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REVIEW

SUBJECT COLLECTION: METABOLISM

Looking beyond the gene network – metabolic and mechanical cell drivers of leaf morphogenesis

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ABSTRACT

The above-ground organs in plants display a rich diversity, yet they grow to characteristic sizes and shapes. Organ morphogenesis progresses through a sequence of key events, which are robustly executed spatiotemporally as an emerging property of intrinsic molecular networks while adapting to various environmental cues. This Review focuses on the multiscale control of leaf morphogenesis. Beyond the list of known genetic determinants underlying leaf growth and shape, we focus instead on the emerging novel mechanisms of metabolic and biomechanical regulations that coordinate plant cell growth non-cell-autonomously. This reveals how metabolism and mechanics are not solely passive outcomes of genetic regulation but play instructive roles in leaf morphogenesis. Such an integrative view also extends to fluctuating environmental cues and evolutionary adaptation. This synthesis calls for a more balanced view on morphogenesis, where shapes are considered from the standpoints of geometry, genetics, energy and mechanics, and as emerging properties of the cellular expression of these different properties.

KEY WORDS: Biomechanics, Cell wall, Leaf morphogenesis, Metabolism, Mechanical feedback, Proprioception

Introduction

Plant seeds hold a variety of nutrient reserves, along with molecular regulators, to feed young seedlings, which start their life cycle upon germination. Early on, young plantlets possess a minimal number of organs, namely the cotyledon, hypocotyl and root. Although cotyledons usually share some features of leaves, true leaves only emerge from the shoot apical meristem (SAM) after germination (Lopes et al., 2021). At that stage, the energy source for *Arabidopsis thaliana* development switches from an exhausted stock of nutrients (mainly in the seed and cotyledons) to a metabolic flux fueled by photosynthesis, the yield of which primarily depends on leaf size, shape and anatomy. In this Review, we focus on leaf morphogenesis.

Unlike stems and roots, which can grow without limit thanks to the uninterrupted supply of cells from the meristems, plant leaves generally do not hold a self-renewing meristem and cannot grow indefinitely. More specifically, leaves are initiated at the periphery of the SAM as a protruding pool of founder cells, known as leaf primordia (Shi and Vernoux, 2022). The leaf primordia retain the ability to divide only for a certain period to fix the cell numbers within the leaf. A meta-analysis of the literature revealed the importance of this step for final leaf size. In particular, a compilation of results obtained in different species, and their linear regression, showed that the final size of plant organs depends on cell number rather than cell size, and that cell number is correlated with meristem size (Gázquez and Beemster, 2017).

After gradually losing their proliferative ability, leaf cells enter a second phase of post-mitotic cell differentiation, marked by a significant increase in cell size that coincides with increased vacuole volume and cell wall synthesis activity. Size increase in plants is an irreversible process given that plant morphogenesis, unlike that of most animals, does not involve cell migration and only marginally involves cell death (Hamant and Saunders, 2020). These features make leaves suitable model organs to tackle key, long-standing questions, such as how do organs know when to stop growing (Vogel, 2013).

Beyond these intrinsic factors, environmental cues also have a strong impact on final leaf size and shape. For instance, the typical etiolated phenotype of leaves in darkness, or under shade, is a long petiole with a small, yellowish blade. The associated molecular pathways are now well known, and notably include photoperception (Fiorucci and Fankhauser, 2017) and hormonal pathways (Yang and Li, 2017). Conversely, changing sucrose availability during early *Arabidopsis* development by transferring seedlings to a sucrose-containing medium strongly affects leaf growth by stimulating cell proliferation and postponing the transition to cell expansion, indicating the central role of chloroplasts, and thus photosynthesis, in sugar-induced leaf growth (Van Dingenen et al., 2016a,b).

At the nexus between developmental and environmental cues, the crucial role of transcription factors in orchestrating leaf formation is widely recognized (see, for example, Du et al., 2018; Kalve et al., 2014). However, deciphering transcription factor regulatory networks alone does not help us understand the principles of morphogenesis, and other regulating factors have often been overlooked. This is the consequence of bias in the gene identification strategy – a genetic screen might miss indirect and diffuse properties that build on complex interactions. For example, compensation, in which cell size compensates for cell division in the establishment of leaf size, cannot be fully explained by transcription alone (Ferjani et al., 2008, 2014, 2018). More fundamentally, growth is defined as an irreversible increase in volume; thus, transcription factors are insufficient for morphogenesis and metabolites need to be available to provide the building blocks of an expanding cell wall. Beyond geometry, growth also implies changes in structure, and thus an essential contribution of mechanics.

Recent developments in plant cell biology now make it possible to have such a systemic view on morphogenesis. For instance, multi-omics approaches have started to uncover novel molecular players,

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including hundreds of small molecular compounds with critical roles spanning major developmental transitions during plant growth and including metabolic components and regulators (Omidbakhshfard et al., 2021). Recent progress in cell biophysics has also revealed how the mechanical properties of cells and tissues not only are the outputs of the molecular network but also provide instructive mechanical cues to channel morphogenesis (Trinh et al., 2021). Thus, we are now ready to integrate genetic, metabolic and mechanical regulation to fully understand development. This Review uses the leaf as a model system to investigate these aspects.

The molecular scale – the example of metabolism and compensation

Metabolism has many impacts on final leaf shape. Certain actors, such as that of the target of rapamycin (TOR) pathway, are positioned at the crossroads of developmental, environmental and metabolic pathways (Wu et al., 2019). Here, we discuss more specifically the case of compensation.

In the simplest scenario, leaf size would be a linear function of cell number and size. However, accumulating evidence in the model plant *Arabidopsis thaliana* suggests the presence of compensatory mechanisms (Tsukaya, 2002), such that when the leaf contains fewer cells, the size of each cell is unusually increased [the so-called compensated cell enlargement (CCE)], which in extreme cases, results in a greater than 2-fold increase in cell area (Horiguchi et al., 2006a; Ferjani et al., 2008, 2010, 2013a,b, 2014; Horiguchi and Tsukaya, 2011). Compensation occurs in several mutant and transgenic plants in which the number of leaf cells is significantly reduced (Mizukami and Fischer, 2000; De Veylder et al., 2001; Ferjani et al., 2007; Hisanaga et al., 2013; Horiguchi et al., 2005, 2006b). This suggests that compensation might reflect a general and primary size regulatory mechanism in plants (Tsukaya, 2002, 2008; Horiguchi et al., 2006a). To that end, compensation offers a framework to investigate the links between cell proliferation and post-mitotic cell expansion at the organ level, which might in turn help elucidate size regulatory mechanisms. However, the mechanism behind this compensation is poorly understood.

Compensation has two phases – the induction phase, which corresponds to a reduction in the number of cells due to decreased cell proliferative activity, and the response phase, during which post-mitotic cell expansion of individual leaf cells is significantly enhanced (Ferjani et al., 2007). In mutants that lack FUGU5, the vacuolar H^+-translocating pyrophosphatase (H^+-PPase), mature cotyledons contain ∼60% fewer, but ∼1.8-fold larger, cells compared with the wild type (Ferjani et al., 2007, 2011; Katano et al., 2016; Asaoka et al., 2016; Takahashi et al., 2017). This involves large-scale metabolic modifications. Indeed, in *fugu5* mutants, loss of vacuolar H^+-PPase activity leads to excess cytosolic pyrophosphate (PPi) accumulation, which partially reduces the triacylglycerol (TAG)-to-sucrose conversion and cotyledon cell numbers (Ferjani et al., 2011). During germination, sucrose is synthesized from the TAG of the oil bodies via β-oxidation, the glyoxylate cycle and the tricarboxylic acid cycle (TCA) cycle, as well as gluconeogenesis (Graham, 2008). The development of *Arabidopsis* seedlings relies on TAG-to-sucrose conversion as the sole energy source before they acquire photosynthetic capacity (Graham, 2008). Consistently, excess PPi interferes with the metabolic reactions that produce sucrose, specifically by inhibiting UDP-glucose pyrophosphorylase activity (Ferjani et al., 2018). Whereas decreased cell numbers in *fugu5* mutants have long been ascribed to reduced sugar biosynthesis from TAG, the molecular mechanisms underlying CCE were elusive until recently.

Two studies have provided some insights into the response phase. In the first study, CCE in *fugu5*-mutant cotyledons was shown to be explicitly suppressed by mutations in peroxisomal enoyl-CoA hydratase 2 (*ech2*), and it was proposed that CCE is likely driven by indole-3-butyric acid (IBA)-derived auxin indole-3-acetic acid (IAA) (Katano et al., 2016 and references therein). The second study validated the above hypothesis and further demonstrated that peroxisomal IBA-to-IAA conversion is a prerequisite for CCE to occur, not only in *fugu5* mutants, but also in other mutants such as *icl-2* and *mls-2* (which have defects in the peroxisomal glyoxylate cycle enzymes), and *pck1-2* (which has defects in the cytosolic glucogenic enzyme) (Takahashi et al., 2017 and references therein). More recently, the contribution of IAA to CCE has been corroborated by (1) generating higher-order mutant lines with up- or down-regulation of the endogenous level of IBA, (2) quantifying the endogenous IAA levels in *fugu5* mutants, (3) genetically dissecting the role of auxin signaling in this process by mutating two key transcription factors (ARF7 and ARF19), and (4) addressing the potential implication of vacuolar turgor pressure in CCE (Tabeta et al., 2021).

The above work shows that IBA-to-IAA conversion is a prerequisite for CCE to occur in *fugu5* mutants. Although IBA is not recognized by the auxin receptor (Lee et al., 2014; Uzunova et al., 2016), by serving as a source of auxin, IBA plays a significant role in the IAA supply to sustain CCE during cotyledon development in *fugu5* mutants. Finally, vacuolar acidification mediated by the V-ATPase complex has been shown to play a crucial role in *fugu5*-mutant CCE (Fig. 1), consistent with the role of turgor pressure in distended vacuoles during post-mitotic cell expansion (Geitmann and Ortega, 2009; Hamant and Traas, 2010). The major events involved in CCE in *fugu5* mutants are summarized in Fig. 1 (Tabeta et al., 2021). From this, it appears that final organ size can be the counterintuitive result of compensation mechanisms at the nexus between signaling and metabolic pathways. This is also a role for cell wall properties and turgor pressure, that is plant cell biomechanics, as discussed next.

The cellular scale – turgor and size

Owing to their stiff cell walls, plant cells can sustain high osmotic pressure to drive water and nutrient uptake without the risk of bursting. Nevertheless, to grow beyond their current size, the cells must overcome the physical confinement of the cell walls. This is achieved by cells accumulating turgor pressure that pushes and stretches the cell walls, causing elastic (reversible) deformation and tensile stress buildup, like inflating a balloon (Fig. 2). When the cell wall is stretched beyond a critical point, it triggers plastic (irreversible) expansion of the cell wall (Fig. 2). This process is captured by the Lockhart–Ortega model, which combines the mechanical and hydraulic aspects of plant cell elongation and expansion (Lockhart, 1965; Ortega, 1985; Cheddadi et al., 2019) that, coupled with the synthesis of new cell walls and physiological maintenance of osmotic pressure, ensure plant cell expansion without sacrificing mechanical integrity.

The plant cell wall is composed of polysaccharides, glycoproteins and water in the form of a fiber-enforced gel-like matrix (Cosgrove, 2018). Past research has revealed that different polysaccharides and their modifications contribute to different biophysical properties. For example, cellulose is composed of a linear glucose chain and is a very stiff polysaccharide that can co-align into microfibrils, which are then tethered by hemicellulose chains and embedded in a pectin

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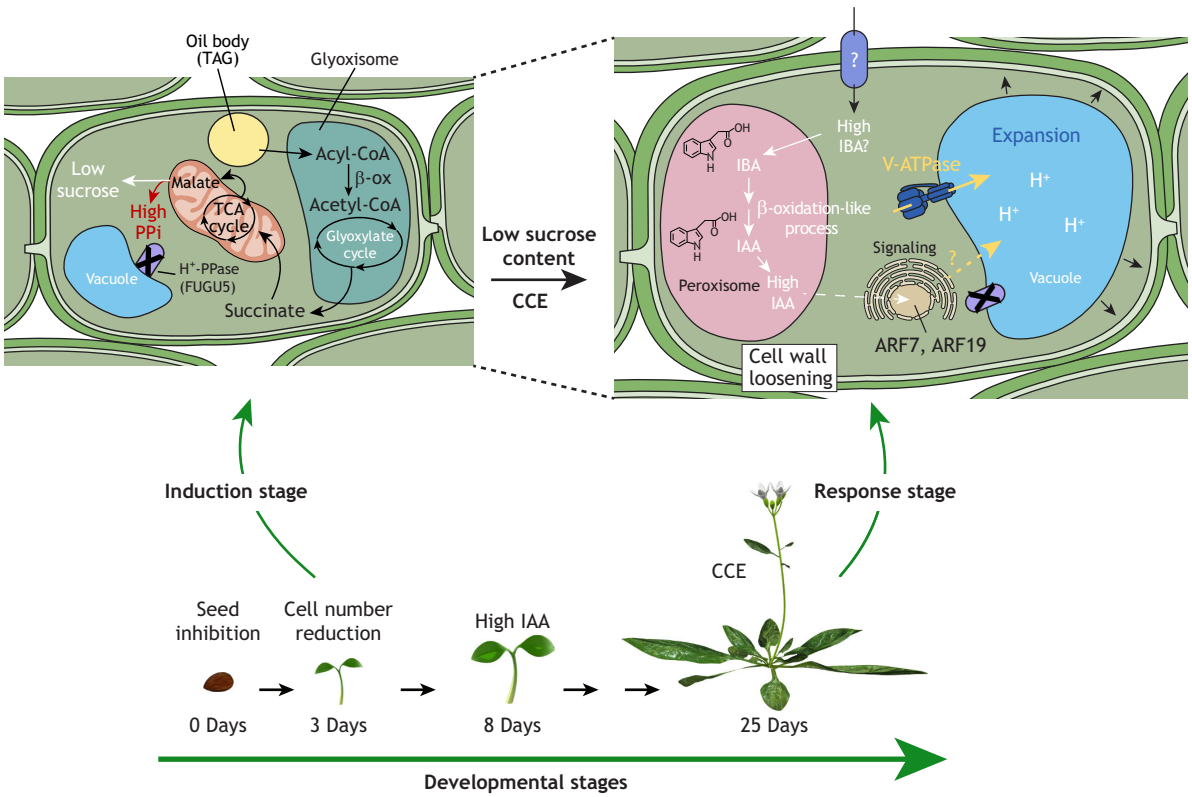

Fig. 1. Proposed model for compensated cell enlargement. The decreased cell number in *Arabidopsis fugu5*-mutant cotyledons is known to be exclusively attributed to a decreased level of TAG-derived sucrose, and IBA-derived IAA has been suggested to mediate CCE as follows. First, upon seed inhibition, excess cytosolic PPi in *fugu5* mutants leads to the inhibition of *de novo* sucrose synthesis from triacylglycerol (TAG), a major seed-storage lipid and substrate for the conversion of fatty acids to acetyl-CoA for glyoxylate bypass that takes place in the glyoxysome. This is owing to inhibition of gluconeogenesis. Second, during seedling establishment, the reduced sucrose content promotes the conversion of indole-3-butyric acid (IBA), an auxin precursor, to indole-3-acetic acid (IAA), the natural phytohormone auxin, leading to an increase in endogenous IAA, which triggers the auxin signaling pathway through the auxin response factors ARF7 and ARF19, transcriptional activators of early auxin response genes. This subsequently activates the vacuolar type V-ATPase, leading to an increase in turgor pressure and cell wall loosening. Ultimately, this drives an increase in cell size and CCE. Figure was modified from Tabeta et al. (2021) where it was published under an CC-BY 4.0 license.

hydrogel (Fig. 2). In a physical model resembling the epidermal wall of the onion scale (modified leaf), a recent study confirmed that cellulose microfibrils are the primary bearers of tensile stress (Zhang et al., 2021). Furthermore, cellulose also accounts for elastic and plastic cell wall deformations by affecting microfibril network reorganization (Zhang et al., 2021). Other studies indicate that pectin controls growth by regulating cell wall elastic modulus, a measurement of ‘stiffness’, depending on its methyl-esterification state (Peaucelle et al., 2011). However, pectin-induced elastic changes in the cell wall were found to be coupled and uncoupled to plastic deformation *in vitro* and *in vivo*, respectively (Kaplan et al., 2019 preprint; Wang et al., 2020). In addition to affecting average stiffness, pectins also differ from cellulose in that the changes they induce are more dynamic than the more long-term and cumulative cellulose-microfibril-derived wall stiffness. In other words, there is increasing evidence supporting the regulatory role of pectins in plant cells (Haas et al., 2021; Wormit and Usadel, 2018). Cell wall composition and biomechanics are complex issues beyond the scope of this Review. For this reason, only the mechanism of directional cellulose deposition will be elaborated on below.

Turgor pressure is the second aspect affecting cell wall expansion. Owing to the high osmolyte concentration and stiff cell walls, plant cells can build up turgor pressure several fold higher than that of atmospheric pressure (Beauzamy et al., 2014). Since turgor pressure is required to drive cell expansion, it would suggest that the higher the pressure, the faster the growth and the larger the cell becomes. However, from an engineering perspective, turgor pressure P and stress in the cell wall σ should be related as follows:

$$\sigma = Pr/2t,$$

where r is the cell radius, and t is the cell wall thickness. So, if the cell wall can bear a comparable amount of stress, determined by its biochemical composition, larger cells should have lower turgor pressure. Indeed, this has been observed recently in the *Arabidopsis* SAM in which larger cells with more neighbors have lower turgor pressure (Long et al., 2020). This may be surprising considering that since plasmodesmata connect most plant cells, any pressure difference between plant cells is expected to equal out. The key realization is that cellular water flux is not instantaneous, and the rate balance between water flux and cell wall expansion is critical for pressure distribution and growth regulation. Higher pressure might further stretch the cell wall, causing faster growth, or repel water uptake, causing slower growth (Long et al., 2020). How this balance is achieved remains an open question. Other studies also suggest that turgor pressure can attenuate growth rate via feedforward regulation (Creff et al., 2021 preprint), that it does not directly associate with oscillatory growth (Kroeger et al., 2011) and that, surprisingly, it is unnecessary for cell wall expansion (Haas et al., 2020). Interestingly, turgor measurements from *Arabidopsis*

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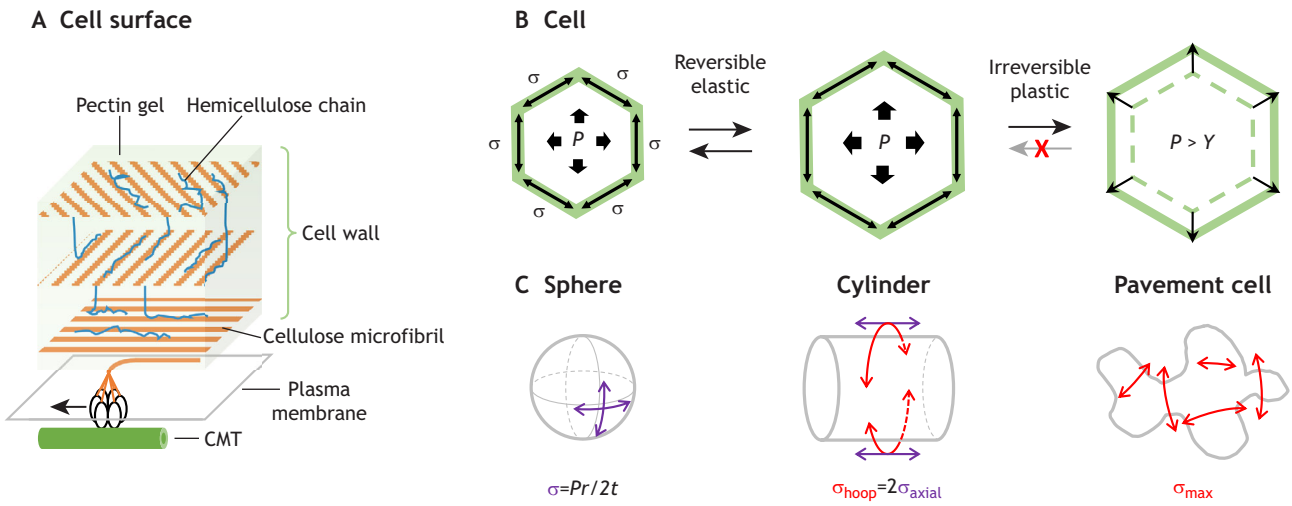

Fig. 2. Cell shape and mechanics. (A) As simplified illustration of the molecular composition of cell wall polysaccharides. Cellulose microfibrils (orange) form co-aligned laminas bound by hemicellulose chains (blue) and embedded in a pectin gel (pale green shaded block). New cellulose microfibrils are synthesized directly above the plasma membrane (grey outline) by the cellulose synthase complex (CSC, white ovals) along cortical microtubules (CMTs, green cylinder). (B) On the cellular level, turgor pressure (*P*) pushes on the cell wall, causing elastic deformation and accumulation of tensile stress (σ). On a longer timescale, if *P* is higher than the threshold *Y*, the cell wall will irreversibly expand, and the cell will grow. (C) Geometry affects stress patterns. Left, a spherical cell has isotropic stress (σ) on its surface. Middle, a cylindrical cell has twice the stress in the hoop direction (σ_{hoop}) than in the axial direction (σ_{axial}), due to local curvature. Right, a pavement cell has maximal tensile stress (σ_{max}) focalized to the neck regions.

cotyledons have revealed that plants can sustain the same turgor level despite growth on media with different water potentials, suggesting the versatility of plant osmoregulation and growth regulation (Verger et al., 2018).

Integrating biochemical signaling and biomechanics

The mechanical effectors of growth are under genetic control. According to the 'acid growth theory', IAA activates the plasma membrane H^+-ATPase, acidifies the apoplast and activates a range of enzymes involved in cell wall loosening (Hager et al., 1971; Hager, 2003). This model was recently consolidated with the identification of a cortical transmembrane auxin signaling pathway that triggers wall acidification (Lin et al., 2021). Although the above theory puts forward the cell cortex as a major driver of cell growth, little is known about the contribution of the tonoplast in the IAA-mediated growth of plant cells. This offers an interesting avenue for future research, especially in light of the previously mentioned contribution of vacuolar acidification by the V-ATPase complex in compensation.

The role of the vacuole in growth might indeed be underestimated. Increased vacuolar occupancy allows cell expansion via a mechanism that requires the leucine-rich repeat extensin–FERONIA receptor-like kinase module, which senses extracellular signals and conveys them to the cell to coordinate the onset of cell wall acidification and loosening, with an increase in vacuolar size (Dünser et al., 2019). More recently, it has been reported that plant vacuoles significantly increased their volume (~2-fold) after incubation with 1 µM IAA (Burdach et al., 2018, 2020), linking auxin and vacuolar acidification, and providing insights into vacuole volume regulation during post-mitotic plant cell expansion.

The integration of biochemical signaling and mechanics might actually provide a much deeper insight into their molecular diversity and related mechanical implications. Living organisms produce thousands of metabolites at varying concentrations and distributions, which underlie the complex nature of metabolic networks. Identifying key metabolites, such as sucrose or IAA precursors, and their roles in regulating cell and organ size provides clues for future studies of the mechanisms of organ size regulation in multicellular organisms. Applying metabolomics would help map the components underlying organ size regulation, and analysis of the related network may help identify the most pertinent targets. However, such molecules must also be considered not only as chemical components but also as physical components. Sugars are also building blocks of the polysaccharides in the cell wall (Verbančič et al., 2018). Most metabolites and ions also play the role of osmolytes, and thus contribute to turgor regulation (Taiz and Zeiger, 2010). Furthermore, the dynamic motion of these molecules can also greatly affect growth. In particular, by using modified versions of myosin proteins, with either longer or shorter arms, it has been shown that leaf growth can be increased and decreased, respectively (Tominaga et al., 2013). To explain this behavior, it was proposed that longer arms would enhance the velocity of cytoplasmic streaming, thus increase metabolic capacities, and ultimately growth rate. This suggests that cell hydraulics, through metabolism, may have a more important role in growth than usually recognized.

However, the multifaceted analysis of molecular and mechanical control of cell growth is not sufficient to understand the formation of a complex, multicellular structure like a leaf. Cell-to-cell interactions are often pictured as essential coordinators of morphogenesis. Here again, this involves signaling, mechanical and metabolic pathways.

Intercellular communication – the case of epidermal pavement cells

Because the epidermis is generally thought to act as a limiting factor for growth in aerial organs (Kutschera and Niklas, 2007; Savaldi-Goldstein et al., 2007; Vaseva et al., 2018; Asaoka et al., 2021), special attention has been paid to epidermal cells. In particular, *Arabidopsis* pavement cells have been a popular model to investigate the role of signaling in cell morphogenesis. Briefly, hormones (notably IAA), Rho GTPase (ROP6 and ROP2), and their respective effectors (RIC1 and RIC4, respectively) pattern the

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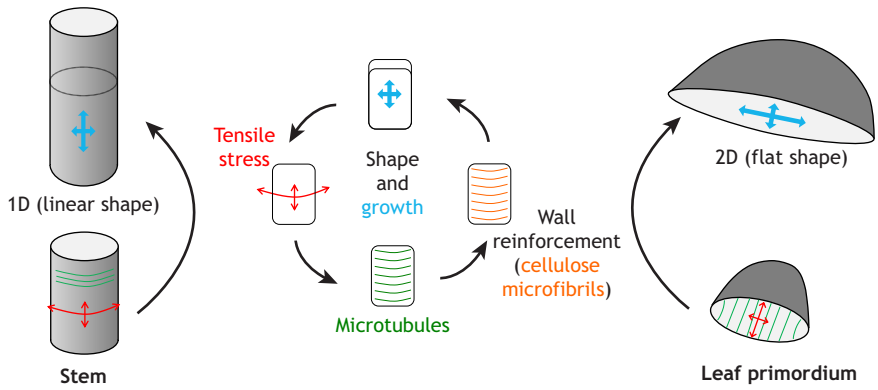

Fig. 3. A mechanical feedback loop maintains symmetric plant shapes. Cortical microtubules align with the direction of the maximal tensile stress in the wall and guide cellulose deposition to allow the wall to resist stress. In turn, this biases cell elongation and stress patterns. Because the maximal tensile stress direction can be prescribed by the tissue shape and growth (tissue stress), this cell-based feedback loop has implications at larger scales. For instance, a cylindrical organ such as a stem exhibits maximal tensile stress in the transverse direction, locking growth along the axis of the cylinder (shown on the left). Similarly, for a flat shape like a leaf, maximal tensile stress can occur across the tissue thickness, thus propagating the flattening of the organ as it grows (shown on the right). Because such symmetric shapes are also highly deformable, they have the additional property of providing proprioceptive cues, further directing organ shapes.

cortical cytoskeleton (microtubules and actin filaments, respectively) to modulate cell wall properties leading to the formation of necks and lobes, respectively (Liu et al., 2021). Metabolic pathways associated with compensation can also affect these shapes, even turning their jigsaw puzzle shapes into twisting S-shaped cells. This is notably the case of the *an-1 fugu-5* double mutant (Gunji et al., 2020).

Interestingly, when assuming that the pavement cell is pressurized by its osmotic pressure, the shape of pavement cells prescribes a stereotypical stress pattern on the outer wall. This notably derives from the fact that a pressurized sphere will have uniform tensile stress on its surface, but a pressurized cylinder will have twice the tensile stress in its circumference than in its longitudinal direction due to its local surface curvature (Fig. 2). Computational finite element models indicate that pressurized pavement cells accumulate maximal stress at the neck regions, which are connected by arrays of cortical microtubules (CMTs) (Sampathkumar et al., 2014). Plant CMTs are proposed to be stress-sensitive and to align along the maximal tensile stress directions (Green and King, 1966; Hamant et al., 2008). In short, the tensile stress pattern emerging from cell shape would induce anisotropic (directional) CMT alignment, which would guide deposition of the stiff cellulose microfibrils as load-bearing fortifications. This deposition will modulate cell growth patterns and inform cell shape and stress patterns, constituting a mechanical feedback loop (Sampathkumar et al., 2014; Bidhendi et al., 2019; Altartouri et al., 2019). Further simulations support this proposition; neck-connecting growth restriction can generate epidermal cell shapes found on the leaf blade (pavement cells), those above the midrib (elongated) and the gradient of shapes in between based on growth directionality (Sapala et al., 2018). Another hotspot of mechanical stress lies in the epidermal wall of the pavement cells, in which the stress magnitude is determined by the largest circle that fits on the cell surface. By having a concave outline, pavement cells can enlarge without accumulating too much stress (Sapala et al., 2018). Conversely, by uncoupling mechanical signaling, for example, by disrupting the function of the putative mechanosensor FERONIA (FER), pavement cells can lose their signature complex shape and risk bursting (Malivert et al., 2021; Tang et al., 2022). This also suggests that mechanical signaling is likely to have a strong interplay with hormonal signaling and metabolic pathways behind pavement cell morphogenesis.

The analysis of the epidermis demonstrates the contribution of a multifactorial intercellular communication to cell shape. However, this gives little insight into the shape of an entire leaf. The jigsaw puzzled shaped cells have been proposed to mechanically reinforce the epidermis, thus contributing to its growth-limiting role for leaf development. However, if leaf cells are like ‘little balloons’, why does the leaf not inflate into a sphere? To address this question, we now need to dig into the three-dimensional anatomy of the leaf.

Regulation of leaf flatness

Most leaves are polar with an upper side containing the palisade parenchyma (where most of light capture occurs) and a lower side containing the spongy parenchyma (where most gas exchanges occur). While this spatial functionalization can make sense in view of the photosynthetic role of this organ, and its selection during evolution, it still does not explain why leaves are usually thin and flat, mechanistically.

In fact, because cells grow at different rates, both within and across layers, one would expect tissues to not be flat or to have a constant thickness by default. In the simplest scenario, mechanical interactions among cells or regions growing at different speeds or in directions would induce buckling events (Coen et al., 2004; Fukushima et al., 2015; Coen and Rebocho, 2016; Sarath et al., 2020). This was formally analyzed in the *cincinatta* mutant in *Antirrhinum* (Nath et al., 2003). This mutant has leaves with ruffled shapes compared with flat leaves in the wild type, and this altered phenotype is related to enhanced growth at the leaf margin. The resulting mechanical interactions generate buckling events, i.e. lead to tissue folding (Nath et al., 2003).

Although this finding suggests that growth must be carefully monitored to ensure flatness, the underlying mechanism remains unknown. The role of biochemical gradients, including metabolic patterning, is likely crucial to coordinate cell behavior and, thus cell mechanics, across larger regions. This coordination involves hormones, miRNAs, peptides, oligosaccharides, reactive oxygen species, metabolite intermediates and lipids, as well as polarity factors (Manuela and Xu, 2020). Mechanical forces might also contribute, notably through feedback mechanisms on these regulators or by acting directly on the effectors of cell growth (Fig. 3). Recently, this hypothesis has been investigated in light of the feedback of tensile stress on cellulose deposition (Zhao et al., 2020).

The deposition of cellulose microfibrils depends on pre-existing cellulose microfibrils in the wall (Chan and Coen, 2020). However, there is also accumulating evidence showing that CMTs guide the trajectory of the cellulose synthase complex, thereby allowing faster and more refined tuning of cellulose deposition (Paredez et al., 2006). In turn, the self-organization of CMTs in oriented arrays is correlated with the predicted pattern of tensile stress in tissues. This provides a feedback loop in which cells resist maximal tensile stress directions by depositing stiff cellulose microfibrils in the same direction (Williamson, 1990; Hejnowicz et al., 2000; Hamant et al., 2008). Interestingly, this behavior has been observed in the epidermis of several organs (the SAM, cotyledons, stem and sepals) and after mechanical perturbations (Trinh et al., 2021). This fits with the idea that, at least in the shoot, the epidermis is load-bearing for the organ (Kutschera and Niklas, 2007; Savaldi-Goldstein et al., 2007; Vaseva et al., 2018; Asaoka et al., 2021; Onoda et al., 2015) and is experiencing high tension (Beauzamy et al., 2015; Verger et al., 2018).

This could also contribute to compensation by providing a supracellular cue for cell growth. Such a role for mechanical feedback has been proposed to contribute to organ growth arrest in sepals, for instance (Hervieux et al., 2016). However, these biomechanical studies are often limited to the epidermis and thus may not be sufficient to determine how leaf flatness arises.

When predicting mechanical stress patterns by computational modeling, it appears that leaves experience strongly anisotropic tensile stress across the organ thickness. Interestingly, CMT analysis along these internal walls also matches this stress pattern. While the CMT pattern matches that of the tensile stress at the outer wall of the epidermis early on during organogenesis, the CMT pattern becomes gradually more disorganized and remains strongly aligned with the predicted maximal tensile direction in internal tissues (Zhao et al., 2020). Computational simulations demonstrate that such an alignment is an essential component of leaf flatness; by resisting stress across the leaf thickness, the organ can expand laterally while coordinating its shape in three dimensions (Zhao et al., 2020).

As mechanical stress is essential for leaf flatness, external perturbations could be expected to affect flatness. This can be observed experimentally; when leaves are stretched artificially, the tissue resists the additional tensile stress and the vasculature modifies its pattern to resist mechanical stress (Bar-Sinai et al., 2016). Albeit at a larger scale, this echoes the CMT–cellulose response to mechanical stress, in which the organ changes its structure to align the stiff elements in the new direction of maximal tensile stress.

Finally, leaves constantly experience mechanical fluctuations, either through variations in their growth (e.g. circadian rhythm or variable osmotic conditions, possibly linked to metabolic fluctuations) or through elastic external deformations (e.g. wind). Could such perturbations also interfere with leaf flattening as they grow? Although this remains open to discussion, a pioneering study suggests that leaves integrate these cues to attain flatness (Derr et al., 2018). In particular, when observing leaf nastic movement, leaf flattening is correlated with leaf movement. In theory, the mechanical interaction between the growth of the midrib and its impact on the leaf lamina could generate leaf movement via buckling. In turn, these mechanical interactions might help the leaf reach flatness while straightening its midrib (Derr et al., 2018).

Leaf morphogenesis integrates different types of cues (e.g. hormones, sugars, turgor and tensile stress) at different scales (molecular, cellular, epidermal and whole organ), and the dynamics of these properties are major determinants of the final leaf size and shape. Further research is needed to understand how the final organ shape and size arises and whether we should pay more attention to standard deviations instead of focusing on the average.

Dynamics – when fluctuating geometries become instructive signals

We have outlined above how plant shapes emerge from molecular and mechanical cues, and that shape itself can be an instructive, proprioceptive cue through geometrical and mechanical feedback on growth regulators (Fig. 3). Such feedback mechanisms have three main properties.

First, whether metabolic or mechanical, feedback mechanisms appear to be mostly conservative, i.e. they maintain the existing geometry, while allowing modifications to the size or aspect ratio (e.g. a longer or wider cylinder). This occurs in the growing stem, in which mechanical feedback on cellulose synthesis maintains its cylindrical geometry while allowing elongation or widening. As metabolism provides osmolytes, it also maintains pressurization and, indirectly, stress feedback.

Second, feedback mechanisms are involved in shape robustness. For example, in the stem, tissue stress is multicellular, providing a coordinating cue for cells. In other words, tissue stress can channel the behavior of many cells independently of their geometry or history. The same would apply to small molecules that diffuse between cells and contribute to regional coordination. This finding questions the scale at which coordination is the most effective for shape robustness (Hong et al., 2016).

Finally, and possibly counterintuitively, feedback mechanisms may explain why the shapes of most plant organs are made of one-dimensional (e.g. stems or petioles) or two-dimensional (e.g. leaves or petals) structures. A more detailed explanation is required for this last property.

One-dimensional and two-dimensional shapes are close to critical points; i.e. they have a high degree of freedom. For example, a stem and leaf can be bent in all directions. This is self-explanatory when observing a young plant in the wind. In contrast, complex three-dimensional shapes are much harder to bend, such as an *Antirrhinum* petal, which would require many buckling events to bend. These structures are usually very stable because their geometry prevents deformation. When shapes fluctuate, they also generate dynamic instructive cues. If it is assumed that fluctuations in shape contribute to proprioception, then we can conclude that feedback mechanisms maintain and channel organ shapes that can fluctuate (Moulia et al., 2021). This may explain why plant organs often have one- and two-dimensional geometries. Symmetric shapes at critical points fluctuate more and therefore provide more proprioceptive cues. In contrast, complex three-dimensional and stable shapes are not fluctuating and are poor providers of proprioceptive cues. Finally, this is consistent with a central paradox of development – organs have reproducible shapes to be able to change all the time.

This conclusion puts forward the reproducibility of shapes within a given species and the role of internal fluctuations (metabolic and mechanical) as instructive cues to monitor shape changes as they occur. However, with the example of nastic movement and the response to wind, the boundary between internal and external fluctuations might not be clear. The same applies at the molecular level – osmolytes can be produced by cells or imported from the environment, and mechanosensing pathways do not appear to differentiate between internal and external forces. Because plant development is mostly postembryonic, organ morphogenesis must also integrate such external fluctuations, whether metabolic or

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Box. 1. Leaf form diversification in evolution – simple leaves

A wider range of leaf forms in nature have often evolved to adapt to the surrounding environment (Tsukaya, 2018). Hence, diversification of leaf morphology is frequently thought to be the result of adaptive evolution to various external stimuli. As illustrated in the figure, *A. thaliana* (Columbia-0) leaves (left) do not display obvious lobes, whereas *A. lyrata* (MN47) (right) develops lobed leaves (scale bar in figure is 1 cm). This unlobed leaf form is a derived trait in the genus *Arabidopsis* (Piazza et al., 2010). A previous study showed that the evolution of the unlobed leaf form in *A. thaliana* from the lobed leaf form involved the loss of expression of *STM* in leaves. The expression of *KNOX1* genes is repressed to suppress the undifferentiated state of the SAM in regions, in which leaf primordia will initiate (Jackson et al., 1994; Long et al., 1996). Such repression is maintained throughout leaf development in the simple-leafed *A. thaliana* (Long et al., 1996). The previous study suggests that the loss of *STM* expression may have been maintained by positive selection (Piazza et al., 2010). Interestingly, *KNOX1* genes are re-activated during leaf development, and re-expression is involved in compound leaf development in *Cardamine hirsuta* (Brassicaceae; Hay and Tsiantis, 2006). Additionally, *REDUCED COMPLEXITY* (*RCO*), an HD-ZIP class transcription factor, is also involved in the promotion of leaflet formation (Vlad et al., 2014). *RCO* arose in the Brassicaceae via a gene duplication event of *LATE-MERISTEM IDENTITY1*, a floral regulator, indicating that *RCO* function was acquired via neo-functionalization. However, *RCO* was secondarily lost in *A. thaliana*, leading to the evolution of a simple leaf phenotype (Vlad et al., 2014). These studies indicate the limitations of using a single model organism, at least in some cases, and highlight the importance of comparative analysis among closely related species, particularly from an evolutionary perspective.

mechanical. Once again, leaves are excellent models to address this question, especially in species with significant differences in morphology according to their habitat.

Environmental responses – plasticity in leaf morphogenesis

In addition to the morphological diversity among species (see Boxes 1 and 2), external perturbations can affect leaf growth and morphology. Indeed, leaf size and form in many plants are variable, to a greater or lesser extent, depending on age and/or environmental factors, such as light, temperature and water availability (Tsukaya, 2005; Fritz et al., 2018). The mechanism underlying such plasticity has been studied (Kim et al., 2005; Poethig, 2010; Fritz et al., 2018). As morphologically intensive cases, heteroblasty often refers to age-related substantial changes, whereas heterophylly refers to significant changes in response to the ambient environment (Zotz et al., 2011). Since heterophylly and heteroblasty involve remarkable modulations in the leaf development, they provide a suitable basis for studying external or internal stimuli and morphogenesis. In this section, we introduce heterophylly in amphibious plants, which is comparatively well-studied in the context of molecular developmental biology.

Amphibious plants are aquatic plants that can grow on land and in water. Amphibious species often exhibit remarkable heterophylly depending on whether the shoot is in the air or water. Generally, leaves produced in water (submerged leaves) are narrower, longer, and sometimes more highly branched compared with those produced in the air (aerial leaves). In addition, the stomatal number and thickness of cuticles are either drastically reduced or absent in submerged leaves. These phenotypes are thought to be adaptive for survival under submerged conditions (Sculthorpe, 1967; Wells and Pigliucci, 2000). The phenomenon of heterophylly in aquatic plants is well-documented, but only recently have the underlying molecular mechanisms been clarified. For instance, *Rorippa aquatica* (a Brassicaceae) and *Hygrophila difformis* (an Acanthaceae) form simple leaves on land and deeply serrated leaves in water (Fig. 4A). In *R. aquatica*, *SHOOT MERISTEMLESS* (*STM*), a *KNOX1* gene, is expressed in the leaves, in addition to the SAM, during the formation of serrated submerged leaves

Box. 2. Leaf form diversification in evolution – compound leaves

The molecular mechanisms underlying compound leaf development have been studied mainly in *Solanum lycopersicum* (tomato) and some legume species. Of these, a variety of insights have been revealed at the genomic and gene levels in tomato research. Unlike *A. thaliana*, tomato *KNOTTED1* (*Tkn1*), a *KNOX1* gene in tomato, is expressed in leaf primordia, and overexpression of *KNOX1* results in a highly complex leaf phenotype (Hareven et al., 1996; Janssen et al., 1998). Tkn1 promotes CK biosynthesis and represses GAs. As mentioned in the main text, these two hormones are involved in the regulation of leaf form. Based on these frameworks, evolutionary developmental studies have also been applied to tomatoes, such as *S. galapagense* (a Galapagean tomato), which displays increased leaf complexity. In this Galapagean tomato, a single nucleotide deletion in the promoter of *PETROSELINUM* was found to alter the KNOX1 protein interaction with BIPINNATA (BIP). The KNOX1–BIP complex regulates leaf complexity through the modulation of *KNOX1* expression. As a result, higher expression of *KNOX1* in the leaves leads to increased leaf complexity in *S. galapagense* (Kimura et al., 2008). Another study demonstrated that Silvery Fir Tree, a Russian heirloom tomato showing increased leaf complexity, has a single nucleotide deletion in the *BIP* gene, leading to higher expression of *KNOX1* in leaves (Nakayama et al., 2021). These studies indicate that metabolic changes might be involved in the diversification of leaf morphology. Although transcription factors orchestrate the initiation of compound leaves, their development is multifaceted and precisely tuned by the dynamic action of phytohormones, whose regulation during biosynthesis and transport cannot be ignored.

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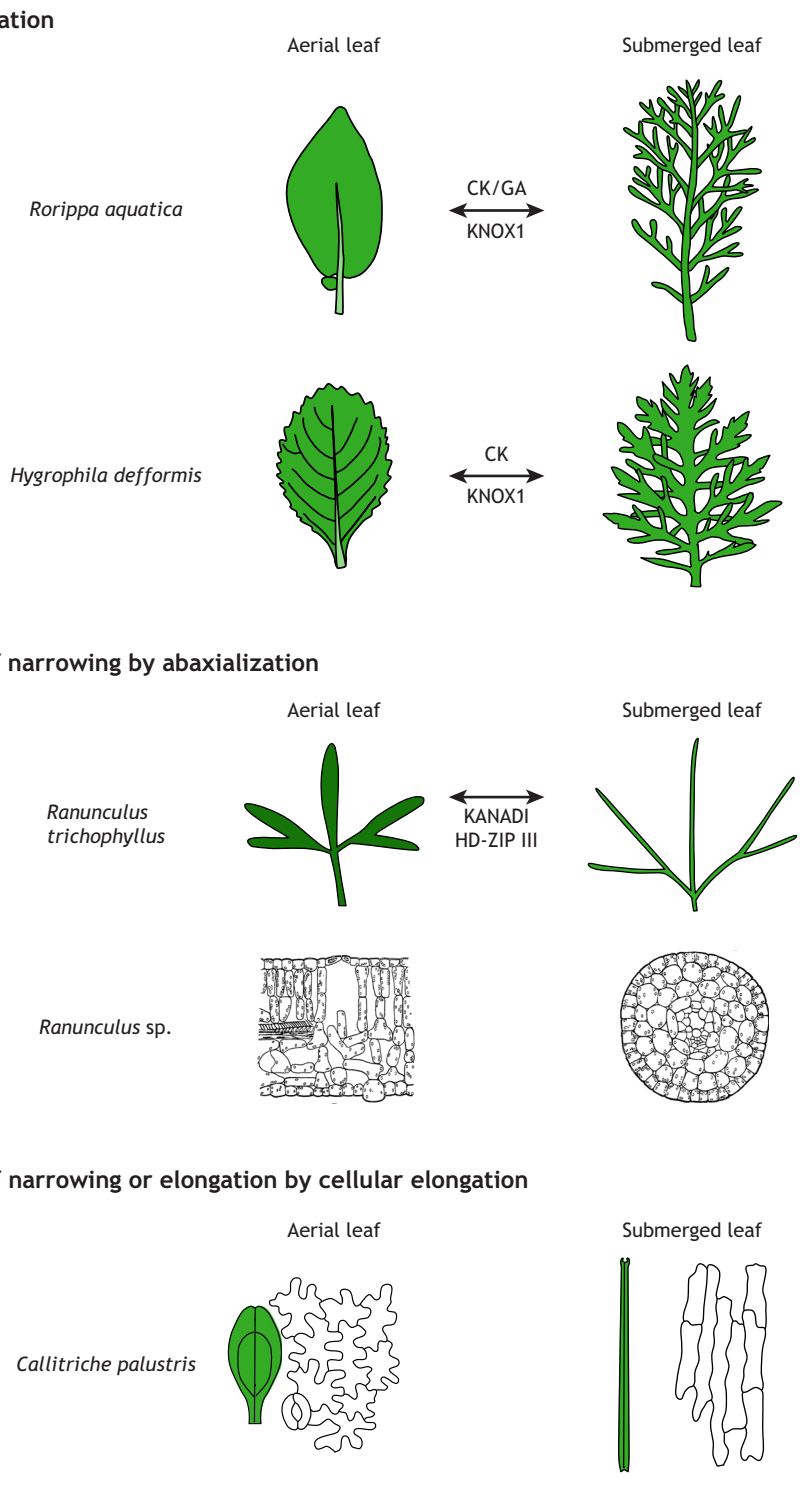

Fig. 4. Changes in leaf morphology in heterophyllous aquatic plants. (A) Heterophylly is associated with deep serration. The plants form a simple leaf in the aerial condition, they form a deeply serrated leaf in the submerged condition. *KNOX1* genes and cytokinin are involved in this type of heterophylly. (B) In *Ranunculus*, the drastic leaf narrowing in the submerged condition has been linked to leaf abaxialization and is supported by the expression patterns of the dorsiventral polarity genes and their anatomy. The leaf section sketches were traced from Sculthorpe (1967). (C) In *Callitriche palustris,* the submerged leaves are more narrowed and elongated than are the aerial leaves. Abaxialization is not observed in this species, but extensive cellular changes, accompanied by changes in CMT orientation, are evident. This type of cellular change may also be involved in other cases.

(Nakayama et al., 2014). The expression of *STM* and the other KNOX1 gene, *BREVIPEDICELLUS*, are also upregulated in the submerged condition in *H. difformis*, suggesting that comparable mechanisms govern the deeply serrated leaf formation in these species (Li et al., 2017). The serrations are also associated with a modulation of the levels of gibberellin (GA) and/or cytokinin (CK) in the leaves of these species (Nakayama et al., 2014; Li et al., 2020). GA can shorten the morphogenetic window in the leaf developmental sequence by promoting differentiation (Bassel et al., 2008; Shwartz et al., 2016). CK is important for prolonged morphogenesis by promoting cell proliferation. Manipulation of CK biosynthesis leads to alterations in leaf complexity (Shani et al., 2010; Shwartz et al., 2016).

There is a prevailing trend for the leaf blade to become narrower as aquatic plants adapt to submergence. The two species mentioned above form compound leaves in water, and each lobe of a submerged leaf narrows dramatically. The molecular mechanism of leaf-blade narrowing, which is not accompanied by serration, has been studied in *Ranunculus* (Ranunculaceae) and *Callitriche* (Plantaginaceae). In *Ranunculus trichophyllus*, which forms

a pedate-compound leaf in the air, each leaflet dramatically narrows and elongates in the water (Fig. 4B). The elongation is correlated with changes in the expression of the homeodomain transcription factors KANADI and HD-ZIP III, which regulate dorsiventral differentiation of the leaves. In submerged leaves, the region of *KANADI* expression, which is usually on the abaxial side, is expanded. The HD-ZIP III gene is expressed on the adaxial side and is restricted (Kim et al., 2018). In other words, the abaxialization prevents leaf flattening and is thought to be responsible for formation of the narrow, submerged leaves. Such anatomical abaxialization in submerged leaves has also been observed in other *Ranunculus* and *Myriophyllum* species (Schenck, 1887; Sculthorpe, 1967) (Fig. 4B).

Interestingly, this is not the case for *Callitriche palustris*, which forms a simple ovate leaf in air and a simple elongated, ribbon-like leaf in water (Koga et al., 2020, 2021) (Fig. 4C). This species shows extensive changes in cell morphology associated with leaf narrowing. For example, although epidermal cells display a jigsaw puzzle shape in aerial leaves, they have a simpler more elongated shape in submerged leaves (Fig. 4C). A similar cellular change, and subsequent simple and elongated submerged leaf formation, are also observed in *Ludwigia arcuata* (an Onagraceae). Interestingly, cellular elongation in submerged leaves is associated with changes in CMT orientation in both species (Sato et al., 2008; Koga et al., 2021). In addition, a previous study argued that the higher turgor pressure in a submerged plant tissue aids cellular elongation of a submerged leaf in a *Callitriche* species (Deschamp and Cooke, 1983). A similar system is possibly involved in the heterophylly of the species mentioned above. Collectively, heterophylly in several aquatic plants likely alters leaf shape via different and possibly synergistic cellular pathways.

The current understanding of heterophyllous leaf development lacks metabolic and biomechanical insights, yet the factors mentioned above are likely key players. The link with CMT seems obvious. Furthermore, plant metabolism can vary greatly upon submergence due to the limitation or modulation of photosynthetic activities (Maberly and Madsen, 2002) and hypoxia (Narsai et al., 2011; Hartman et al., 2021). Furthermore, the different physical properties of the medium surrounding the plant (air or water) may also profoundly affect leaf development. The following questions remain: is leaf morphogenesis in heteropyllous plants a manifestation of metabolic and mechanical reprogramming, and if so, does it start shortly after submergence? Which genetic framework would enable a flexible response to such a mechanical shift? Research using heterophyllous plants, in which leaf development can be addressed in a genetically identical background, would be advantageous, as it would allow to understand the modulability of leaf morphogenesis in a broad context in which metabolic, biomechanical and environmental cues are integrated.

Conclusions and future prospects

Leaf morphogenesis is a multiscale, dynamic and highly variable process that is modulated by metabolism, biomechanics and environmental cues, via and in addition to the core gene regulatory networks. To dissect the relative contributions of these factors, future research will benefit from the promise of quantitative biology (Autran et al., 2021). This includes mapping morphological descriptors, biomaterial and energy flow, mechanical stress fields and environmental fluctuations, as well as their underlying connections. Particular attention should be paid to the relevant spatiotemporal patterns and their variability. Combining precise measurements with modeling approaches will also help us understand how their interactions lead to emergent properties, such as shape reproducibility or physiological adaptability (Roeder, 2021). Such analyses may facilitate revisiting the role of fluctuations as a primary determinant of leaf shape.

Most evolutionary developmental studies have been inspired by modifying or extending data and concepts obtained using model plants. In that sense, revisiting the fields of biomechanics and metabolism will likely inspire evolutionary developmental studies in the future. This is because all developmental phenomena obey the laws of physics. To extend Dobzhansky's famous phrase, "nothing in biology makes sense except in the light of evolution", we might add, "nothing in morphogenesis makes sense except in the lights of biomechanics and metabolism". For instance, CCE, which is involved in the sugar and IAA metabolic pathways in *A. thaliana*, is also induced in response to environmental stimuli in *R. aquatica* (Amano et al., 2015). Reactive oxygen species are increasingly viewed as major regulators of cell wall stiffness and shape changes, suggesting that mitochondrial metabolism is likely to contribute to organ shape, as shown in sepals (Hong et al., 2016). The findings discussed here suggest that metabolic and mechanical studies are essential to understanding the mechanisms underlying the rich morphological diversity of the natural world.

Finally, if morphogenesis is analyzed solely from the point of view of morphometry and genetics, the following two key drivers are missed – the metabolic and mechanical energy underlying shape changes. These new fields have proved challenging to study, especially compared with molecular genetics. Today, developments in quantitative plant biology have made this field accessible to experimentation and modeling of plants. With such an integrative (genetic, metabolic, and mechanical) approach, new ecological and evolutionary development questions can be addressed in non-model plants. This is a revolution in plant science that will likely lead to novel insights in the coming years.

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Competing interests

The authors declare no competing or financial interests.

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