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REVIEW PAPER

Mechanical control of morphogenesis at the shoot apex

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Abstract

Morphogenesis does not just require the correct expression of patterning genes; these genes must induce the precise mechanical changes necessary to produce a new form. Mechanical characterization of plant growth is not new; however, in recent years, new technologies and interdisciplinary collaborations have made it feasible in young tissues such as the shoot apex. Analysis of tissues where active growth and developmental patterning are taking place has revealed biologically significant variability in mechanical properties and has even suggested that mechanical changes in the tissue can feed back to direct morphogenesis. Here, an overview is given of the current understanding of the mechanical dynamics and its influence on cellular and developmental processes in the shoot apex. We are only starting to uncover the mechanical basis of morphogenesis, and many exciting questions remain to be answered.

Key words: Cell wall; growth; material properties; mechanical stress and strain; mechanics; morphogenesis; shoot apex.

Introduction

The process of development transforms the fertilized egg cell into a complex three-dimensional structure. While genetic investigations continue to reveal more about the molecular mechanisms of pattern formation, understanding their relationship with consecutive morphogenetic events remains a central question in developmental biology. New technologies have made it possible to study mechanical properties in young tissues, such as the shoot apex, and have brought back into focus the study of biological forms from a mechanical perspective (Moulia *et al.*, 2011).

When considering tissue mechanics, one needs to consider both stress and strain. Stress is force acting per unit area, and strain is the deformation resulting from the stress; the two parameters are related according to the material properties of the tissue (see Terminology box). In general, there are two modes by which mechanics may instruct morphogenesis (Moulia *et al.*, 2011). From the definition of stress and strain, it follows that local differences in the material properties of a tissue, or the stress the tissues is subjected to, can lead to differential tissue deformation and, therefore, directly control morphogenic events. How such differences in tissue properties and stress patterns are regulated at the molecular level in order to ensure correct morphogenesis is still not well understood and is an active area of research that we will address in this review.

In addition, tissue deformation may feedback via mechanical signals to specify spatial information and alter

Terminology

Stress: force per unit area, (unit: Pascal, Pa=1⁄4 N m^{-2}). The force maybe generated internally or externally.

Strain: the relative change in length of a domain. It can have an elastic and a plastic component.

Elastic: state of materials in which they quickly return to their original state once the stress is removed.

Viscous: state of materials in which they resist shear flow and strain linearly with time when a stress is applied.

Visco-elastic: state of materials in which the relationship between stress and strain depends on time. Exhibiting both viscous and elastic characteristics, they initially resist strain then deform reversibly. Elasticity is usually the result of bond stretching, while viscosity is the result of the diffusion of atoms or molecules inside a material.

Visco-plastic: state of materials that differ from viscous fluids in that they undergo permanent deformation only once the yield stress has been reached. Below that, the material exhibits elastic behaviour.

Isotropic: the property does not depend on direction; the opposite is anisotropic. In terms of growth, isotropic means growth is equal in all directions. In terms of material properties, it means that the stress–strain relationship is independent of the material orientation with respect to the stress. However, this does not exclude differences in the behaviour of the structure due to geometry.

Homogeneous: a property that is the same or similar throughout; the opposite is heterogeneous. In the context of this review, this can refer to the variability in rates of growth but also to the composition of the material, or the material properties.

developmental patterning mechanisms. Plant cells contain considerable turgor pressure and are physically connected to each other by their cell walls. This connection permits the propagation of mechanical signals that may arise due to turgor pressure and differential growth (Boudaoud, 2010). Both stress and strain can be isotropic, if their magnitude is equal in all directions, or anisotropic, if their magnitude varies in different directions. Therefore, mechanical signals can provide vectorial information to each cell, as well as signal gradients over multiple cells. In both animals and plants, developmentally important cellular processes such as cell division, expansion, differentiation, polarity establishment, and fate specification have been found to be sensitive to mechanical stimuli (e.g. Engler *et al.*, 2006; Hamant and Traas, 2010; Mammoto and Ingber, 2010; Moulia *et al.*, 2011; Bosveld *et al.*, 2012). Research is currently focused on assessing the extent to which physical force can act as a pattern-imposing mechanism over large distances, either separately or in addition to the more conventional chemical gradient mechanisms.

The shoot apex (Fig. 1) is a tissue in which mechanical control of morphogenesis has been postulated since the 19th century (Hofmeister, 1868; Thompson, 1942; Turing, 1952; Green, 1992) and has become a representative model for studying the mechanics of primary growth in plant development. In this review, we give an overview of mechanical regulation of morphogenesis at the shoot apex, bringing together knowledge from a range of subjects and thus reflecting the multidisciplinary nature of the field. We first describe the mechanical parameters of the shoot apex and then summarize their effects on cell and tissue growth, cell division plane orientation, and developmental pattern formation, with particular emphasis on the open questions and future challenges.

The structure of the shoot apex

The shoot apex is the growing tip of the plant shoot and has a crucial role in morphogenesis: this is where new leaves or flowers emerge and rapidly expand (Fig. 1A, B) (Steeves and Sussex, 1989; Ha *et al.*, 2010). The shoot apex comprises young organ primordia and the shoot apical meristem proper (Fig. 1C). The meristem can be divided into two distinct functional domains: the central zone and the peripheral zone (Fig. 1D). The stem cell-harbouring central zone consists of isotropically and slowly growing cells, with a low rate of cell division. Meanwhile, the peripheral zone is the site of organogenesis where new organs are initiated and start to differentiate; growth is more anisotropic, and growth and division rates are higher.

In angiosperms, the shoot apical meristem is also organized into distinct domains called the tunica and the corpus (Fig. 1B, E). The tunica consists of the surface (L1) layer and a few cell layers below (L2 and so on). While the number of tunica layers can vary among species or even within a species depending on the developmental phase and growth rate, it is typically two (Fig. 1E) (Steeves and Sussex, 1989). The corpus corresponds to the mass of cells below the tunica (called L3 in the case of meristems with two tunica layers). This internal organization of the meristem reflects the eventual fate of cells; the L1 layer differentiates to form epidermis and, depending on the organ types and species, the L2 and L3 layers make up the ground tissue and vasculature, respectively (Jenik and Irish, 2000).

Dynamics of mechanical stress and strain in the shoot apex

Stress distribution at the shoot apex

The shoot apex, like the whole plant body, is a mechanically stressed structure (Green, 1962). The stress generated within the tissues is sometimes called 'tissue stress' (Peters and Tomos, 1996; Hejnowicz, 1997; Kutschera and Niklas, 2007), or more specifically residual stress (Vandiver and Goriely, 2008), autostress (Moulia and Fournier, 2009), or pre-stress (Ingber 2003). For simplicity, we will refer to it as residual stress, while we will use the term 'tissue stress' to refer to all mechanical stresses that a tissue is subjected to; i.e. the combination of the residual stress and external loads due to gravity and external mechanical stimuli (e.g. wind and touch). In a mechanically protected and light-weight tissue like the shoot apex, however, tissue stress is dominated by the residual stress.

Predicting the mechanical stress in plant cell walls is challenging at both the cellular and tissue levels, as the magnitude

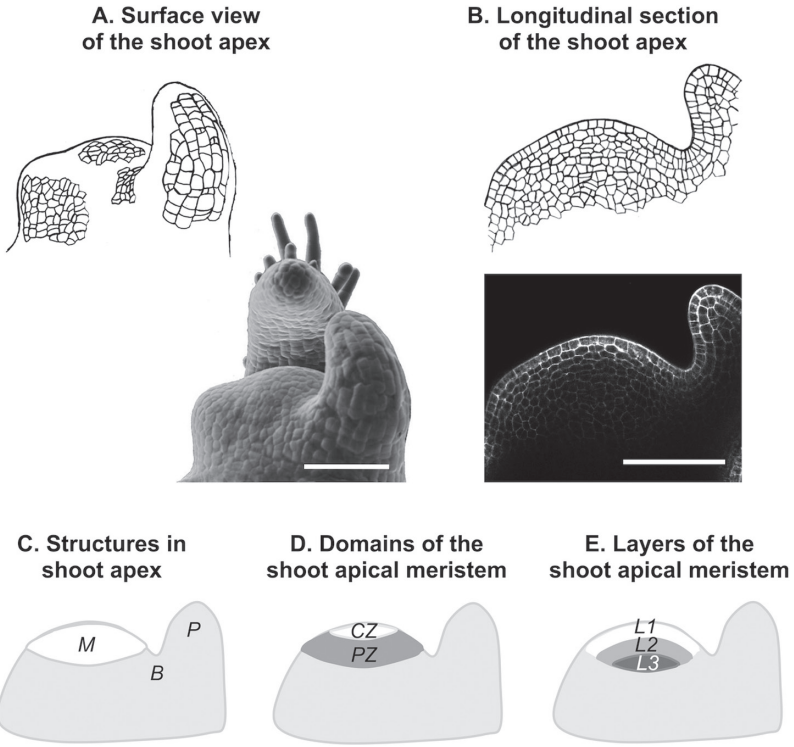

Fig. 1. Structure of the shoot apex. (A, B) Structures of the tomato vegetative shoot apex. Scanning electron micrograph showing the surface view (A) and longitudinal confocal micrograph showing the cells inside (B), both accompanied by line drawings of traced cell definitions. Bars, 100 μm. (C–E) Generalized schematics of the specified information within a typical dome-shaped shoot apex bearing cylindrical young primordia. (C) Major structures. M, shoot apical meristem; P, organ primordia; B, boundary between the meristem and organs. (D) Morphological domains of the shoot apical meristem. CZ, central zone, which harbours the stem-cell population; PZ, peripheral zone, where new organs are generated. (E) Internal (tunica-corpus) organization of the shoot apical meristem. L1, the presumptive epidermis; L1 and L2, tunica layers; L3, corpus.

and distribution of stress is a complex outcome of several factors and stress can only be defined in specific sites and in comparison to some reference state. A model of a single isolated cell can be simplified to a pressure vessel in which the stress in the plane of its wall depends on turgor pressure, cell-wall thickness, cell size, and cell geometry; the bigger the cell or the thinner its walls, the higher the stress. In spherical cells, the stress is isotropic, while in elongated cells it is anisotropic. Plant cells are physically connected by a continuous system of cell walls; therefore, the stress in the plane of the cell wall depends on the mechanical properties of each cell, as well as tissue- or organ-level factors. The stress in the wall of a cell within a tissue can be different from that predicted for an isolated cell of the same shape (Hejnowicz *et al.*, 2000). However, turgor pressure is the dominant force in most cells and results in their cell wall being under tension.

At the tissue level, we can consider homogenized tissue stress, which compares the tissue with their state if they were separated. In the shoot apex, the stresses at this scale may be generated by differential growth between the inner and outer cell layers or between adjacent regions within the same tissue. For example, the faster-growing internal tissues may be compressed compared with their isolated state, while the epidermis may be under tension (Hussey, 1971; Green and Poethig, 1982), and compressive stresses may build up in rapidly growing regions of the tissue that are adjacent to slower growing regions (Selker *et al.*, 1992; Green *et al.*, 1996). Tissue stresses can also be a consequence of variations in the mechanical properties of different cell layers, as has been shown for the stem (Hejnowicz and Sievers, 1996). In the case of the sunflower hypocotyl, tissues described to be under compression relative to their isolated state still shrink if plasmolysed (Peters and Tomos, 2000), reminding us that in most cases the turgor pressure is the dominant force and that cell walls are under tension if compared with an isolated piece of wall.

The pattern of residual stress, especially stress in the surface layer, can be indirectly assessed by observing the consequence of cuts made in the tissue surface. If the tissue surface is under tension, the cut will open; if under compression, the cut will close. Using such a technique, the surface of the shoot apex was revealed to be under tensile stress, although several exceptions were noted where cuts closed, suggesting compression (Snow and Snow, 1951; Hussey, 1973; Dumais and Steele, 2000). In theory, if the tissue behaves like an inflated shell made of a homogeneous and linear material; the stress distribution within the tissue can be estimated from its geometry. Therefore, the discrepancies between the behaviour of the cuts may be due to differences in the size or shape of the shoot apices or cyclic local changes in the geometry

and growth patterns during development (Kwiatkowska and Nakielski, 2011).

The stress distribution for the surface of the shoot apex has been deduced, with the supposition that it is determined mainly by the tissue geometry (Lintilhac, 1974; Dumais and Steele, 2000; Hamant *et al.*, 2008). In the case of the apex of the *Arabidopsis* inflorescence, the tensile stress in the outer cell wall of the L1 layer is predicted to be isotropic at the top of the apical dome, whereas at the meristem flanks, the stress is anisotropic with maximal tensile stress in the circumferential direction (Fig. 2A) (Hamant *et al.*, 2008). In the saddle-shaped boundary between the apical dome and a primordium, the stress is predicted to be strongly anisotropic: the stress is tensile in the circumferential direction and compressive in the radial direction. This unique mechanical status of the boundary, namely the presence of compressive stress, has been confirmed experimentally (Hussey, 1971, 1973).

Interestingly, a different distribution of tissue stress has been described for the sunflower capitulum meristem, which is shaped like a saucer. Using both microsurgical manipulations and computer simulations based on the geometry, Dumais and Steele (2000) showed that circumferential compressive stress occurs in the meristem region where new primordia are initiated, while in the central undifferentiated region there is the tensile stress in both radial and circumferential directions. As various plant species exhibit different meristem shapes and sizes (Cutter, 1971), the stress distribution may be different as well. Alternatively, stress might also be modulated in time and space by other chemical or physical factors, resulting in similar local stress distributions at particular developmental stages.

Strain distribution at the shoot apex

A consequence of stress acting in the cell wall can be an elastic or visco-elastic (reversible) strain, and, provided the stress is sufficiently large, a plastic or visco-plastic (irreversible) strain of the cell walls. In biophysical terms, the latter is growth. The strain can be described in terms of its magnitude (i.e. growth rate), its principal directions (i.e. directions in which growth rates attain the maximal and minimal values), and anisotropy (i.e. the degree to which growth occurs preferentially in any direction) (Hejnowicz, 1984; Dumais and Kwiatkowska, 2002; Coen *et al.*, 2004). Growth anisotropy can be expressed by the ratio of growth rates in the two principal directions. In practice, we often consider growth as the total strain, i.e. how much the material has deformed from one time point to another, as this is easier to measure. It is possible to separate the plastic strain from the elastic strain by looking at the deformation of the tissue during plasmolysis (Peters and Tomos, 2000).

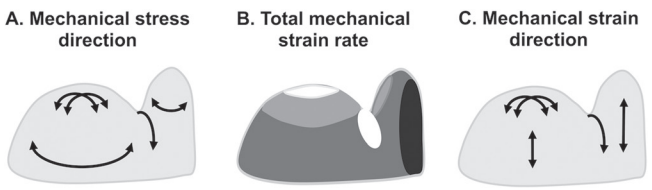

Fig. 2. Distributions of mechanical stress and strain. (A) Directions of mechanical stress. The arrows point to the direction of the maximal stress, according to Hamant *et al.* (2008). (B) Distribution of mechanical strain rate (i.e. cell growth rate measured as a total strain rate), according to studies such as Lyndon (1998), Kierzkowski *et al.* (2012), and Uyttewaal *et al.* (2012). The darker the shading, the higher the strain. (C) Directions of mechanical strain, according to Dumais and Kwiatkowska (2002) and the literature cited in Kwiatkowska (2008).

The total strain rate of the shoot apical meristem has been quantified using time-lapse imaging and replica methods (Dumais and Kwiatkowska, 2002; Kwiatkowska, 2006, 2008; Kierzkowski *et al.*, 2012; Uyttewaal *et al.*, 2012) and will be referred to here as growth. The growth rate is relatively low at the meristem centre and much higher at the periphery, where primordium formation takes place (Fig. 2B) (Lyndon, 1998). In *Arabidopsis* and *Anagallis*, growth at the centre of the meristem is nearly isotropic (see review, Kwiatkowska, 2008, and references therein), while at the periphery, growth is generally anisotropic (Fig. 2C). Growth in the periphery is also highly variable, depending on the developmental stages of adjacent primordia (e.g. Kierzkowski *et al.* 2012; Uyttewaal *et al.*, 2012). At the region where no organogenesis takes place and the meristem periphery is rebuilding following primordium formation, maximal growth occurs in the meridional (radial) direction.

The magnitudes and principal directions of growth at the meristem periphery are highly heterogeneous, with gradients and mixtures of growth rates occurring during primordium or boundary emergence. This is consistent with the existence of residual stress, not only at the tissue level, for instance between the internal layers under compression and the surface layer under tension, but also at a local level between adjacent cells. This heterogeneity is thought to be maintained, at least in part, by the ability of cortical microtubules (i.e. microtubules that form on the inner side of the plasma membrane) to respond to mechanical stress. By reacting to the stress and modifying growth, the microtubules could act to amplify the local growth variability (Uyttewaal *et al.*, 2012) (see below for more details on how cortical microtubules guide cell growth). In the *katanin* mutant, in which a microtubule-severing protein is impaired, the response of the microtubules to mechanical stress is weaker, and growth is more homogeneous than in the wild type. Growth heterogeneity seems to have a biological role, as reducing it has morphogenetic consequences. In particular, organ emergence is delayed, and tissue folding at the boundary is reduced in the *katanin* mutant. In other words, growth heterogeneity may potentiate organogenesis.

Regulation of growth via tissue material properties

Organ initiation at the shoot apical meristem is thought to result from localized accumulation of auxin at the sites of future primordium (Fig. 3A; Reinhardt *et al.*, 2000, 2003). Auxin peak formation is predicted to result from the directional transport

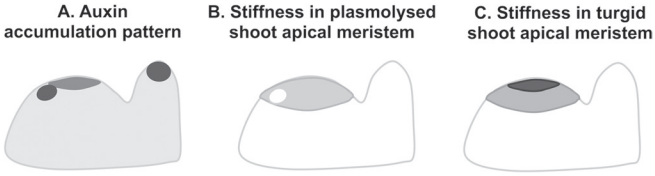

Fig. 3. Material properties of the shoot apex. (A) Schematics of auxin accumulation pattern, based on the data from the *Arabidopsis* inflorescence apices (De Reuille *et al.*, 2006; Smith *et al.*, 2006; Heisler *et al.*, 2008; Vernoux *et al.*, 2011) and tomato vegetative shoot apex (Nakayama *et al.*, 2012). The darker the shading, the higher the concentration. (B) Local stiffness of plasmolysed shoot apical meristem, which mostly reflects the cell wall rigidity, based on he findings from the apex of the *Arabidopsis* inflorescence (Peaucelle *et al.*, 2011; Braybrook and Peaucelle, 2013). The darker the shading, the higher the stiffness. (C) Local stiffness of turgid shoot apical meristem, which indicates deformability of the normal tissue that contains turgor pressure, based on findings from the shoot apical meristem of the *Arabidopsis* inflorescence (Milani *et al.*, 2011) and the tomato vegetative shoot apex (Kierzkowski *et al.*, 2012). The darker the sahding, the higher the stiffness.

of auxin by its efflux carrier PIN1 and the cellular polarity of PIN1 towards neighbours with the highest auxin content (Reinhardt *et al.*, 2003; Jönsson *et al.*, 2006; Smith *et al.*, 2006; Vernoux *et al.*, 2011). Local application of auxin on a *pin1* meristem can restore organogenesis (Reinhardt *et al.*, 2000), but what does this mean in terms of the physical changes required for organogenesis?

Changes in tissue mechanical properties underlying organogenesis

When studying mechanical properties and growth, it is important to distinguish the different behaviours of the cells or tissues. The cell wall is often modelled as having visco-plastic behaviour, whereby, below the plastic yield stress (the threshold stress for the onset of plastic deformation), a cell behaves like a pressurized balloon. It expands elastically if turgor pressure increases and shrinks if turgor pressure decreases. Turgor pressure itself results from a gradient in osmotic potential across the plasma membrane. If osmotic conditions change, cells import or export water, causing turgor pressure to increase or decrease, until a new equilibrium is established within seconds to minutes (Dumais and Forterre, 2012). How much the cell deforms for a given change in turgor pressure depends on a number of factors, including cell size, geometry, and the material properties (i.e. the relationship between stress and strain) of the cell wall. In the simplest case, the cell wall is modelled as being made of a homogeneous material that is linearly elastic and isotropic. More sophisticated models include anisotropic and non-linear material properties (Vogler *et al.*, 2012); however, many models still tend to treat the cell wall as a homogeneous elastic material.

Above the yield stress, the cell walls undergo 'irreversible flow' (Lockhart, 1965) in which they behave like visco-plastic fluids, and the rate of strain is a function of wall stress (Goriely *et al.*, 2008). In the simplest case, this relationship is described by a single extensional viscosity, which is called extensibility. It is important to distinguish the material properties that control elastic and visco-plastic deformation, because they involve different molecular mechanisms (Proseus *et al.*, 1999). The cell wall is a composite, heterogeneous material, with different components exhibiting different properties, and fully capturing its behaviour remains a challenge.

The cell wall is a network of sugar polymers and proteins, among which cellulose microfibrils and hemicelluloses are thought to be the load-bearing elements. While elastic deformation relates to stretching of the cell-wall polymer network, irreversible deformation results from the release of heat energy due to a change in the rates of bonding and unbonding of load-bearing links in the cell wall. Both stiffness and extensibility have been shown to be affected by various proteins within the cell wall such as expansins that are thought to break the hydrogen bonds between cellulose and hemicellulose (Cosgrove, 2005). Glycosylated proteins such as extensins and arabinogalactan proteins self-aggregate and cross-link to other cell-wall components and are likely to play an important role in cell-wall extension (Showalter, 1993; Velasquez *et al.*, 2011; for a recent review of the cell wall, see Cosgrove and Jarvis, 2012, and references therein).

Of particular relevance to organogenesis at the shoot apex, application of auxin has been shown to lower the pH of the cell wall, which in turn activates cell-wall remodelling and softening (Rayle and Cleland, 1970; Cleland, 1973). Auxin also directly induces the expression or activity of cell-wall remodelling factors (Overby *et al.*, 2005), such as expansins and pectin methylesterases (Reinhardt *et al.*, 1998; Braybrook and Peaucelle, 2013). Pectin is an abundant polymer in the cell wall with a versatile mechanical status: its demethylesterification can lead to degradation (and thus wall softening), while in the presence of divalent cations such as Ca^{2+}, higher-order polymerization leads to wall stiffening (for more details, see the recent review by Peaucelle *et al.*, 2012). Localized activation of expansin or pectin modification mimics the application of auxin and can induce primordium outgrowth (Fleming *et al.*, 1997; Pien *et al.*, 2001; Peaucelle *et al.*, 2008). Auxin is not sufficient for organ induction in the absence of pectin modification; similarly, pectin demethylesterification cannot lead to organogenesis in the *pin1* mutant background (Braybrook and Peaucelle, 2013). This interdependence between auxin and the pectin modification suggests a feedback relationship between the mechanical modification of the cell wall and the auxin-mediated developmental patterning.

Using atomic force microscopy on wild-type *Arabidopsis* shoot apical meristems, Peaucelle *et al.* (2011) showed that the sites of organ initiation are elastically softer than the surrounding tissue (Fig. 3B) when making larger (500 nm) indentations with a 5 µm tip. Milani *et al.* (2011) made smaller indentations (40–100 nm) and found the centre of the meristem to be stiffer than the flanks (Fig. 3C). The small tips are thought to be able to measure the local stiffness of the cell wall within a cell face, whereas the larger tips are likely to detect the stiffness of composites of cell-wall materials at cellular

or tissue-level resolutions. It should be noted that determination of the material properties is not a straightforward task and requires models to interpret the results; additionally, the complex composite structure and heterogeneity of the cell wall makes the task all the more challenging (more details on methods of measuring mechanical properties can be found in Milani *et al.*, 2013, this issue). Although elastic and plastic deformations are usually considered to be independent processes, both experiments show correlations between measured elastic properties and eventual plastic growth.

The analogy between the mechanical properties and elastic deformation was investigated in another recent study, in which the deformability of domains within the tomato shoot apical meristem was assessed following osmotically induced inflation and deflation. A non-linear response was observed in the central zone, as the region was easy to shrink yet hard to expand. Consequently, the domain was modelled to be strain stiffened (Kierzkowski *et al.*, 2012). Strain stiffening is a common phenomenon in polymer physics and causes a material to become stiffer when it is stretched beyond a certain threshold. By comparison, the peripheral zone was equally easy to expand and shrink. The inflation/deflation-induced deformations were mostly elastic in both domains, indicating again that elastic deformability correlated with growth rates.

If we are to illustrate the current understanding of how differential tissue mechanical properties relate to regional morphogenic events in the shoot apex, it is as follows. The cell reservoir in the centre of the shoot apex remains relatively slow-growing, because the surface of the domain is made of a material that is strain stiffened under the influence of the tissue internal pressure. In the peripheral zone, the surface becomes stiffer, yet more elastically deformable under the normal pressure from the internal tissues. Within the peripheral zone, auxin accumulates and induces organ formation by making the cell wall softer and much more elastically deformable than the rest of the peripheral zone; this change in cell-wall properties is dependent on pectin modification. In general, growth seems to occur as a consequence of elastic deformation, which transforms into plastic deformation over time, probably via cell wall remodelling.

Turgor pressure is critically important for growth. Therefore, quantifying its strength, particularly if it is uniform or regionally variable within the shoot apex, is important in order to better understand growth regulation. Although turgor pressure can be measured in large cells using pressure probe technology, cells at the shoot apex are too small (~1 mm^3) for conventional pressure probe set-ups (Hüsken *et al.*, 1978; Steudle, 1993). Development of high-resolution pressure probe or micro-indentation methods to measure internal pressure in turgid cells would help to determine whether turgor pressure varies among the different regions of the shoot apex (see Milani *et al.*, 2013, this issue, for more details).

Which layer controls morphogenesis?

So far, mechanical characterization of the shoot apex has focused on the surface layer (i.e. the L1 layer). But is the L1 sufficient to control the rate and orientation of growth of the whole structure? In other words, can we really assume that the shoot apex behaves like a pressurized shell or balloon?

There is theoretical and experimental evidence supporting the hypothesis of epidermal-driven morphogenesis (Kutschera and Niklas, 2007). PIN1-based models of organ formation and spatial positioning typically assume that patterning takes place in the L1, as PIN1 expression is mainly restricted to this layer (Green *et al.*, 1996; Jönsson *et al.*, 2006; Smith *et al.*, 2006). In addition, chimaeric floral organ primordia, in which different cell layers confer different organ identity, developed into a shape according to the identity of the L1 layer (Jenik and Irish, 2001). Brassinosteroid signalling in the L1 both promotes and restricts aboveground tissue growth non-autonomously, possibly via changes in the tissue mechanical properties (Savaldi-Goldstein *et al.*, 2007). Furthermore, L1-specific inhibition of cell division also reduces tissue expansion (Serralbo *et al.*, 2006; Bemis and Torii, 2007).

However, in the peripheral zone of the shoot apical meristem, pectin-dependent tissue softening, which is sufficient and necessary for organ outgrowth, begins in the internal L2 and L3 layers before spreading to the L1 (Peaucelle *et al.*, 2008, 2011). When pectin demethylesterification was limited to the L1, no morphological change was observed. This result confirmed the earlier observation that expansin activity is necessary in all three layers for induction of proper organogenesis (Fleming *et al.*, 1997; Pien *et al.*, 2001). Taken together, these results raise the possibility that the epidermis may not be the only layer limiting growth at the site of organ formation.

Alternatively, the presence of multiple tunica layers in the meristem may suggest that more than the epidermal layer is involved. Accumulating evidence also shows that microtubule and cellulose orientations correlate better with the pattern of growth anisotropy on the inner side of the epidermis in hypocotyls (Crowell *et al.*, 2009; Chan, 2011) and stems (Fujita *et al.*, 2011) (see below for more details). It is therefore possible that, depending on the tissues or the developmental stages, the inner wall of the epidermis, i.e. the one contiguous to the L2, plays a dominant role over the outer wall of the epidermis. While mechanical characterization of the shoot apex has mostly focused on the surface, there is clearly a need to better investigate the inner tissues.

Currently, there are no methods to directly measure the mechanical properties of the inner layers in a non-invasive manner; however, micro-indentation techniques may provide information, as in Peaucelle *et al.* (2011), especially in conjunction with tissue-scale three-dimensional mechanical models to help interpret the data. In animals, differential responses to high-frequency oscillation have been used to characterize the elasticity of inner tissues, as rigid cells vibrate at a higher frequency than softer cells (e.g. Cai *et al.*, 2012), and such methods might be applicable to plants.

Mechanical influence on cell growth direction and cell division plane orientation

Organogenesis is a complex process that also requires directional cell elongation and cell division. Both processes are

responsive to the mechanical environment in which the cells are embedded.

Impact of mechanical cues on cellulose orientation and directional growth

The main cause of growth anisotropy is the anisotropic structure of the cell wall, in particular of the network of cellulose microfibrils. Growth is restricted in the direction parallel to the microfibril hoops due to their high stiffness (Green and Poethig, 1982; Schopfer, 2006). Accordingly, in the primary cell wall, the direction of maximal growth is generally perpendicular to the net orientation of cellulose microfibrils (Baskin, 2005).

As the orientation of cellulose in the cell wall closely resembles the orientation of cortical microtubules in the underlying cytoplasm, it has been hypothesized that the microtubules guide the deposition of the microfibrils (Giddings and Staehelin, 1991). A close coupling between the microtubules and microfibrils has been observed in many plant organs, including the shoot apical meristem and primordia (Hardham *et al.*, 1980). Further studies showed that the microtubules guide the movement of cellulose synthase complexes in the plasma membrane (Paredez *et al.*, 2006; Gutierrez *et al.*, 2009; Li *et al.*, 2012).

Studies of anisotropic growth have focused mainly on stems and roots (Baskin, 2005; Crowell *et al.*, 2010; Chan, 2011) and the mechanisms that regulate anisotropic growth at the shoot apex have not yet been fully explored. Nevertheless, it is known that microtubules are crucial in maintaining anisotropic growth at the apex: the disruption of microtubule organization in the *katanin* mutant results in a significant decrease in growth anisotropy (Uyttewaal *et al.*, 2012), while the depolymerization of microtubules by oryzalin treatment resulted in additional homogenization of the cell size and shape, converting the meristem into a cellular froth (Hamant *et al.*, 2008; Corson *et al.*, 2009).

The organization and dynamics of cortical microtubules at the surface of the meristem is different in different regions of the shoot apical meristem (Fig. 4A) (Hardham *et al.*, 1980; Sakaguchi *et al.*, 1988; Marc and Hackett, 1989; Hamant *et al.*, 2008; Uyttewaal *et al.*, 2012), and may at least in some regions be related to the local growth pattern (Fig. 4B). At the meristem centre, where growth is isotropic, the microtubules are disordered and undergo a constant reorientation, while at the meristem periphery, where growth is anisotropic and maximal in the meridional (radial) direction (Fig. 2A), microtubules are more ordered and mostly oriented circumferentially.

At the boundary between the primordium and the apical dome, where growth is strongly anisotropic, cortical microtubules are consistently oriented along the boundary. In contrast to the other domains of the shoot apex, the microtubules in the boundary are oriented parallel to the principal direction of growth. This unusual correlation shows that growth anisotropy is not only determined by the structural anisotropy of the cell wall, i.e. the orientation of cellulose microfibrils, but might also be related to properties of the microfibrils (Wasteneys, 2004; Fujita *et al.*, 2011), interactions between the microfibrils and the other cell wall components, or the mechanical stress (Baskin, 2005). At the boundary, the stress is predicted to be strongly anisotropic (Fig. 2A) (Hamant *et al.*, 2008), and the high tensile stress along the boundary might specify both the growth direction and the microtubule orientation. This specific orientation of cortical microtubules in the boundary favours the hypothesis that microtubules align according to the maximal stress in the cell wall (Hejnowicz *et al.*, 2000; Hamant *et al.*, 2008). This hypothesis is part of a more general concept that a mechanical signal provides the information necessary to organize cortical microtubules in each cell and coordinate their organization at the tissue level (Williamson, 1990, 1991; Cyr, 1994; Zandomeni and Schopfer, 1994; Wymer *et al.*, 1996; Uyttewaal *et al.*, 2012).

Mechanical influence on cell division

Complex tissue shapes can arise exclusively through the control of cell elongation. The example of the unicellular algae *Caulerpa taxifolia*, which morphologically resembles a fern, demonstrates that elaborate structures can be formed in a single cell (e.g. Harrison *et al.*, 2002). None the less, regulation of cell division plays a crucial role in the complex morphogenesis of most multicellular organisms.

Just like cell elongation, cell division parameters also vary across the shoot apex (Fig. 4C). The central zone of the meristem is defined by a low cell-division rate, while the

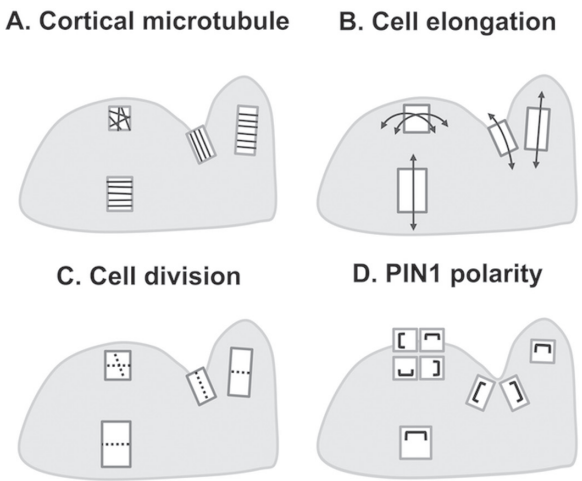

Fig. 4. Directionality of morphogenic processes in the shoot apex. (A) Cortical microtubule orientation, which guides the orientation of the cellulose deposition, based on the patterns observed in the *Arabidopsis* inflorescence apex (Hamant *et al.*, 2008; Uyttewaal *et al.*, 2012). (B) Directions of cell expansion, which are highly anisotropic except for the centre of the meristem. The arrows indicate the direction of the maximal growth. Based on Kwiatkowska (2008) and the literature cited within. (C) Cell division plane orientation, based on observations from the *Arabidospis* inflorescence apex (Breuil-Broyer *et al.*, 2004; Reddy *et al.*, 2004; Rast and Simon, 2008). (D) Polarity of PIN1 proteins within the cell, which direct auxin accumulation dynamics at the shoot apex, according to observations in the *Arabidopsis* inflorescence apex (Reinhardt *et al.* 2003; Heisler *et al.*, 2005, 2010) and the tomato vegetative shoot apex (Bayer *et al.*, 2009).

peripheral zone and the emerging organs contain rapidly dividing cells (Laufs *et al.*, 1998; Reddy *et al.*, 2004). In the boundaries between the primordia and the meristem, cells also divide slowly, mostly along the circumference of the primordia (Breuil-Broyer *et al.*, 2004; Rast and Simon, 2008). In addition, the orientation of the division plane demarcates the meristem into distinct internal cell layers (Fig. 1B, E): in the outermost (L1 and L2) layers, cells continue to divide perpendicularly to the surface of the meristem, whereas in the inner corpus tissue, the L3 layer, there is no preferential orientation for the cell-division planes (Fig. 1B).

How mechanical forces regulate plant cell division is a long-standing question. For instance, bending an explant can increase the cell proliferation rate (Yeoman and Brown, 1971), and externally applied mechanical stress influences the orientation of cell division planes on plant tissue (Lintilhac and Vesecky, 1981, 1984). However, depending on the set-up, new division planes orient either parallel or perpendicular to the maximal stress direction (Lynch and Lintilhac, 1997); this observation suggests that other factors might interfere with mechanical stress.

In particular, cell geometry might be sufficient to dictate the overall cell-division plane via Errera's rule, whereby cells divide at the plane that corresponds to the wall of least area. Mechanistically, transvacuolar strands containing microtubules bridge the nucleus to the cortex and tend to span the shortest distance, possibly acting as geometry sensors (Flanders *et al.*, 1990; Lloyd, 1991). Based on the observation that cells of the same shape do not necessarily display the same division plane, Besson and Dumais (2011) proposed a generalization of Errera's rule, whereby the selected path corresponds to the wall of least area locally. Interestingly, the revised rule is valid only in the absence of external cues. Moreover, the proposed underlying mechanism relies on the tensile status of the cytoplasmic strands, which therefore relates cell geometry back to its mechanical status.

The link between mechanical stress and cell division might be more universal. In animal cells, mechanical stress induced by adhesion to the extracellular matrix is known to influence the orientation of retraction fibres, which in turn modify the orientation of the mitotic spindle (Théry and Bornens, 2006; Théry *et al.*, 2007; Fink *et al.*, 2011). Moreover, mechanical stress has also been shown to influence the mitotic cell-cycle progression (Chen *et al.*, 1997; Montel *et al.*, 2011). Compression of the spindle accelerates the transition between metaphase and anaphase, whereas extension has the opposite effect (Itabashi *et al.*, 2012).

As described above, mechanical stress can guide microtubule orientation in some plant cell types (Fischer and Cyr, 2000; Hamant *et al.*, 2008), most clearly in the boundary domain of the shoot apex (Fig. 4A). This also implies that cell divisions in this domain are oriented preferentially along the crease, via cortical microtubule alignment along the stress (Figs 1A and 4A, C). This preferential orientation reinforces the main stress orientation along the crease, and thus contributes to further deepening of the crease and shaping of the meristem and organ primordia. The division plane in plant cells is delimited by the pre-prophase band, a bundle of cortical microtubules that forms along the circumference of the future division plane (Mineyuki, 1999). An important question to be addressed is whether the cortical microtuble orientation during interphase determines the orientation of the pre-prophase band.

Mechanical regulation of developmental pattern formation

Beyond the role of mechanical signals in orientating growth and cell division, one of the key questions in development and biomechanics is whether physical forces and/or the geometry and deformation of tissues can instruct specification of cell identity.

Can tissue mechanics influence developmental patterning?

Studies in *Drosophila* revealed that the dorsoventral patterning gene *TWIST* is mechanosensitive in the embryo (Farge, 2003). The fates of some animal stem cells have also been shown to depend on their mechanical environment; for example, they can become either neuroblasts or osteoblasts in the presence of either a soft or a stiff matrix, respectively (Engler *et al.*, 2006).

There is some experimental evidence suggesting that similar types of regulation occur in plant development. For example, compressing the sunflower inflorescence apex resulted in the formation of ectopic leaves or modification of the identity or position of the florets (Hernandez and Green, 1993; Green, 1999). In *Arabidopsis*, local application of expansin proteins induced organogenesis accompanied by primordium gene expression (Fleming *et al.*, 1997), indicating that changes in cell-wall properties can turn on suites of developmental pathways. Similarly, a new lateral root can be induced by bending of the primary root (Ditengou *et al.*, 2008; Richter *et al.*, 2009). Such mechanically induced lateral root formation was preceded by the relocalization of the auxin efflux carrier PIN1 within the protoxylem cells near the site of induction, suggesting that mechanical cues may control developmental patterning via the polarity of auxin transport (Ditengou *et al.*, 2008) and/or be integrated with the endogenous patterning mechanism (Moreno-Risueno *et al.*, 2010).

Although the molecular mechanisms controlling PIN1 polarity and thus the dynamics of auxin accumulation in the shoot apex (Figs 3A and 4D) are still largely unknown, two studies have recently pointed out roles of mechanics in this process (Heisler *et al.*, 2010; Nakayama *et al.*, 2012). Through laser ablation and cell-wall weakening (Heisler *et al.*, 2010), or a combination of osmotic treatments, mechanical perturbations, and growth inductions (Nakayama *et al.*, 2012), the level and distribution of PIN1 proteins within the cell, including the polarity, were shown to be affected by the orientation and the magnitude of mechanical stress and/or strain acting on the cell. The mechanical modulations also impaired, although weakly, the auxin accumulation pattern and the downstream organ growth (Nakayama *et al.*, 2012). However,

exactly how cells perceive the mechanical signals and alter PIN1 polarity is as yet unknown. It has been shown that PIN1 and cortical microtubules respond to mechanical stress independently of each other (Heisler *et al.*, 2010); therefore, the two molecular responses may be two parallel outputs of a sensing mechanism or may act downstream of two separate mechanosensing factors.

External mechanics alters plant morphology

Periodical mechanical perturbations, such as wind or touch, can influence plant morphology and anatomy through a process named thigmomorphogenesis (Chehab *et al.*, 2009; Moulia *et al.*, 2011). Plants alter their structure generally to become physically stronger and more able to withstand future mechanical challenges, for example by producing shorter and wider stems and generating more supportive cells such as secondary xylem cells. Removal of the shoot apex did not eliminate the phenotypic conversion of bean (*Phaseolus vulgaris* L.) stem, indicating that the thigmomorphogenic effect does not occur via the shoot apex (Erner *et al.*, 1980). However, in wheat, mechanical agitation of young leaves leads to the production of future stems that are mechanically robust, which suggests that the shoot apex mediates this response. The mechanical environment also affects whether the axillary meristems will develop into new shoot apices (Prasad and Cline, 1987).

The structures within the shoot apex, especially primordia, tend to be in contact at some point in time. The idea that physical interactions among growing organs may contribute to determination of organ shape was first proposed in 1819 by Turpin (cited in Williams, 1975). By comparing the volume and arrangement of primordium at different stages in the shoot apex of *Nerium oleander*, Williams *et al.* (1982) suggested that physical constraints restrict primordia growth. Leaf primordia grow within a bud and therefore grow until they fill the available space (Couturier *et al.*, 2011). In many species of eudicotyledons, the enclosed primordia fold along the major veins (Couturier *et al.*, 2009, 2012), so that they press their lamina margins against one another, reciprocally limiting lamina growth. Taken together, the folding pattern and the growth inhibition by physical constraint can explain palmate leaf shapes. The effects of the physical constraint can be demonstrated by removing one of the leaves in the bud; in the shoot apex of the maple *Acer pseudoplatanus*, the remaining leaf rapidly grows into the newly available space (Couturier *et al.*, 2012). How the cells sense spatial constraints is still an open question.

Mechanisms of mechanoperception and response

Although plants do detect intrinsic and external mechanical stimuli and respond through developmental modifications, the details of the underlying molecular processes remain unclear. Mechanosensing in plants is thought to involve a cytoskeleton–plasma membrane–cell wall network and/or stretch-activated ion channels located in the plasma membrane. Telewski (2006) and Monshausen and Haswell (2013, this issue) provide detailed reviews on this topic; here we summarize the information on mechanosensing that is relevant to this review.

Putative mechanosensors and signalling pathways in the shoot apex

In animals, mechanosensing has been well characterized, and the transmembrane protein integrin has been identified as a major mechanosensor in developmental control. It forms a link between the extracellular matrix and the cytoskeleton (Ingber, 1991, 1993; Ingber and Jamieson, 1985). The hypothesis that integrins are involved in mechanosensing was proven experimentally by the imposition of mechanical forces on integrins using magnetic microbeads controlled by a magnetic field (Wang *et al.*, 1993; Wang and Ingber, 1994; Alenghat *et al.*, 2004; Matthews *et al.*, 2006; Overby *et al.*, 2005). Although no direct homologues of animal integrins have been found in plants, proteins that, like integrins, bind the Arg-Gly-Asp (RGD) motif have been identified, and RGD-containing peptides can cause a range of defects when applied to plant cells (Schindler *et al.*, 1989).

In plants, the strain in the cell wall can be transferred directly to the cytoplasm via the cell wall–cytoskeleton connections. Indeed, such cell wall–cytoplasm connections, mediated by cellulose synthase complexes, were found to specify the polar distribution of the PIN proteins in *Arabidospis* roots (Feraru *et al.*, 2011). However, in the shoot apex, the PIN1 proteins and the cortical microtubules that position the cellulose synthase complex are localized in a mutually exclusive manner (Heisler *et al.* 2010), reducing the likelihood that this mechanism is also taking place in the shoot apex.

Stretch-activated ion channels are thought to participate in mechanosensing (Monshausen and Gilroy, 2009), reading the state of tension of the plasma membrane as a function of the mechanical stress applied to the cell wall and the resulting mechanical strain. A large number of ion currents have been described as responsive to mechanical stimulation in plants (Cosgrove and Hedrich, 1991; Ding and Pickard, 1993). These currents are attributed to mechanosensitive channels, such as of the MscS-like family, which transports anions (Haswell and Meyerowitz, 2006; Maksaev and Haswell, 2011), and Mid1-complementing activity 1 and 2, which are responsible for Ca^{2+} currents (Nakagawa *et al.*, 2007; Yamanaka *et al.*, 2010; Furuichi *et al.*, 2012). Whole organs or single cells show a transient elevation in cytoplasmic free Ca^{2+} when stimulated mechanically (Monshausen *et al.*, 2008; Monshausen and Gilroy, 2009). This elevation in cytosolic Ca^{2+} further triggers the accumulation of reactive oxygen species, the increase in cell-wall pH, and the acidification of the cytoplasm (Monshausen *et al.*, 2009; Monshausen and Gilroy, 2009). These changes result in both transcriptional responses (Apel and Hirt, 2004; Lapous *et al.*, 1998) and rigidifying of the cell wall (D'Avino *et al.*, 2003; Kerr and Fry, 2004). Both the elevation in cytosolic Ca^{2+} and the further downstream responses to mechanical stimuli can be

impeded by Ca^{2+} channel blockers, such as Gd^{3+} and La^{3+} (Sato *et al.*, 2001, 2003).

Interestingly, the PIN1 relocalization upon the induction of lateral root initiation via bending of the primary root could be inhibited by blocking Ca^{2+} influx (Richter *et al.*, 2009). Calcium-binding proteins have been shown to be highly upregulated in response to touch (Braam, 2005); among these proteins, the calmodulin-related protein able to bind calcium, TOUCH3 (Sistrunk *et al.*, 1994), has been shown to interact with PINOID (Benjamins *et al.*, 2003), a protein involved in the control of PIN1 polarity (Christensen *et al.*, 2000; Friml *et al.*, 2004). Based on these data, one could draw a model in which mechanical stress via strain would lead to the activation of stretch-activated channels, generating an influx of calcium in the cell that would bind to TOUCH3 and influence PINOID activity and therefore PIN1 polarity.

Most candidate mechanosensors measure strain, not stress. Stress and strain are related and difficult to uncouple; however, plants may be able to sense the two mechanical signals separately. Hamant *et al.* (2008) reported that, where the orientations of the effects of the two mechanical signals are contradictory (e.g. in the boundary), cell division, as well as PIN1 polarity and cortical microtubule orientation, appear to align better with mechanical stress than with strain (Figs 1E and 3B). But how do plants recognize the mechanical stress independently of mechanical strain? Other reports suggest that strain is a more likely candidate for mechanical signalling; this question has been addressed in detail by Moulia *et al.* (2011).

Candidate sensors of mechanical stress are receptor-like kinase proteins, which have been proposed to detect cell-wall fragments or changes in the cell-wall composition that result from mechanical loading (Marshall and Dumbroff, 1999; Nakagawa and Sakurai, 2001; Baluška *et al.*, 2003). These proteins thus use the integrity of the cell wall as an indirect indicator of mechanical stress. A group of receptor-like kinases—Theseus1, Hercules, and Feronia (Hematy *et al.*, 2007; Hematy and Hofte, 2008; Guo *et al.*, 2009)—have been suggested to sense cell-wall integrity during development, and mutations in these three proteins cause dwarf phenotypes. The roles of the receptor-like kinase proteins in the shoot apex remain to be elucidated.

Distinguishing mechanical signals from other signals

If we are to understand the role of mechanical signalling in development or identify signalling cascades downstream of mechanical stimuli, we must be able to distinguish mechanical signalling from other types of signalling. Previously, mechanical measurements have been performed on dead or fixed tissues to avoid additional complications from the response of the living tissue (Cosgrove, 2011). However, current methods to study mechanics in the shoot apex utilize living tissues, which has many benefits but may make the results harder to interpret. For example, osmotic treatments are used to determine and manipulate the mechanical properties of plant cells (Kierzkowski *et al.*, 2012; Nakayama *et al.*, 2012), and many mechanical measurements are performed on plasmolysed tissues (Peaucelle *et al.*, 2011; Routier-Kierzkowska *et al.*, 2012). However, plants rapidly respond to osmotic stress (Mikolajczyk *et al.*, 2000), and the possible impact of osmoregulation or osmotically induced cellular responses in the aforementioned studies, or more importantly lack thereof, has not been clarified.

Similarly, some methods of mechanical perturbation, such as laser ablation, also involve wounding of the tissue (Hamant *et al.*, 2008; Heisler *et al.*, 2010). Plants are able to detect and respond to being wounded. For example, levels of the phytohormone jasmonic acid increase within minutes of wounding (Glauser *et al.*, 2008), and responses include alterations to plant growth and development (Pauwels and Goossens, 2011). Can we distinguish the response to these chemical signals from the response to the mechanical signals? Furthermore, as most biotic and abiotic stresses induce mechanical changes in plant cells (e.g. Walley *et al.*, 2007; Hamann, 2012), separation of mechanical effects from other environmental effects is a major challenge in understanding plant responses to the changing environment. Dissecting the differential response pathways is likely to involve precise mechanical application and careful isolation of the molecular responses.

Integration of mechanical data into developmental models

Ultimately, we want to understand the genetic control of mechanical stress and material properties in plant development. We can better investigate such phenomena by coupling mechanical measurements with time-lapse data on gene-expression patterns, cortical microtubule orientation, and growth. The collection of such data must go hand in hand with the development of computer models capable of integrating this data. We would also like to investigate the role of mechanical stress in long-term and long-range signalling to alter developmental patterning. This requires perturbation of the system and an extension of the modelling environments to allow bidirectional feedback between genes and mechanics.

Commercial software such as Abaqus and ANSYS have proven useful for building mechanical models (Fayant *et al.*, 2010; Routier-Kierzkowska *et al.*, 2012; Vella *et al.*, 2012); however, these software were not designed for biological tissues and can be limited when it comes to integrating gene expression data and growth. Other custom-built environments exist that are able to deal with the visualization or the modelling (Heisler and Jönsson, 2007; Hamant *et al.*, 2008; Dupuy *et al.*, 2010; Green *et al.*, 2010; Heisler *et al.*, 2010; Kennaway *et al.*, 2011; Kierzkowski *et al.*, 2012; Kuchen *et al.*, 2012; Nakayama *et al.*, 2012, and many others), but we are not aware of any complete suites capable of integrating four-dimensional data, including complex three-dimensional geometry, growth, and gene expression, into mechanical models with feedback regulation of development. There is clearly also a need for more environments that are able to reflect the complex heterogeneous composite nature of the cell wall.

Many models of plant development exist that can accurately reproduce plant form without including feedback from mechanical signals onto the pattern formation (e.g. Jönsson

et al., 2006; Smith *et al.*, 2006; Dupuy *et al.*, 2010; Green *et al.*, 2010; Kuchen *et al.*, 2012). This raises the question of whether mechanical signalling is necessary or not, and if it is, in what circumstances it plays a role. There is also a move to extend these models to produce a more accurate representation of the physical materials to understand fully how gene expression patterns generate the final form of the plant. Can we integrate existing models of development with the mechanical data? Interestingly, existing models for the spatial patterning of organ formation at the shoot apex do not simulate the pattern as robustly as is observed in plants, implying that additional stabilizing factor may exists. Tissue mechanics has been suggested to play a role in positioning PIN1 in such models (de Reuille *et al.*, 2006), but it would be interesting to know whether it could act in addition to the other patterning mechanism to stabilize them. The shoot apex provides us with an opportunity to study organ formation *de novo*, including the transition from isotropic to anisotropic growth, and may help to answer these questions.

Conclusion

Using the shoot apex as a case study, we have described how mechanics can influence morphogenesis in two ways: by altering the tissue properties and though mechanical signalling. Mechanical characterization of growth is a classic subject of plant biology, for which much knowledge and insight have been accumulated for specific tissues or organs, such as the etiolated hypocotyl. The recent surge in the integration of biological and physical sciences is enabling mechanical studies in a broader range of systems, including meristematic tissues, such as the shoot apex, that are undergoing complex morphogenesis and developmental patterning. This advancement has put forth the platform on which we can address how mechanical signals may instruct developmental regulation. However, there are still many challenges ahead: to fully characterize the mechanical properties of the tissue, to understand how the different properties are generated and how they impact morphogenesis, to elucidate the molecular mechanisms behind mechanical sensing and signalling, and to appreciate the interplay between mechanical signals and other signals. Understanding morphogenesis at the shoot apex remains a fascinating endeavour.

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