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REVIEW

Getting leaves into shape: a molecular, cellular, environmental and evolutionary view

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ABSTRACT

Leaves arise from groups of undifferentiated cells as small primordia that go through overlapping phases of morphogenesis, growth and differentiation. These phases are genetically controlled and modulated by environmental cues to generate a stereotyped, yet plastic, mature organ. Over the past couple of decades, studies have revealed that hormonal signals, transcription factors and miRNAs play major roles during leaf development, and more recent findings have highlighted the contribution of mechanical signals to leaf growth. In this Review, we discuss how modulating the activity of some of these regulators can generate diverse leaf shapes during development, in response to a varying environment, or between species during evolution.

KEY WORDS: Morphogenesis, Evolution, Environmental cues, Transcription factors, Hormones, Mechanics

Introduction

Leaves have been defined morphologically as plant lateral organs with a determinate growth, a vasculature, a polarity and a specific arrangement along the stem, called phyllotaxy. Functionally, leaves are defined as the main photosynthetic organs, although many exceptions exist (Tomescu, 2009; Fukushima and Hasebe, 2014; Tsukaya, 2014). Although most tracheophytes (vascular plants) bear organs that can be defined as leaves, these organs appeared many times during land plant evolution in a convergent manner and are therefore not all homologous (Tomescu, 2009). As such, leaves are believed to have evolved from undifferentiated branches that switched to a determinate growth, transformed their original 3D arrangement to become mainly planar and subsequently fused (Beerling and Fleming, 2007).

Leaves are good models for studying how complex organs arise from simple structures. Indeed, all leaves are initiated at the shoot apical meristem (SAM) as simple rod-like primordia, which later acquire their final shape. This happens via three intertwined processes: morphogenesis (defined as shape acquisition), growth (defined as irreversible increase in size) and differentiation (the production of specialised cell types). Additionally, leaf shape shows great diversity across species (Fig. 1A). The leaf blade can be either simple or compound (divided into units called leaflets, connected by a rachis) and the leaf margin can be smooth, serrated (toothed) or lobed. Moreover, leaves show shape diversity within individuals: leaves from higher nodes usually become sequentially bigger and more serrated, until flowering, when the pattern reverts (Fig. 1B). Many species also exhibit plasticity with regard to leaf development in response to environmental conditions (Fig. 1C). Understanding how molecular and genetic networks control proliferation, growth and differentiation to allow a small primordium to turn into a complex organ with a consistent size and shape (Fig. 1D), how these networks respond to both endogenous and environmental signals to generate plasticity, and how they have been modified by evolution for such diversity in shape and function to arise, is of interest to a broad array of biologists.

In this Review, we show how recent findings challenge or shed new light on classical knowledge about leaf development. We focus on the morphogenesis of flowering plant leaves, with a strong emphasis on flat, bifacial leaves, such as those of *Arabidopsis* or tomato, because they have been studied extensively. Insights from other models, in particular from grasses, will be discussed when specific developmental processes are at play. Some major aspects of leaf development, such as venation patterning, specific cell-type differentiation or leaf aging and abscission, have been reviewed recently elsewhere (Kalve et al., 2014; Hepworth and Pautot, 2015; Schippers, 2015; Han and Torii, 2016; Linh et al., 2017) and are thus not considered here. We start by describing how leaves are initiated at the SAM, and discuss how they acquire their fate and polarity to grow as mainly flat structures. We then highlight new findings focusing on how lateral organs acquire their size in a reproducible manner. Next, we detail the molecular factors leading to leaf shaping and explain how leaf shape diversity arises within and across species. Finally, we highlight some recent insights into the molecular processes controlling leaf shape plasticity in response to the environment.

Leaf initiation at the shoot apical meristem

Hormonal and genetic factors control the formation of new organs

Shoot organs, such as leaves, are continuously produced post-embryonically by the SAM (Fig. 2). During vegetative growth the SAM gives rise to shoot-bearing leaves whereas during reproductive growth the SAM becomes an inflorescence meristem and produces flowers. Direct observation of the vegetative meristem in the model species *Arabidopsis* has proven to be technically challenging. However, as we discuss below, studies of *Arabidopsis* inflorescence meristems and vegetative meristems in other species, such as tomato, have provided insights into how organ growth is initiated.

The SAM is divided into different regions, including a central zone containing stem cells, a peripheral zone where organ primordia are initiated, and a rib zone that forms stem tissues. Organ primordia are initiated at the SAM periphery by bulging caused by local increases in cell division (Reddy et al., 2004). Organ initiation is intimately linked with the plant hormone auxin. Auxin is required for many developmental processes in plants (Vanneste and Friml, 2009)

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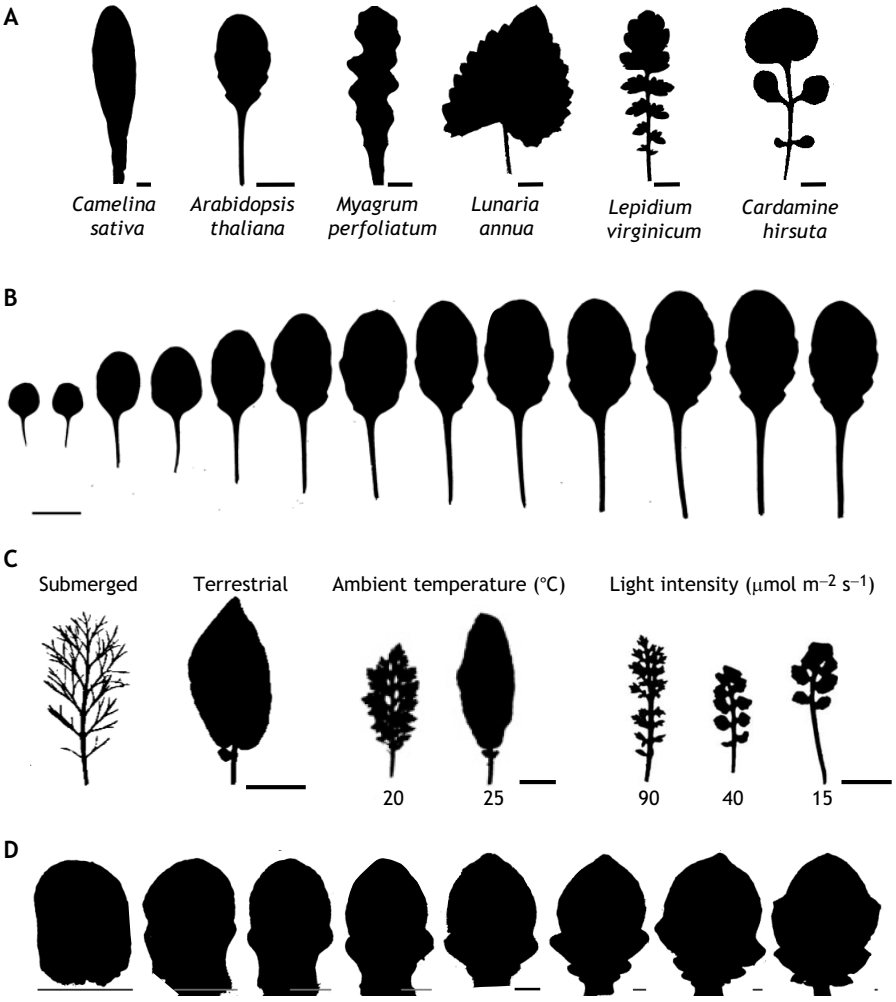

Fig. 1. Leaf shape diversity in the Brassicaceae family. (A) Leaf shape diversity among Brassicaceae species. The family contains species with simple leaves and smooth margins (*Camelina sativa*), serrated margins (*Arabidopsis thaliana* and *Lunaria annua*) or lobed margins (*Myagrum perfoliatum* and *Lepidium virginicum*), as well as species with compound leaves (*Cardamine hirsuta*). (B) Leaf shape diversity (also known as heteroblasty) in *Arabidopsis*; leaves from higher nodes usually become sequentially bigger and more serrated. (C) Leaf shape plasticity in response to environmental conditions (also known as heterophylly) in *Rorippa aquatica*. (D) Successive stages of *Arabidopsis* leaf 11 morphogenesis. Scale bars: 1 cm (A,B); 2 cm (C); 500 µm (D). The image shown in C is modified, with permission, from Nakayama et al. (2014).

and can form local maxima as a result of coordinated cell-to-cell transport. In particular, auxin can be exported from cells by the PIN-FORMED (PIN) efflux-transporters, which often themselves exhibit a polar localisation (reviewed by Armengot et al., 2016). Within the SAM epidermal layer, PIN1 repolarisation dynamically creates convergent auxin flows at the meristem surface, which then leads to a high local auxin response thereby promoting primordium formation (Fig. 2; Reinhardt et al., 2000, 2003; Heisler et al., 2005). In the absence of PIN1, lateral organ initiation is perturbed during both the vegetative and inflorescence phases, but this can be restored by external auxin application (Reinhardt et al., 2000; Vernoux et al., 2000; Guenot et al., 2012).

Organ primordia are separated from the meristem by a groove formed by small slow-dividing and slow-expanding cells, which together form a boundary domain (Breuil-Broyer et al., 2004; Kwiatkowska, 2004; Reddy et al., 2004). This boundary domain is genetically defined by several factors, including those of the NO APICAL MERISTEM/CUP-SHAPED COTYLEDON (NAM/CUC) transcription factor family. In line with this, mutants for NAM/CUC genes show fusions of lateral organs and often lack a SAM (Souer et al., 1996; Aida et al., 1997; Hibara et al., 2006), indicating that, in addition to allowing organ separation, this boundary domain contributes to meristem formation. The boundary domain also shows a specific hormonal status with low auxin and brassinosteroid (BR) levels (Fig. 2; Heisler et al., 2005; Gendron et al., 2012).

Mechanical cues influence lateral organ initiation

In the growing meristem, a feedback loop linking tissue morphology, stress patterns and cortical microtubule network organisation constrains morphogenesis (Hamant et al., 2008). Indeed, whereas cortical microtubules adopt no preferential orientation at the top of the meristem, where the stress pattern is isotropic, microtubules align along the principal stress axis in regions where stress is anisotropic, such as within the boundary domain. Microtubule alignment feeds back on morphogenesis though microtubule-directed cellulose deposition (Paredez et al., 2006). Additionally, mechanical cues perceived at the plasma membrane affect PIN1 levels and localisation, thus creating a positive feedback between growth and auxin accumulation during organ formation (Heisler et al., 2010; Nakayama et al., 2012). Such a feedback mechanism could also be at play during lateral organ initiation following cell wall relaxation by tampering with the pectin methyl-esterification status (Peaucelle et al., 2008). This indicates that mechanical cues control cell growth, contribute to tissue folding, and even define cell identity (Landrein et al., 2015).

Acquisition of a lateral organ fate

The primordium and SAM express different genes and have different hormonal signalling levels that contribute to define their identity and cellular behaviour while keeping these domains separated (Fig. 2). The class I KNOTTED-like homeodomain

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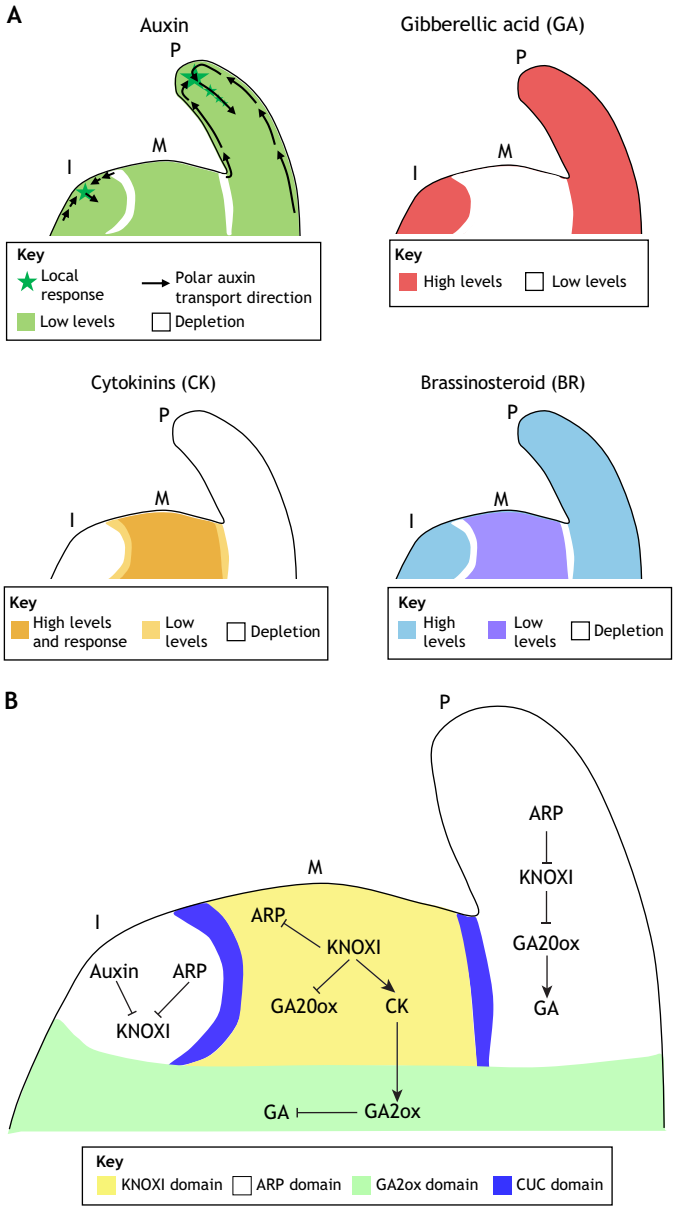

Fig. 2. Genetic and hormonal factors that control leaf primordium initiation. (A) Overview of the distribution and response to the main hormones in the SAM. The meristem (M) shows high CK and low GA levels. An initium (I) is formed due to convergent polar auxin transport, which leads to the formation of a local auxin response maximum. The initium and primordium (P) exhibit high levels of GA, which promotes their growth and differentiation. By contrast, boundary domains are globally depleted for hormones, which maintains their growth at a low level. (B) Genetic and hormonal factors controlling the identity switch in the primordium from an indeterminate to a determinate fate. Two main opposing domains are formed: a KNOXI indeterminate meristematic domain and an ARP domain that has a leaf identity. KNOXI proteins maintain high CK levels and low GA levels (by inhibiting GA20ox biosynthetic enzymes in the meristem). By contrast, GA levels are high in the leaf primordium. Please note that this is a non-exhaustive list of regulators. Panel A is based on Hepworth and Pautot (2015) and B is based on Hasson et al. (2010).

(KNOXI) transcription factors, for example, are expressed in the SAM and act partially redundantly to initiate and maintain the SAM (Long et al., 1996; Byrne et al., 2002; Belles-Boix et al., 2006). KNOXI gene expression is repressed in organ primordia, and ectopic expression in the primordium leads to strongly lobed leaves with occasional ectopic meristems (Ori et al., 2000; Spinelli et al., 2011). A similar leaf phenotype is observed in *asymmetric leaves 1* (*as1*) and *as2* mutants (Byrne et al., 2000; Ori et al., 2000). *AS1* encodes a MYB domain transcription factor that is specifically expressed in the leaf primordium and is homologous to maize *ROUGHSHEATH2* and *Antirrhinum majus PHANTASTICA* (*PHAN*), which together are named the ARP genes. AS1 interacts with the LATERAL ORGAN BOUNDARIES DOMAIN protein AS2 and together these recruit Polycomb Repressive Complex 2 (PRC2) to KNOXI loci to stably repress their expression (Lodha et al., 2013). Conversely, KNOXI factors also repress ARP gene expression in the SAM (Byrne et al., 2000), thus creating two mutually exclusive domains. In parallel with the ARP complex, an auxin response maximum in the initium also contributes to downregulation of KNOXI gene expression (Hay et al., 2006). In addition to this transcriptional control, KNOXI activity on downstream factors is antagonistically regulated by a sister group of proteins, the class II KNOX proteins, which are expressed in the leaf primordium where they promote differentiation. Accordingly, class II KNOX mutants show increased leaf complexity, as observed in KNOXI-overexpressing lines (Furumizu et al., 2015).

KNOXI proteins act through the modulation of hormonal pathways: the cytokinin (CK) pathway, which is mainly involved in promoting meristematic fate, and the gibberellic acid (GA) pathway, which promotes cell differentiation. KNOXI proteins inhibit GA biosynthesis by directly downregulating GA20ox biosynthetic enzymes in the SAM (Hay et al., 2002). The absence of KNOXI expression in the leaf primordium thus de-represses the GA pathway. Conversely, KNOXI proteins promote CK biosynthesis and response in the SAM, which maintains active proliferation and inhibits differentiation. CK and GA pathways are interconnected, as CK promotes the expression of GA catabolic genes (Jasinski et al., 2005; Yanai et al., 2005; Scofield et al., 2013). In rice, the KNOXI protein OSH1 promotes the expression of several BR catabolism genes to keep the BR pathway low in the SAM (Tsuda et al., 2014).

The observations presented above indicate how the SAM controls primordium initiation, but recent data provide insights into the mechanisms by which lateral organs feedback on the SAM. In maize, a CLAVATA3/ESR-related (CLE) peptide expressed in organ primordia signals to FASCIATED EAR3 (FEA3), a leucine-rich-repeat receptor expressed in the SAM, to limit the stem cell population and SAM size (Je et al., 2016). In *Arabidopsis*, lateral organs feedback on SAM size via modulation of auxin transport (Shi et al., 2018).

In summary, during the last decade important progress has been made with regard to linking previously identified key genes in SAM function with multiple interconnected hormonal pathways. In parallel, studies performed at the cellular level have identified feedback mechanisms linking growth and mechanical signals. A major challenge for the next coming years will be to reinforce the bridges between these fields in order to provide a more comprehensive view of SAM function and organ formation. Understanding how environmental factors control plant architecture through modulation of SAM activity, as shown recently for nitrate (Landrein et al., 2018), is also an exciting perspective with agronomical applications.

Leaf polarity

As mentioned in the Introduction, a shared feature of many leaves is their flat shape, with clear tissue differentiation between the upper and lower faces. For instance, the mesophyll differentiates into an adaxial (or dorsal) palisade parenchyma with densely packed cells

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that have a high chloroplast content to maximise light interception, whereas the abaxial or (ventral) spongy parenchyma is formed by loose cells, leaving spaces between them to facilitate gas circulation. Polarisation is also observed in the epidermal layer, which often contains more stomata on the abaxial side, and in the vasculature, which is formed by xylem on the adaxial side and phloem on the abaxial side. Such a complex leaf anatomy has an impact on many factors that are crucial for photosynthesis, for instance light or water distribution, CO_2 diffusion within the leaf and leaf temperature (Tholen et al., 2012). A number of studies have set out to improve understanding of how leaf polarity is established. As we highlight below, these studies have revealed that transformation of a small finger-shaped leaf primordium into a flat polarised structure requires an adaxial/abaxial polarity axis to be defined, the orientation of this axis to be coordinated with cues external to the leaf primordium, and this polarity to be translated into differential growth patterns to form the leaf lamina – the expanded portion (or blade) of the leaf.

Defining adaxial/abaxial leaf polarity

The adaxial and abaxial domains of the leaf primordium are defined by several redundant factors that are expressed specifically in their respective domains and define their identity (Fig. 3). At the heart of this complex network lie class III HD-ZIP transcription factors, such as PHABULOSA, PHAVOLUTA and REVOLUTA, and the GARP family transcription factors KANADI 1-4 (KAN1-4), which determine adaxial and abaxial identities, respectively (Eshed et al., 2001; Kerstetter et al., 2001; McConnell et al., 2001; Emery et al., 2003). In addition, class II HD-ZIP proteins and the AS1/AS2 complex determine adaxial identity (Husbands et al., 2015; Merelo et al., 2016), whereas the auxin response factors ARF2, ARF3 (also known as ETTIN) and ARF4 contribute to abaxial identity (Pekker et al., 2005; Guan et al., 2017). The expression patterns of the adaxial and abaxial determinants resolve into complementary domains as a result of different regulatory mechanisms. First, two types of small RNAs restrict the expression of adaxial and abaxial determinants. The miR165/166 genes expressed in the epidermis of the abaxial domain produce mobile miRNAs that restrict class III HD-ZIP gene expression to the adaxial domain (Kidner and Martienssen, 2004; Tatematsu et al., 2015; Skopelitis et al., 2017), and mobile trans-acting siRNAs (ta-siRNAs) produced in the adaxial domain limit ARF3 and ARF4 expression to the abaxial domain (Chitwood et al., 2009; Schwab et al., 2009; Skopelitis et al., 2017). In addition, regulatory cross-talk between adaxial and abaxial factors contributes to the mutual inhibition of their expression. For instance, KAN1 directly represses *AS2* (Wu et al., 2008) and, in turn, the AS1-AS2 complex represses the abaxial factors *MIR166* and *ARF3* (Husbands et al., 2015). In parrallel, class III HD-ZIP genes are repressed by miR165/166, and the genes coding for these miRNAs are repressed by a class II-class III HD-ZIP protein complex (Merelo et al., 2016). Finally, auxin also plays a role in establishing polarity: its depletion from the adaxial domain, which results from auxin flow from the young leaf primordium to the meristem, is proposed to establish a transient low auxin domain that contributes to leaf polarity (Qi et al., 2014).

Coordinating adaxial/abaxial leaf polarity with external cues

The establishment of leaf polarity is intimately associated with both the recruitment of leaf founder cells (a population of cells that gives rise to the leaf primordium) and the associated repression of meristematic genes: for instance, AS1 contributes both to leaf polarity and to KNOXI gene repression in the primordium. Microsurgical manipulation of the apex led to the suggestion that

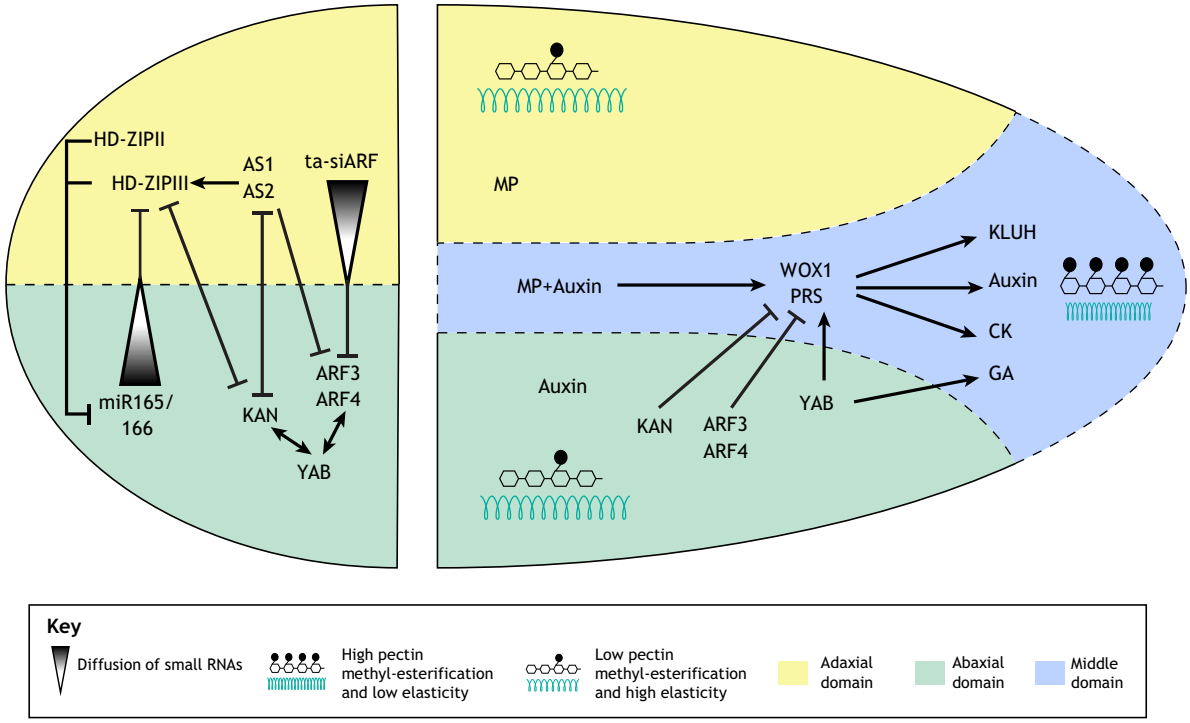

Fig. 3. Regulatory network controlling leaf polarity and lamina outgrowth. The network controlling leaf polarity (left) is based on the mutual inhibition of factors controlling adaxial or abaxial identities (shown in yellow and green, respectively). For the sake of clarity, not all interactions are shown. Leaf lamina formation (right) is associated with the establishment of a third domain, the middle domain (blue). Interactions between adaxially and abaxially expressed factors contribute to the expression of factors defining middle domain identity. These factors in turn promote lamina growth via the modulation of multiple hormonal pathways. Mechanical heterogeneity resulting from differential pectin methyl-esterification also contributes to leaf polarity and lamina formation. Please note that this is a non-exhaustive list of regulators and that this figure integrates results from different species.

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the establishment but not the maintenance of leaf polarity results from an inductive signal, known as the Sussex signal (Kuhlemeier and Timmermans, 2016), that is produced by the SAM, transmitted by the epidermal layer and perceived by the leaf primordium to promote adaxial identity (Sussex, 1954; Reinhardt et al., 2005). This signal might be an as-yet-unidentified lipidic signal that binds to a putative binding domain in class III HD-ZIP proteins and modifies their activity (McConnell et al., 2001). Alternatively, drainage of auxin out of the adaxial domain by the meristem has been proposed to initiate organ polarisation (Qi et al., 2014). Such scenarios propose that feedback between the meristem and the leaf primordium is at the base of a progressive polarisation mechanism, whereas another view suggests that leaf polarity is a local read-out of a pre-pattern that is intrinsic to the meristem (Husbands et al., 2009). Such a view originates from observations that the expression of leaf polarity factors is often polarised early on, before primordium outgrowth, and coincides with a larger expression pattern of these regulators in different domains of the meristem (Heisler et al., 2005; Caggiano et al., 2017). Hence, patterning of the meristem into a central class III HD-ZIP-expressing domain and a peripheral KAN-expressing domain could canalise the formation of auxin response maxima to the region located between these domains. Such canalisation could define the position of the auxin response maximum along the radial axis, whereas its angular position would result from interactions with the two neighbouring organs. Importantly, in such a scenario, the organ primordium would already be patterned at its initiation into two domains with different identities (Caggiano et al., 2017). A possible multi-step scenario emerges from these different observations: the patterning of the meristem would at the same time contribute to the position and to the pre-patterning of the incipient primordium; then, this pattern would be reinforced and stabilised initially by interactions between the primordium and meristem and later by the complex mutual inhibitory interactions between adaxial and abaxial determinants.

Lamina outgrowth

Following its emergence from the meristem, the finger-shaped leaf primordium starts to expand along the medio-lateral axis to form the leaf lamina. A dramatic reduction in lamina expansion, leading in the most extreme cases to the formation of cylinder-shaped leaves, is observed in plants in which adaxial/abaxial polarity is compromised and hence are either adaxialised (Eshed et al., 2001; McConnell et al., 2001) or abaxialised (Waites and Hudson, 1995; Eshed et al., 2001; Kerstetter et al., 2001; Emery et al., 2003). Furthermore, the outgrowth of ectopic structures resembling leaf laminas can also occur at the junction between cellular patches of ectopic abaxial tissues on the adaxial side of the *A. majus phantastica* mutant (Waites and Hudson, 1995). Based on an analogy between wing growth from *Drosophila* imaginal discs and lamina formation from leaf primordia, this observation led Waites and Hudson (1995) to propose that the juxtaposition of adaxial and abaxial domains triggers blade outgrowth, possibly via the activation of downstream signals. Since then, several factors that act downstream of polarity leaf determinants and promote lamina formation have been identified (Fig. 3).

The first factors that were identified to be involved in lamina formation were transcription factors of the YABBY family. In *Arabidopsis*, these factors are expressed in the abaxial and marginal domains, and their expression is promoted by abaxial factors such as KAN (Eshed et al., 2004) and ARF3/4 (Garcia et al., 2006). *Arabidopsis* quadruple mutants for the four YABBY genes expressed in leaves show reduced lamina growth but only limited polarity defects, indicating that these genes act downstream of the network establishing leaf polarity to promote lamina formation (Sarojam et al., 2010). Furthermore, the role of a YABBY gene in the lamina growth of *Juncus prismatocarpus* leaves, which are unifacial and abaxialised, supports a wide role for YABBY genes in lamina formation that is independent of polarity establishment (Yamaguchi et al., 2010).

Further studies revealed the importance of a middle domain that forms between the adaxial and abaxial domains and is marked by the expression of WUSCHEL-RELATED HOMEOBOX (WOX) genes. These include *PRESSED FLOWER* (*PRS*, *WOX3*) and *WOX1* in *Arabidopsis* (Vandenbussche et al., 2009; Nakata et al., 2012) and *STENOFOLIA* (*STF*) in *Medicago truncatula* (Tadege et al., 2011). The middle domain is also marked by a high auxin response, which results from local overlap between a high auxin level in the abaxial domain and the specific expression of activator ARFs such as *MP* in the adaxial domain (Qi et al., 2014; Guan et al., 2017). In turn, MP can directly activate the expression of *PRS* and *WOX1* (Guan et al., 2017). YABBY factors also upregulate the expression of *WOX1*, and the expansion of the middle domain is restricted by abaxial factors such as KAN (Nakata et al., 2012) or ARF3/ARF4, which bind to the same elements as MP in the WOX gene promoters and inhibit their expression (Guan et al., 2017). Inactivation of the WOX genes expressed in the middle domain leads to moderate-to-strong inhibition of lamina outgrowth, depending on the species (Vandenbussche et al., 2009; Tadege et al., 2011; Nakata et al., 2012). Although only mild polarity defects limited to the leaf margin are observed in *prs wox1* mutants, mutations in these genes enhance the polarity defects of partially adaxialised or abaxialised mutants (Nakata et al., 2012). Altogether, these observations lead to a model in which interaction between the abaxial and adaxial domains leads to the formation of third domain – the middle domain – that in turns feeds back to separate the abaxial and adaxial domains while at the same time promoting lamina formation.

Lamina formation depends on cell proliferation and cell expansion, which are regulated both spatially and temporally (Donnelly et al., 1999; Andriankaja et al., 2012). High cell proliferation at the margin of the young leaf primordium is observed when the lamina forms (Donnelly et al., 1999). More recently, the idea that lamina formation relies mostly on the activity of the margin was further pushed forward with the hypothesis that, like the shoot and the root, the leaf contains a meristem at its margins (Alvarez et al., 2016). Following leaf primordium formation, the activity of this meristem would be rapidly restricted to the marginal and proximal domains of the leaf blade and its modulation could contribute to leaf shape diversity. This idea is essentially similar to the previously described concept of a ‘blastozone’ (Hagemann and Gleissberg, 1996). At later stages, growth occurs in a rather dispersed manner along the developing lamina as shown by clonal analysis (Poethig and Sussex, 1985; Kuchen et al., 2012). Whether lamina formation involves a leaf meristem or not, however, may depend on how the concept of meristem is defined. From a molecular point of view, lamina formation involves members of the WOX family that are known to contribute to stem cell fate in the SAM, root apical meristem and cambium (Miyashima et al., 2013), which speaks in favour of the presence of a meristem. However, so far not all members of the WOX family have been associated with stem cell fate, and the pattern of divisions typical to stem cells and their progeny has not yet been recognised in leaf primordia, which argues against the presence of a bona fide meristem. It therefore appears that, because they evolved from branched shoots, leaves in

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seed plants have conserved some of the regulatory pathways acting in branches. However, whether these pathways set up a transiently active meristem is still an open question.

Factors that define the identity of leaf primordium subdomains promote lamina growth in a non-cell-autonomous way, suggesting that their effect may be mediated by downstream mobile signals (Nakata et al., 2012). Auxin appears to be an important factor. Indeed, at the leaf margin, the juxtaposition of adaxial/abaxial domains promotes the expression of YUCCA genes, which encode flavin monooxygenase-like enzymes involved in auxin biosynthesis and which are required for proper lamina expansion (Wang et al., 2011). More generally, HD-ZIPIII and KAN transcription factors antagonistically regulate multiple genes involved in auxin biosynthesis, transport and signalling, thus providing a regulatory module that links leaf patterning to leaf growth (for a recent review, see Merelo et al., 2017). Other factors such as the *M. truncatula WOX STF* gene and the *YABBY FILAMENTOUS FLOWER* (*FIL*) gene also modulate auxin homeostasis (Tadege et al., 2011; Douglas et al., 2017). In addition to auxin, *STF* also controls cytokinin homeostasis (Tadege et al., 2011), and the rice OsYABBY4 protein modulates gibberellin signalling (Yang et al., 2016). Furthermore, the middle domain-specific factors PRS and WOX1 promote the expression of *KLUH*, which encodes a cytochrome P450 that promotes cell proliferation in a non-cell-autonomous manner (Nakata et al., 2012). Together, these observations suggest that the recruitment of multiple hormonal pathways is at play during lamina outgrowth. Finally, patterning of the leaf domain into different domains is associated with differential methyl-esterification of cell wall pectins, leading to mechanical heterogeneity (Qi et al., 2017). Modelling studies and experimental manipulations of the pectin methyl-esterification status further show that partitioning of the leaf primordium into a middle domain with a low cell wall elasticity surrounded by two domains with a higher elasticity is sufficient for asymmetric growth of the primordium and lamina initiation.

In conclusion, leaf polarity is a wonderful example of how a group of cells is progressively organised into different domains, as a result of both extrinsic and intrinsic regulatory mechanisms involving multiple types of molecular players, and how these different domains in turn guide morphogenesis and differentiation to set up a functional and complex organ. Although the link between the polarity of a leaf tissue and its physiology has often been put forward, how the leaf polarity network directs cellular differentiation to form different cell types is not understood. A particular issue related to this general question is how leaf polarity impinges on leaf angle in grasses. Leaf angle, the angle between the blade and the main stem, is an important agronomical trait impacting many factors, such as light interception efficiency and planting density (Mantilla-Perez and Salas Fernandez, 2017). A link between leaf polarity and leaf angle is indicated by the observation that BRs modulate cell proliferation and expansion differentially in the adaxial and abaxial domains at the junction between the blade and the sheath of grass leaves, and thus control leaf angle (Sun et al., 2015). Another remaining question is how variations in the framework described here for bifacial dicot leaves contributes to the formation of other types of leaves, such as unifacial leaves (Yamaguchi et al., 2010), or to leaf shape evolution (Kim et al., 2003b).

Leaf size regulation

A cellular perspective on leaf growth

After a leaf primordium has been specified and its lamina begins to outgrow, it expands dramatically before reaching its final size. *Arabidopsis* shares a basipetal gradient of growth with other species, such as tomato and maize, i.e. these leaves grow for a longer time at their base compared with their apex (Nath et al., 2003; Ori et al., 2007; Nelissen et al., 2012). However, this gradient is not universal within angiosperms, as some species show diffuse growth, acropetal or bidirectional growth gradients (Fig. 4A) (Das Gupta and Nath, 2015).

Two basic cellular processes support the increase in leaf size: cell proliferation (defined as combined cell growth and cell division) and cell expansion (cell growth without cell division). Initially, cells proliferate throughout the entire primordium. Later, a transition from cell proliferation to cell expansion occurs, following a cell cycle arrest front that, in species with a basipetal growth gradient, migrates towards the leaf base before abruptly disappearing (Fig. 4B; Donnelly et al., 1999; Kazama et al., 2010; Andriankaja et al., 2012). After proliferation arrest, a modified cell cycle leads to an increase in cell ploidy driven by DNA replication cycles without cell division. Such endoreduplication is associated with cell expansion in a cell identity-dependent manner. In the epidermis, the correlation between ploidy level and cell expansion is high compared with other leaf cell layers, and is due to the specific expression of AtML1 is this layer (Katagiri et al., 2016). Additionally, specific epidermal cells, called meristemoid cells, have the ability to remain in a proliferative state after surrounding cells have switched to an expansion programme and contribute to almost half of the leaf epidermal cells (Geisler et al., 2000). Thus, three factors contribute to leaf size modulation: the rate and duration of cell proliferation, the rate of cell expansion, and the rate and duration of meristemoid cell proliferation. Although the duration of cell proliferation is known to be related to progression of the cell cycle arrest front (Gonzalez et al., 2012; Hepworth and Lenhard, 2014), how these basic cellular processes are coordinated within an organ remains unclear (see Box 1).

Hormones regulating leaf size

Among the most prominent hormones regulating leaf size are the GAs. GA biosynthesis or signalling mutants have smaller leaves, whereas overexpression of the GA20ox1 biosynthetic enzyme leads to larger leaves (Huang et al., 1998; Richards et al., 2001). Such larger leaves are due to simultaneous increases in cell size and cell number (Gonzalez et al., 2010), the latter being related to the positive effect of GAs on cell proliferation via repression of the cell cycle inhibitors KIP-RELATED PROTEIN 2 (KRP2) and SIAMESE (SIM) (Fig. 5; Achard et al., 2009).

Leaf growth is also promoted by BRs. Like GA mutants, BR biosynthesis or signalling mutants show reduced leaf size, whereas overexpressing the BR receptor gene *BRI1* or the BR biosynthesis gene *DWARF 4* leads to larger leaves (Gonzalez et al., 2010; Zhiponova et al., 2013). These changes in leaf size can be traced back to modulation of cell proliferation rates and cell expansion, with the larger leaves observed in BRI1 overexpressors specifically resulting from an increased cell number, likely through downregulation of the transcription factors PEAPOD1 (PPD1) and PPD2, which limit meristemoid cell proliferation (Gonzalez et al., 2015). Conversely to the situation in the SAM, CK signalling acts alongside GA and BR to promote cell proliferation in leaf primordia. Indeed, artificially decreasing CK levels in the leaf reduces organ size as a result of lower cell division rates (Shani et al., 2010).

Auxin also regulates leaf size, and seems to act primarily on cell expansion, although it also acts on cell proliferation. However, overall auxin effects on leaf growth appear to be equivocal. On the one hand, auxin has been shown to promote leaf cell expansion:

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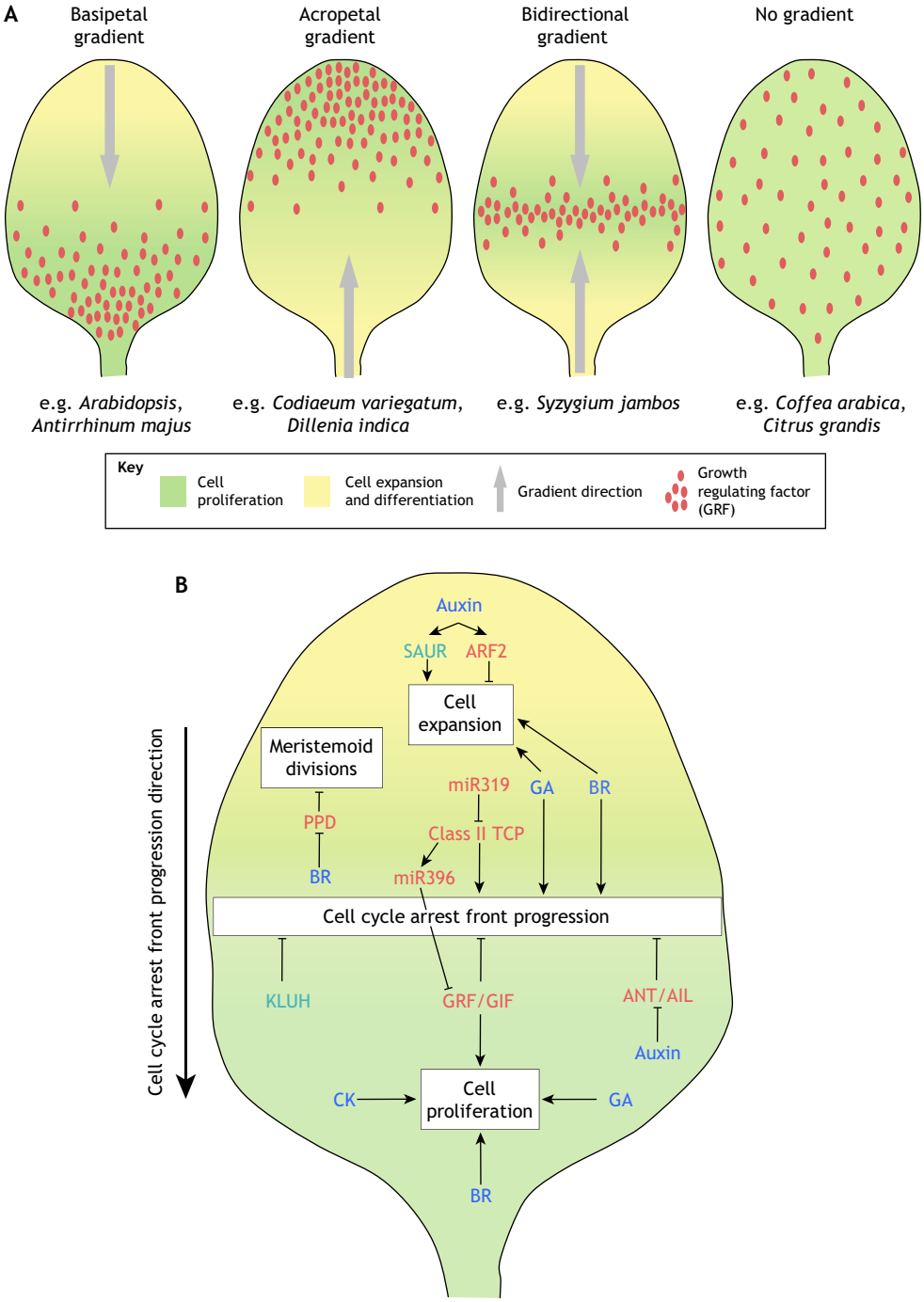

Fig. 4. Growth pattern gradients and regulation. (A) Diverse gradients of growth patterns exist among angiosperms. These growth gradients might be controlled by the miR396/GRF module, which regulates cell proliferation. The area containing proliferating cells is shown in green, and the area with expanding/differentiating cells is represented in yellow. This panel is inspired by experiments reported by Das Gupta and Nath (2015). (B) Schematic view of an *Arabidopsis* leaf during the cell proliferation-to-expansion transition: the cell cycle arrest front migrates from the apex to the blade petiole junction (i.e. a basipetal growth gradient is observed). Three cellular processes control final leaf size: cell proliferation, cell expansion and meristemoid cell proliferation. Factors that regulate these processes and progression of the cell cycle arrest front are shown; hormones are shown in blue, transcription factors in pink and other types of protein in light blue. Please note that this is a non-exhaustive list of regulators. ANT/AIL, AINTEGUMENTA/ANT-LIKE.

larger leaves with larger cells are observed following overexpression of the auxin-induced genes *SAUR19-24* (Spartz et al., 2012) or downregulation of the auxin response repressor IAA28 (Wang and Guo, 2015). On the other hand, auxin may repress cell expansion in leaves, as in plants overexpressing the kinase PINOID (which controls polar auxin transport), in which a higher auxin content and response correlates with a repression of cell expansion (Saini et al., 2017). These lines also show reduced proliferation rates, although it has also been shown that auxin may promote cell proliferation via the induction of ARGOS, AINTEGUMENTA and CYCD3 expression (Hu et al., 2003). Overall, these observations suggest that auxin can have contrasting effects on cell proliferation and expansion. This may, for instance, reflect variations in signalling pathways depending on developmental context or opposite responses to different auxin levels.

Beside these classical phytohormones, an unknown mobile signal that acts downstream of the cytochrome P450 78A-encoding gene *KLUH* has also been suggested to regulate leaf growth. *KLUH* promotes cell proliferation duration in many lateral organs (Anastasiou et al., 2007; Eriksson et al., 2010) and, accordingly, *kluh* mutants form smaller organs due to premature cell proliferation arrest, and *KLUH* overexpressors show prolonged cell proliferation. Because the expression of *KLUH* in a few cells is sufficient to promote whole organ overgrowth, *KLUH* has been proposed to exert a non-cell-autonomous effect, although the nature of the mobile factor through which it acts remains unknown.

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Box 1. The regulation of collective cell behaviour

One central question regarding the development of many multicellular organs is how they reach their final size (Vogel, 2013). Indeed, although individual cell behaviours are variable, final organ size and shape is generally robust (Day and Lawrence, 2000). In the case of the leaf, this can be partly illustrated by compensation mechanisms: genotypes with decreased cell proliferation usually show increased cell size, partially restoring organ size (Tsukaya, 2008). Thus, final organ size appears to be regulated via a non-cell-autonomous mechanism initiated by overall size-sensing mechanisms. However, the molecular mechanisms that generate collective cell behaviours are unclear. The diffusion of a mobile signal could coordinate such cell behaviours. For instance, a KLUH-derived signal has been proposed to be produced from the blade petiole junction and to control progression of the cell cycle arrest front in the leaf (Kazama et al., 2010). The AN3 protein also coordinates cell proliferation through different cell layers in leaves, as it moves from the mesophyll where it is produced to the epidermis (Kawade et al., 2013). Mobile small RNAs also allow sharp transitions between cells not expressing their target and cells expressing it, and in parallel reduce cell-to-cell variations in target gene expression levels, thus buffering noise in gene expression (Skopelitis et al., 2017). Another possible mechanism for sensing organ size is the mechanical stress associated with heterogeneous growth. For instance, at the scale of the entire sepal, differences in growth between regions leads to mechanical stress, with cortical microtubules aligning with the maximal tensile stress at the sepal tip. This, in turn, feeds back on morphogenesis and ultimately controls organ size and shape (Hervieux et al., 2016). Growth can also be heterogeneous at the cellular scale. The epidermis is formed by different cell types, with fast-growing cells such as trichomes that may lead to organ shape distortion. These cells are, however, mechanically isolated by stress-induced cortical microtubule rearrangement in the surrounding cells, thus buffering growth heterogeneity (Hervieux et al., 2017). Therefore, multiple signals contribute to the integration of individual cells into organs.

Transcription factors and miRNA modules regulating leaf growth

Two classes of opposing transcriptional regulators control the switch between cell proliferation and cell expansion: the class II TEOSINTE BRANCHED/CYCLOIDEA/PROLIFERATING CELL FACTORs (class II TCP factors) and the GROWTH REGULATING FACTORs (GRFs). Class II TCP genes promote the switch from cell proliferation to cell differentiation, and their inactivation leads to overproliferation of the leaf margin and hence larger, crinkly leaves with strongly serrated margins (Nath et al., 2003; Efroni et al., 2008). Conversely, GRFs delay the transition from proliferation to differentiation (Kim et al., 2003a; Gonzalez et al., 2010). At the molecular level, GRFs interact with GRF-INTERACTING FACTOR 1 (GIF1; also known as ANGUSTIFOLIA 3, AN3), GIF2 and GIF3 to form a transcriptional module that regulates cell proliferation by controlling CYCLIN B (CYCB) expression (Fig. 5; Lee et al., 2009).

The proper spatiotemporal control of class II TCP and GRF gene expression is in turn regulated by two classes of miRNAs that are essential for leaf development. The regulation of five TCP genes by miR319 (also known as jaw) prevents their ectopic expression and miR396 restricts GRF gene expression to the leaf base (Palatnik et al., 2003; Rodriguez et al., 2010). Interestingly, TCP4 activates *MIR396* thus creating a miRNA-mediated complementary expression pattern between class II TCPs and GRFs (Rodriguez et al., 2010). Furthermore, differences in the *MIR396* expression pattern leading to differential GRF repartitioning along the leaf proximodistal axis could be instrumental for the variable growth gradients observed among angiosperms (Fig. 4A; Das Gupta and Nath, 2015).

Despite the identification of many factors having positive or negative effects on cell proliferation and/or expansion, it is still unclear how they interact and how their effects are integrated over time and space. How a cell switches from proliferation to differentiation in the context of a multicellular organ is still not fully understood. A further understanding of these processes will require simultaneous quantification of the different cellular processes and gene or hormonal signalling activities contributing to organ growth. The recent development of imaging techniques together with new reporters of hormonal activities will no doubt facilitate these investigations.

Leaf shape acquisition

The shapes of angiosperm leaves are diverse. Although the last common ancestor of all flowering plants almost certainly had simple leaves, compound leaves have emerged and been lost several times during evolution (Bharathan et al., 2002). This observation indicates that neither all compound, nor all simple leaves, are homologous to each other. Additionally, the ancestral Brassicaceae had lobed margins, which indicates that the serrated margins of *Arabidopsis* are a derived state (Piazza et al., 2010).

One of the proposed fundamental differences between compound and simple leaves is the level of morphogenetic competence of the leaf margin from which elaborated structures such as leaflets and lobes can be initiated. This level of morphogenetic competence is related to two linked parameters: the size of a morphogenetic region along the leaf margin, called the blastozone (Hagemann and Gleissberg, 1996), and the duration of the morphogenetic window for which this region in maintained in a competent stage. Increasing any of these two parameters leads to more-complex leaves. The morphogenetic competence is linked to progression of the cell cycle arrest front: once cells have entered the differentiation process, morphogenetic competence is reduced. This is supported by the fact that delaying the cell cycle arrest front in *Arabidopsis* greatly increases leaf dissection (Palatnik et al., 2003; Blein et al., 2013; Alvarez et al., 2016). Below, we discuss key molecular factors that are involved in controlling this morphogenetic competence and the subsequent patterning and growth of the leaf margin.

Increased meristematic activity in the leaf promotes dissection

Many regulatory pathways that control the formation of compound leaves are similar to those involved in the maintenance of an indeterminate fate in the meristem (Brand et al., 2007) and in the formation of axillary meristems (Busch et al., 2011; Naz et al., 2013). Notably, a great number of species showing compound leaves exhibit KNOXI gene reactivation in their leaves i.e. KNOXI gene expression is not confined to the meristem (Fig. 6; Bharathan et al., 2002). Such KNOXI gene reactivation in the leaf is required for leaflet emergence (Hay and Tsiantis, 2006; Shani et al., 2009). However, KNOXI gene expression reactivation is not automatically associated with the formation of mature compound leaves, as compound leaf morphology can be lost during secondary morphogenesis (Bharathan et al., 2002; Champagne et al., 2007) or lobes rather than true leaflets can be formed (Piazza et al., 2010). Given the number of times compound leaves have emerged, it is likely that KNOXI activity has been recruited into leaf primordia several times during angiosperm evolution (Bharathan et al., 2002). However, a clade from the Fabaceae family develops compound leaves through an alternative pathway. In this group of complex-leaved species, KNOXI proteins are not found in the leaves and, instead, leaflet emergence is promoted by LEAFY (LFY) orthologues (Hofer et al., 1997; Champagne et al., 2007; Wang et al., 2008). Yet, these species are still sensitive to KNOXI gene expression in the leaf (Champagne et al., 2007), indicating that

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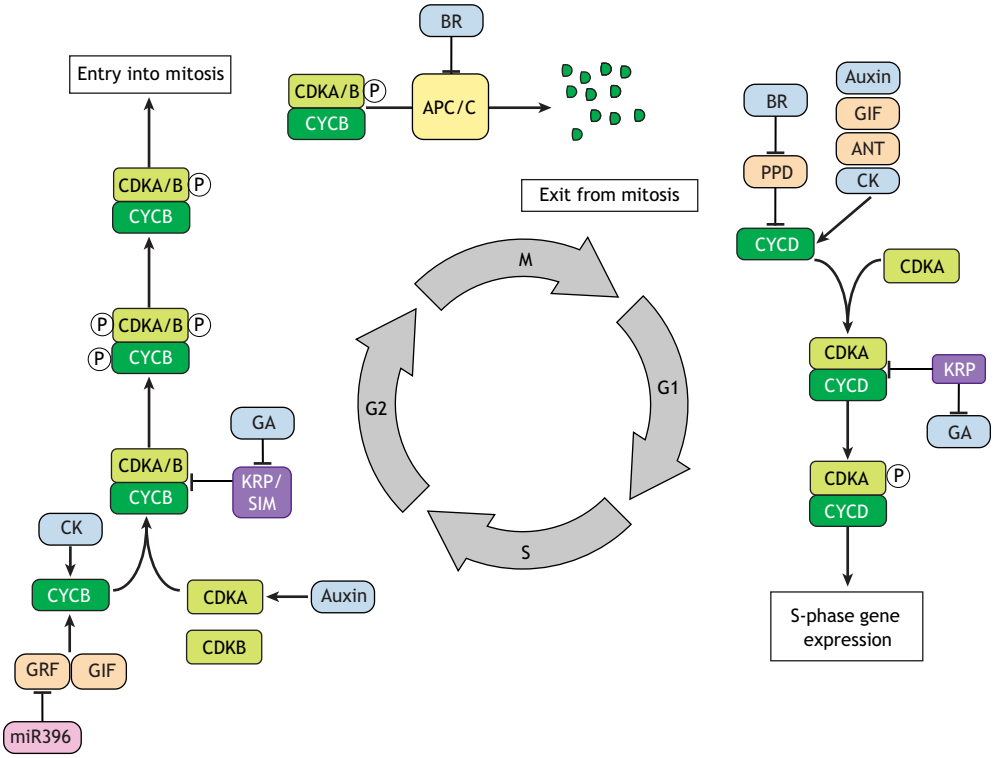

Fig. 5. Cell cycle regulation and leaf size modulation. The cell cycle is divided into four phases: the Gap 1 phase (G1), the synthesis phase (S phase) during which DNA replication occurs, the Gap 2 (G2) phase, and the mitosis phase (M phase) when the cell divides. Cell cycle regulation is achieved via checkpoints between successive phases. These transitions are controlled by CYCLIN (CYC; dark green)/CYCLIN-DEPENDENT KINASE (CDK; light green) protein complexes and their phosphorylation levels. The G1/S transition is operated by the CYCD/CDKA complex, whereas the G2/M transition is mediated by the CYCB/CDKA/B complex. KRPs (purple) are negative regulators of the CYC/CDK complex. Mitosis exit requires anaphase-promoting complex/cyclosome (APC/C; yellow)-mediated CYCB degradation. Regulation by leaf size regulators, including hormones (blue) or transcription factors (orange), is indicated.

KNOXI and LFY functions may have co-existed before LFY function took over leaflet formation promotion in these species.

Compound leaf dissection is also promoted by CKs, which act downstream of KNOXI factors. Indeed, artificial modulation of CK levels in tomato leaves show that CK levels promote leaf complexity and can at least partially compensate for the effects of KNOXI misexpression (Shani et al., 2010). Additionally, the *clausa* classical tomato mutant, which shows increased leaf dissection due to prolonged morphogenetic activity, disrupts a MYB domain transcription factor that negatively regulates CK signalling at the leaf margin (Bar et al., 2016). Thus, CKs act downstream of both KNOXI genes and *CLAU* and extend the morphogenetic window to allow leaflet formation.

A cell proliferation-to-expansion transition reduces leaf dissection

As mentioned above, leaf cells have to be maintained in a proliferative state to be able to induce a new growth axis. Factors that promote cell differentiation are thus negative regulators of leaf dissection. Accordingly, GAs that promote the early cell proliferation-to-expansion transition negatively regulate leaf dissection. For example, mutation of the PROCERA DELLA protein, which is involved in GA signalling, leads to fewer leaflets and smoother margins in tomato (Jasinski et al., 2008). Moreover, modulation of GA signalling partly mediates the effect of the class II TCP protein LANCEOLATE in promoting tomato leaf differentiation (Yanai et al., 2011). Finally, in *Rorippa aquatica*, a semi-aquatic plant from the Brassicaceae family, GA application is sufficient to decrease leaf dissection, and uniconazole (a GA biosynthesis inhibitor) promotes leaf serration (Nakayama et al., 2014).

Molecular factors controlling leaf margin patterning

Whereas the molecular mechanisms regulating growth potential maintenance are diverse, the molecular factors controlling leaf margin patterning events are conserved. Of note, the NAM/CUC/miR164 module and auxin are conserved factors that are required to promote leaf dissection in angiosperms with different types of leaf morphologies (Figs 6 and 7). The NAM/CUC3 genes promote all levels of leaf dissection including margin serration, leaflet emergence and separation (Nikovics et al., 2006; Blein et al., 2008; Berger et al., 2009; Hasson et al., 2011; Cheng et al., 2012). Polar auxin transport-generated auxin maxima are required for leaflet emergence in many different species (Hay et al., 2006; Barkoulas et al., 2008; Koenig et al., 2009; Bilsborough et al., 2011; Zhou et al., 2011). Additionally, elevated CUC gene expression is sufficient to induce leaflet formation in *Arabidopsis* (Hasson et al., 2011), indicating that NAM/CUC3 genes are not only required but are also sufficient to promote leaflet formation in certain contexts.

The interaction between CUC genes and auxin signalling has been thoroughly studied in *Arabidopsis* serrated leaf margins (Fig. 7). The CUC genes seem to have a prominent function, as *cuc2* mutant leaves are smooth (Nikovics et al., 2006). It has also been shown that *CUC2* expression is tempered by *MIR164A*; *mir164a* mutants, as well as plants expressing a miR164-resistant version of *CUC2*, show increased serration (Nikovics et al., 2006). Interestingly, a local increase in auxin response is detected at the site of tooth outgrowth and forms an alternate pattern with CUC2, which is expressed in the sinuses (the spaces between the teeth) (Bilsborough et al., 2011). This alternate pattern is generated via a negative-feedback loop between *CUC2* and auxin: *CUC2* allows PIN1 repolarisation, which creates convergent auxin fluxes that promote the local increase in auxin response at the tooth emergence site, and, conversely, local high auxin response in the tip restricts *CUC2* expression to the sinuses. However, it is still unclear whether the interactions between auxin and the NAM/CUC genes are conserved across species. Interestingly, this CUC/auxin regulatory module is not only involved in leaf shaping and primordium initiation at the SAM, but is also involved in ovule initiation and separation (Galbiati et al., 2013; Gonçalves et al., 2015) and in ligule formation in maize (Johnston et al., 2014), showing that it is

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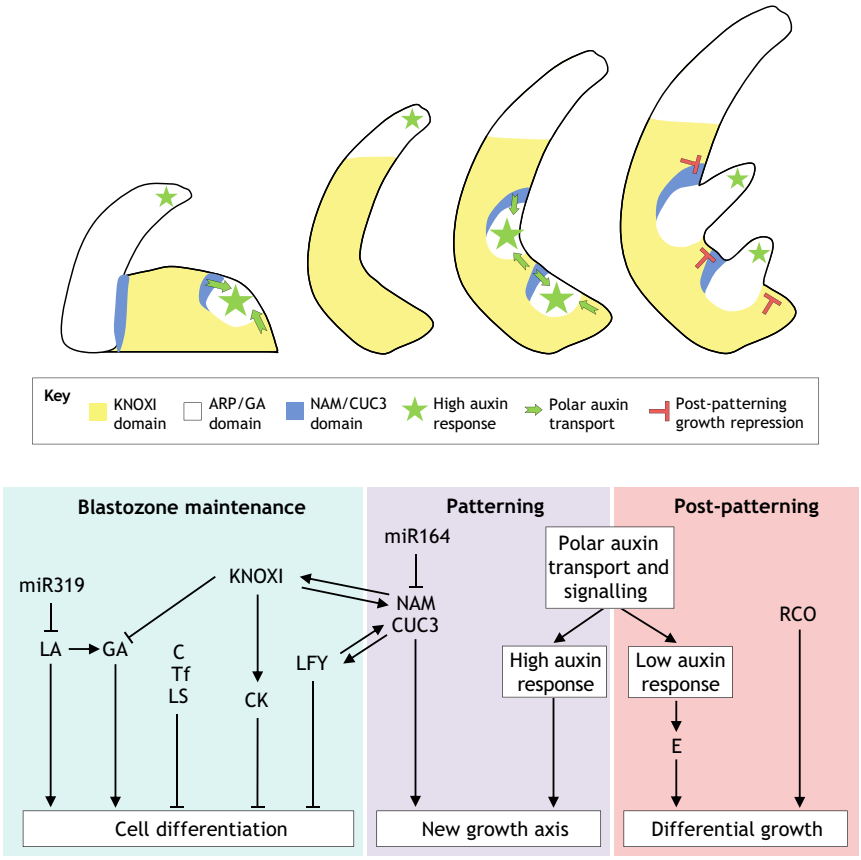

Fig. 6. The control of compound leaf development. A model for primordium initiation and leaflet emergence is shown (top). A summary of the molecular factors that act during the various stages of leaflet emergence is provided (bottom). Three main processes control leaflet emergence: maintenance of the blastozone, patterning of the new growth axis at the margin, and post-patterning differential growth of the new axis. The blastozone is a pseudomeristematic region of the primordium from which a leaflet can emerge. Its maintenance is thus crucial to leaflet emergence and is favoured by meristematic factors. Conversely, factors promoting cell differentiation contribute to blastozone shrinkage. Similarly to simple leaf serration, leaflet patterning involves alternate NAM/CUC3 expression and high auxin response domains. Post-patterning lateral blade growth inhibition can be promoted via two main pathways: low auxin response and *RCO* expression. Note that this figure represents pathways that have been demonstrated to be important in different species. The following factors are conserved: NAM/CUC3, high auxin response, KNOXI expression, the effect of CK and GA. The *LFY* effect is specific to one Fabaceae clade. The effects of *LA*, *C*, *Tf*, *LS* and the low auxin response mediated by *E* were demonstrated in tomato and could be conserved in other species. The *RCO* effect was demonstrated in Brassicaceae, but was likely recruited in different species (see text for more details). Top panel is modified, with permission, from Blein et al. (2010). C, POTATO LEAF; E, ENTIRE; LA, LANCEOLATE; LS, LATERAL SUPPRESSOR; Tf, TRIFOLIATE.

repetitively used for the formation of new axes in the shoot and flowers.

In addition to PIN1, other factors contribute to generating local auxin maxima within the leaf margin. The AUXIN RESISTANT1 (AUX1) and LIKE-AUX1 (LAX) polar auxin importers are expressed at the tips of serrations in a CUC2-dependent manner and contribute to maintaining auxin polarity convergence points (Kasprzewska et al., 2015; Abley et al., 2016). A sharp auxin response is also maintained during tooth growth by the interaction between EPIDERMAL PATTERNING FACTOR-LIKE 2 (EPFL2) and receptor kinases of the ERECTA (ER) family (Tameshige et al., 2016). The receptors are present at the serration tips as their expression is activated by auxin. Their ligand EFP2, which is repressed by auxin, is expressed at the base of the serration. Interaction between the ligand EFP2 and its receptors can only occur in a small region where these proteins are both present and this interaction represses the auxin response, thus forming a negative-feedback circuit that restricts the auxin response to the tip of the serration.

The control of leaf margin differential growth

In addition to the factors described above that regulate the early stages of leaf dissection, other factors act later to maintain the separation between adjacent outgrowths (serrations or leaflets; Figs 6 and 7). One such factor is the *Arabidopsis CUC3* gene, which contributes to sustained tooth outgrowth (Hasson et al., 2011). The *Arabidopsis IAA8* and *IAA9* genes are also involved, and act to inhibit the auxin response to restrict blade outgrowth between outgrowing serrations, whereas in tomato a similar inhibition of the auxin response is required for leaflet separation and results from the partially redundant actions of *SlIAA9*/*ENTIRE* and the miR160-regulated *SlARF10A* gene (Koenig et al., 2009; Ben-Gera et al., 2016). In addition to local inhibition of the auxin response, regions between the outgrowths may have a low auxin content as a result of PIN1-mediated auxin fluxes to the outgrowths. This auxin depletion is partially balanced by YUCCA1-mediated local auxin biosynthesis in the sinuses (Wang et al., 2011; Abley et al., 2016).

Another distinct pathway that contributes to leaflet separation was discovered by a genetic screen in *Cardamine hirsuta* and a quantitative trait loci approach on two *Capsella* species (Sicard et al., 2014; Vlad et al., 2014). These studies revealed that the *REDUCED COMPLEXITY* (*RCO*) gene (also known as *LATE MERISTEM IDENTITY1-LIKE2*, *LMI1-LIKE2*), which encodes a HD-ZIP transcription factor, promotes separation of leaflets by specifically repressing growth between them, via a mechanism that remains to be uncovered.

Beside these intrinsic molecular factors that control post-patterning shape acquisition, external factors have been reported to control organ shape specifically for leaves that are encapsulated into buds. In these cases, three-dimensional folding of the blade combined with mechanical constrains imposed in the bud by neighbouring organs were proposed to be the main determinants of leaf shape (Couturier et al., 2009).

Molecular mechanisms underlying the morphological evolution of leaves

One common way to generate morphological diversity is to modify the expression of key developmental regulators. Such modifications can be mostly quantitative, as for instance in two wild Galapagean tomato species in which increased leaf complexity results from increased *PETROSELIUM* (*PTS*) expression level, which perturbs the balance between the different KNOXI-containing protein complexes formed (Kimura et al., 2008). In other instances,

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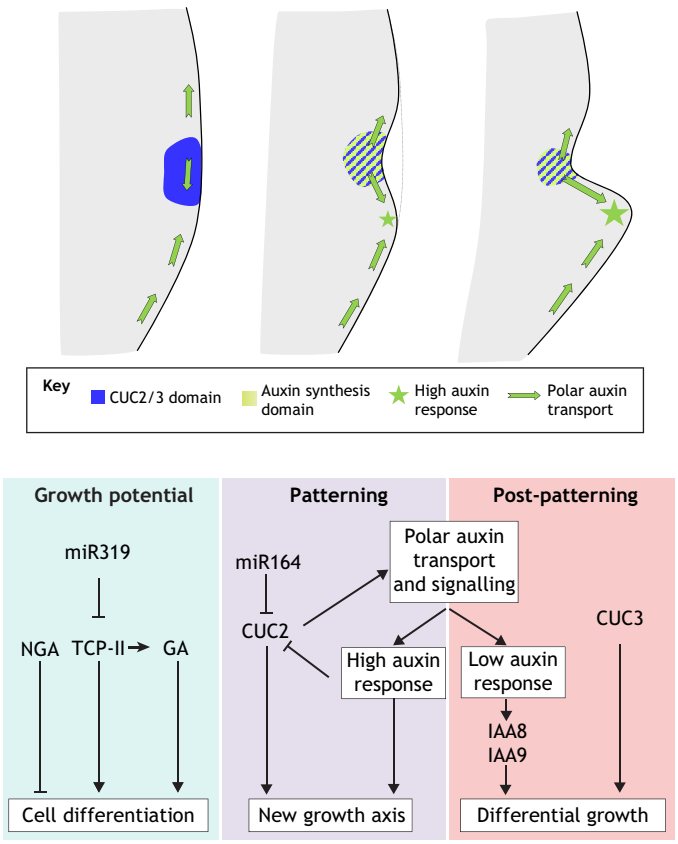

Fig. 7. Genetic and hormonal networks controlling *Arabidopsis* leaf margin serration. A model for serration emergence and tooth growth dynamics at the leaf margin is shown (top), along with an overview of the genetic and hormonal factors that control leaf margin serration (bottom). Precocious cell differentiation does not allow for teeth to be formed whereas delayed differentiation increases serration levels. Sinus and tooth tip patterning is controlled by CUC2 and a localised high auxin response, which alternate along the leaf margin. CUC2 promotes PIN1 convergence point formation and, in turn, a high auxin response restricts *CUC2* expression to the sinuses. Additionally, a low auxin response and *CUC3* expression in the sinuses both promote post-patterning differential growth. NGA, NGATHA.

expression patterns can be modified in a more qualitative way, as for example in the primordia of compound Brassicaceae leaves in which KNOXI gene reactivation is due to changes in upstream regulatory sequences (Hay and Tsiantis, 2006; Piazza et al., 2010). Novel gene functions can also be derived from complex gene histories associated with gene duplication and neofunctionalisation, as for instance is the case of the *RCO* gene. *RCO* appeared after an *LMI1-LIKE* gene duplication followed by a switch in its expression from the tips of marginal outgrowths to their sinuses, due to cis-regulatory modifications (Sicard et al., 2014; Vlad et al., 2014; Vuolo et al., 2016). *RCO* was later lost in *Arabidopsis*, which contributed to leaf shape simplification. In the Brassicaceae family, *RCO* expression levels also explain leaf margin dissection differences between two *Capsella* species (Sicard et al., 2014). Interestingly, *LMI1-LIKE* genes are also involved in leaf shape evolution in domesticated cotton species (Andres et al., 2017). This designates *LMI1-LIKE* genes (along with the KNOXI genes) as evolutionary hotspots that have been recruited several times in angiosperms to modify leaf shape.

Interestingly, in the case of RCO, the possible pleiotropic effects resulting from the modification of its expression pattern are limited by modifications to the RCO protein sequence that reduce its stability (Vuolo et al., 2016). In the same vein, modifying the expression of the *C. hirsuta* gene *ChBP*, which has less pleotropic roles than *ChSTM*, has more potent effects with regard to modifying leaf morphology (Rast-Somssich et al., 2015). In particular, expression of ChBP in *C. hirsuta* leaves makes it susceptible to negative and positive regulation by *ChAS1* and *ChCUC2*, respectively, thus creating an interaction between *ChAS1* and *ChCUC2* that is important for leaf development (Rast-Somssich et al., 2015). Altogether, this suggests a trade-off between gene pleiotropy and gene potency, and indicates that genes with low pleiotropic effects form flexible genetic reservoirs that can contribute to the modification of pre-existing regulatory networks, thereby allowing morphological innovations.

Leaf shape diversity within individuals: heteroblasty

Heteroblasty, a change in the shape of leaves formed on successive nodes (see Fig. 1B), is a general feature of most plant species. In *Arabidopsis*, for example, juvenile leaves are round and have a rather smooth leaf margin whereas adult leaves show a more elliptical shape with small serrations. Such morphological differences result from early divergence of the developmental trajectories of leaf primordia of different nodes (Biot et al., 2016). In recent years, a number of factors that control this divergence have been identified. Of note, these include miRNAs and other small RNAs that target regulators of phase transition and together play a striking role in regulating heteroblasty.

The first pathway described to regulate heteroblasty involves the ta-siRNA-mediated regulation of *ARF3* and *ARF4* (Hunter et al., 2006). The ta-siRNA ta-siARF downregulates the expression of *ARF3* and *ARF4*, which promote juvenile traits. However, expression levels of *ta-siARF*, *ARF3* and *ARF4* do not change as the plant ages, indicating that this pathway is not responsible for the phase transition but rather sets a threshold for the transition to happen. The second key pathway regulating heteroblasty involves the SQUAMOSA BINDING PROTEIN-LIKE (SPL) genes, which are targeted by miR156. SPL9 and SPL10 are transcription factors that promote the juvenile-to-adult phase transition (Wu et al., 2009). Some SPLs are negatively regulated by miR156, which is expressed in a pattern complementary to SPLs and the levels of which decrease during plant maturation, thus allowing de-repression of the SPL genes. Interestingly, miR156 expression is repressed by sugar, which provides a molecular link between plant nutritional status and developmental timing (Yang et al., 2013; Yu et al., 2013). How SPL proteins coordinate multiple aspects of developmental timing is slowly beginning to be elucidated. For instance, it has been shown that SPLs control the expression of miR172, which inhibits AP2 transcription factors and promotes adult characteristics while repressing the competence to flower (Wu et al., 2009). Furthermore, by interacting with TCP proteins, increasing levels of SPL proteins could release CUC proteins from their inhibitory interaction with TCP proteins and thus indirectly promote leaf serration in *Arabidopsis* and leaflet formation in *C. hirsuta* during plant maturation (Fig. 8; Rubio-Somoza et al., 2014).

Leaf shape plasticity: heterophylly

The phenomenon of heterophylly – leaf shape changes in response to abiotic conditions – has been reported consistently in many species (Fig. 1C). For instance, submerged leaves of aquatic plants are more dissected than aerial ones (Bharathan et al., 2002; Nakayama et al., 2014). Plants grown in cold temperatures also tend to have more dissected leaves than plants grown in hotter temperatures (Nakayama et al., 2014; Sicard et al., 2014; Chitwood

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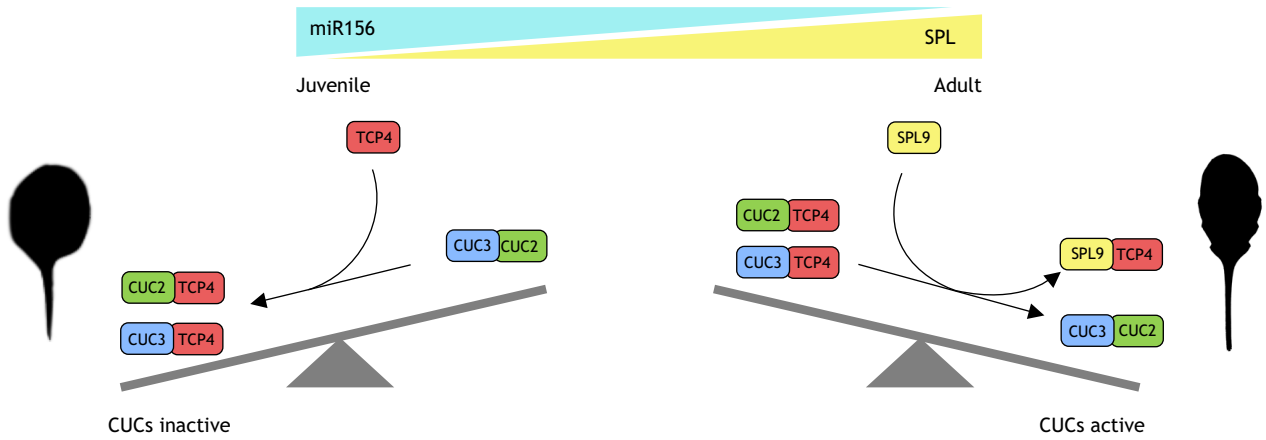

Fig. 8. Molecular regulators of heteroblasty in *Arabidopsis*. The regulation of heteroblasty can be explained by a shift in protein complex availability. The miR319-regulated TCP4 physically interacts with CUC2 and CUC3 proteins thus preventing CUC2-CUC3 active heterodimers from forming. Additionally, TCP4 can physically interact with SPL9, which accumulates during development and destabilises TCP4-CUC heterodimers. As plant age advances, more SPL9-TCP4 and fewer TCP4-CUC complexes are formed, allowing CUC2-CUC3 dimers to promote leaf dissection.

et al., 2016); this correlation has even been used in paleobotany to reconstruct past climates based on fossil records (Greenwood, 2005). Shade avoidance in tomato also increases leaf dissection (Chitwood et al., 2015), although the effect of light is equivocal as decreased light intensity and more closed canopy structures lead to reduced leaf dissection in other species (Nakayama et al., 2014; Ostria-Gallardo et al., 2015).

From a molecular point of view, two pathways have been shown to control leaf dissection in response to temperature (Fig. 9): the KNOXI/NAM pathway in *Rorippa aquatica* and the RCO pathway in different *Capsella* species (Nakayama et al., 2014; Sicard et al., 2014). Interestingly, the KNOXI/NAM pathway is also known for controlling differences in leaf dissection in aquatic plants (Nakayama et al., 2014), and changes in KNOXI/NAM gene expression levels have also been linked to increases in tomato leaf dissection in shade, suggesting that the increase in leaf dissection in tomato shade avoidance is a true heterophilic response (Chitwood et al., 2015). By contrast, decreased leaf dissection levels in reduced light intensities can be traced back to increased class II TCP and decreased SPL gene expression levels (Ostria-Gallardo et al., 2015) and would thus correspond to more juvenile leaves being produced. This is probably due to decreased sugar production in low light and is interpreted as a disguised heteroblastic change (Chitwood and Sinha, 2016). Overall, leaf shape plasticity seems to be mediated by changes in the expression of genes controlling leaf morphogenesis, although the molecular pathways that sense these environmental changes and modify target gene expression remain to be characterised.

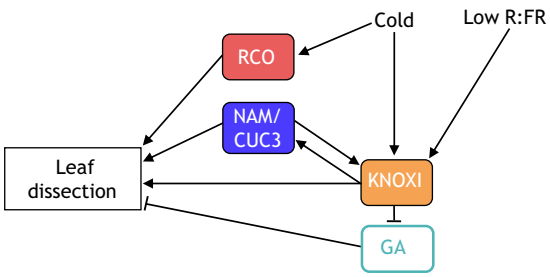

Fig. 9. Molecular regulators of heterophylly. A schematic network showing the main factors that regulate heterophylly, highlighting the abiotic conditions that modify their expression and their effects on leaf dissection. Low R:FR indicates low Red:Far Red light ratio.

Leaf shape is not only variable between different species, it is also plastic. This shows that not only has leaf shape changed during evolution, but plants have also acquired the ability to modify it in response to short time-scale local changes in the environment, suggesting that leaf shape changes might be adaptive (see Box 2). A striking conclusion resulting from many studies is that key regulators of leaf development are also the targets that generate leaf shape plasticity. However, some regulators, such as the RCO genes, are not conserved in all species, which underlines the need to develop studies on diverse, non-model species to widen our knowledge of the repertoire of the actors and mechanisms at play during leaf shape plasticity.

Conclusions

The leaf provides a wonderful biological framework with which to study fundamental questions in developmental and evolutionary biology. Indeed, during recent years tremendous progress has been made in identifying the genetic and molecular networks at play during the transition of a small primordium into a mature leaf. Furthermore, some links have been established between these regulators and the machinery controlling cell proliferation and expansion, which now allows us to envisage how mechanisms acting at multiple levels (molecules and signals, cells and organs) are integrated, and how we can obtain a more comprehensive view of plant organ development. In this respect, computational approaches will no doubt be essential for addressing the particular challenge that leaves contain numerous cells of multiple identities and that their development at the cellular level can be highly flexible. Furthermore, following the full developmental sequence of leaves remains a technical challenge as it is a long process (for example, more than 1 month from primordium to fully grown leaf in *Arabidopsis*) and ranges over several magnitudes of size. Non-destructive observation methods such as tomography methods could provide valuable insights (Dhondt et al., 2010; Lee et al., 2017).

Our expanding knowledge of the regulatory networks at play during leaf development has also highlighted that some of the nodes of this network, or some interactions between network components, are targeted by environmental factors or have been modified during evolution. Such studies are starting to provide a molecular basis for the developmental plasticity of leaves and their variability between plant species. Beside these questions, another one remains largely

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Box 2. Linking leaf shape to leaf function

There is perhaps one striking question that remains to be tackled: what is the function of different leaf shapes? Although this is difficult to test, one possible answer has to do with the main function of leaves: photosynthesis. Many leaf morphological parameters influence photosynthesis and, ultimately, biomass production (Tholen et al., 2012). One study focusing on lobed leaves in temperate trees has suggested that lobed leaf margins could be important for early season photosynthesis, as the leaf margin seems to incorporate more CO_2 (Baker-Brosh and Peet, 1997). More recently, specific studies on tomato and cotton cultivars found a positive correlation between leaf complexity and the expression of genes involved in photosynthesis, which could explain the previously described increase in photosynthesis efficiency and fruit sugar content (Chitwood et al., 2013; Vuolo et al., 2016; Andres et al., 2017). Thus, leaf dissection could increase photosynthesis, at least under some particular environmental conditions. Another hypothesis is that leaf dissection could regulate leaf temperature. One striking observation is that individual leaf temperatures vary little compared with outside temperatures (Helliker and Richter, 2008), which indicates that leaves thermoregulate to some level. Leaf dissection is one of the parameters that affects thermal exchanges between the leaf and its environment, thus explaining why sun leaves (i.e. those developing at the outer part of a plant crown) tend to be more dissected than shade leaves (Vogel, 2009). These are only some possible links between leaf morphology and physiology, and future studies bridging different scientific fields will be required to deepen our understanding of the relationship between leaf shape and function.

unanswered: what is the relationship between leaf development and leaf function (see Box 2)? Leaves are the main photosynthetic organs of plants and have therefore developed numerous cellular, physiological or biomechanical adaptations to fulfil this function. However, despite these general observations, a deeper understanding of how leaf characteristics such as size, shape or structure impact, and are impacted by, photosynthesis will require interdisciplinary work between developmental biologists, physiologists and ecologists. Such a deeper understanding of the interplay between development and biological function will contribute to the design of plant systems with increased photosynthetic performance and thus help increase crop productivity and bioenergy production (Ort et al., 2015).

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Competing interests

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