the plant journal

SEB Society for Experimental Biology

The Plant Journal (2013) **75,** 324–338 doi: 10.1111/tpj.12188

A GLORIOUS HALF-CENTURY OF MICROTUBULES

How mechanical stress controls microtubule behavior and morphogenesis in plants: history, experiments and revisited theories

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Received 29 October 2012; revised 11 March 2013; accepted 20 March 2013; published online 28 March 2013.
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SUMMARY

Microtubules have a key role in plant morphogenesis, as they control the oriented deposition of cellulose in the cell wall, and thus growth anisotropy. The idea that mechanical stress could be one of the main determinants behind the orientation of microtubules in plant cells emerged very soon after their discovery. The cause of mechanical stress in plant cells is turgor pressure, which can build up to 1 MPa and is restrained by cell wall stiffness. On the tissue scale, this can lead to regional patterns of tension, in particular in the epidermis of aerial organs, which resist the stress generated by cells in internal tissues. Here we summarize more than 50 years of work on the contribution of mechanical stress in guiding microtubule behavior, and the resulting impact on growth anisotropy and growth heterogeneity. We propose a conceptual model on microtubule dynamics and their ability to self-organize in bundles parallel to the direction of maximal stress, as well as a synthetic representation of the putative mechanotransducers at play.

Keywords: mechanics, microtubule orientation, morphogenesis, growth, tensile stress.

THE BIOPHYSICS OF GROWTH IN PLANT CELLS

Growth in plant cells corresponds to an irreversible increase in volume, which is controlled by two antagonistic parameters: turgor pressure and cell wall stiffness. Turgor pressure (P) corresponds to the hydrostatic pressure exerted on the cell wall by the cytoplasm (Box 1). It results from the difference in osmotic potential π between the apoplastic and the symplastic compartments (Cosgrove, 1993; Peters and Tomos, 1996; Schopfer, 2006). As long as it is under the so-called yielding pressure threshold (*Y*) of the wall, turgor pressure does not lead to a plastic deformation of the cell (Cosgrove *et al.*, 1984; Boyer *et al.*, 1985; Cosgrove, 1987; Matyssek *et al.*, 1988); however, beyond that threshold, growth can occur (Figure 1a).

Plant cells maintain a state of turgidity, in which turgor pressure can be as high as 10 times the atmospheric pressure (Husken *et al.*, 1978). Because it pushes the wall outwards, this pressure is inducing tension in the wall, which can be described by vectors of tensile stress. These vectors follow a minimal and a maximal direction, and their norm is measured as a force per unit of area, to take into account the geometrical features of the wall (Box 1). For instance, in an isolated turgid plant cell, the exact value of tensile stress will depend on the surface area on which it is normal to (i.e. the thickness of the wall). In the simplest scenario, where cell walls are mechanically homogeneous, tensile stress increases as wall thickness decreases. However, as cell walls are multilayered and heterogeneous, both chemically and mechanically, this relationship might not always be so straightforward.

The ability for a cell to deform elastically and plastically can be regulated by two means. First, the cell may modulate its osmotic pressure, for instance by synthesizing osmolites (a strategy that is used in drought- and freezing-tolerant species) or by exchanging osmolites with the apoplastic compartment (Proseus *et al.*, 1999). A classic example of such regulation is the elastic deformation of guard cells in stomata, in which a decrease in osmotic potential is induced by an intake of K^+ ions from the apoplastic compartment, lead-

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Box 1: Glossary

Turgor pressure: hydrostatic pressure exerted on the cell wall by the content of the cell and generated by cell osmotic pressure.

Yield threshold: level of stress that needs to be applied to a structure to induce an irreversible deformation.

Stress: force applied on a surface normalized by the surface area upon which it is exerted. Stress has the units of pressure (N m^{-2}), and can be either tensile if the force pulls on the surface or compressive if the force pushes on the surface.

Anisotropy: the existence of directions with distinctive properties: *anisotropic growth* reflects the maximal and minimal direction of growth; *microtubule anisotropy* reflects a dominant microtubule orientation over a population of microtubules; *stress anisotropy* means that a maximal and a minimal direction of stress can be defined. The degree of anisotropy measures the ratio between the maximal and minimal directions. Isotropy, or the absence of anisotropy, corresponds to a ratio equal to one.

Strain: relative deformation of an object induced by stress pattern (strain is a number with no unit). Strain rate measures the level of strain over time, and corresponds to the growth rate in living organisms.

ing to the deformation of the guard cells through an increase of turgor pressure and the opening of the pore (Humble and Raschke, 1971). Plastic deformations using turgor regulation are less documented, probably because of the averaging effect of symplastic communications between cells and the technical difficulties behind turgor pressure measurements and osmolite exchange quantifications in tissues. Nonetheless, the rapid growth of the cotton fiber (*Gossypium* spp.) has been associated with an increase of turgor pressure coupled with the dynamic closing of plasmodesmata (Ruan *et al.*, 2001), and the emergence of a lateral root in Arabidopsis has been recently correlated with a blockage of water transport in the root through the downregulation of aquaporins (Peret *et al.*, 2012). Although these represent specific and quite extreme cases in which a strong shift in growth rate is measured, it is also possible that turgor pressure is not the main variable through which growth is usually regulated (e.g. see Spollen and Sharp, 1991).

Second, the modification of the mechanical properties of the wall can impact on the yielding pressure Y of the cell wall by, for instance, softening the wall and thus stimulating the plastic deformation of the wall under turgor pressure. In that case, growth may last as long as cells are able to match the difference in osmotic potential π with the synthesis of new wall components to compensate for the thinning resulting from the stretching of the wall (Boyer *et al.*, 1985). In this framework, growth directly results from stress relaxation through wall loosening, and is not linked to a change in turgor pressure (Cosgrove, 1986). The Lockhart equation summarizes these concepts (Lockhart, 1965), notably by reflecting how growth, which corresponds to an increase of cell volume over time, dV/dt, is driven by the difference between the osmotic pressure ($\Delta\pi$) and the yielding pressure Y, and is modulated by both cell wall extensibility (φ) and a water conductance coefficient (L):

$$\frac{dV}{V\,dt} = \frac{\varphi L}{\varphi + L}(\Delta\pi - Y)$$

This equation allows a fine description of the growth rate in a single turgid cell, and has also been used to study growth rates in plant tissues as a continuous model of multicellular growth (Schopfer, 2006).

One of the remarkable features of plant cells is also their ability to grow significantly along a given direction. This is not reflected in the Lockhart equation, as turgor pressure is by definition non-directional. The theoretical and experimental analysis of plant microtubules, in relationship to the mechanical anisotropy of the cell wall and growth, has provided significant advances to better understand the cell biophysics behind shape changes in plants.

THE RELATIONSHIP BETWEEN MICROTUBULES AND GROWTH

Strain describes the deformation of an object, and like stress, it can be defined by vectors that integrate strain directions (maximal strain direction; minimal strain direction) and strain intensity (e.g. areal strain rate) (Box 1). In contrast to stress, strain can be easily measured when following the evolution of shapes over time. For instance, the displacement of landmarks such as cell edges can be measured in order to quantify shape changes in tissues during growth (Coen *et al.*, 2004; Mirabet *et al.*, 2011). This method has been widely used to measure strain profiles in organs such as leaves, petals, roots or vegetative meristems in various plant species (e.g. Dumais and Kwiatkowska, 2002; Kwiatkowska and Dumais, 2003; Rolland-Lagan *et al.*, 2003; van der Weele *et al.*, 2003; Kuchen *et al.*, 2012). By definition, when the maximal and minimal strains at one point differ, growth is anisotropic (Figure 1b). As turgor pressure is isotropic in essence (i.e. does not have any specific direction), growth anisotropy in single cells is only related to the mechanical properties of the cell wall and to the geometrical features of the cell.

The description of the complexity of the plant cell wall (Somerville *et al.*, 2004; Cosgrove, 2005) is beyond the scope of this review. In short, the primary cell wall forms a highly organized and complex structure, mainly composed of cellulose microfibrils, tethered by hemicellulose and embedded in a matrix of pectins as well as structural proteins (Somerville *et al.*, 2004). Cellulose is thought to be the tension-bearing component of the wall, forming microfibrils with stiffness comparable with that of steel. Hemicelluloses are complex polymers of polysaccharides that

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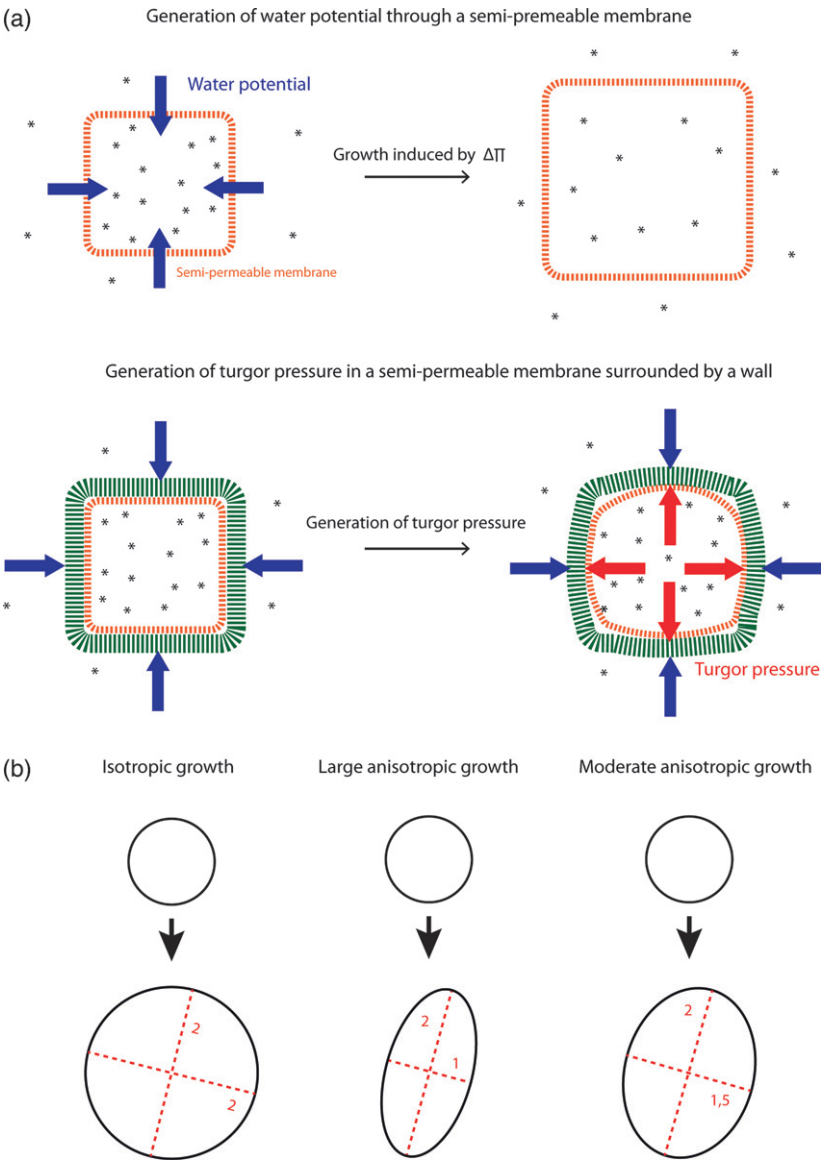

Figure 1. Biophysics of growth in plant cells: the role of the wall.
(a) Schematic view of the generation of turgor pressure in plant cells. Upper panel: the difference in concentration between the apoplastic and symplastic compartments of the cell generates a water potential that drives an influx of water into the cell, and leads to an increase in volume until the concentration of metabolites in the two compartments becomes equal. Lower panel: the influx of water into the cell is restricted by the stiffness of the cell wall. The difference in water potential therefore constantly generates a force on the cell wall surface, which corresponds to turgor pressure. Growth occurs when turgor pressure exceeds the yielding pressure of the cell wall.
(b) Depending on the mechanical anisotropy of the cell wall, cell growth can be isotropic or anisotropic. Most differentiating plant cells exhibit anisotropic growth.

crosslink the cellulose microfibrils. Their mechanical contribution is, however, debated, notably as mutants with reduced levels of hemicellulose do not show much alteration in their phenotype (Cavalier *et al.*, 2008). Last, pectins maintain wall hydration and they can change their mechanical status depending on their association with calcium, which is regulated by their methylesterification status (Pelloux *et al.*, 2007; Mohnen, 2008; Palin and Geitmann, 2012; Peaucelle *et al.*, 2012).

Cellulose microfibrils are thought to be the main determinant controlling growth anisotropy (Green, 1962). In elongating cells, the preferential orientation of cellulose microfibrils in parallel arrays at the innermost side of the cell wall generates mechanical anisotropy, thus allowing directional growth to occur under isotropic turgor pressure (Gertel and Green, 1977; Baskin *et al.*, 1999; Wei *et al.*, 2006). It is commonly believed that this control also occurs on a multicellular scale (Baskin *et al.*, 1999); however, the analysis of cellulose deposition in organs such as the root, the hypocotyl or the inflorescence stem has recently shown that this might be more complex than what is generally described in single cell systems like *Nitella* (Chan *et al.*, 2011; Crowell *et al.*, 2011; Fujita *et al.*, 2011, and see below).

Soon after the discovery of microtubules in higher plants (Ledbetter and Porter, 1963), the orientation of cellulose microfibrils in the walls was correlated with the orientation of the cortical microtubule arrays (CMTs; Baskin *et al.*, 1999; Lloyd *et al.*, 1985). Furthermore, it is now well established that growth becomes isotropic when microtubules are disrupted, either with microtubule depolymerizing drugs such as colchicine or oryzalin (Baskin *et al.*, 1994;

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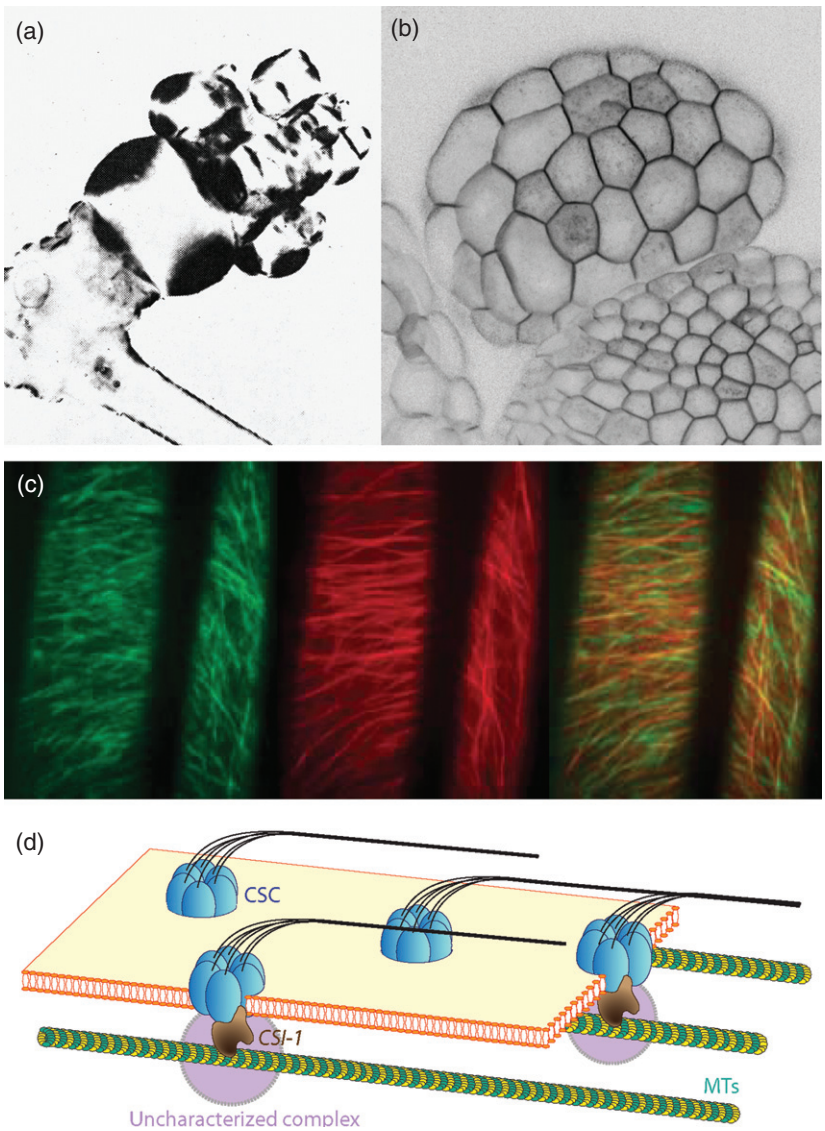

Figure 2. Cortical microtubules control cellulose deposition.
(a) *Nitella* exhibiting spherical cells after incubation with the spindle (microtubule) depolymerizing drug colchicine. This is consistent with a shift towards isotropic growth. Adapted from Green (1962).
(b) Young primordium from a shoot meristem treated with the microtubule depolymerizing drug oryzalin: cell growth becomes isotropic.
(c) Co-localization between the orientation of cortical microtubules and the trajectory of cellulose synthase complexes in hypocotyl cells; green, YFP-CESA6 fusion marking CESA trajectories; red, CFP-TUA1 fusion marking cortical microtubules. The figure is a superposition of 30 frames that were acquired every 10 s. Adapted from Paredez *et al.* (2006).
(d) A classical model recapitulating how cellulose synthase complex (CSC) movement is guided on the plasma membrane by cortical microtubules. This motion may be driven by cellulose synthesis and anchorage of nascent cellulose microfibrils in the wall. CSI1 (brown) acts as one of the physical linkers between CMTs and CSC. The list of all CSC partners is still incomplete (purple).

Green, 1962; Figure 2a) or in mutants with disorganized CMTs (for a review, see e.g. Buschmann and Lloyd, 2008).

In fact, in the absence of microtubules, cells even behave geometrically like soap bubbles, which are purely isotropic (Corson *et al.*, 2009; Figure 2b). The correlation between CMTs and cellulose microfibril orientations approached causality with the isolation of cellulose synthase (CESA) sequences (Arioli *et al.*, 1998; Fagard *et al.*, 2000; for reviews, see Endler and Persson, 2011; Somerville, 2006; Taylor, 2008) and the observation of tagged CESA traveling along CMTs in living hypocotyl cells (Paredez *et al.*, 2006; Figure 2c). Furthermore, the correlation between CESA trajectories and CMT orientations was also observed in the presence of rotating CMTs in young hypocotyls (Chan *et al.*, 2010). Therefore, through guidance of cellulose deposition, the orientation of cortical microtubules in the cell may dictate the direction of cell growth, allowing anisotropic growth to occur, a fundamental prerequisite of plant morphogenesis (Figure 2d).

Although the molecular mechanism behind the coupling between microtubule orientation and cellulose deposition is still largely unknown, the identification of the *cellulose synthase interactive protein 1* (*CSI1*), which acts as a linker protein between CESA complexes and microtubules in Arabidopsis, has further confirmed the relationship between cellulose deposition and microtubule orientation, while uncoupling the microtubule functions in CESA guidance from CESA insertion into the plasma membrane (Bringmann *et al.*, 2012; Li *et al.*, 2012; Mei *et al.*, 2012).

Importantly, the relationship between microtubules and cellulose microfibril orientation may not always be so simple. A disruption of the microtubule arrays does not necessarily lead to an alteration of the orientation of the cellulose microfibrils in the wall (Sugimoto *et al.*, 2003),

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and it was also observed that cellulose synthase complexes can still move along linear trajectories in the absence of microtubules, although at a slower speed and only in the short term (Paredez *et al.*, 2006). It was thus proposed that microtubules may only define a primary scaffold for the deposition of cellulose microfibrils, and self-organization processes occurring between microfibrils in the wall would later on lead to their parallel organization (Emons, 1994; Emons and Kieft, 1994; Baskin, 2001). However, as cellulose can reorganize into parallel arrays in the absence of both a primary scaffold and without microtubules (Himmelspach *et al.*, 2003), other microtubule-independent self-organization processes might also contribute to cellulose oriented deposition. Conversely, microtubules do not only determine the orientation of cellulose deposition but also affect the length of the cellulose microfibrils (Wasteneys, 2004), the rate of cellulose deposition (Sugimoto *et al.*, 2003), the insertion site of CESA complexes in the membrane (Crowell *et al.*, 2009; Gutierrez *et al.*, 2009), the relative degree of crystallinity of cellulose (Fujita *et al.*, 2011) and the delivery of other proteins such as the GPI-anchored protein COBRA, to the membrane (Crowell *et al.*, 2009; Gutierrez *et al.*, 2009; Fujita *et al.*, 2012).

The tissue scale adds another layer of complexity to this picture. In particular, microtubules and cellulose microfibril orientations can be very different at the inner side and the outer side of the epidermal cells in hypocotyls (Chan *et al.*, 2011; Crowell *et al.*, 2011; Fujita *et al.*, 2011). More specifically, in elongating hypocotyls, microtubules near the outer wall display highly variable orientations and remain transverse only for a short time. This may lead to the deposition of an isotropic polylamellate cell wall, which cannot simply be correlated with the high anisotropic growth of the hypocotyl cells. In contrast, microtubules near the inner side of the epidermal cells are stably transverse and their orientation can be more directly correlated with the growth pattern. Interestingly, the overexpression of a microtubule binding domain of mammalian MAP4 fused to GFP, which leads to an even more pronounced opposition between the inner and outer side of the wall, can be correlated with larger growth defects too (Crowell *et al.*, 2011). Nevertheless, the apparent dominant role of the inner side of epidermal cells in controlling growth anisotropy in hypocotyls might not be relevant to other organs.

As microtubule orientation has such a dramatic impact on the mechanical properties of the cell wall, and thus on growth anisotropy, it is essential to understand how CMT orientations are controlled.

THE CONTROL OF MICROTUBULE ORIENTATION

The orientation of the CMTs may depend on the geometry of the cell (Williamson, 1990). This has been largely supported by experiments and modeling approaches, based on direct observation of CMTs or when deduced by the orientation of the cell division plane (Dixit and Cyr, 2004; Cosentino Lagomarsino *et al.*, 2007; Allard *et al.*, 2010a,b; Dupuy *et al.*, 2010; Eren *et al.*, 2010, 2012; Hawkins *et al.*, 2010; Nakamura *et al.*, 2010; Ambrose *et al.*, 2011; Besson and Dumais, 2011). Briefly, CMTs tend to follow the shortest path in the cell (transverse orientations), and bend along the cell edges with the most open angles. Mechanistically, this behavior may be related to a global energy minimum configuration prescribed by the cell shape, and to the apparent high stiffness of microtubules (see below).

Although geometry may predict microtubule orientation by default, this is not sufficient to explain the behavior of microtubules in certain cases. For instance, in *Graptopetalum*, microtubules reorient circumferentially around the future site of organ emergence in a way that is independent of cell geometry (Hardham *et al.*, 1980). Furthermore, Hush and colleagues also showed that microtubules in cells surrounding a wound in *Pisum sativum* (pea) roots reorient circumferentially, independently of cell geometry (Hush *et al.*, 1990; Figure 3a), also demonstrating that CMT orientations can be regulated, despite the geometrical cues from the cell. This also raises the question of the mechanism behind cell geometry sensing, and whether it could be modulated by other cues. Many signals have been shown to impact microtubule orientation. For instance, blue light can switch microtubule orientation from transverse to longitudinal in hypocotyl cells (Fischer and Schopfer, 1997). Similarly, hormones have been shown to impact on microtubule orientation or anisotropy (e.g. Bouquin *et al.*, 2003). Here, we discuss how mechanical stress provides a directional cue to the microtubules. First, we explore the self-organizing properties of the CMTs in relation to mechanical stress.

A CONCEPTUAL MODEL TO EXPLAIN HOW MECHANICAL STRESS COULD ORIENT THE MICROTUBULES

Microtubules have the ability to self-organize in coherent arrays in plants. We might argue that this is the rule rather than the exception for all living organisms, as, for instance, microtubules in axons still organize in parallel arrays in the absence of a neighboring centrosome. Depending on the quality of their encounters, microtubules can zip up, if the encountering angle is small, or can crossover or shrink, if the angle is larger than 40°C (Dixit and Cyr, 2004). Several recent studies, using either particle-based models (Dixit and Cyr, 2004; Eren *et al.*, 2010, 2012; Tindemans *et al.*, 2010; Ambrose *et al.*, 2011) or probability-based models (Hawkins *et al.*, 2010), have demonstrated how these interaction rules can be sufficient to generate parallel orientations in cells. Some of these models have been tested and validated in mutant backgrounds too (Eren

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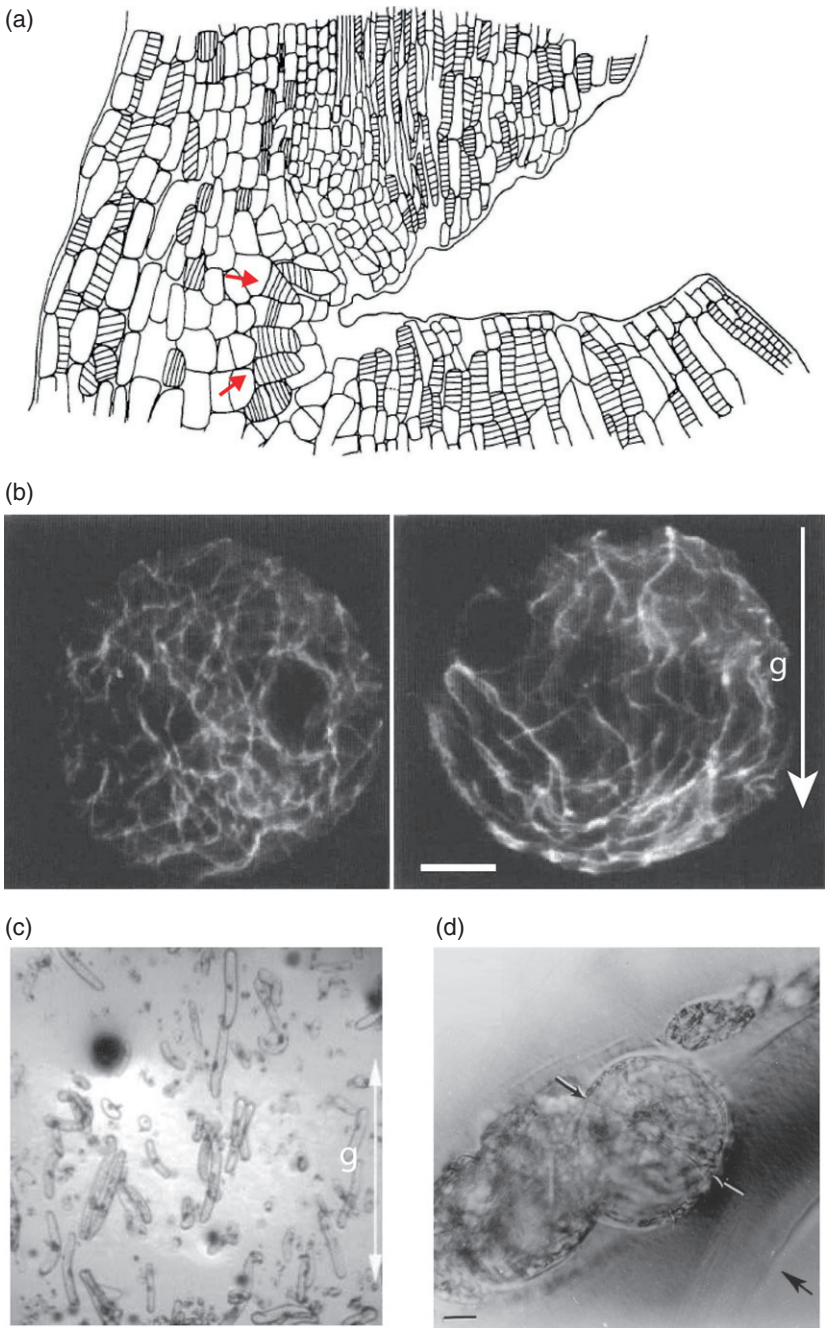

Figure 3. Three experiments suggesting that microtubule orientation may be controlled by mechanical stress.
(a) Drawing showing the reorientation of interphase microtubules (arrows) 5 h after wounding in roots of *Pisum sativum* (pea). Adapted from Hush *et al.* (1990).
(b) Microtubule reorientation after centrifugation in *Nicotiana tabacum* (tobacco) protoplasts. Left panel: before centrifugation, protoplasts exhibit random cortical microtubule (CMT) orientations. Right panel: after centrifugation (30 ***g*** for 15 min), most of the microtubules orient parallel with the centrifugal force. The white arrow represents the orientation of the force vector (scale bar: 5 μm). Adapted from Wymer *et al.* (1996).
(c) Long time effect of stretching on tobacco protoplasts. Protoplasts were embedded in agarose, and then stretched by wrinkling the membrane supporting the agarose for 2 h. After 7 days, the impact upon cell elongation was recorded. Adapted from Fisher and Cyr (2000).
(d) Influence of mechanical loads on the division plane in single cells. When single cells embedded in agar are submitted to compressive forces, their division plane is correlated with the force vector (arrows). Adapted from (Lynch and Lintilhac, 1997).

et al., 2010; Ambrose *et al.*, 2011). While this shows how microtubule anisotropy can emerge from their encounters, it does not provide a mechanism to explain how a preferential orientation is chosen.

Because of their hollow structure, microtubules are particularly resistant to bending from external forces: the second moment of cross-sectional area, and thus the flexural rigidity, is higher in a hollow cylinder than in a solid rod of the same mass. The elastic modulus of microtubules has been estimated to be around 1 GPa *in vitro*, which is comparable with that of Plexiglas (Bicek *et al.*, 2007). In the cell, microtubules often appear curved, suggesting that molecular motors and other associated proteins force the bending of microtubules and/or decrease the elastic modulus *in vivo* (Bicek *et al.*, 2009). Nonetheless, the microtubule intrinsic stiffness allows them to store mechanical energy, notably in their bent form. This is actually the mechanical basis for the propulsion of ciliated cells. This property might also be relevant to growth and morphogenesis in plant tissues: in addition to micro-

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tubule-associated proteins, microtubule dynamics can be affected by mechanical stress. In particular, compressive forces tend to induce microtubule shrinking, whereas tensile forces favor microtubule extension (Zheng *et al.*, 1993; Putnam *et al.*, 1998; Janson and Dogterom, 2004a, b). Thus, in principle, the direction of maximal tension may bias microtubule self-organization in a preferred orientation. Furthermore, studies on migrating animal cells have shown that microtubule dynamics depend on the stiffness of the extracellular matrix, with the softest matrix being associated with increased microtubule dynamics (Myers *et al.*, 2011). As microtubule dynamics is at the root of their encounters and self-organization, mechanical stress intensity may thus also act as a regulator of microtubule behavior. Whereas the link between microtubule self-organization and mechanical stress has not been formalized in a model so far (for a scheme, see Figure 4), a number of cell biology-based studies have explored whether mechanical stress impacts on microtubule behavior *in vivo*, and these are displayed chronologically in the following discussion.

THE CONTRIBUTION OF MECHANICAL STRESS IN MICROTUBULE ORIENTATION: EARLY WORK

The possibility that mechanical cues guide morphogenesis was proposed, notably, by German anatomist Julius Wolff, who observed that bones change their internal architecture in response to an imposed mechanical load (Wolff, 1892). This concept was then expanded to animal and plant development by D'Arcy Thompson in his book *On growth and Form*, in which the shape of living organisms is essentially described as the consequence of the laws of physics (D'Arcy Thompson, 1917). Whereas these propositions were largely too one-sided, recent work on animal systems has revealed a widespread role of mechanical forces in development, in parallel with biochemical signaling. For instance, it is now well established that cell polarity (for a review, see Asnacios and Hamant, 2012), division plane orientation (Thery *et al.*, 2007) or fate (Farge, 2003; Engler *et al.*, 2006) depend on the mechanical environment.

In plants, Castle proposed that mechanical stress could provide plant cells with a positional cue to align cellulose microfibrils and generate anisotropic growth (Castle, 1937). Green and King later used the green alga *Nitella* to study the emergence of growth anisotropy in cells. They proposed that differences in the expansion rates of isotropic walls may lead to an anisotropic stretching of these walls that could serve as a cue to orient cellulose through strain sensing (Green and King, 1966). Preston proposed that cellulose fibers in cell walls must be able to orient according to longitudinal and transverse forces, to explain the diversity of cellulose orientation in the wall in a mechanism independent from the one controlling microtubule orientation (Preston, 1988). The relationship between microtubules and strain was also analyzed (Wasteneys and Williamson, 1987, 1989), and based on these data the hypothesis that stress pattern can control the orientation of microtubule arrays, and thus the oriented deposition of cellulose, was fully theorized (Williamson, 1990). This hypothesis was further tested later on.

When centrifuging protoplasts, microtubules reorient parallel with the centrifugal force vector (Figure 3b). This reorientation further impacted growth, as the maximal strain rate became mostly perpendicular to this axis in the

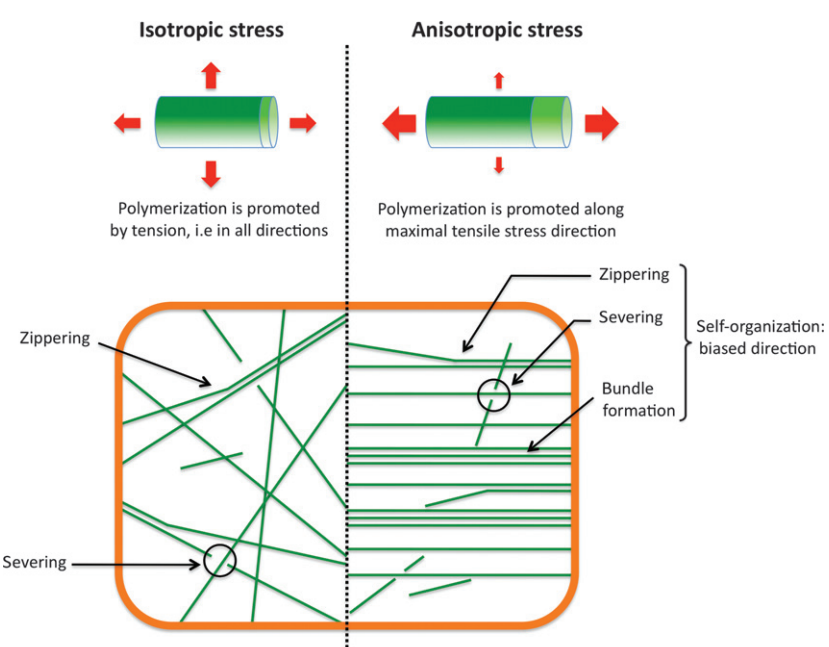

Figure 4. A conceptual and speculative model relating mechanical stress and microtubule dynamics. Left: microtubule dynamics and self-organization is not biased in a given direction, when patterns of tension and compression are isotropic. Right: in the presence of anisotropic tensile stress, microtubules which are aligned parallel to the direction of maximal tensile stress become extended, whereas microtubules in the other directions are polymerized at a slower rate. If the cell is in addition under compression along one direction, and under tension along the orthogonal direction (i.e. strong anisotropic stress, e.g. in the boundary domain of the shoot apical meristem; Figure 5), microtubules may even depolymerize along the axis of maximal compression. Based on existing microtubule self-organization models, this would be sufficient to induce bundling parallel to the direction of maximal tensile stress.

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recovered cells (Wymer *et al.*, 1996). A similar outcome was observed in protoplasts that were embedded in agarose and stretched: microtubule arrays and the axis of elongation were reoriented (Fisher and Cyr, 2000; Figure 3c). Using the same method, Lynch and Lintilhac also showed that the orientation of the cell division plane is controlled by both the geometry of the cell and its state of compression (Lynch and Lintilhac, 1997), consistent with the observation that the cell division plane orientation in tissues grown *in vitro* can be influenced by mechanical loads (Lintilhac and Vesecky, 1984; Figure 3d). Stretching hypocotyl peels also induced a reorientation of microtubules parallel to maximal stress direction (Hejnowicz *et al.*, 2000).

These data strongly suggest that microtubules are able to reorient in response to mechanical stress *in cellulo*. The recent development of live imaging techniques and modeling approaches further confirmed this finding *in planta.*

MECHANICAL STRESS ORIENTS CMTS AND CONTROLS GROWTH ANISOTROPY *IN PLANTA*

Although mechanical stress direction can be relatively easily inferred in single cell systems, it is much more difficult to calculate a stress pattern within tissue. Hofmeister proposed in 1859 that the outer cell layers of plant tissues may mechanically restrict the growth of the inner tissues, which would put the outer cell layers under tension and the inner tissues under compression (Hofmeister, 1859). This concept has been supported experimentally. For instance, in sunflower hypocotyls, inner tissues tend to elongate immediately after peeling out the outer layers, thus suggesting that they were under compression beforehand (Figure 5a). Similarly, outer layers exhibit the opposite behavior, suggesting that the epidermis in plants is usually under tension. This epidermal growth

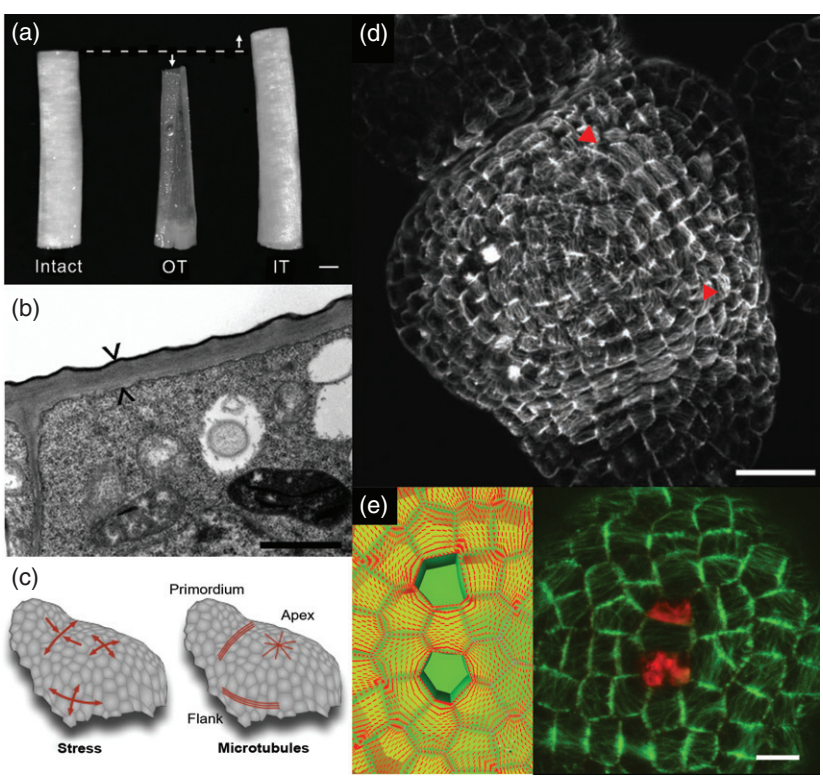

Figure 5. A mechanical feedback loop controlling microtubule orientation in the shoot apical meristem.
(a) Experimental support for the epidermal theory of growth: outer teguments (OTs) were peeled from etiolated sunflower hypocotyls: they retracted instantly while in water. In contrast, the remaining inner tissues (ITs) expanded, suggesting that the OTs were under tension before their removal, whereas the ITs were under compression (scale bar: 1 mm). Adapted from Kutschera and Niklas (2007).
(b) Experimental support for the epidermal theory of growth: TEM micrographs of tomato shoot apices showing epidermal cells with thick outer cell walls (scale bar: 1 μm). Adapted from Kierzkowski *et al.* (2012).
(c) The pattern of mechanical stress, as calculated assuming that the epidermis is under tension, correlates with cortical microtubule array (CMT) behavior at the surface of the shoot apical meristem. Adapted from Dumais (2009).
(d) CMT orientation in the shoot apical meristem, showing a supracellular alignment in the boundary domain (red arrows) parallel with the predicted stress pattern, and isotropic CMTs at the tip of meristem where mechanical stress is also isotropic (scale bar: 20 μm). Adapted from Hamant *et al.* (2008).
(e) Microtubules reorient after cell ablation in the meristem. Left panel (finite-element model of the meristem surface): if two cells are ablated, the mechanical stress pattern should be reinforced between the ablated cells. In contrast, the diffusion of biochemical signals from both ablated cells would not provide a directional cue between the ablated cells. Right panel: microtubules reorient parallel with the predicted mechanical stress pattern after ablation (scale bar: 10 μm). Adpated from Hamant *et al.* (2008).

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control hypothesis is also consistent with the observation that the outer walls of stems are much thicker than the walls in inner tissues (Kutschera and Niklas, 2007). Therefore, to take a simple analogy, plant tissues can be compared with pressure vessels in which an envelope under tension encases content that is under compression. An important consequence is that the stress pattern becomes highly dependent on the shape of the tissue. For instance, in a cylindrical stem, the circumferential stress in the epidermis is twice higher than the axial stress, although this is debated notably for the hypocotyl (see e.g. Hejnowicz and Sievers, 1995). This pressure vessel analogy can be applied to the shoot apical meristem, where complex morphogenetic events occur (Hamant *et al.*, 2008).

The shoot apical meristem is a group of dividing cells that is responsible for the initiation of all the aerial organs of the plant. It is shaped as a dome with two distinct zones: a central zone containing slowly dividing stem cells and a peripheral zone containing cells that are dividing faster, and where new organs are initiated (for reviews, see Barton, 2010; Vernoux *et al.*, 2010). As in the sunflower hypocotyl, the epidermis exhibits a thicker outer wall (Kierzkowski *et al.*, 2012; Figure 5b). Assuming that the epidermis is under tension, a mechanical stress pattern can be derived in this tissue, based on its geometry: in this scenario, tensile stress is isotropic in the central zone at the top of the meristematic dome, as it exhibits a hemispherical shape. Interestingly, microtubules are highly dynamic and present unstable orientations in this domain (Figure 5c,d). In the boundary between the emerging organ and the meristem, the tissue is folded and mechanical stress is predicted to be highly anisotropic along the axis of the boundary. In that domain, in contrast, microtubules are relatively stable and oriented along the axis of the boundary (Figure 5c,d). Altogether, this suggests that microtubules may orient along maximal stress orientation in the meristem. This hypothesis was further consolidated in a cell-based model in which the main CMT orientation in each cell depends on the local stress pattern. The simulations reproduced the observed supracellular CMT orientation, notably in the boundary domain. To test this hypothesis experimentally, the stress pattern was locally modified either through compression or laser-induced cell ablation, and the microtubules reoriented parallel with the new stress pattern (Hamant *et al.*, 2008; Figure 5e). Interestingly, cells that are more prompt to respond to a change in mechanical stress are the ones with the most dynamic CMTs, which is consistent with the involvement of self-organization processes, including polymerization, shrinking and severing (and not a true reorientation) in this response (Uyttewaal *et al.*, 2012).

Based on these data, a feedback loop can be proposed where microtubules are affecting morphogenesis through oriented cellulose deposition and anisotropic growth, which in turn defines a global mechanical stress pattern that influences microtubule orientation.

A PLAUSIBLE ROLE FOR CMTS IN GROWTH COORDINATION VIA THEIR RESPONSE TO MECHANICAL STRESS

Beyond the stress pattern associated with the global shape of the tissue, mechanical stress can also be induced more locally when growth is heterogeneous, e.g. when a cell tends to grow faster than its neighbors. As plant cells are glued to each other by their pectic lamella, this stress can be compensated by wall synthesis; however, as this response is not instantaneous and as stress can even build up, it is also possible that cells use this information to drive their own growth. This was explored in the framework of the microtubule response to mechanical stress (Uyttewaal *et al.*, 2012).

Growth rates in the epidermis of the shoot apical meristem can be extremely heterogeneous: neighboring cells usually exhibit different growth rates (Kwiatkowska and Dumais, 2003). Assuming that cells are able to reorient their microtubules in response to this residual stress, a model was built in which the input was the reorientation of growth anisotropy in response to stress, and the output was the local heterogeneity of growth. Strikingly, the dominant response of the model was an increase in growth heterogeneity when cells are highly responsive to mechanical stress. In other words, when cells respond more effectively to mechanical stress, local differences in growth rates increase. To test this hypothesis, growth heterogeneity was measured in the microtubule severing *katanin* mutant. In this background, microtubules exhibit a decreased dynamics, notably in response to mechanical stress. Interestingly, growth was locally more homogeneous in the mutant, consistent with a scenario in which mechanical stress promotes growth heterogeneity via its impact on the microtubules (Uyttewaal *et al.*, 2012).

What could be the role of such growth heterogeneity in the meristem? As organ outgrowth is triggered by a rapid change in growth parameters, the presence of heterogeneous growth could provide the tissue with the ability to induce quick shifts in growth rates. In other words, if growth homogeneity were reinforced, it would be harder for the tissue to induce local outgrowth. Interestingly, growth heterogeneity is highest in the boundary domain of the meristem, i.e. a domain with the highest anisotropic stresses and where the most dramatic shape changes occur. In the katanin mutant, this local heterogeneity was reduced and organs remained longer within the meristem domain (Uyttewaal *et al.*, 2012).

This concept remains to be explored in other tissues and experimental set-ups but, if confirmed, this would reveal a mechanism by which CMTs coordinate growth between

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adjacent cells, in addition to their regulatory role in growth anisotropy.

A POSSIBLE LINK BETWEEN CMTS, AUXIN AND MORPHOGENESIS THROUGH MECHANICAL STRESS

Beyond microtubules, the key player in plant morphogenesis is the plant hormone auxin. Among other signals, auxin can influence microtubule organization and orientation. For instance, auxin can induce the reorientation of microtubules from longitudinal to transverse in *Vigna angulari* epicotyl segments, even in non-growing cells (Takesue and Shibaoka, 1999). Interestingly, this mechanism has been related to the deposition of polylamellate walls in the epidermis (Mayumi and Shibaoka, 1996). The same effect has been observed in *Zea mays* (maize) coleoptiles, where it was shown that the microtubule pattern was also impacted by auxin application as well as by both auxin-dependent growth changes and mechanical stress (Fischer and Schopfer, 1997). Last, it has been reported that ABP1-dependent auxin signaling promotes the ordering of CMTs in pavement cells via the activation of Rho GTPase ROP6, its effector RIC1 and finally katanin (Fu *et al.*, 2005, 2009; Xu *et al.*, 2010; Nagawa *et al.*, 2012; Lin *et al.*, 2013). However, although some interactions exist between auxin perception and CMT behavior, auxin transport and microtubule behavior seem to be rather uncoupled. The polar localization of the auxin efflux carrier PIN1 relies on actin and not microtubules (Geldner *et al.*, 2001). Furthermore, in the absence of microtubules, PIN1 can still polarize in meristematic cells and reorient so as to generate auxin peaks at organ initiation sites. Conversely, CMT orientation appears rather normal in meristems treated with napthylphthalamic acid (NPA), where polar auxin transport is inhibited (Heisler *et al.*, 2010).

Nonetheless, CMTs and PIN1 often exhibit consistent patterns: in the shoot apical meristem (SAM), PIN1 is usually polarized on the membrane that is parallel with the main CMT orientation, as viewed from the top. As the interaction between CMTs and PIN1 is not direct, it was proposed that mechanical stress could act as the common input controlling both CMT orientation and PIN1 polarity. In this scenario, PIN1 would be recruited to the membrane exhibiting the highest tensile stress (Heisler *et al.*, 2010). Recent experimental tests further support this hypothesis (Nakayama *et al.*, 2012). Note that this shows that mechanical stress only contributes to PIN1 polarity, and it is very likely that other factors such as cell geometry or molecular-based mechanisms trigger PIN1 polarity in parallel with mechanical stress.

To fully understand these interactions, it seems important to go beyond the sole epidermal layer. In particular, auxin regulates many cell wall remodeling proteins (Overvoorde *et al.*, 2005). Furthermore, some of these regulators, like pectin methylesterases, seem to act in the subepidermal layer of the meristem to soften cell walls and trigger organ initiation (Peaucelle *et al.*, 2011a). It would be interesting to investigate the behavior of CMTs in these deeper layers too. Another area for future research is to understand how mechanical stress can be perceived and transduced to the CMTs. Several putative pathways are described below.

PUTATIVE MECHANOTRANSDUCTION PATHWAYS CONTROLLING MICROTUBULE BEHAVIOR

Although the precise chain of events leading to the reorganization of the microtubule array in response to a mechanical cue is still largely unknown, we present here a synthetic view of the putative effectors at play (Figure 6).

The initial sensor of mechanical stress could be a component of the wall that would have the property to deform proportionally to the stress applied to the wall. Being coupled to a transmembrane receptor, this mechanical deformation in the wall would thus translate into a protein conformation change, amenable to be transduced inside the cell via classic transduction pathways (Williamson, 1990; Vogel and Sheetz, 2006). The possibility that stress sensing starts in the wall is supported by experiments showing that degradation of the cell wall using enzymes is sufficient to induce a disruption of the microtubule array (Hasezawa *et al.*, 1988), and that mutants hypersensitive to the microtubule depolymerizing drug oryzalin are wall synthesis effectors (Paredez *et al.*, 2008). Among the different components of the wall, cellulose itself could play a role in mechanosensing, notably in the so-called amorphous region of the wall. As the glycan chains of cellulose present in these specific regions are freer than in the crystalline regions, they would be more amenable to deformation in the presence of stress (Williamson, 1990). Consistent with this hypothesis, inhibiting cellulose synthesis using isoxaben leads to the disorganization of microtubules in *Nicotiana tabacum* (tobacco) culture cells (Fisher and Cyr, 1998) and in pollen tubes (Lazzaro *et al.*, 2003); however, this is not observed in the SAM, as treatment with isoxaben instead promotes microtubule bundling, consistent with a hyper response to stress as a result of wall weakening (Uyttewaal *et al.*, 2012). Although it is difficult to analyze the role of cellulose in stress sensing without interfering with cellulose synthesis, these conflicting results might reveal that mechanical stress can be perceived from different entry points.

Other components of the wall such as pectins are also good candidates. The methyl-esterification status of these pectins affect the degree of elasticity of the wall, as revealed by atomic force microscopy and its impact on organogenesis in the shoot apical meristem (Peaucelle *et al.*, 2008, 2011a,b). Interestingly, cells are able to read this pectin state as a marker of cell wall integrity, and they respond through a feedback loop involving brassinosteroid signaling (Wolf *et al.*, 2012b). This suggests that pectins may serve as baits for stress sensing too, in conjunction

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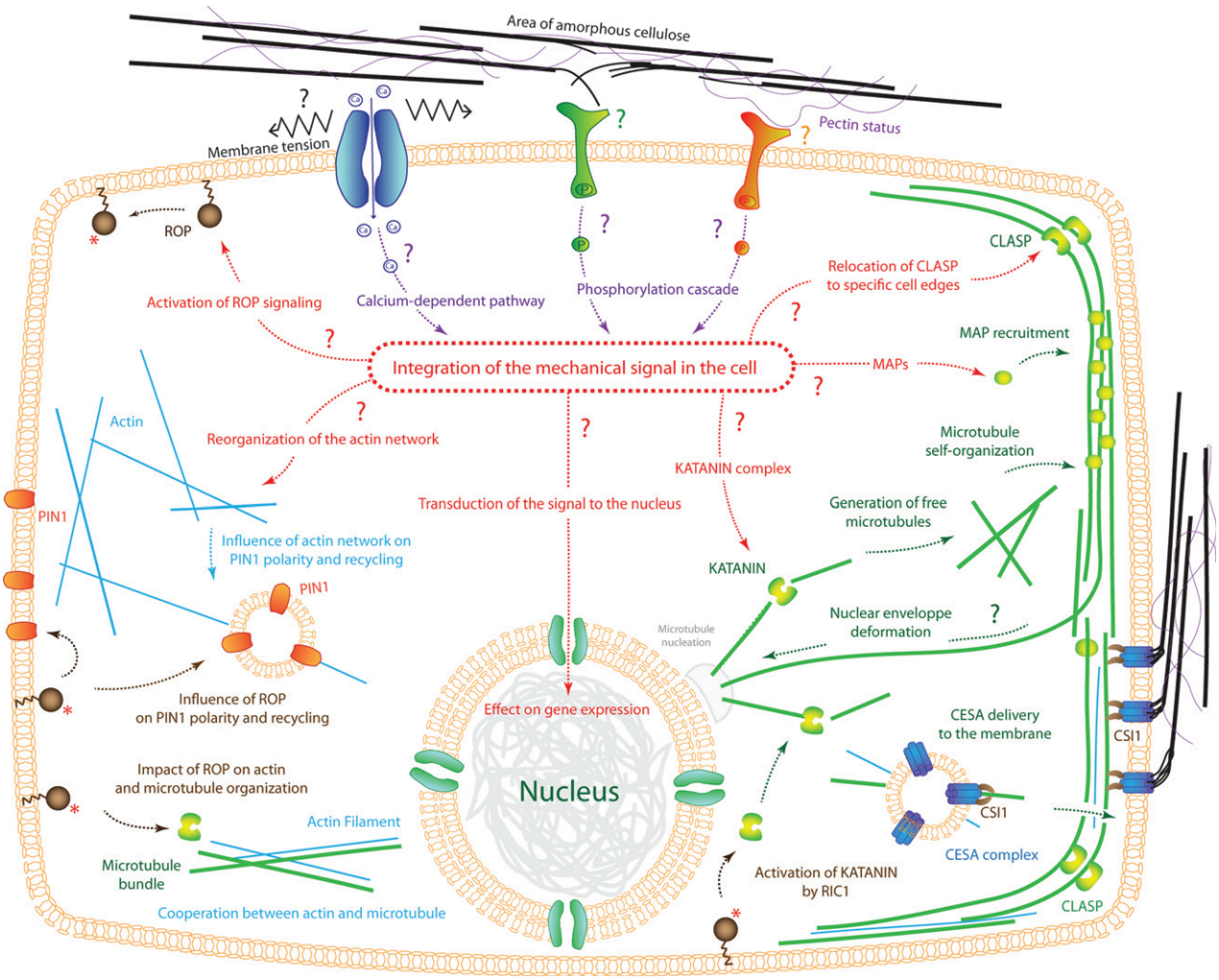

Figure 6. Several candidates may contribute to the mechanotransduction mechanisms behind microtubule reorganization. Mechanical stress in the wall may lead to the modification of a specific component, such as glycan chain deformation in amorphous cellulose or a modified methyl-esterification status of pectins. This may induce a conformation change in a transmembrane receptor kinase and/or increase membrane tension, leading to the selective opening of a stretch--activated channel. Signal intermediates include kinases, calcium or the small Rho GTPases. This ultimately may lead to the relocation and/or activation of specific factors such as KATANIN, CLASP, MAP65 or actin filaments, which would impact on microtubule dynamics and/or organization. Mechanical cues could also act on gene expression, either through the signaling cascade described above or more directly through the deformation of the nuclear envelope, which may in turn influence microtubule organization in a feedback loop.

with other signaling pathways (see also Proseus and Boyer 2007).

A long list of transmembrane receptor kinases and other membrane-associated proteins have been shown to interact with different components of the cell wall; however, none of them has been associated with the perception of mechanical stress so far (Monshausen and Gilroy, 2009; Ringli, 2010; Wolf *et al.*, 2012a). Nevertheless, the transmembrane receptor kinase THESEUS1 has received more attention as it has been proposed to act as a sensor of growth, to maintain cell wall integrity, in relation to Brassinosteroid signaling (Hematy *et al.*, 2007; Guo *et al.*, 2009). Other receptors are likely to be involved, such as COBRA, because of its predicted interaction with cellulose microfibrils (Schindelman *et al.*, 2001; Roudier *et al.*, 2005; Fujita *et al.*, 2012).

In turgid cells, the plasma membrane is pushed against the cell wall and is anchored at specific sites via plasmodesmata. Interestingly, the axis of elongation and division displayed by single cells in response to mechanical deformation is dependent of the integrity of the microtubule array as well as on the presence of adhesion points between the plasma membrane and the cell wall (Zhou *et al.*, 2007). Therefore, tensile stress in the cell wall could also be converted into membrane tension. By analogy with what is observed on motile animal cells, this might be another entry point for the transduction of a mechanical signal (Asnacios and Hamant, 2012). In fact, the observed reorientation of microtubules in wall-less centrifuged protoplasts (Wymer *et al.*, 1996) is consistent with a role of the plasma membrane and the cytoplasm independent

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from the cell wall in mechanical stress sensing. Several stretch-activated channels have been studied. Calcium fluxes have been shown to be modified by various mechanical cues, such as gravity (Plieth and Trewavas, 2002; Toyota *et al.*, 2008), hypo-osmotic shocks (Takahashi *et al.*, 1997) or response to touch (Knight *et al.*, 1991; Legue *et al.*, 1997). The MCA1 (*mid1*-complementing activity) protein was isolated in a complementing assay of the yeast calcium channel MID1, and was shown to be associated with a stretch-activated calcium influx in Arabidopsis hypocotyls (Nakagawa *et al.*, 2007; Yamanaka *et al.*, 2010; Furuichi *et al.*, 2012). Two members of the MSL (MscS-like) family, *MSL9* and *MSL10*, orthologs of the bacteria mechanosensitive small channels (MscS), were also isolated and characterized (Haswell *et al.*, 2008; Peyronnet *et al.*, 2008; Maksaev and Haswell, 2012); however, the corresponding mutant phenotypes are rather weak, suggesting that other mechanosensors are involved, and/or that these mechanosensors are mostly associated with the organelle response to osmotic stress (Braam and Davis, 1990; Braam, 2005; Veley *et al.*, 2012).

Downstream of these transmembrane sensors, a number of proteins associated with microtubules could be involved in the organization of the microtubules according to a directional cue (Sedbrook and Kaloriti, 2008; Buschmann *et al.*, 2010). For instance, two microtubule-associated proteins, MAP65 and CLASP, have been involved in the reorientation of microtubules, leading to periclinal divisions in Arabidopsis roots (Dhonukshe *et al.*, 2012). Interestingly, CLASP has been proposed to facilitate and stabilize the bending of microtubules at cell edges (Ambrose *et al.*, 2011). Because of microtubule self-organization processes, this has important consequences on the 3D organization of microtubules in cells, and this puts CLASP as a potential target of the mechanotransduction pathway, leading to microtubules aligning parallel with maximal stress directions. Similarly, regulators of microtubule dynamics, such as severing proteins, could be controlled by mechanical stress too, in order to increase the number of free microtubules amenable to self-organize according to stress directions (Uyttewaal *et al.*, 2012). Last, TEM images as well as live-imaging data have demonstrated that microtubules and actin are often associated with one another, and are mutually interdependent (Traas *et al.*, 1987; Takesue and Shibaoka, 1998; Collings *et al.*, 2006; Sampathkumar *et al.*, 2011). Interestingly, these associations are maintained in response to mechanical stress: actin filaments reorient circumferentially around wounds, as observed for CMTs (Goodbody and Lloyd, 1990). Actin filaments may thus contribute to the observed response of CMTs to mechanical stress.

The link between mechanical stress perception and microtubule regulators might rely on various transducers, such as kinases (e.g. downstream of a receptor kinase associated with the cell wall) or calcium (e.g. downstream of a stretch-activated channel). It might also be directly translated to the microtubules, e.g. by affecting their stability. Because of their documented involvement in cytoskeleton reorganization in response to hormones (Fu *et al.*, 2005, 2009; Xu *et al.*, 2010; Nagawa *et al.*, 2012), the Rho GTPases might also play a crucial role in mechanotransduction.

Fifty years of research on various systems, from the green algae *Nitella* to the angiosperm Arabidopsis, has supported the idea that mechanical stress reorganizes the microtubule arrays in cells to control morphogenesis. This somehow echoes the initial observation by Wolff on bones, in which the architecture of the bone was remodeled in response to mechanical stress, in resistance to it, thus revealing some interesting multiscale similarities across kingdoms.

ACKNOWLEDGEMENTS

We thank Arezki Boudaoud for his critical reading of the article. This work was supported by a grant from Agence Nationale de la Recherche ANR-10-BLAN-1516.

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