Fig.1). Detailed analysis of

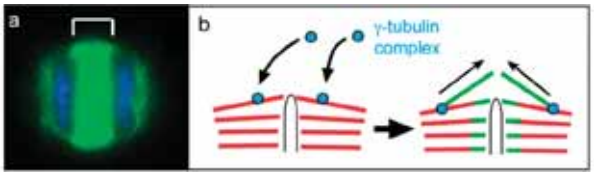

Figure 1. Phragmoplast development in flowering plants. a). A phragmoplast of tobacco BY-2 cells visualized with anti-α-tubulin antibody (green). Daughter nuclei are stained with DAPI (blue). A schematic explanation of a microtubule formation in a white bracket is shown in b. b). A scheme of microtubule nucleation during phragmoplast expansion. Red: preexisting microtubules. Green: newly formed microtubules. Blue: γ-tubulin complexes.

microtubule dynamics and functional analyses of γ-tubulin are in progress. T. Murata mainly performed this study.

III. Evolution of molecular mechanisms in plant development

3-1 Stem cell initiation and maintenance

Postembryonic growth of land plants occurs from the meristem, a localized region that gives rise to all adult structures. Meristems control the continuous development of plant organs by balancing the maintenance and proliferation of stem cells and directing their differentiation. Meristem initiation and maintenance is a fundamental focus of plant development research. Three lines, exhibiting reporter gene (*uid*A) expression preferentially in the apical cells, were isolated from previously established gene- and enhancer-trap lines, and identified as encoding kinesin-like protein (API1) and ubiquitin-like protein (PUBL1), as well as an unknown protein. Functional analyses of these genes are currently under investigation, mainly by Y. Hiwatashi. A distortion of phragmoplast was observed in double disruptants of *API1* and its sister gene. This suggests that these kinesin-like proteins are indispensable for the proper formation of phragmoplast. On the other hand, double disruption of *PUBL1* and its sister gene, *PUBL2,* retarded the collapse of phragmoplasts, suggesting that these ubiquitin-like genes likely regulate the stability of phragmoplast microtubules. Further analyses are in progress.

3-2 Function of gametophytic MADS-box genes

Land plants are believed to have evolved from a gametophyte-dominant ancestor without a multicellular sporophyte; most genes expressed in the sporophyte were probably co-opted from those used in the gametophyte during the evolution of land plants. To analyze the evolution and diversification of MADS-box genes in land plants, eight MADS-box genes predominantly expressed in *Arabidopsis thaliana* pollen, male gametophyte, were analyzed, mainly by N. Aono. Four of eight genes belonged to MIKC*-type MADS-box genes and quadruple disruptants of these genes were formed by multiple crossings of four single disruptants. The quadruple disruptants showed a defect in pollen germination both in vivo and in vitro.

3-3 Function of class 1 KNOX genes in a moss *Physcomitrella patens*

We performed a functional analysis of class 1 KNOX genes to investigate the co-option hypothesis on the evolution of body plans in sporophyte generation.

The shoot apical meristem repeatedly forms stem and lateral organs, such as leaves, in vascular plant sporophytes. The sporophyte of a moss forms a single sporangium without lateral organs, while leafy shoots are formed in the gametophyte generation. We aimed to reveal whether or not the homologues of vascular plant genes are involved in shoot apical meristem formation in gametophytic shoot formation in the moss *Physcomitrella patens* and whether or not the homologues are involved in sporophyte development of *P. patens* without leafy shoots. Recent genetic analyses in *Arabidopsis thaliana* showed that *SHOOT MERISTEMLESS*, which is a member of the class 1 KNOX subfamily of the homeobox gene superfamily, is involved in the formation and maintenance of the shoot apical meristem. Three class 1 KNOX genes, *MKN2*, *MKN4*, and *MKN5,* were isolated from *P. patens*. We inserted a GUS reporter gene at the end of every one of the three genes to investigate the expression patterns.

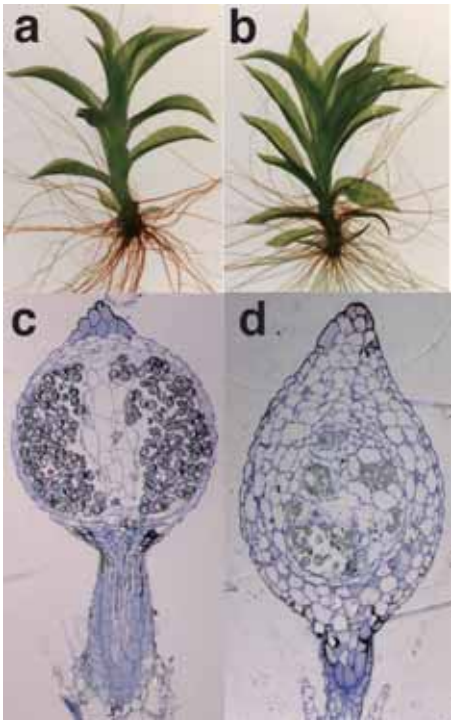

Figure 2. A leafy shoot (gametophore) of a wild type (a) and a triple class 1 KNOX disruptant (b). Longitudinal sections of sporophytes in the triple disruptant (c) and a wild type (d). Photos by Dr. Keiko Sakakibara.

The transgenic *P. patens* showed GUS activity in sporophytes but not in gametophytes, including leafy shoots. GUS activity was detected in an apical cell until the apical cell stops dividing. Later, GUS activity was detected in a seta meristem until a sporangium matured. We constructed triple disruptants of the three genes. They formed normal gametophytes, but aberrant sporophytes (Fig. 2). These results shows that the three class 1 KNOX genes function in the sporophyte development, but not in gametophytic shoot development. In spite of the significant morphological difference, KNOX class 1 genes are involved in the development of diploid generations in the moss and vascular plants, suggesting that the molecular mechanisms of gametophytic shoot formation are different from sporophytic shoot formation. We need to revisit the co-option hypothesis. This work is a collaboration with Dr. Keiko Sakakibara and Prof. Hironori Deguchi of Hiroshima University.

3-4 nuclear genome project of the moss *Physcomitrella patens*

A comparison of developmental genes among major land plant taxa would facilitate our understanding of their evolution, although it was not possible because of the lack of genome sequences in basal land plants. We established an international consortium for a genome project of the basal land plant; the moss *Physcomitrella patens* and its entire genome has been mostly sequenced as a collaborative work with the Joint Genome Institute at the U.S. Department of Energy. To facilitate the contig assembling and the gene annotation, we performed (1) the EST analyses of several libraries of cDNAs isolated from different developmental stages, (2) the construction of full-length cDNA libraries and sequencing in their full length, (3) the construction of BAC libraries and their end-sequencing, and (4) 5'-end serial analysis of gene expression (5' SAGE) as collaborative works with Dr. Tomoaki Nishiyama (Kanazawa Univ.), Prof. Asao Fujiyama (National Institute of Informatics), Prof. Sumio Sugano (Univ. Tokyo), and Prof. Yuji Kohara (National Institute of Genetics) groups. This work was mainly performed by T. Tanahashi.

We developed a system to construct phylogenetic trees efficiently with whole genome shotgun sequence data in public databases before their assembly. We collected homologs of approximately 700 *Arabidopsis thaliana* genes involved in development, and their phylogenetic analyses are in progress.

3-5 functional characterization of polycomb genes in the moss *Physcomitrella patens*

Polycomb group (PcG) proteins regulate chromatin modification and function as a cellular memory system to maintain the repressed state of developmental genes in both animals and plants. PcG genes are involved in phase changes of *Arabidopsis thaliana* development, such as vegetative to reproductive and haploid to diploid transitions. Bryophytes have dominant haploid generation, while sporophyte generation is dominant in angiosperms. The change of dominant generations was one of most conspicuous evolutionary aspects of land plants. To elucidate the molecular mechanisms underlying the evolution in alteration of generations, we characterized functions of PcG genes in *P. patens*. *A. thaliana CLF*, *MSI1*, *EMF2,* and *FIE* homologs were cloned in *P. patens*. We inserted a GUS reporter gene at the end of every one of the PcG genes to investigate the expression patterns. Disruptants for each gene were established and their characterization is in progress. This work was mainly performed by N. Aono.

IV. Molecular mechanisms of speciation

Sexual isolation is an important step for speciation, although the molecular mechanisms governing the isolation in plants are mostly unknown. A proper pollen tube guidance is essential for reproduction in angiosperms, and sexual isolation is often related to the arrest of guidance. In spite of the long history of studies on the pollen tube guidance, few guidance factors have been reported because of the difficulty of genetic analyses. We focused on receptor like kinases (RLKs), which function to receive extra cellular ligands and transmit the signal into a cell. We postulated that RLKs involved in pollen tube guidance are likely expressed more abundantly in pollen and/or pollen tube than in other tissue. Gene expression profiles between *A. thaliana* pollen and pollen tube were compared with those of other tissue using microarray. Pollen and Pollen tube expression profiles were similar to each other and 95 % of expressed genes were overlapped within the 4 fold differences. We focused on 45 RLKs predominantly expressed in pollen or germinating pollen to characterize signaling mechanisms during fertilization. Characterization of single and double T-DNA insertion lines are in progress. This work was mainly done by S. Miyazaki.

Publication List:

Original papers

Machida, M., Takechi, K., Sato, H., Chung, S.J., Kuroiwa, H., Takio, S., Seki, M., Shinozaki, K., Fujita, T., Hasebe, M., and Takano, H. (2006). Genes for the peptidoglycan synthesis pathway are essential for chloroplast division in moss. Proc. Natl. Acad. Sci. USA *103*: 6753-6758.

Shigyo, M., Shindo, S., Hasebe, M., and Ito, M. (2006). Phylogenetic analysis of AP2 domain-containing genes. Gene *366*: 256-265.