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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. Origin of the plant cell

The first green alga cell evolved via symbiosis between an ancestral non-photosynthetic eukaryote and a cyanobacterium. Cyanobacteria now exist as chloroplasts in the host cell. The factors and mechanisms of chloroplast movement are being investigated to reveal the molecular mechanisms used to "domesticate" cyanobacteria as organelles. Analyses of (1) cytosolic calcium ion concentration and cytoskeleton organization during chloroplast movement in the moss *Physcomitrella patens* and (2) the functional divergence of photoreceptors and motor proteins involved in chloroplast movement between the moss and angiosperms are in progress by a team directed by Y. Sato.

II. 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* is in progress by a team directed by T. Fujita. 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. Thus far we identified 58 cDNAs, whose overexpression caused the defects in asymmetric cell divisions in two repeated experiments. Overexpression of the genes in protoplasts with GFP-tubulin or GFP-talin, expression analyses of each gene-cytrin fusion protein under its native promoter, loss of function experiments using RNAi are now in progress to characterize what processes these genes are involved in. Functional analyses of these genes should help us to understand the molecular mechanisms of how plants generate distinct cell lineages to build their multicellular bodies.

III. 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, which are regulated by cortical microtubules, determine the morphology of differentiated tissues and organs.

3-1 Microtubule-dependent microtubule nucleation

Almost all microtubule arrays are organized by one or more microtubule organizing centers, such as centrosomes, that regulate nucleation spatially and temporally. It has long been puzzling how, despite the absence of conspicuous organizing centers, higher plant cells form well-organized cortical microtubule arrays, which are essential for cell morphogenesis. A recent report suggests that microtubule nucleation sites for the array are capable of associating with and dissociating from the cortex. We showed that nucleation requires extant cortical microtubules, onto which cytosolic

γ-tubulin is recruited. Microtubule-independent nucleation is rarely observed in living tobacco BY-2 cells and nucleation is minimal in the absence of original microtubules in a cell-free system. In both living cells and the cell-free system, microtubules are nucleated as branches on the extant cortical microtubules. The branch points contain γ-tubulin, which is abundant in the cytoplasm, and microtubule nucleation in the cell free system is prevented by inhibiting γ-tubulin function with specific antibodies. When isolated plasma membrane with microtubules is exposed to purified neuro-tubulin, no microtubules are nucleated, but when the membrane is exposed to a cytosolic extract, γ-tubulin binds microtubules on the membrane, and after a subsequent incubation in neuro-tubulin, microtubules are nucleated on the pre-existing microtubules. We propose that a cytoplasmic γ-tubulin complex shuttles between cytoplasm and the side of a cortical microtubule and has nucleation activity only when bound to the microtubule (Murata *et al*. 2005). T. Murata mainly directed this study.
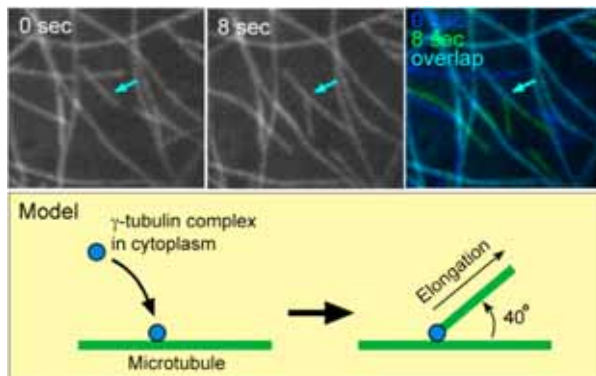

Figure 1. Microtubules in plant cortical arrays nucleate on existing microtubules as branches. Top panels show microtubule nucleation visualized by GFP-alpha-tubulin in living leaf epidermal cells of *Arabidopsis thaliana*. An arrow in each panel indicates a microtubule nucleation site. Images at 0 (left) and 8 (center) seconds, and merged image (right) showing changes of microtubules in 8 seconds are shown. Bottom panel shows proposed model of microtubule nucleation on existing microtubules.

IV. Evolution of molecular mechanisms in the plant development

4-1 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 question in 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- and ubiquitin-like proteins,

and an unknown protein. Functional analyses of these genes are currently under investigation by a team directed by Y. Hiwatashi. The disruption of the kinesin-like gene did not show any phenotypic differences from the wild type. This is likely caused by the functional redundancy of closely related genes, and the analyses of double disruptions are in progress. Disruption of the gene encoding ubiquitin-like protein suggests that the gene is involved in cell division and elongation through microtubule organization with the proteasome complex.

4-2 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 probably originated 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 pollen, male gametophyte, are analyzed by a team directed by N. Aono.

4-3 Origin of floral homeotic MADS-box genes

The MADS-box genes of land plants are extensively diverged to form a superfamily and are important in various aspects of development including the specification of floral organs as homeotic selector genes. The closest relatives of land plants are the freshwater green algae charophyceans. To study the origin and evolution of land plant MADS-box genes, we characterized these genes in three charophycean green algae: the stonewort *Chara globularis* (*CgMADS1*), the coleochaete *Coleochaete scutata* (*CsMADS1*), and the desmid *Closterium peracerosum-strigosum-littorale* complex (*CpMADS1*). Phylogenetic analyses suggested that MADS-box genes diverged extensively in the land plant lineage after the separation of charophyceans from land plants. The stonewort *CgMADS1* mRNA was specifically detected in the oogonium and antheridium together with the egg and spermatozoid during their differentiation. The expression of *CpMADS1* increased when vegetative cells began to differentiate into gametangial cells, and decreased upon fertilization. These expression patterns suggest that the precursors of land plant MADS-box genes originally functioned in haploid reproductive cell differentiation, and that the haploid MADS-box genes were recruited into a diploid generation during the evolution of land plants (Tanabe *et al*. 2005).

4-4 Ancestral function of the floral regulator *FLO/LFY*

After fertilization, the zygote undergoes dynamic changes in the chromosomal and cytoplasmic organizations and begins the cell cycles that eventually lead to formation of multicellular embryo. Specific transcription factors that initiate this cascade of events in land plants have not been identified. We have identified two *FLO/LFY* genes, *PpLFY1* and *PpLFY2*, that regulate

the first cell division after formation of the zygote in the moss *Physcomitrella patens*. The deduced amino acid sequences of the two *PpLFY* genes are 94.8% identical to each other and show similar expression patterns. While fertilization occurred in the *PpLFY* disruptants, the development of double disruptant zygotes was arrested at the single-cell stage. When the double disruptants, as female parent, were crossed with the wild type, as male parent, normal sporophytes were formed, supporting the notion that the *PpLFY* genes function after fertilization to regulate the first mitotic cell division in zygotes. The rare sporophytes that formed on the *PpLFY* double disruptants showed mostly normal organogenesis, but had abnormalities in the pattern of cell division, supporting a role of *PpLFY* genes in regulating cell division. The *FLO/LFY* genes in angiosperms are conserved master regulators of floral identity without any obvious effects on cell division. In contrast, our study suggests that *FLO/LFY* genes have functions throughout sporophyte development in the basal land plant lineages (Tanahashi *et al*. 2005).
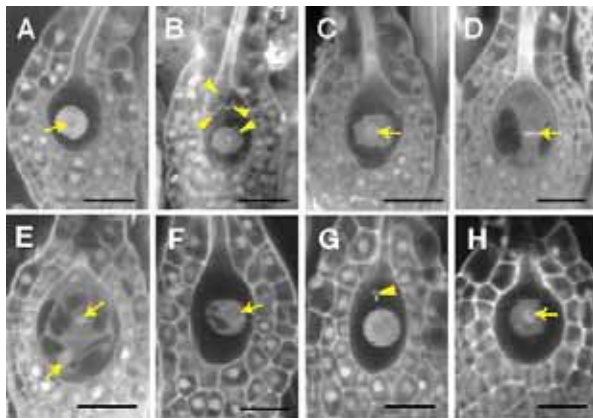

Figure 2. Development of the egg cell, zygote, and embryo. Ventral parts of archegonia in the wild type (A-E) and the *PpLFY* double disruptants (F-H) were observed by confocal laser scanning microscopy. Nuclei and sperm are indicated with arrows and arrowheads, respectively. (A, B, F, G) An egg cell before (A, F) or after (B, G) sperm invasion. (C, H) An unexpanded zygote. (D) An expanded zygote at the first cell division. (E) A two-cell embryo. An expanded zygote and multicellular embryos were hardly observed in the double disruptants. Bars = 25 μm.

4-5 Evolution of the floral regulator FLO/LFY in land plants

The plant-specific transcription factor FLO/LFY controls general aspects of the life cycle in a basal plant, the moss *Physcomitrella patens*. In contrast, FLO/LFY has more specialized functions in angiosperms, where it specifically induces floral fate during the reproductive phase. This raises the question of a concomitant change in the biochemical function of FLO/LFY during the evolution of land plants. We report that the DNA binding domain of FLO/LFY, although largely conserved, has diverged in activity. On the contrary, other, more rapidly evolving portions of the protein have few effects on FLO/LFY activity (Maizel *et al*. 2005).

VI. Molecular mechanisms of speciation

Reproductive isolation is the first step in speciation. To obtain insights into reproductive isolation, several receptors specifically expressed in the pollen tube are being studied to screen for the receptors involved in pollen tube guidance by a team directed by S. Miyazaki.

Polyploidization is a major mode of speciation in plants, although the changes that occur after genome duplication are not well known. Polyploid species are usually larger than diploids, but the mechanisms responsible for the size difference are unknown. To investigate these mechanisms, tetraploid Arabidopsis was established and its gene expression patterns are being compared to those of diploid wild-type plants using microarrays.

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Original papers

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