Meeting Report

The Development of Plant Leaves

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Most leaves appear simple at first sight and they consist of only a few cell types. Despite that, many developmental processes are involved in leaf ontogeny, including positioning and initiation of leaf primordia, specification of leaf identity, establishment of dorsiventrality, the control of cell division and expansion, and pattern formation. This report highlights and reviews some of what we know about the genetic circuitry that underlies leaf form, as discussed at a workshop on "Leaf Development" held February 11 through 13, 2002, at the Instituto Juan March (Madrid).

LEAF INITIATION

Shoot meristems produce leaves on their flanks in regular patterns called phyllotaxy. As leaves are initiated, meristem cells divide and replace the cells that have just been committed to initiating a leaf primordium. Thus, the meristem balances self-renewal with organ initiation. The regular pattern of leaf initiation allows one to predict where the next leaf will appear. Leaves that have just appeared as bumps from the meristem are in Plastochron 1 and are referred to as P_1 leaves. The cells in the meristem that will become the next leaf are designated P_0. The P_0 cells, although still part of the meristem, soon become radically different from adjacent cells. They divide at higher rates, their growth axis changes from isodiametric to axial, they loose their indeterminate nature, and they gain leaf cell identity. Even more amazing, the boundary that establishes the P_0 cells from the meristem is continually being remade and reinterpreted with every initiating leaf.

The identification of genes that are expressed in the P_0 cells or are specifically excluded from these cells has been useful in understanding meristem function. In many plant species such as maize (*Zea mays*) and Arabidopsis, *knox* (*knotted1-like homeobox*) genes are expressed in shoot meristems but not in P_0 cells. Recessive, loss-of-function mutations in these genes impair the generation of organs from the shoot apical meristem (SAM), whereas their overexpression leads to the production of meristems on leaves (Lincoln et al., 1994; Long et al., 1996).

Miltos Tsiantis (Oxford) has explored the relationship of the gibberellin pathway and *KNOX* genes such as *SHOOTMERISTEMLESS* (*STM*), which is required for meristem maintenance (Long et al., 1996). Gibberellic Acid (GA) is a diterpenoid plant growth hormone that is important for germination, stem elongation, and flowering time. Previous studies have shown that *KNOX* gain-of-function phenotypes in tobacco (*Nicotiana tabacum*) could be suppressed by the addition of GA. They also demonstrated a direct link between NTH15, the tobacco KN1 ortholog, and the promoter of *Ntc12*, a gene encoding a GA 20-oxidase required for GA biosynthesis (Sakamoto et al., 2001). Tsiantis and colleagues demonstrated the negative regulation of the GA-oxidase gene, *GA5*, by *KNOX* genes in Arabidopsis. He showed that the leaves of the *asymmetric leaves1-1* (*as1-1*) Arabidopsis mutant, which are lobed and misexpress *KNOX* genes, become more severe and gain meristematic activity when grown on media supplemented with paclobutrazol, an inhibitor of GA biosynthesis. In addition, the phenotype of *stm-2* is enhanced by the *spindly-5* (*spy-5*) mutation, which increases GA signaling (Jacobsen and Olszewski, 1993). Furthermore, the expression of a *GA5::β-glucuronidase* transgene is excluded from the wild-type SAM, but is found in the region where a meristem would have been in *stm* mutants. Taken together, these results indicate that one of the roles of the *STM* gene is to exclude *GA5* expression. They also lead to the interesting hypothesis that *KNOX* genes negatively regulate GA in the meristem, thus keeping cells from expanding and differentiating. Cells that do not express *KNOX* genes, as found in P_0 and later leaf stages, have increased GA biosynthesis, resulting in increased cell expansion and differentiation.

It has been proposed that differential regulation of *KNOX* genes may be involved in the generation of dissected leaf morphology (Hareven et al., 1996; Janssen et al., 1998). Tsiantis also reported that the dominant *Mouse ear* mutation of tomato (*Lycopersicon esculentum*) increases leaf dissection, a phenotype that is suppressed by the constitutive GA response *procera* mutation, suggesting again that GA acts antagonistically to *KNOX* genes in simple (Arabidopsis) and compound (tomato) leaves.

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LEAF SHAPE

Maize leaves are characterized by clear proximal/distal domains as seen in the morphological differences of the sheath and blade. Not as obvious to the eye are the lateral domains. Mike Scanlon (University of Georgia, Athens) discussed establishment of lateral domains in the maize leaf and the role of *narrow sheath* genes, *ns1* and *ns2*, in the process (Scanlon et al., 1996). Deletion of a lateral domain characterizes the narrow sheath phenotype. A single copy of the wild-type allele of either *ns* gene is sufficient for normal leaf development (Scanlon et al., 1996; Scanlon, 2000). Clonal analysis has demonstrated that NS1 is required at two discrete foci in the meristem to act in a noncell autonomous way directing recruitment of marginal, leaf founder cells (Scanlon, 2000). NS1 is not required for development of the central domain of the leaf. With the aim of identifying the NS recruitment signal, *ns2* was cloned by transposon tagging and was found to encode a protein with similarity to nitrilases, enzymes that are known to participate in the biosynthesis of auxin in plant shoots. This finding, together with the narrow sheath-like phenotype of tobacco and petunia (*Petunia hybrida*) transgenic plants overexpressing iodoacetamide biosynthetic genes, suggests a role for auxin synthesis and/or transport in the regulation of lateral growth (Berleth and Sachs, 2001; Reinhardt et al., 2000). In the model proposed by Scanlon (2000), iodoacetamide initiates founder cell recruitment of the central domain, and the NS signal completes recruitment of founder cells in the NS domain.

One of the properties of a leaf is that it is a bifacial organ that has dorsiventral asymmetry as soon as it emerges from the flanks of the SAM. This asymmetry allows plants to capitalize on the distribution of specialized cell types such as trichomes, photosynthetic cells, and stomata. Beginning with the analysis of the *phantastica* (*phan*) mutant in *Antirrhinum majus* by Waites and Hudson (1995), where the leaves are partially abaxialized, a number of mutations affecting other genes have been found that produce leaves with different degrees of perturbation in dorsiventrality.

The *PHAN* gene of *A. majus* encodes a MYB transcription factor, the mRNA of which is expressed throughout lateral organ anlagen and primordia (Waites et al., 1998). Its activity is partially redundant with that of *Handlebars* (*Hb*), and the *phan hb* double mutant displays abaxialized, radialized cotyledons and leaves (Waites and Hudson, 2001). In Arabidopsis, semidominant *phabulosa* (*phb*) and *phavoluta* (*phv*) mutations adaxialize lateral organs, which are filamentous and radially symmetrical in *phb* homozygous plants (McConnell and Barton, 1998; McConnell et al., 2001). On the other hand, *revoluta* (*rev*) mutants lack axillary meristems, a trait that can be considered as a partial loss of adaxial identity (Talbert et al., 1995). The *PHB*, *REV*, and *PHV* genes encode members of the homeodomain/Leu zipper (HD-ZIP) family that are expressed adaxially in lateral organs. *PHB* is first expressed throughout lateral organ anlagen and is later restricted to adaxial regions as the lateral organ primordia emerge (McConnell et al., 2001; Ratcliffe et al., 2000).

John Bowman (University of California, Davis) emphasized that the adaxial and abaxial sides of lateral organ primordia are, respectively, adjacent to and at a distance from the apical meristem from which they derive. Such a positional relationship was proposed by Wardlaw as early as 1949 (Wardlaw, 1949) to underlie the asymmetry of leaves, and was supported by surgical experiments performed by Sussex (1955) and Snow and Snow (1959). These classical analyses, together with recent molecular studies, indicate that the SAM produces an adaxial-promoting signal (Sussex, 1955), the perception of which is likely to be mediated through the HD-ZIP proteins PHB, PHV, and REV (McConnell and Barton, 1998; McConnell et al., 2001).

Abaxial cell fate, which is likely to be the default state in the absence of the adaxial signal produced by the SAM (Bowman et al., 2002), is promoted by the *YABBY* and *KANADI* (Kerstetter et al., 2001) genes. *YABBY* gene expression is restricted to abaxial organ domains by the activity of *PHB* and related genes. Based on the epistasis displayed by double mutants, *KANADI* genes are assumed to act upstream of the *YABBY* genes, but their precise relationships remain to be determined. Andrew Hudson (University of Edinburgh) highlighted the fact that in *A. majus*, the activity of *PHAN* is required in adaxial domains for the expression of *PHB-like* genes and the repression of *YABBY-like* genes. These observations suggest a role for *PHAN* in the promotion of organ asymmetry via PHB-like functions.

Several *KNOX* homeobox genes are known to be targets of *PHAN* regulation in *A. majus* as well as of its ortholog, *AS1*, in Arabidopsis (Byrne et al., 2000). *AS1* represses several genes, including *KNAT1* and *KNAT2*, although their loss-of-function mutations do not suppress the phenotype of *as1* mutants. This observation suggested to Hudson that *AS1* has other targets than *KNAT1* and *KNAT2*. Thus his group screened for suppressors of the *as1* mutant phenotype, and identified the *SYMMETRICA* (*SYM*) gene, which may function between the *AS1* and *KNOX* genes, as indicated by epistasis experiments.

Mary Byrne (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) spoke on the partial redundancy of *KNAT1* and *STM* in regulating stem cell function (Byrne et al., 2002). By a series of genetic studies, she was able to place *AS1* and *AS2* between different *KNOX* genes. Her results suggest that *AS1* and *AS2* are negative regulators of *KNAT1* and *KNAT2*, but are negatively regulated by *STM*. A suppressor screen performed to isolate genes redundant with *STM* allowed her to identify the *QUASIMODO* (*QUASI*) gene, which encodes a BELL1-like homeodomain protein. Triple mutant analysis suggests that *QUASI* is

required for meristem function in the absence of *STM* and *AS1*.

Hirokazu Tsukaya (National Institute for Basic Biology, Okazaki, Japan) described his analysis of *AS2* and *BLADE-LIKE PETIOLE* (*BLP*) two genes that are, in his view, involved in the positioning of the central and proximodistal leaf axes in Arabidopsis. Whereas abnormal vein patterning is displayed by leaves of *as2* mutants, which also develop leaflet-like structures (Semiarti et al., 2001), the *blp* mutant develops leaf lamina-like structures on the petioles. *AS2* and *BLP* are required in the leaf for repression of *KNAT* genes. The recent cloning of *AS2* revealed that it encodes a novel protein with Cys repeats and a Leu-zipper-like sequence. This nuclear-localized protein (Iwakawa et al., 2002) belongs to a novel family that has been named LOB, after the expression of gene family members at lateral organ boundaries (Shuai et al., 2002).

Tsukaya (1994) studied two mutations, *angustifolia* (*an*) and *rotundifolia3* (*rot3*), which, respectively, narrow or widen the leaves of Arabidopsis (Tsuge et al., 1996). His group identified the *ROT3* gene product as a CYP90 cytochrome of the P450 family, which may be involved in steroid biosynthesis (Kim et al., 1998; 1999). However, they also discovered that the *rot3-1* mutation only affects the size of leaf cells, whereas their number and size are reduced in mutants impaired in brassinosteroid biosynthesis (Nakaya et al., 2002). This group has recently cloned the *AN* gene, which encodes the first known plant member of the CtBP family (Kim et al., 2002). The *AN* gene is likely to regulate the polarity of cell growth by modulating the arrangement of cortical microtubules, which is abnormal in leaf cells of the *an* mutant. Positional cloning of *AN* has also been carried out by Martin Hülskamp's group (University of Köln). They demonstrated a role for *AN* in the regulation of microtubule organization (Folkers et al., 2002), together with its genetic and physical interaction with *ZWICHEL* (*ZWI*), which encodes a kinesin motor molecule involved in trichome branching.

Dorsiventral patterning of the maize leaf was discussed by Marja Timmermans (Cold Spring Harbor Laboratory) on the basis of her studies on two mutants: *leafbladeless1* (*lbl1*) and *Rolled1-O* (*Rld1-O*). Whereas severe *lbl1* mutants are embryo lethal and shoot meristemless, ectopic laminae arise at the boundaries of abaxial sectors on the adaxial leaf surface of weak *lbl1* mutants, suggesting a role for LBL1 in the specification of adaxial leaf identity. Although the number of founder cells recruited from the meristem and incorporated into the leaves of *lbl1* mutants is strongly reduced, the semidominant *Rld1-O* mutation inverts the dorsiventral polarity of leaves but has no apparent effect on founder cell recruitment. Timmermans proposed that these two genes negatively regulate each other or act in opposite ways on the same pathway, given that their phenotypes are mutually suppressed in double mutants. To characterize the phenotypes of *lbl1* and *Rld1-O*, Timmerman's group isolated several maize homologs of the Arabidopsis *YABBY* genes; their protein products are highly conserved in the Zn-finger and YABBY domains. As already shown in Arabidopsis, the *yabby* genes of maize are expressed throughout the incipient leaf primordium. It is interesting that later in development, their expression becomes restricted to the adaxial domain of the leaf, contrary to what happens in Arabidopsis. These results, taken together with the demonstration that *yabby* genes are downstream of *lbl1*, indicate that the *lbl1*, *rld1*, and *yabby* genes are involved in dorsiventral patterning of the monocotyledonous maize leaf, whose polarity may be inverted relative to that of the dicotyledonous Arabidopsis leaf.

QTL MAPPING AS A TOOL FOR ANALYZING LEAF DEVELOPMENT

José Luis Micol and colleagues (Universidad Miguel Hernández, Elche, Spain) analyzed variations in the architecture of vegetative leaves in a large sample of Arabidopsis accessions. Crosses between accessions that displayed extreme and opposite variations in leaf architectural traits revealed that the F_2 progeny could not be placed into discrete phenotypic classes. Because these results suggested that intraspecific variability in Arabidopsis leaf morphology arises from an accumulation of mutations at quantitative trait loci (QTL), a mapping population of recombinant inbred lines (RILs; Lister and Dean, 1993) was studied. One hundred RILs were grown, and the third and seventh leaves of several plants from each RIL were analyzed. More than 20 QTL were identified, harboring naturally occurring alleles that contribute to natural variations in the architecture of leaves (Pérez-Pérez et al., 2002).

A similar approach has been followed by Andrew Hudson and colleagues using interfertile hybrids in the genus *Antirrhinum majus*. The 20 species under study display marked variations in morphology such as large leaves in *A. majus* and small leaves in *A. molle*. Analysis of hybrids between these species suggests that overall leaf size or length and width are independently controlled by several genes. Their identification is under way by following a QTL mapping approach.

CELL BIOLOGY IN THE LEAF

The relationship between cell cycle and leaf development has been explored with different techniques. Gerrit Beemster (University of Gent) has quantified cell division, cell expansion, and endoreduplication as well as their variation with time, in the abaxial surface of the first vegetative leaves of Arabidopsis. Cell division rates remain relatively constant throughout 9 d after initiation, and decline over a 4-d period, in which the onset of endoreduplication becomes apparent.

With the aim of determining the effect of altered cell cycle regulation on the interplay between the division and expansion of leaf cells, Beemster obtained transgenic lines overexpressing the *KRP2* gene; its product is a Kip-related protein with cyclin-dependent kinase binding specificity (De Veylder et al., 2001). Overexpression of *KRP2* inhibited cell cycle progression in leaf primordia cells without affecting the temporal pattern of cell division and differentiation and resulted in markedly serrated leaves, which consisted of enlarged cells. Based on the comparison of the effects of the overexpression of *KRP2* and a number of other cell cycle genes, which differentially affect individual growth parameters, Beemster concluded that they are independently controlled.

The switch from the mitotic cell cycle to endoreduplication is well known in the differentiation of trichomes and guard cells in the developing leaf epidermis. Yuki Mizukami (University of California, Berkeley) described experiments aimed at determining whether or not the cell cycle can be uncoupled from cell differentiation in the epidermis of Arabidopsis and tobacco leaves. She overexpressed cell cycle regulator genes such as D-type cyclins and found that cell division was accelerated in tobacco Bright Yellow 2 cells. In transgenic Arabidopsis plants, the mitotic cycle continued, leading to the development of multicellular trichomes and multicellular guard cells with essentially normal morphology, although stomatal patterning was affected.

Mizukami also overexpressed the Arabidopsis homologs of the FZY/FZR (Fizzy/Fizzy-related) proteins, which are known to target Cyclin B for degradation, and found that they induced endoreduplication in tobacco Bright Yellow 2 cells. Tobacco plants expressing a *35S::AtFZR* transgene developed unicellular trichomes instead of the wild-type multicellular trichomes. However, the branching pattern of such hair cells remained undisturbed. Taken together, these results led Mizukami to conclude that the entry into endoreduplication is not essential for trichome cell morphogenesis and that patterned cell cycle transition can be uncoupled from determination and progression of cell differentiation in leaf epidermis.

Hülskamp's group has studied the *TRIPTYCHON* (*TRY*) gene, which encodes a MYB-related transcription factor that negatively regulates trichome patterning (Schnittger et al., 1999) and is a homolog of the root-hair patterning gene *CAPRICE* (*CPC*). Given that these two genes act redundantly in trichome development and in root-hair cell fate choice, Hülskamp proposed a genetic model for trichome patterning based on a reaction-diffusion model (Meinhardt and Gierer, 1974) that couples a short range autocatalytic activator process with a long range inhibitory process. According to Hülskamp, *TRY* and *CPC* fit as inhibitors in this model.

Aiming to ascertain the nature of the controls in the switch from mitosis to endoreduplication, Hülskamp and colleagues have generated transgenic lines that express known cell cycle genes in a trichome-specific manner. In agreement with the results of Mizukami, they demonstrated that the ectopic expression of specific B-type and D-type cyclins suppresses endoreduplication (Schnittger et al., 2002a; 2002b), thereby producing multicellular trichomes but not substantially modifying trichome shape.

Laurie Smith (University of California, San Diego) has isolated mutations in three *brick* (*brk*) genes that are required for formation of the lobes that characterize the margins of epidermal pavement cells in maize (Frank and Smith, 2002). The lobed shape is assumed to be determined by the arrangement of cortical microtubules. Expanding pavement cells of *brk* mutants display apparently normal microtubule organization, but lack the patches of cortical F-actin that colocalize with the tips of elongating lobes and the sites of lobe initiation, suggesting a role for *brk* genes in multiple, actin-dependent cell polarization in the developing maize leaf epidermis. Evidence from clonal analyses and genetic interactions indicate that the *brk* genes play different roles but act in a common process. The *brk1* gene has been cloned and found to encode a small protein of 8 kD belonging to a family highly conserved throughout plants and animals. Smith speculated that the BRK1-like proteins function in actin-dependent aspects of cell polarization, and that, at least in maize, BRK1 regulates localized actin polymerization.

THE FORMATION OF LEAF PATTERNS

A striking aspect of leaves is their venation pattern. Veins differentiate from procambial cells that form de novo from meristematic precursors within the leaf primordium (Nelson and Dengler, 1997; Dengler and Kang, 2001). Procambial strands arise from ground cells, which elongate, proliferate, and finally differentiate into veins. Aiming to ascertain how positional signals translate into procambium, Nancy Dengler (University of Toronto) has used a *β-glucuronidase* reporter construct for the HD-ZIPIII homeobox gene *ATHB-8* (Baima et al., 2001), a member of the above mentioned family, including *PHB*, *PHV*, and *REV*, as a molecular marker of early procambial development. An almost exact correlation in spatial and temporal patterns of cell cycling and *ATHB-8* expression was found, suggesting that this gene might be involved in the maintenance of procambium-specific patterns of cell cycling during leaf development.

Tim Nelson's group (Yale, New Haven, CT) is studying venation patterning by identifying mutants, one of which, *cotyledon vascular pattern1* (*cvp1*; Carland et al., 1999), displays discontinuous cotyledon veins. The *CVP1* gene encodes a sterol methyltransferase involved in sterol biosynthesis (*SMT2*; Schaeffer et al., 2001), and its lack of function is rescued by the overexpression of a related gene, *SMT3*. Another

of the mutants found by Nelson is *cvp2*, which encodes a synaptojanin-like phosphatidylinositol (4, 5)-bisphosphate phosphatase (McPherson et al., 1996). They also identified genes expressed specifically or predominantly in provascular cells. One of these is *VASCULAR HIGHWAY1* (*VH1*), which encodes a protein product that resembles CLAVATA1 and BRASSINOSTEROID INSENSITIVE1, in having an extracellular domain with a Leu-rich repeat and an intracellular Ser/Thr kinase domain. A possible role for this putative receptor is to receive signals for differentiation versus proliferation in provascular cells.

The patterning of stomata, the cellular structures of the epidermis that mediate gas exchange, is under genetic and environmental control (Serna and Fenoll, 2000; van Groll and Altmann, 2001). Thomas Altmann's group (University of Potsdam, Golm, Germany) has isolated ethyl methanesulphonate-induced mutants with altered stomatal traits to unravel the mechanisms regulating stomatal distribution. Several of the mutants found display clustered stomata compared with stomata on wild-type leaves, which are surrounded by a stomata-free area (Berger and Altmann, 2000). The *stomatal density and distribution1-1* (*sdd1-1*) mutation causes a 2- to 4-fold increase in the density of stomata, many of which are not separated by intervening pavement cells. The *SDD1* gene encodes a subtilisin-like Ser protease that accumulates near the extracellular surface associated to the plasma membrane of meristemoids and guard mother cells, two successive stages of the stomatal developmental pathway. Based on the previously known activities of eukaryotic subtilases, Altmann proposed that SDD1 is a processing protease involved in the creation of a signal emanating from meristemoids or guard mother cells, which regulates the development of cell lineages forming stomatal complexes. *SDD1* overexpression results in reduced stomatal density and premature arrest of divisions in stomatal precursor cells. *SDD1* overexpression has no effect on *too many mouths* (*tmm*; Yang and Sack, 1995), a mutant that displays clustered stomata. The epistasis of *tmm* on the overexpression of *SDD1* suggests that the genes act in the same pathway.

Carmen Fenoll (University of Castilla-La Mancha, Toledo, Spain) presented results on the influence of light in stomata formation, which they studied by means of cell lineage analyses in constitutive photomorphogenesis mutants, affected in the pleiotropic CONSTITUTIVE PHOTOMORPHOGENIC/DE-ETIOLATED/FUSCA (*COP/DET/FUS*; for review, see Hardtke and Deng, 2000) loci. They found that these mutants display perturbations in the development and positioning of stomata that are remarkably similar to those described for mutants specifically altered in stomatal patterning. These results strongly suggest that the genes involved in the repression of photomorphogenesis play a crucial role in stomatal pattern formation in a light-independent manner.

THE EVOLUTION OF LEAF SHAPE

Leaf shape varies from simple, such as found in the Arabidopsis leaf, to dissected as in tomato. Two different groups presented work on genetic mechanisms that lead to dissected leaves. In each case, gene expression, normally excluded from simple leaves, was found expressed in species with dissected leaves and correlated with the dissected leaf shape.

Neelima Sinha (University of California, Davis) described how most species with dissected leaves express *KNOX* genes and species that have simple leaves do not express *KNOX* genes (Bharathan and Sinha, 2001). What appeared to be exceptions to the rule, i.e. species with a simple leaf that express *KNOX* genes, were shown by scanning electron microscopy to actually be dissected at leaf initiation. *KNOX* gene expression is found along the leaf margins of young leaves, leading to the elaboration of the lobes and leaflets. As found in plants with simple leaves, *KNOX* expression disappears in the P_0 cells of the meristem, consistent with the hypothesis that *KNOX* genes are needed to be repressed to initiate a leaf.

Julie Hofer (John Innes Centre, Norwich, UK) examined the dissected leaf of pea (*Pisum sativum*). She showed that the *unifoliata* mutant of pea, which no longer has a dissected leaf, is a mutation in a *LEAFY* ortholog (Hofer et al., 1997). *LEAFY* was first discovered as a gene that regulates the initiation of flowers from the inflorescence meristem in Arabidopsis (Weigel et al., 1992). Her analysis shows that pea leaves do not express *KNOX* genes, also discovered by Sinha, but instead express *LEAFY*. She has expanded this research to the related species of *Medicago*. A *Medicago* mutant was described that has unifoliate leaves and again the mutation is in a *LEAFY* ortholog. From the combined work of Sinha and Hofer, it appears that the phylogenetic clade that includes pea and *Medicago* gained dissected leaves, not by expressing *KNOX* genes, but expressing genes normally restricted to the floral meristems.

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