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Mechanisms and variation in plant development: sorting the wood from the trees in Vermont

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The biannual FASEB summer research conference 'Mechanisms in Plant Development' was recently held in Saxtons River, Vermont and was organised by Neelima Sinha and Cris Kuhlemeier. Although most of the work discussed at the meeting concentrated on developmental mechanisms and on studies in *Arabidopsis* and maize, the meeting also emphasised the importance of variation between species and the elaboration of a broader range of model systems.

Introduction

The FASEB meeting 'Mechanisms in Plant Development' covered a broad range of themes, from developmental topics, such as meristems and organogenesis, gametophytic development, signalling, small RNAs and mathematical modelling, to the field of variation and evolution, which was discussed in sessions on natural variation, seed-free plants and the evolution of development. This review highlights several of the emerging issues covered in the meeting.

Leaf development: roles for small RNAs and auxin

The development of leaves and the topic of how their morphology changes as the shoot grows were addressed by several speakers. The plant shoot exhibits indeterminate growth, and continuously gives rise to new organs from its growing tip, called the shoot apical meristem (SAM). As leaf primordia grow away from the shoot meristem, the upper side closest to the SAM takes on an adaxial identity, whereas the other side acquires abaxial identity (Fig. 1). Extensive genetic studies in maize and *Arabidopsis* have provided a regulatory framework for understanding how these abaxial and adaxial identities are conferred (Chitwood et al., 2007). The exploitation of both species has made the system more accessible, because some pathways have more prominent roles in one species than in the other. Marja Timmermans (Cold Spring Harbor, New York, USA) described the complex interplay that occurs between transcription factors and small RNAs in establishing adaxial and abaxial leaf identity. She explained that adaxial identity involves class III homeodomain leucine zipper (HD-ZIPIII) transcription factors, the mRNAs of which become restricted to the adaxial side of the leaf primordium as it emerges. By contrast, abaxial identity involves *Arabidopsis* genes that encode three KANADI (KAN) proteins and three YABBY proteins. The KAN proteins, as well as two AUXIN RESPONSE FACTORS (ARF3 and ARF4), activate the expression of the *YABBY* genes in the abaxial region. The HD-ZIPIII proteins repress *KAN* gene expression in the adaxial region, while the KAN proteins repress the HD-ZIP genes in the abaxial region. In parallel to this transcription factor system, small RNAs act to confer both adaxial and abaxial identity. MicroRNA166 (*miR166*) acts in abaxial tissues and inhibits the expression of HD-ZIP proteins in both maize and *Arabidopsis*, while trans-acting short interfering RNAs that target ARF3 and ARF4 (*tasiR-ARFs*) and that reduce the domain of expression of the *miR166* precursor confer adaxial fate and have a more prominent role in maize (Chitwood et al., 2007; Nogueira et al., 2007). The distinct activities of the two small regulatory RNAs suggest that *tasiR-ARF* and *miR166* oppose each other in creating the boundary between adaxial and abaxial regions, respectively. An issue discussed at the meeting was the extent to which the movement of small RNAs contributes to the position of the boundary between the abaxial and adaxial regions. This issue was addressed for two small RNAs: *tasiR-ARF* and *miR390*, a miRNA required for *tasiR-ARF* biogenesis (Allen et al., 2005). The Timmerman's group showed that a graded abundance of *tasiR-ARF* is created by the localised activity of *miR390* on the adaxial side of the developing leaf primordia, with the low abundant *tasiR-ARF* moving abaxially into deeper layers. The extent of movement of this microRNA would contribute to the position of the boundary between the adaxial and abaxial regions.

The juxtaposition of adaxial and abaxial identities is also required for the extension of the leaf in the laminar plane to produce the typical flat structure of a leaf (Waites and Hudson, 1995). Sarah Hake (Plant Gene Expression Center, Albany, CA, USA) interpreted the phenotype of the *milkweed pod1* (*mwp1*) mutant of maize in the context of these processes (Candela et al., 2008). The leaves of *mwp1* mutants show normal abaxial and adaxial identities, but sectors that exhibit characteristics of adaxial tissues arise on the abaxial side. Outgrowths occur at the junctions between these sectors and the surrounding abaxial tissues, in agreement with other indications that the juxtaposition of these two tissue types promotes growth. The *MWP1* gene is expressed in the abaxial domain of the leaf and encodes a KAN protein, consistent with the data from *Arabidopsis* indicating a role for KAN proteins in conferring abaxial identity. Furthermore, the *rolled leaf1* (*rld1*) gene encodes an HD-ZIPIII protein homologous to those that confer adaxial fate in *Arabidopsis*, and *rld1-N* mutants contain a mutation in the *miR166* binding site of *rld1* mRNA (Juarez et al., 2004). In *rld-N* mutants, *MWP1* expression is reduced in abaxial tissues. As Hake discussed, the *mwp1* mutant also shows an interesting phenotype in the prophyll, a specialised leaf produced by the axillary meristem that gives rise to the ear of maize. The prophyll grows around the ear and is keeled, such that two laminae extend from the abaxial surface of the prophyll to wrap around the stem. In the *mwp1* mutant, the keel is not formed, suggesting a role for abaxial identity in producing the keel. Prophylls develop from two separate leaf primordia that fuse at the margins, leading to the proposal that keels may be outgrowths stimulated by the juxtaposition of abaxial and adaxial tissues, which are not formed in *mwp1* mutants because of impaired abaxial identity. This would then be an example of boundaries between abaxial and adaxial tissues being used to stimulate growth in a different context from that of promoting laminar growth in typical leaves. Chuck Gasser (UC Davis, CA, USA) described that, in another context, the outgrowth of the integument in the developing ovule, the boundary between layers specified by the

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Fig. 1. Adaxial-abaxial sides of a leaf. The adaxial side of an *Arabidopsis* leaf is dark green and rich in hair-like outgrowths called trichomes, whereas the abaxial leaf surface is matt grey-green and does not have many trichomes. Reproduced, with permission, from Chitwood et al. (Chitwood et al., 2007).

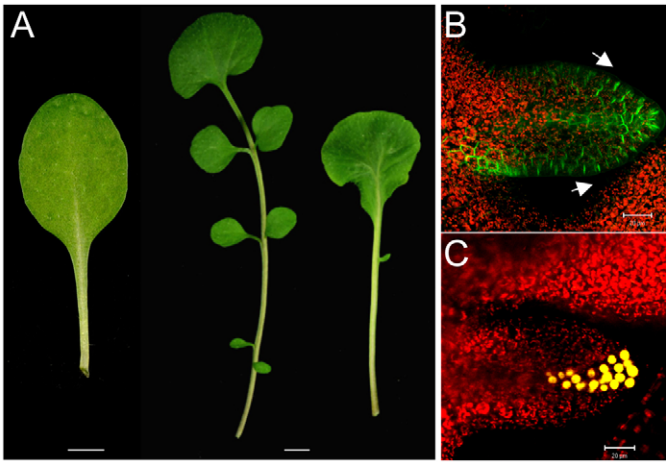

Fig. 2. Simple and dissected leaves. (**A**) Mature adult leaves from *A. thaliana* (left), *C. hirsuta* wild type (middle) and *C. hirsuta pin1* mutant (right). (**B**,**C**) Confocal laser-scanning microscope images showing expression of PIN1::PIN-GFP (B) and the auxin activity reporter DR5::VENUS (C) in *C. hirsuta* developing leaflets. Arrows indicate the predicted direction of auxin transport. Scale bars: 0.5 cm in A; 20 μm in B,C. Images courtesy of Miltos Tsiantis (Oxford, UK).

HD-ZIPIII, KAN and YABBY proteins, is also required to promote growth, indicating that this system has been co-opted in different contexts during plant evolution.

Miltos Tsiantis (University of Oxford, Oxford, UK) also described an example of alterations in leaf morphology, using *Cardamine hirsuta*, a wild relative of *Arabidopsis*, as a model for compound leaf formation (Barkoulas et al., 2008; Hay and Tsiantis, 2006). *Arabidopsis* forms simple leaves in which the leaf blade is undivided, whereas *C. hirsuta* produces dissected leaves in which the blade is divided into leaflets (Fig. 2). In both species, the leaves develop from similar primordia produced on the flanks of the SAM. However, the morphology of the developing leaves diverges when two subsequent leaf primordia have been formed by the SAM. The Tsiantis group could show that only a small number of cells at the leaf margin give rise to the leaflet in dissected leaves. In simple leaves, by contrast, the leaf margin plays only a minor role in leaf growth. To identify genes required for leaflet formation, the group has conducted a screen for *C. hirsuta* mutants that produce simple leaves, which identified a mutation in the auxin efflux carrier PIN FORMED 1 (PIN1), indicating a role for auxin transport in leaflet formation. A PIN1-GFP fusion protein localises in the developing leaflet, and the polarity of its accumulation suggests that auxin accumulates at the tip of the developing leaflet. KNOTTED1-LIKE HOMEOBOX (KNOX) proteins also promote leaflet initiation in *C. hirsuta* (Barkoulas et al., 2008), and from further findings Tsiantis and co-workers conclude that PIN1 acts downstream of the KNOX genes, leading them to a model in which KNOX genes act to maintain cells at the margins of *C. hirsuta* leaves in an undifferentiated state, allowing these cells to respond to auxin accumulation generated by PIN1. These auxin maxima would then stimulate leaflet outgrowth. Different varieties of *C. hirsuta* show alterations in the number of leaflets formed; an issue now being addressed is whether these variety-specific differences are caused by allelic variation at the same *PIN1* and *KNOX* gene loci responsible for the species-specific differences between *A. thaliana* and *C. hirsuta*.

Leaf development can also vary within a single plant as the shoot progresses through different phases of growth. George Chuck (Plant Gene Expression Center, Albany, California, USA) and Scott Poethig (University of Pennsylvania, Philadelphia, PA, USA) described the mechanisms that control the progression of the shoot from juvenile to adult phases, and its striking conservation between maize and *Arabidopsis*. In the maize shoot, development is divided into juvenile, adult and reproductive phases, and the leaves formed during these phases differ in, for example, their overall shape, the organs formed from the meristems present in the axils of leaves, and their cell morphology and wax deposition. Mutations have long been known that alter the duration of these phases (Poethig, 1988a). George Chuck described the basis of one of the most intriguing of these mutations, *corngrass1* (*cg1*) (Chuck et al., 2007). The *cg1* mutation in maize greatly extends the juvenile phase, so that several juvenile characteristics are observed in leaves throughout the shoot, such as a slender morphology, production of epidermal wax and tillers (stems produced from axillary meristems). Furthermore, the nodes on the stem at which the leaves arise continuously produce prop roots, a characteristic of the stem normally found during juvenile development. The *cg1* mutant also shows altered development of the inflorescence, and specialised leaves (bracts) that are present in the ear and tassel are much larger than those of wild-type maize. As Chuck and colleagues have previously reported (Chuck et al., 2007), the *cg1* locus comprises a tandem arrangement of two genes that encode two microRNAs, *zma-miR156*b and *zma-miR156c*. In *cg1* mutants, *zma-miR156* is continually expressed at elevated levels, whereas in wild-type plants it is highly expressed only during the juvenile phase and is not expressed in the adult phase. These results indicate that *zma-miR156* has a major role in conferring juvenile characteristics. *zma-miR156* targets mRNAs of the SQUAMOSA PROMOTER BINDING LIKE (SPL) class of transcription factors, seven of which are reduced in the *cg1* mutant. One such target is *tassel sheath4* (*tsh4*); *tsh4* mutants show much larger inflorescence bracts than in wild-type plants, as is also observed in *cg1* mutants, suggesting that this aspect of the *cg1* mutant can be explained by increased *zma-mir156* levels causing the downregulation of *tsh4*. The timing of the transition from juvenile to adult phase possibly involves an interplay between *zma-mir156* and a second class of microRNA: *miR172* (Chuck et al., 2007). As Chuck discussed, *miR172* targets mRNAs of the APETALA2 (AP2)-LIKE transcription factor class, such as GLOSSY15 (GL15), a

transcription factor required in maize for wax formation on juvenile leaves. During the phase change from juvenile to adult, *zma-miR156* levels fall, while *miR172* levels rise, and in *cg1* mutants (in which *zma-mir156* levels are higher for longer during shoot development), *miR172* levels are strongly downregulated. This leads to ectopic GL15 expression in *cg1* shoots (Evans et al., 1994; Moose and Sisco, 1996). Although the mechanism by which *miR156* controls the expression of *miR172* is unclear, these findings suggest that these two miRNAs interact to control the timing of the transition between juvenile and adult development. Scott Poethig described how his work on phase change led independently to the identification of *miR156* and *miR172* as determinants of juvenile and adult development in *Arabidopsis* (Willmann and Poethig, 2007; Wu and Poethig, 2006), and how this enabled him to return to his pioneering work on *cg1* and related mutants (Poethig, 1988a; Poethig, 1988b). In addition, he showed the function of specific *SPL* and *AP2-like* genes in controlling adult and juvenile traits, respectively. Furthermore, he pointed out that in maize, mutations in *teopod* genes (which are similar to *cg1* and also cause elevated levels of *mir156*) are non-cell autonomous because sectors of tissue that do not contain the mutation express the mutant phenotype because of the presence of the mutation in neighbouring tissues (Poethig, 1988b). The basis of this non-cell autonomous signal and the role of *mir156* in the signalling mechanism remain unclear.

Technological advances

Venugopala Reddy (University of California, Riverside, CA, USA) and Ueli Grossniklaus (University of Zürich, Zürich, Switzerland) presented their technological approaches to identifying patterns of gene expression in specialised tissues, such as the shoot apical meristem (Reddy) and the female gametophyte (Grossniklaus). The SAM is maintained partly by the activity of the CLAVATA3 (CLV3) and WUSCHEL (WUS) proteins (Laux, 2003). Although these proteins act in different cells of the meristem, a feedback loop exists between their activities, such that WUS (a homeobox transcription factor that promotes cell divisions in the organising centre of the meristem) promotes *CLV3* expression, maintaining the presence of stem cells, whereas CLV3 (a small peptide that is expressed in apical cells located above the organising centre) represses *WUS*. These interactions maintain a homeostasis in cell number within the different regions of the meristem. Reddy presented his cell-sorting strategy to identify further genes that act within this system. Transgenic plants that express *pCLV3:mGFP-ER*, *pWUS:RFP-ER* and *pFIL::dsRED-N7* were created. The GFP and RFP fluorescent markers were then used to sort the cells that comprise the *CLV3* or *WUS* expression domains in a mutant plant that produces many floral meristems, thus providing access to a much larger number of cells of the required types than would otherwise be possible. The resulting gene expression profiling experiments showed that over 400 genes are apparently specific to the SAM. Moreover, the expression of over 700 genes was detected, the expression patterns of which had not previously been described. This appears to be a promising way of identifying genes that act specifically in these cell types and that have not so far been identified by genetics or other genomics approaches. A related technical problem was experienced by Ueli Grossniklaus, who was interested in identifying genes expressed in specific cell types in the female gametophyte. He described using laser capture microscopy to isolate individual cell types, including the egg cell, which has a diameter of only 7-8 μm. Depending on the cell type, amplifications were performed with RNA extracted from 250-800 cells and carried out in triplicate. In this way, Grossniklaus and his co-workers could compare the transcriptome of the central cell, egg cell and synergid, and they found that between 5000 and 7000 genes were expressed in a given cell type, with about 200 genes being specific to each cell type. Such genomics-based approaches will form the basis of future reverse genetics analyses to identify genes that contribute to the identity of each of these cell types.

Patterning the female gametophyte

The mechanisms conferring cell identity to the female gametophyte were also discussed by Venkatesan Sundaresan (UC Davis, California, USA). The female gametophyte is produced from a haploid megaspore, which goes through three rounds of mitosis to form a syncytium that contains eight nuclei (Brukhin et al., 2005). This syncytium then undergoes cellularisation to form seven cells of four different cell types: an egg cell, two synergid cells, a bi-nucleate central cell and three antipodal cells. One of the problems studied by Venkatesan Sundaresan is how different cell identities are conferred during cellularisation. He proposed that a signalling gradient might provide the information that is required for the specification of different cell identities within the female gametophyte. Experiments carried out to test this model revealed that the identities of cells could indeed be influenced by varying the concentrations of a small molecule to which they were exposed. The mechanisms by which such a gradient could be established and maintained were the subject of lively debate.

Variation and evolution

Several talks discussed the exploitation of natural genetic variation to study developmental processes and of establishing further model systems to study how such processes are shaped by evolution**.** Kirsten Bomblies (Max Planck Institute for Developmental Biology, Tübingen) described a molecular analysis of hybrid necrosis, a surprisingly common phenomenon in plants, in which the progeny of a cross between two accessions show necrosis and are much less vigorous (Bomblies and Weigel, 2007). Typically, two loci are required to confer the necrotic effect. She studied this phenomenon in *Arabidopsis* by first performing a remarkable 1487 crosses between 311 *Arabidopsis* accessions. In 25 of these crosses, the hybrid progeny were different, varying from small with necrotic lesions but still producing seeds, to tiny and sterile. These phenotypes were observed when plants were grown at 16°C (at 23°C, they appeared normal). In one cross between two accessions, UK1 and UK3, two loci, dubbed *DANGEROUS MIX 1* (*DM1*) from UK3 and *DM2* from UK1, were shown to interact to confer the necrosis (Bomblies et al., 2007). The *DM1* locus mapped to chromosome 5 and encodes a Toll interleukin receptor (TIR)-NB-LRR protein homologous to proteins that commonly confer resistance to pathogens. The UK3 allele of *DM1* encodes a full open reading frame (ORF), whereas in UK1 *DM1* only contains incomplete ORFs. Gene-swapping experiments demonstrated that the leucine-rich repeat region of DM1 is required for the hybrid necrosis effect. *DM2* maps to a 168 kb region of chromosome 3 that contains a cluster of resistance gene homologues. This observation raises the possibility that hybrid necrosis is caused by incompatibility between two resistance proteins, and that this combination does not arise in either parent. Why the effect is temperature dependent is not clear but might be due to the differential stability of the resistance protein homologues at different temperatures or to a trade off between stress signalling pathways so that the resistance-gene signalling pathway is suppressed if the plant is stressed at higher temperatures. In a second analysis, accessions of *Arabidopsis* were analysed from 95 sites in the Tübingen area

using 455 single nucleotide polymorphism (SNP) markers. The degree of polymorphism present at each site varied tremendously, but generally those from urban sites showed little variation within the population, whereas those from rural sites contained many variants. These results suggested that wild populations are highly differentiated at the local scale, and that rural meadow populations are where this variation can be detected most clearly.

Jody Banks (Purdue University, IN, USA) presented a first glimpse of the *Selaginella moellendorffii* genome (see http://selaginella.genomics.purdue.edu). This species is a lycophyte, a group believed to have diverged from seed plants and ferns around 400 million years ago. The lycophytes appeared before roots and true leaves had arisen in the higher plant lineage, and as they represent the earliest surviving vascular plant lineage, they provide an insight into the early evolution of land plants. The genome is around 130 Mb, has been sequenced to 14× coverage and encodes around 22,000 proteins, of which over 3000 have been manually curated. Early analysis of the gene content identified over 13,000 gene clusters, of which 120 are absent in seed plants.

Elena Kramer (Harvard, Cambridge, MA, USA) described how *Aquilegia* (Columbine) can be used as a model to study the evolution of unusual floral structures, which in this case includes five whorls of organs rather than the usual four (Kramer et al., 2007). She explained how genes that encode homologues of the *Arabidopsis* genes *APETALA3* and *PISTILLATA*, which are required for petal and stamen identity in *Arabidopsis*, show more complex patterns of expression in the *Aquilegia* flower, and how virus-induced gene silencing was used to demonstrate the activity of one of them in conferring the identity of a novel structure: the stamenodium.

Conclusions

The power of comparative biology in more rapidly deciphering the common features of plants and in describing their species-specific differences was strongly evident at this meeting, particularly in the discussions on leaf polarity and phase transitions. Similarly, comparisons of *A. thaliana* and *C. hirsuta* provided an example of how common regulatory mechanisms can be co-opted to generate specific characters. The concept of opposing activities of different small RNAs to provide spatial and temporal boundaries of gene expression in developmental processes may also prove to be much more common in plants. I also expect that genomic approaches to investigating the RNA transcriptome of specialised cell types; for example, in conjunction with cell sorting or laser dissection, will have a much more general impact in the future. The development of new model systems and the availability of more genome sequences will also provide access to a broader range of developmental problems. The next meeting in this series is planned for August 2010 and will no doubt reflect how the themes discussed at this meeting develop in the intervening period.

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