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TEACHING TOOLS IN PLANT BIOLOGY: LECTURE NOTES

Leaf Development 2: Cell Proliferation and Differentiation

The commitment of a group of cells to form a leaf primordium initiates a sequence of events that ultimately leads to the development of a mature, functional leaf. These events include the establishment of the major axes of the leaf, cell division and expansion programs in the leaf blade, and patterning and differentiation of cells including those that make up the vascular tissues and specialized epidermal cells, such as the trichomes and guard cells. Throughout the development of the leaf, the temporal and spatial control of cell proliferation and cell differentiation is tightly controlled through positional information involving auxin and microRNAs (miRNAs) and expression of regulatory transcription factors. Most of our understanding of the molecular underpinnings of leaf development is based on studies in *Arabidopsis thaliana*, rice (*Oryza sativa*), and maize (*Zea mays*) and a few other model organisms, such as *Antirrhinum majus*, tomato (*Solanum lycopersicum*), and pea (*Pisum sativum*). Comparative genomics analyses have allowed the models developed based on these angiosperms to be investigated in other plants as well, to uncover the evolutionary origins of the developmental pathways.

LEAF EVOLUTION AND DIVERSITY

Leaves evolved from modified branches. Branches and shoots are indeterminate; they are capable of ongoing growth. By contrast, (most) leaves are determinate; they grow to a genetically predetermined size and shape. Leaf development requires a suppression of indeterminate growth, but in some cases this suppression is incomplete. Leaves have evolved multiple times independently. Nonseed vascular plants, such as *Lycopodium* and *Selaginella*, make small leaves with a single vascular strand; these leaves are sometimes called microphylls. The larger leaves in ferns and seed plants evolved independently and are often called megaphylls.

WHAT DETERMINES LEAF SIZE AND SHAPE?

Leaf primordia do not differ greatly in shape and size, but their mature forms vary tremendously. Leaf size and shape is largely a consequence of differential patterns of cell division and cell expansion. In long, thin monocot leaves, cell division and expansion primarily occur with a unidirectional orientation. By contrast, the more rounded and complex dicot leaf shapes are formed by more complex patterns of cell proliferation. In most leaves, cell division stops earlier at the leaf tip than the base, in a wave of cell cycle arrest. Once cells complete their division cycles, they begin to expand and differentiate into their final forms.

Several mutations that affect cell cycle persistence have been identified. The *AINTEGUMENTA* (*ANT*) gene, which encodes an AP2-type transcription factor, is positively correlated with cell proliferation. The loss-of-function *ant* mutant has smaller but morphologically normal leaves with fewer cells. When overexpressed, *ANT* causes enlarged but morphologically normal-looking leaves. ANT is thought to maintain meristematic competence of cells, in part through its regulation of the cyclin *CYCD3* gene, which is a key driver of the cell cycle.

Other mutations have been identified that affect cell division unequally across the leaf blade. For example, mutation of the *CINCINNATA* (*CIN*) gene in *Antirrhinum* causes prolonged cell cycling in the margins but not the center of the leaf. Loss-of-function *cin* leaves have too much margin, or a negative curvature, and so buckle and bulge. *CIN* encodes a TCP-type transcription factor, named for TEOSINTE BRANCHED1 from maize, CYCLOIDEA from *Antirrhinum*, and PROLIFERATING CELL FACTOR from rice. Each of these proteins has an effect on cell cycle persistence. Expression of the *CIN*-like genes is regulated by miRNA expression; the miRNA miJAW is a negative regulator of *CIN-TCP* genes, and overaccumulation of miJAW in *Arabidopsis* (*jaw-D*) causes crinkly leaves, similar to the *cin* loss-of-function phenotype. These mutants demonstrate that spatial cell cycle control across the leaf contributes to final leaf shape.

Differential persistence of cell divisions similarly controls leaf margin morphology. Leaf margins can be smooth, serrated, or lobed. In serrated or lobed leaves, the protruding regions are separated by sinuses. In smooth leaves, cell divisions persist in the sinuses, causing a smooth, uniform enlargement of the leaf margin. By contrast, in lobed or serrated leaves, the cells in the sinus region arrest earlier than those in the adjoining regions. Polar auxin transport contributes to leaf margin morphology. The auxin efflux carrier PIN1 orients so that an auxin maximum is produced at the region of outgrowth, and interfering with this differential accumulation of auxin produces smooth-margined leaves. Mutations that convert lobed leaves to smooth and vice versa have revealed a role for boundary genes, such as *CUP-SHAPED COTYLEDONS2* (*CUC2*), which encodes plant-specific transcription factor. *CUC2* is part of the large *NAM*/*CUC3* multigene family, of which several members affect developmental patterns. The expression patterns of *CUC2* and related genes are modified by the action of regulatory miRNAs.

WHAT DETERMINES LEAF COMPLEXITY?

Angiosperm leaves are derived from a simple ancestral leaf. Compound leaves have evolved many times within the angiosperms and may contribute advantages to light harvesting or airflow under certain conditions. Genetic studies in compound-leaf producing plants, such as pea, tomato, and a compound-leaved close relative of *Arabidopsis*, *Cardamine hirsuta*, indicate that the same signaling pathways that operate in the development of simple leaves underlie the development of these more complex leaves.

Like the outgrowth of leaf lobes or serrations, leaflet initiation is preceded by a local auxin maximum, and interfering with its formation produces simple leaves. Thus, auxin plays a conserved role in leaf development, from the specification of the site of primordium outgrowth in the shoot apical meristem to the specification of outgrowths of leaflets and lobes.

Similarly, class I *KNOX* (*KNOTTED*-like homeobox) genes have a recurring role in leaf and leaflet initiation in most taxa. These *KNOX-1* genes encode transcription factors, many of which promote indeterminacy. They are expressed in the shoot apical meristem, which is an indeterminate structure. In most but not all plants, *KNOX-1* genes are inactivated in the cells destined to form a leaf primordium. In plants with compound leaves, *KNOX-1* gene expression resumes in the developing leaves. (Interestingly, however, some plants whose leaves express *KNOX-1* nevertheless produce simple leaves because secondary morphogenesis causes the leaf blade to grow out). Usually, *KNOX-1* expression is strongly correlated with leaf complexity; *Arabidopsis* plants that overexpress *KNOX-1* genes can produce lobed or compound-like leaves, due to increased proliferation of cells at the leaf margins, whereas *KNOTTED* expression in tomato stimulates ultracompound leaves, and loss of *KNOX-1* function in plants with compound leaves can cause them to produce simple leaves.

Pea plants have compound leaves and a rich genetic history. Many pea mutants with abnormal patterns of leaf development have been identified. In pea, the *unifoliata* mutant produces simple leaves. *UNIFOLIATA* encodes a transcription factor, but not a *KNOX-1* type. Instead, in pea and other closely related legumes, a protein of the FLO/LFY family specifies compound leaf development. This finding is consistent with the numerous evolutionary origins of compound leaves. Interestingly, the subsequent events in pea leaf development are the same as those of other compound-leaved plants.

Additional studies suggest that KNOX-1 transcription factors require a window of morphgenetic potential in which to act. In tomato, the *LANCEOLATE* (*LA*) gene controls this potential. Overexpression of the *LA* gene converts the normally compound tomato leaf into a smooth, simple one, by suppressing outgrowth of the leaflet progenitor cells, even in plants that are overexpressing *KNOTTED*.

Boundary genes, such as *CUC2* and *CUC3*, are required to suppress cell divisions at the boundary between an initiating leaf primordium and the shoot apical meristem. CUC2 expression is also required for proper sinus formation in serrated or lobed leaves. Similarly, boundary genes are required for the formation of leaflets in compound leaves; suppression of their expression prevents the leaves from achieving their proper dissected form. Thus, boundary genes have a recurring role in leaf development.

Taken together, these studies reveal that leaflet initiation parallels leaf initiation, with *KNOX-1* or *UNI* expression promoting indeterminacy, a local auxin maximum specifying the position of leaf or leaflet outgrowth, and boundary genes restricting cell proliferation as appropriate.

These genes and pathways have been identified in angiosperms, whose leaves are derived from a single evolutionary event. Improved techniques for examining gene function and the completion of several genome sequencing projects in nonangiosperms are allowing scientists to investigate developmental pathways in these plants. Ongoing studies are revealing that some of the same genes are involved in leaf development, although in some cases their precise function is not identical. These kinds of comparative studies help us to understand the genetic resources available to the plant common ancestor and how these resources were modified over time to produce diverse plant forms.

WHAT CONTROLS CELL DIFFERENTIATION?

Patterns of cell division underlie the development of leaf morphology, but the development of a functional leaf also depends on the differentiation of proper cell types. Leaves consist of mesophyll cells, which carry out the photosynthetic reactions, vascular tissues, and epidermal tissues, including specialized epidermal hairs (trichomes) and guard cells. Genetic studies primarily in *Arabidopsis* have uncovered some of the developmental pathways that contribute the proper cell differentiation during leaf development. These include the activation of transcription factors and cell-specific genes and positional information conferred by auxin or direct cell-to-cell communication.

VASCULAR TISSUES

Vascular tissues are the conduits through which water and photosynthate move through the plant body. Leaves are extensively vascularized, so that the photosynthetic activities of the mesophyll cells are well supported with water from the xylem, and the photosynthate is readily moved into the phloem. Vascular tissue differentiation during leaf expansion follows a consistent developmental program, with cells of the preprocambium differentiating into procambium cells, which then differentiate into the cells of the xylem and phloem.

The plant hormone auxin plays an important role in specifying the pattern of vascular tissue formation, as it does in specifying the placement of leaf primordia. During leaf initiation, a local auxin maximum, formed by the polar placement of the PIN1 auxin efflux carrier, precedes primordium formation. Once the primordium is initiated, PIN1 redistributes, directing auxin flow basipetally. The locally elevated auxin flow in the center of the leaf primordium causes these cells to differentiate into preprocambium cells and then into procambium cells. These processes can be monitored by expression of cell-specific genes. Preprocambium cells look like the cells that surround them (roughly cube-shaped) but initiate expression of the HD-ZIPIII transcription factor–encoding gene *Athb8*. These cells subsequently elongate and divide to form long narrow cells and begin to express a procambium-specific marker ET1335. From the procambium, xylem and phloem cells are produced, with the xylem forming on the adaxial side of the procambium and the phloem on the abaxial.

Following the specification of the midvein, the direction of auxin transport in the primordium epidermal layer changes, causing auxin maxima to form midway along the margins of the primordium. From these new auxin maxima, the flow of auxin again shifts to an inward direction, ultimately giving rise to the secondary veins.

Monocot leaves have veins that are largely parallel, running along the long axis of the leaf. Cell divisions occur primarily in a zone at the base of the leaf, and cells enlarge and differentiate as they mature, resulting in a gradient of leaf and vein differentiation from tip to base. Mathematical and computer models have shown that parallel veins can be generated by a simple polar auxin transport mechanism. Interfering with auxin transport interferes with vein patterning, suggesting that vascular patterning in monocots is probably specified by auxin transport patterns as it is in dicots.

TRICHOMES

Trichomes are shoot epidermal hairs. On leaves, trichomes can reduce transpirational water loss or form a reflective surface to reduce heat or UV light absorption. Trichomes on seeds can aid in their dispersal; cotton seed trichomes are the most important natural fiber for textile production. Glandular trichomes produce diverse compounds that contribute to defense against herbivory or pathogens. Many products of glandular trichomes are useful chemicals for humans too, including fragrances and flavors (e.g., lavender and mint), medicinal compounds (e.g., artemisinin, an antimalarial), and natural insecticides. Modifying gene expression levels in trichomes can increase production of these compounds, facilitating their purification or making the plants themselves more resistant to insects or pathogens.

In *Arabidopsis*, trichomes are dispensable, facilitating genetic screens for trichome abnormalities. The mechanisms by which trichome spacing are controlled in *Arabidopsis* were identified through mutational analysis and the identification of plants with too few, too many, or abnormally clumped trichomes. In cells destined to produce trichomes, transcriptional complexes form and induce expression of GL2, a homeodomain transcription factor that causes cells to differentiate into trichomes. Formation of these transcriptional complexes in adjoining, nontrichome producing cells is inhibited by one or more trichome inhibitors that are produced in the trichome cell and move intercellularly to repress trichome formation in adjoining cells. In *Arabidopsis*, some trichome spacing mutations also affect spacing of epidermal root hairs, which use a similar patterning mechanism. However, it has become clear that most plants use other genetic pathways for trichome production and that the mechanism used by *Arabidopsis* is a recent innovation, derived from an anthocyanin biosynthetic pathway. Even within a single plant, different types of trichomes are produced via distinct pathways, indicating that trichomes are a diverse set of analogous but not homologous differentiated cell types.

STOMATA

A stomatal pore is a hole in the leaf epidermis that is covered by a pair of guard cells that change size to open or close the pore. When the *Arabidopsis* leaf is $\sim$200 μm in length, stomata begin to appear at the distal end, and stomatal maturation continues in a wave of differentiation from tip to base. Stomatal development is regulated by environmental parameters, cell intrinsic information, and signals emanating from nearby cells, including other guard cells or guard cell precursors and cells in the mesophyll.

Mature guard cells are formed through a regular pattern of cell divisions that differ in monocots and dicots. In monocots such as *Z. mays*, guard cells form in cell files in between veins. The first step is an asymmetric division that produces a guard mother cell that ultimately divides again to produce the two guard cells. However, the stomatal complex also includes a pair of subsidiary cells that are formed from cells adjacent to the guard mother cell. How these cells are induced to divide and differentiate is not fully known, but a recently identified mutant that interferes with this process, *pan1*, encodes a putative receptor that could be involved in perceiving a signal from the guard mother cell.

In dicots such as *Arabidopsis*, the first stage of guard cell development is also an asymmetric division occurring in a protodermal cell. The two daughter cells of the asymmetric division have different cell states; the larger retains its protodermal identity, while the smaller cell becomes a meristemoid mother cell (MMC). The MMC divides asymmetrically one or more times to produce a meristemoid. The meristemoid differentiates into a guard mother cell (GMC), which divides symmetrically to produce a pair of guard cells precursors, which differentiate into guard cells.

Mutational analysis in *Arabidopsis* has identified several basic helix-loop-helix transcription factors that are key regulators of each step in guard cell differentiation. SPEECHLESS (SPCH) is required for asymmetric division of the protodermal cell into the meristemoid mother cell; as the name implies, mutant plants that do not produce SPCH do not produce stomata (they don't make mouths, so they are speechless). The *MUTE* gene is required for the next step, which is the differentiation of the MMC into a GMC; loss of function of *MUTE* leads to the production of stomatal precursors but no stomata. The GMC divides once more and then differentiates into a pair of guard cells; these are separable processes, as shown by the phenotype of the *fama* mutant, in which the GMC divides repeatedly but the daughter cells do not differentiate, producing a row of parallel cells.

In dicots such as *Arabidopsis*, stomata normally form with one or more cells between them. This patterning is based on a system of cell signaling that involves a repression of guard cell differentiation. The current model suggests that one or more peptide signals are secreted by stomatal precursors, which are perceived by adjoining cells and serve to repress their differentiation via a mitogen-activated protein kinase signaling pathway. Guard cell density is also affected by environmental conditions, with lower stomatal densities in conditions of low humidity or high concentrations of CO_2. Environmental signals are perceived by mature leaves, which transduce information to the developing leaves via an unknown mechanism. The *hic* mutant affects environmental stomatal density responses. HIC is thought to contribute to the synthesis of the epicuticular wax that coats leaves and prevents gas and water exchange, but it's not yet known precisely how this gene contributes to stomatal patterning control.

CONCLUSIONS

The outgrowth of a leaf primordium from a tiny bulge of homogenous cells into a large, complex, and physiologically functional organ involves the sequential and coordinated

activities of numerous regulatory genes. Transcriptional regulators that control cell determinacy, division, and differentiation across the leaf blade are themselves positionally regulated by both auxin and small RNAs. Within the expanding leaf, vascular tissues form, while in the epidermis, trichomes and guard cells are formed through regular programs of differentiation and division; analysis of the patterning of these cells reveals a role for cell-to-cell communication as well as environmental factors. The sophisticated developmental programs that have evolved that enable plants to produce leaves are matched only by the amazing photosynthetic processes that occur within them.

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