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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 networks and their functions of different organisms, we hope to infer the genetic changes that caused the evolution of cellular and developmental processes.

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 based on molecular mechanisms 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. Based on live imaging of microtubules, we have hypothesized that the formation of oblique microtubules, which elongate to the outside of a phragmoplast, drives the centrifugal expansion. We examined the role of γ-tubulin in phragmoplast expansion, because γ-tubulin binds onto existing microtubules and nucleates a new microtubule as a 40 degrees branch in interphase cells. We isolated phragmoplasts from synchronized tobacco BY-2 cells and labeled with anti-γ-tubulin antibody. γ-tubulin was detected at the branched points of microtubules in isolated phragmoplasts (Figure 1). We proposed a hypothesis that cytosolic γ-tubulin complexes are recruited onto existing phragmoplast microtubules and nucleate new microtubules as branches, and the branched microtubules drive phragmoplast expansion. Inhibition of γ-tubulin function during phragmoplast expansion is in progress. T. Murata mainly performed this study.

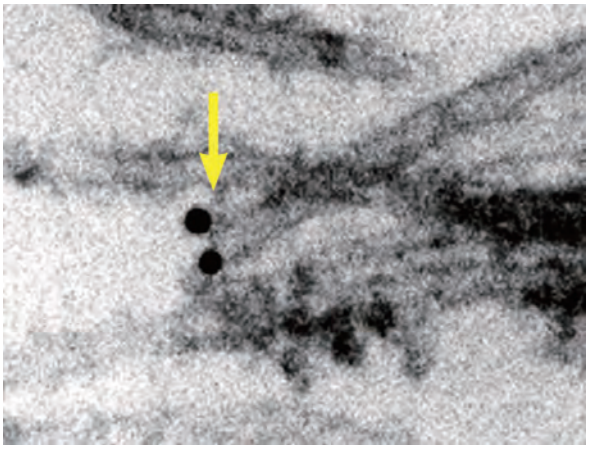

Figure 1. Phragmoplast development in flowering plants. γ-tubulin detected at a microtubule end in an isolated phragmoplast by electron microscopy (yellow arrow).

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 phragmoplast, suggesting that these ubiquitin-like genes likely regulate the stability of phragmoplast microtubules. Further analyses are in progress.

Formation of several types of stem cells to produce different types of differentiated cells is properly regulated during the development of multicellular organisms. However, molecular mechanisms for the stem cell characterization have been largely unknown. We showed that *AINTEGUMENTA/PLETHORA/BABY BOOM* (*APB*) orthologs *PpAPB*s (*PpAPB1*, *2*, *3*, and *4*) are involved in the stem cell characterization in the moss Physcomitrella patens. Gametophore stem cells were induced by exogenous cytokinin in the wild type, while the quadruple disruptants did not form any gametophore stem cells with exogenous cytokinin application. These results suggest that the *PpAPB*s play a critical role in the characterization of a gametophore stem cell. Meanwhile, the expression of *PpAPB*s is regulated by auxin, not cytokinin. This study was mainly performed by Tsuyoshi Aoyama and Yuji Hiwatashi.

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. 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 Evolution of phytohormone regulation

Phytohormones are important regulators for plant development and we investigated their evolution, focusing on polar auxin transport (Fujita et al. in press), cytokinin synthesis (Sakakibara et al. submitted), and gibberellic acid (GA) signal transduction (Hirano et al. 2007).

The shoot is a repeated structure made up of stems and leaves and is the basic body plan in land plants. Vascular plants form a shoot in the diploid generation, whereas non-vascular plants such as mosses form a shoot in the haploid generation. It is not clear whether all land plants use similar molecular mechanisms in shoot development or how the genetic networks for shoot development evolved. The control of auxin distribution, especially by polar auxin transport, is essential for shoot development in flowering plants. We did not detect polar auxin transport in the gametophytic shoots of several mosses, but did detect it in the sporophytes of mosses without shoot structure. Treatment with auxin transport inhibitors resulted in abnormal embryo development, as in flowering plants, but did not cause any morphological changes in the haploid shoots. We fused the soybean auxin-inducible promoter *GH3* with a GUS reporter gene and used it to indirectly detect auxin distribution in the moss *Physcomitrella patens*. An auxin transport inhibitor NPA did not cause any changes in the putative distribution of auxin in the haploid shoot. These results indicate that polar auxin transport is not involved in haploid shoot development in mosses and that shoots in vascular plants and mosses are most likely regulated differently during development (Fujita et al. in press).

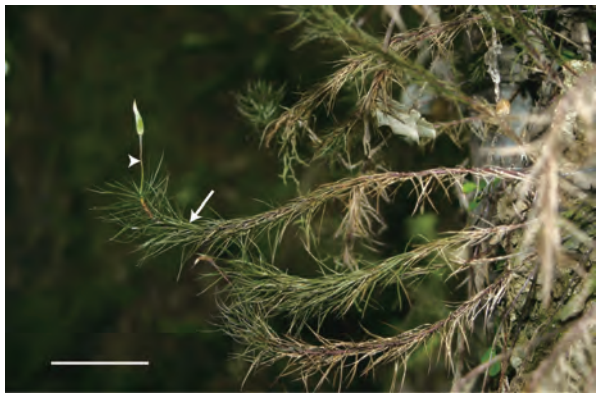

Figure 2. Basipetal polar auxin transport was not detected in a gametophytic shoot (arrow) but in a sporophyte axis (arrow head) of *Dawsonia superba*, which is the biggest moss in the world. Scale bar = 3 cm.

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 international consortium.

We compared the features of *P. patens* genome to those of flowering plants, from which it is separated by more than 400 million years, and unicellular aquatic algae. This reveals genomic changes concomitant with the evolutionary movement to land, including a general increase in gene family complexity, loss of genes associated with aquatic environments, acquisition of genes for tolerating terrestrial stresses, and the development of the auxin and abscisic acid signaling pathways for coordinating multicellular growth and dehydration response. The *P. patens* genome provides a resource for phylogenetic inferences about gene function and for experimental analysis of plant processes through this plant's unique facility for reverse genetics (Rensing et al. in press).

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, (4) 5'-end serial analysis of gene expression (5' SAGE), and (5) a collection of 3' UTR and small RNA sequences as collaborative works with groups associated 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).

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.

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〕

- Hirano, K., Nakajima, M., Asano, K., Nishiyama, T., Sakakibara, H., Kojima, M., Katoh, E., Xiang, H., Tanahashi, T., Hasebe, M., Banks, J. A., Ashikari, M., Kitano, H., Ueguchi-Tanaka, M., and Matsuoka, M. (2007). The GID1-mediated GA perception mechanism is conserved in the lycophyte *Selaginella moellendorffii* but not in the bryophyte *Physcomitrella patens*. Plant Cell *19*, 3058-3079.
- Odahara, M., Inouye, T., Fujita, T., Hasebe, M., and Sekine, Y. (2007). Involvement of mitochondrial-targeted RecA in the repair of mitochondrial DNA in the moss, *Physcomitrella patens*. Genes Genet. Syst. *82*, 43-51.
- Tsuji, S., Ueda, K., Nishiyama, T., Hasebe, M., Yoshikawa, S., Konagaya, A., Nishiuchi, T., and Yamaguchi, K. (2007). The chloroplast genome from a lycophyte (microphyllophyte), *Selaginella uncinata*, has a unique inversion, transpositions and many gene losses. J. Plant Res. *120*, 281-290.

〔Review articles〕

- Murata, T., and Hasebe, M. (2007). Microtubule-dependent microtubule nucleation in plant cells. J. Plant Res. *120*, 73-78.
- Murata, T., Tanahashi, T., Nishiyama, T., Yamaguchi, K., and Hasebe, M. (2007). How do plants organize microtubules without a centrosome? J. Integr. Pl. Biol. *49*, 1455-1463.