The Cell Surface 7 (2021) 100054

Contents lists available at ScienceDirect

The Cell Surface

journal homepage: www.sciencedirect.com/journal/the-cell-surface

The role of pectin phase separation in plant cell wall assembly and growth

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ARTICLE INFO

Keywords:
Plant cell wall
Phase separation
Volume transition
Pectin
Cell expansion

ABSTRACT

A rapidly increasing body of literature suggests that many biological processes are driven by phase separation within polymer mixtures. Liquid-liquid phase separation can lead to the formation of membrane-less organelles, which are thought to play a wide variety of roles in cell metabolism, gene regulation or signaling. One of the characteristics of these systems is that they are poised at phase transition boundaries, which makes them perfectly suited to elicit robust cellular responses to often very small changes in the cell's "environment". Recent observations suggest that, also in the semi-solid environment of plant cell walls, phase separation not only plays a role in wall patterning, hydration and stress relaxation during growth, but also may provide a driving force for cell wall expansion. In this context, pectins, the major polyanionic polysaccharides in the walls of growing cells, appear to play a critical role. Here, we will discuss (i) our current understanding of the structure–function relationship of pectins, (ii) *in vivo* evidence that pectin modification can drive critical phase transitions in the cell wall, (iii) how such phase transitions may drive cell wall expansion in addition to turgor pressure and (iv) the periodic cellular processes that may control phase transitions underlying cell wall assembly and expansion.

1. Phase separation in plant cell walls

Liquid-liquid phase separation (LLPS) of polymers occurs when the interaction energy favoring like neighbours and disfavoring unlike neighbours exceeds their mixing entropy (For an excellent introduction into the physics of phase separation in biology, see (Gucht et al., 2011; Hyman et al., 2014)). Many regulatory processes in the cell are thought to impinge on phase separation, for instance through posttranslational modifications (phosphorylation, acylation, etc) that modify the interaction strength among cellular components (Shin and Brangwynne, 2017). Increasing the affinity between polymers converts liquid droplets in the cytosol into a gel, where the crosslink lifetime distinguishes between liquid (viscous) or solid (elastic) behavior. Interestingly, gels can show, in response to small environmental changes, often spectacular reversible volume transitions caused by phase separation of the gel into domains of high and low density (For a nice introduction into volume transitions in gels see (Tanaka, 1981)). Phase separation also occurs in the semi-solid environment of plant cell walls and contributes to their remarkable nanoscale organisation and material properties that combine strength and extensibility. A striking example is the formation of patterns in the outer (exine) layer of pollen grains (Fig. 1) (Radja et al., 2019). Exine formation during pollen development takes place in a space created between the plasma membrane and a transient callose layer. In this space, an initially uniform polysaccharide (primexine) layer is deposited. This layer subsequently phase separates, through a so far unknown mechanism, creating more dense aggregates, which form highly reproducible patterns upon reaching an equilibrium. These surface patterns are consolidated through impregnation with sporopollinin, a highly resistant polymer consisting of long chain fatty acids and phenolics, which is secreted by the adjacent tapetum cells (Radja et al., 2019). In some species, the primexin phase separation is kinetically arrested, presumably by premature sporopollinin release, leading to variable deposition patterns. Such arrested patterns appear to evolve more rapidly than equilibrium patterns. Another example is the maturation of the cell wall of the single-celled freshwater Desmid alga *Penium margaritaceum* (Domozych et al., 2009; Palacio-Lopez et al., 2020). Desmids are Charophycean green algae, closely related to land plants (Rensing, 2018) with a similar cell wall composition, including an important role for pectins in cell wall architecture and growth (Sørensen et al., 2011). Desmids are characterized by a typical cellular symmetry based on "equal" cell halves or semicells, which are formed by the transverse division of a mother cell (Fig. 2A). The pectin. homogalacturonan (HG) is a linear polymer of α-1,4-linked galacturonic acid (GalA), which is synthesized in the Golgi apparatus as a methylesterified

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https://doi.org/10.1016/j.tcsw.2021.100054
Received 15 March 2021; Received in revised form 28 April 2021; Accepted 28 April 2021
Available online 6 May 2021

and hence largely uncharged polymer (Atmodjo et al., 2013). In Penium, "High degree of Methylesterification HG (HG^{HM})" is deposited at the separation between semicells, after which it is demethylesterified by pectin methylesterases (PME) in a narrow growth zone of the semicells (Fig. 2B) (Green et al., 2014; Palacio-Lopez et al., 2020). This creates polyanionic polymers or polymer domains, which can form Ca^{2+} crosslinks and phase separate from the rest of the cell wall forming a typical pattern of surface protuberances on the mature cell wall (Fig. 2C-F) (Domozych et al., 2009; Palacio-Lopez et al., 2020). This example nicely illustrates how enzymatically induced phase transitions in previously deposited pectin assemblies can govern cell wall patterning. Before discussing less obvious pectin-induced phase separation processes in plant cell walls, we will summarize some relevant features of cell wall structure and pectin metabolism.

2. Cell wall structure and pectin metabolism

The most condensed phase in the cell wall is cellulose, which consists of semi-crystalline microfibrils consisting of multiple ß-1,4 linked glucan chains (Moon et al., 2011). The microfibrils are synthesized from hexameric complexes in the plasma membrane, where each globule contains 3 distinct catalytic subunits (Purushotham et al., 2020). The proximity of these subunits inside the complex ensures the aggregation of 18 parallel glucan chains into typically 3 nm diameter elementary microfibrils. These elementary microfibrils can form higher order aggregates depending on the nature of the matrix polymers that are present during the deposition (McFarlane et al., 2014). Indeed, some matrix polymers or polymer domains readily condense onto cellulose such as certain xyloglucan (Cosgrove, 2014), xylan (Simmons et al., 2016) and pectin species (Fig. 3A) and act either as a double-sided tape crosslinking microfibrils (shown for xyloglucans, (Cosgrove, 2014)) or instead prevent such crosslinking by creating a sterically and/or electrostatically repulsive surface on the microfibril (Jaafar et al., 2019; Simmons et al., 2016). The latter was shown for xylans, which, depending on the substitution pattern, can bind cellulose while exposing neutral or negatively charged substituents to the medium (Jaafar et al., 2019; Simmons et al., 2016). Pectin can also bind to cellulose through xylan (Ralet et al., 2016) and perhaps arabinan/galactan (Verhertbruggen et al., 2009; Zykwinska et al., 2005) side chains or even covalent interactions (Broxterman and Schols, 2018) and thus prevent microfibril aggregation. The majority of the pectins, however, is not bound to cellulose (Atmodjo et al., 2013). Pectins are characterized by the presence of GalA and are the major charged polysaccharides in expanding cell walls of Charophytes and land plants (35%), with the exception of Commelinid monocots, the cell walls of which accumulate smaller amounts (10%) of pectins (Atmodjo et al., 2013). HG is the most abundant pectin polymer in growing cells (20% of cell wall dry weight in Arabidopsis leaves (Zablackis et al., 1995)) and exists in different contexts: as part of the proteoglycan ARABINOXYLAN PECTIN ARABINOGALACTAN PROTEIN1 (APAP1) (Tan et al., 2013), as block co-polymers with rhamnogalacturonan (RG)I or RG-II or as isolated polymers (Fig. 3A,B) (Atmodjo et al., 2013; Round et al., 2010). APAP1, may actually be the precursor for most if not all RG-I in the cell wall (Tan et al., 2013). As mentioned above, the charge density of HG is regulated through selective de-methylesterification by cell wall associated Pectin MethylEsterases (PMEs) (Hocq et al., 2017a). Higher plants produce an enormous diversity of PMEs (e.g 66 annotated members in *Arabidopsis thaliana*) (McCarthy et al., 2014; Sénéchal et al., 2014). This diversity reflects in part differences in gene expression patterns but presumably also variation in enzyme activity. Very little is known, however, about substrate preferences, pH, Ca^{2+} dependence and other characteristics in part due to the difficulty to produce active PMEs in heterologous systems (Sénéchal et al., 2014). The PMEs that have been studied so far *in vitro* typically have a high pH optimum that is further sharpened by endogenous proteinaceous PME inhibitors (PMEIs), most of which are only active at low pH (Hocq et al., 2017b). The situation is more complex *in situ*, where PME activity affects the properties of the pectin gel and, vice versa, the pectin gel modifies the enzyme characteristics (Bonnin et al., 2019; Vincent et al., 2013). This can lead to interesting emergent properties. For instance, the so far studied plant PMEs have processive activities and create polyanionic stretches in HG, which can form cooperative Ca^{2+} crosslinks forming a stiff gel (Fig. 3C, middle). Interestingly, the processivity is lost when the enzyme is incorporated in HG^{HM} in the presence of Ca^{2+} creating a more elastic gel with randomly distributed Ca^{2+} cross links (Fig. 3C, left) (Vincent et al., 2009). Inversely, the incorporation of cellulose into a pectin gel converted an otherwise non-processive fungal PME into a

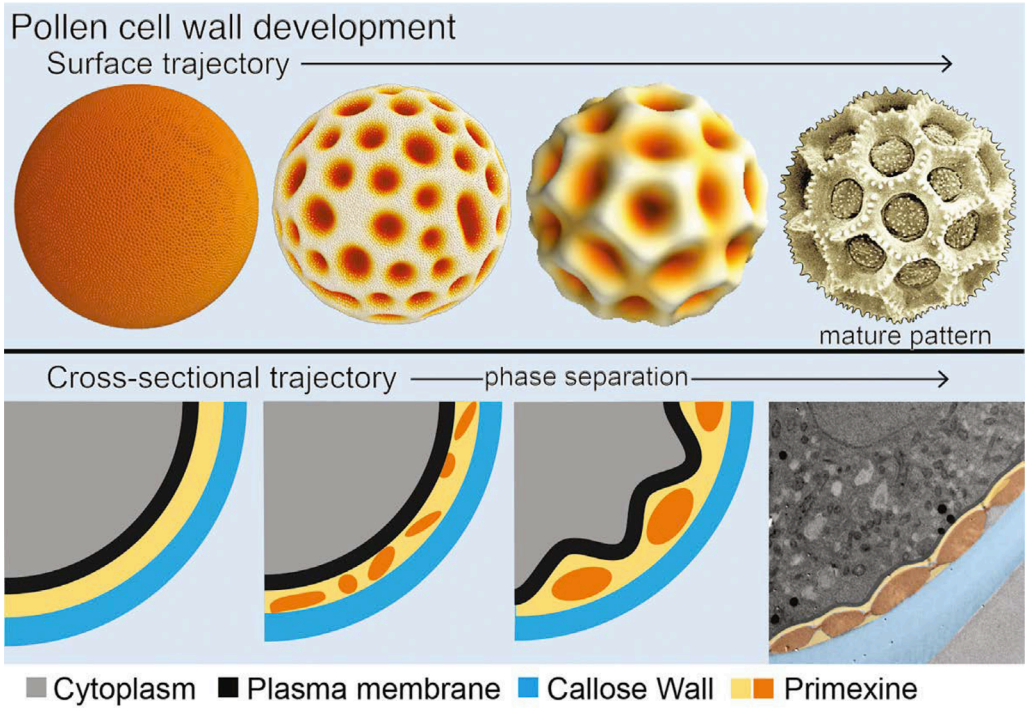

Fig. 1. Phase separation underlies pollen surface patterns. Top row: Numerical simulation of the development of pollen exine patterns. Simulated surface of a foam pattern that matches the SEM images of real pollen grains. The average wavelength of the simulations increases from left to right up to values of around 15 μm. The cross-sectional trajectory (bottom row) shows how phase separation of an initially homogenous primexine layer that is mechanically coupled to the plasma membrane leads to the formation of the patterned pollen surface (Radja et al., 2019). First 3 panels are drawings, last panel is a TEM image.

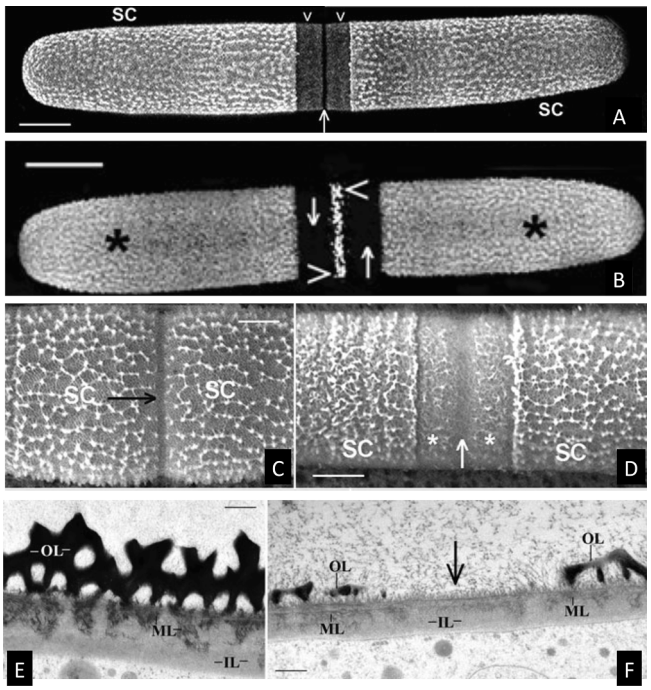

Fig. 2. Pectin phase separation underlies surface patterning of *Penium margaritaceum*. (A) 3-D reconstruction of 0.2 μm CLSM sections of a premitotic cell, live-labeled with a monoclonal antibody against de-methylesterified HG (JIM5) and cultured for 1 h. The two equivalent-sized semicells (SC) are found on either side of the isthmus (arrow). The projections on the cell wall surface are clearly labeled on each semicell. An unlabeled band (i.e., the zone where new CW materials are secreted) is notable in the isthmus (arrow). Between the labeled cell wall and the unlabeled central band (arrowheads) are two ''transition'' zones where the outer projections have yet to form on the cell wall. Scale bar = 9 μm. (B) Premitotic cell labeled first with JIM5, recultured for 2 h, and then labeled with a monoclonal antibody against highly methylesterified HG (JIM7). A thin, JIM7-labeled band refered to as the homogalacturonan secretion band is found in the isthmus (arrowheads). This band is surrounded by two unlabeled zones (arrows) where new pectin was secreted and demethylesterified during the reculture stage. The typical JIM5 labeling is found on the cell walls at the polar zones of the two semicells (*). Scale bar, 15 μm. (C and D) Variable pressure scanning electron microscopy images of the isthmus zone of premitotic cells. (C) A cell in the early stages of cell expansion. The isthmus zone possesses a narrow band (arrow) devoid of the outer cell wall projections that are found on each semicell. (D) A cell in later stages of expansion. The band lacking the cell wall projections in the isthmus is still visible (arrow) together with flanking transition zones (*) that lead to the typical cell wall of the two semicells. Scale bar for (C), 3.5 μm; (D), 4 μm. (E,F) TEM images. (E) ''mature'' cell wall; highlights the three main layers: the inner fibrillar layer (IL), a more-electron-dense fibrillar median layer (ML), and an outer layer (OL), which forms outer cell wall surface projections. Scale bar, 250 nm. (F) isthmus region (large arrow) of a premitotic cell where HG secretion is occurring. In this isthmus zone, only the inner layer (IL) is found. As one moves toward both poles, the median layer (ML) and then the outer layer (OL) become apparent. Scale bar, 450 nm. ().
Adapted from Domozych et al., 2009

processive enzyme and enhanced the mobility of the enzyme in the gel (Bonnin et al., 2019). Also, PME activity might be regulated by a negative feedback loop through the buffering effect of free carboxylic acid groups (pKa of GalA = 3.2), which might create lower pH islands within the wall. Another level of complexity is that de-methylesterification creates a substrate that can be degraded by polygalacturonases (PGs) or pectate lyases (PLs), also represented by large enzyme families, with different biochemical characteristics. The presence of methylesters protects pectin against turnover, although some fungi produce *pectin* (as opposed to *pectate*) lyases (PNLs) that degrade methylesterified pectin *Voxeur et al., 2019). No plant PNLs have been reported so far. Finally, HGs can carry other substitutions: xylosyl groups or acetyl groups (on position 2 or 3), which may also form patterns (Voxeur et al., 2019) and potentially change the physicochemical properties and/or enzyme susceptibilities of the polymer.

3. Pectin-driven phase transitions *in vivo*

3.1. Pectin-mediated rehydration of pollen grains and seed mucilage.

One of the attributes of the success of seed plants in colonizing terrestrial habitats is their remarkable capacity of pollen grains and seeds to dehydrate and rehydrate. The examples below show that these rehydration processes can involve volume transitions in pectin gels. Dicot pollen grains have below their exine layer an intine layer rich in pectin. The de-methylesterification of this pectin prior to germination is essential for normal pollen hydration and germination. Indeed, a mutant for one of the 13 PME genes that are expressed in *Arabidopsis* pollen (*PME48*) showed greatly delayed pollen swelling and germination[1] (Leroux et al., 2015). A recent study on chemically de-esterified sunflower pollen confirms that swelling is driven by the charges of the pectin and limited by Ca^{2+} crosslinks (Fan et al., 2020). Indeed, neutralizing the charges at low pH prevented the swelling and removal of Ca^{2+} with a chelator at neutral pH further promoted swelling (Fig. 4). These results corroborate theoretical and experimental studies on synthetic polymer gels, which show that the extent of the volume transition increases with increasing polymer charges ((Tanaka, 1981; Zhao and Moore, 2001)). It is also interesting to note that the swelling occurred in two steps: a rapid totally reversible step, involving the elastic opening of pores in the exine and a slower, irreversible step involving the rearrangement of the polymers in the exine layer (Fig. 4) (Fan et al., 2020). As discussed below, analogous processes may take place in the walls of growing cells.

Another example is the rehydration of seed mucilage. The seeds of many plant species release a mucilage gel from the seed coat epidermis upon exposure to water (North et al., 2014). *Arabidopsis* seeds for instance, release mucilage consisting of two layers, a free outer layer consisting primarily of RG-I and an inner layer, which remains attached to the seed coat cells via cellulose-containing rays (Šola et al., 2019a). Genetic approaches have given us a valuable insight into the mechanism of mucilage release as follows: In the inner layer, RG-I is connected to cellulose via xylan side chains (Ralet et al., 2017). RG-I also carries galactan side chains, the terminal residues of which can be enzymatically oxidized and form hemiacetal crosslinks with the OH of neighboring sugars during dehydration (Šola et al., 2019b). A critical step for mucilage swelling appears to be the removal of this terminal galactose (and hence the crosslink ability) by a ß-galactosidase, since mucilage swelling is lost in mutants for this enzyme (Macquet et al., 2007). Again the enzymatic modification of the polysaccharide controls the volume transition of the mucilage gel.

3.2. Pectin-dependent enzyme recruitment

Mucilage release from the *Arabidopsis* seed coat is facilitated by the presence of a specialized outer cell wall, which yields to the pressure of the swelling gel at predefined fragile spots at cell edges, where the cell wall is thinner (Fig. 5A,C). A recent elegant study showed that the creation of these fragile spots requires the local action of the peroxidase PRX36 (Francoz et al., 2019). The thinning of the cell wall at this location can be explained by cell wall densification caused by PRX36-catalysed oxidative crosslinking of cell wall polymers, although other

[1] Interestingly, de-esterified pectin also appears to provide an extracellular reservoir for Ca^{2+} required for pollen germination, as suggested by the rescue of the *in vitro* germination defect of *pme48* by a small increase in external Ca^{2+} concentration (from 5mM to 7.5mM).

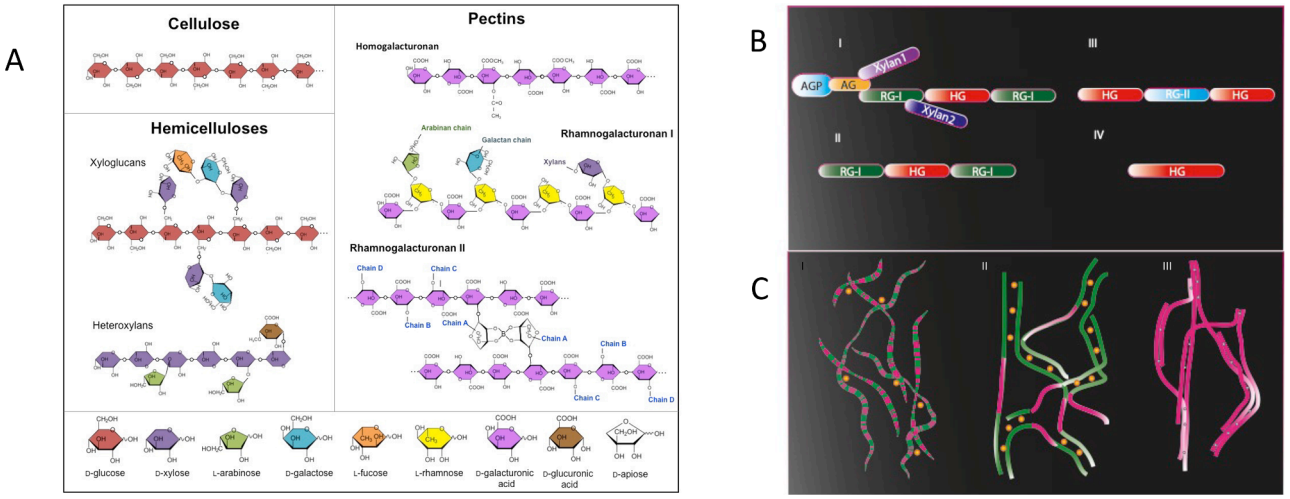

Fig. 3. Common polysaccharides and homogalacturonan species in primary plant cell walls. (A) Most common cell wall polysaccharides and monosaccharides (Höfte and Voxeur, 2017). (B) Homogalacturonan (HG) is found as part of different block co-polymers. (I) APAP consisting of a arabinogalactan protein (AGP) an arabinogalactan (AP), connected to a xylan (xylan1) and a rhamnogalacturonan (RG)-I, which in turn is connected to a xylan (xylan2) and a HG, which in turn is connected to a RG-I; (II) as HG/RG-I copolymers, (III) HG/RG-II copolymers or (IV) as isolated HG chains. Adapted from (Atmodjo et al., 2013). (C) Higher-order organisation of HG chains. Pink and green zones represent methylesterified and demethylesterified domains, respectively and orange spheres Ca^{2+} ions. (I) Partially demethylesterified HG can form a Ca^{2+} crosslinked elastic network (Vincent et al., 2009), (II) HG with large (>9 residues) demethylesterified blocks form cooperative Ca^{2+} crosslinks (so-called eggboxes), (III) Highly methylesterified HG can form fibers crosslinked by hydrophobic interactions between methyl groups and hydrogen bonds involving rare free carboxylic acids (Walkinshaw and Arnott, 1981b). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

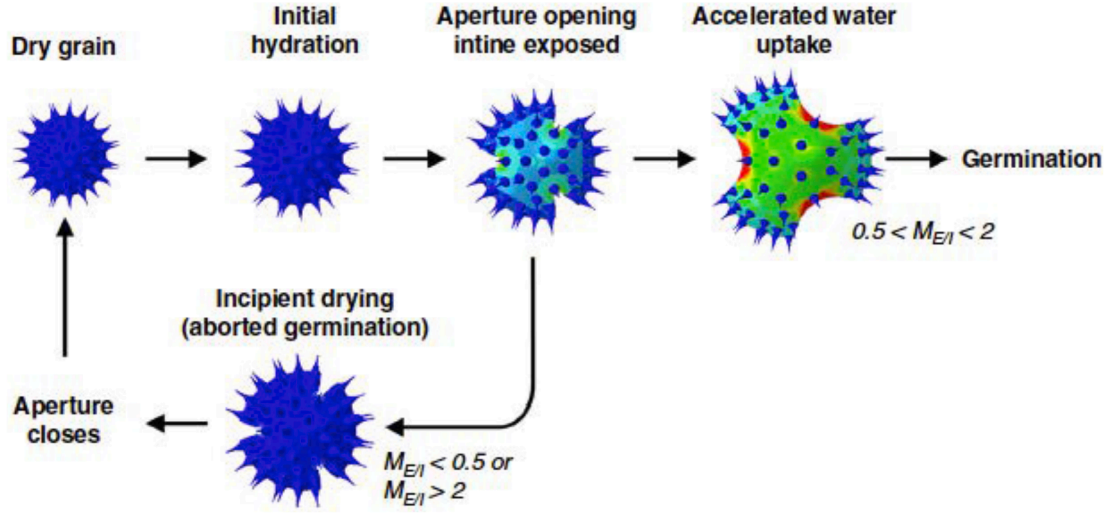

Fig. 4. Pectin volume transition drives dicot pollen hydration (Fan et al., 2020). Pollen hydration/dehydration cycle of angiosperms with corresponding finite element analysis simulation images. The top sequence shows a dry pollen grain undergoing the pectin dependent water uptake process during germination. In situations where complete hydration is not possible, germination might be aborted, and the apertures close again as shown in the bottom sequence.

scenarios, involving hydroxyl-mediated cleavage of polymers cannot be excluded. Interestingly, the authors showed that targeting of RFP-tagged PRX36 (PRX36:RFP) to the cell edges requires the PME inhibitor PMEI6. FRET/FLIM[2] immunofluorescence experiments showed that this inhibitor, presumably in combination with an unidentified PME, creates a defined, partially de-methylesterified pectin epitope, which is recognized by a specific subset of antibody probes (Fig. 5B). This pectin epitope forms a binding site for PRX36 (Francoz et al., 2019). Removal of this epitope in a *pmei6* mutant or by chemical bulk de-esterification of the developing seeds, led to the diffusion of PRX36:RFP into the underlying mucilage layer (Francoz et al., 2019). In this context, it is interesting to make a parallel with the phase separation of proteins into liquid-like condensates in the cytosol or nucleus, which is driven by multivalent weak interactions with RNA or other proteins (Gucht et al., 2011; Hyman et al., 2014). A similar process might underlie the dynamic recruitment of PRX36 into local gel domains formed by specific pectin species. It remains to be determined what drives the creation of the PMEI6-dependent pectin epitopes specifically at the cell edges. One candidate is the Rab-A5c-dependent targeted secretion of cell wall components to the cell corners (Kirchhelle et al., 2016). In general, it will be interesting to see to what extent phase separation contributes to the compartmentalization of other wall modifying proteins within the cell wall and how this relates to their 3D structure and surface charge distribution on these proteins (Blocher McTigue and Perry, 2019).

[2] Foerster Resonance Energy Transfer/Fluorescent Life Time Imaging Microscopy

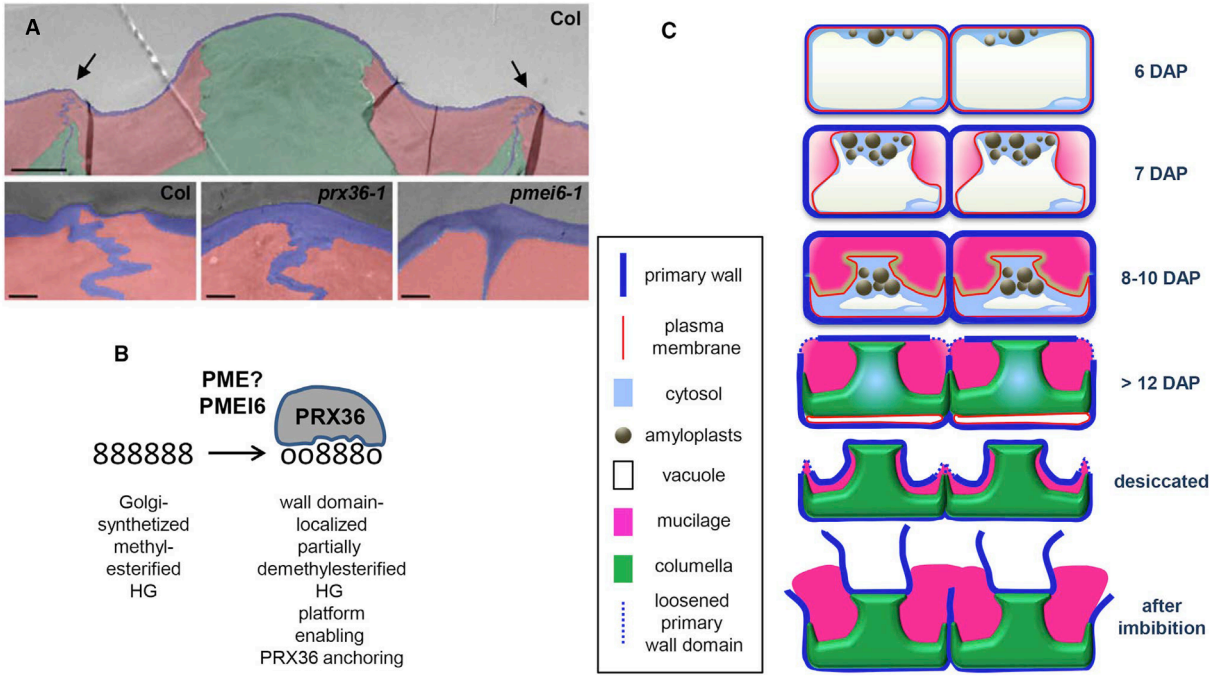

Fig. 5. Pectin pattern-dependent targeting of cell wall-modifying enzyme to a cell wall domain. (A) False colored TEM view of a section through a mucilage-secreting cell of a dry Arabidopsis seed coat. Blue: primary wall; green: columella secondary wall; pink: mucilage; arrows: thin outer wall domain that yields during mucilage hydration. Upper image: lower magnification of a WT (Col). Lower images: higher magnification of WT (Col) and mutants for *prx36-1* (peroxidase) and *pmei6-1* (pectin methyl esterase inhibitor). The thin cell wall domain is absent in both mutants. Scale bars: 5 mm (wide view); 0.5 mm (zooms). (B) simplified model showing the molecular relationship between PMEI6 and PRX36. 8: methylesterified galacturonic acid; o: demethylated galacturonic acid. (C) Schematic view of mucilage secreting cell differentiation, dessication and rehydration. DAP: days after pollination. (). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Adapted from Francoz et al., 2019

3.3. Phase transitions in walls of growing cells

A major enigma in plant biology is how cell wall metabolism can control cell expansion and plant growth. According to the acid growth theory, the growth hormone auxin favors turgor-driven cell expansion through promoting cell wall acidification, which enhances the extensibility of the cell wall (Cosgrove, 2018). Numerous studies have observed acid promoted "creep", i.e. long-term plastic (=irreversible) deformation of the cell wall on isolated cell walls. Expansins were identified as the proteinaceous agents that promote such acid-induced creep (Cosgrove, 2018). These proteins appear to specifically remove the, above-mentioned, cellulose-xyloglucan-cellulose crosslinks through a still mysterious, non-enzymatic mechanism. This activity, characterized on isolated and heat-inactivated walls, appears to be only a part of the picture in the context of living expanding cells. Indeed, cell wall acidification is also expected to have pronounced effects on pectin properties by neutralizing the negative charge of carboxylic acids. A recent study used solid state NMR to compare never dried cell walls at pH 4 and pH 7 (Phyo et al., 2019). The results showed that acidification induced a phase transition, where HG chains became more aggregated and less bound to cellulose, thus increasing the porosity and the mobility of the interfiber polymers. These changes, in addition to the expansin-catalysed removal of cellulose-XG crosslinks, are correlated with an increased wall hydration and cell expansion (Phyo et al., 2019).

3.4. Pectin phase transition as a driving force for cell wall expansion

A role for pectins in growth control was corroborated in a series of experiments based on the *in vivo* manipulation of PME activity. The results showed that HG de-methylesterification promotes cell wall expansion as observed at the organ (Peaucelle et al., 2008; Qi et al., 2017), cellular (Jonsson et al., 2021; Peaucelle et al., 2015) or subcellular scale (Haas et al., 2020a). In addition, Atomic Force Microscopy (AFM) indentation of the cell wall perpendicularly to the growth direction showed that HG de-methylesterification enhanced cell wall elasticity (reversible deformation) (Peaucelle et al., 2015, 2011). This finding was surprising for several reasons. First, it was expected that Ca^{2+} crosslinking of poly-anionic HG would reduce, not enhance cell wall elasticity (Phyo et al., 2017). In addition, HG de-methylesterification did not promote tension-induced wall expansion (irreversible "creep") on isolated onion cell walls (Wang et al., 2020). Finally, the correlation of cell growth with cell wall elasticity but not with creep was also puzzling since growth by nature corresponds to an irreversible, not an elastic deformation (Boudaoud, 2010).

A series of recent studies addressed the relation between cell wall elasticity and morphogenesis by focusing on the intriguing puzzle-shaped cells of the Arabidopsis epidermis (Fig. 6A) (Majda et al., 2017; Bidhendi et al., 2019; Haas et al., 2020b). To investigate the mechanism underlying the formation of lobes in these cells, Majda et al. (Majda et al., 2017) carried out AFM measurements on anticlinal (perpendicular to the organ surface) walls in resin embedded sections of epidermal cells. This revealed alternating stiff (curved) and soft (straight) regions along the wall and across the wall thickness, with stiff concave and soft convex zones. The variation in stiffness was correlated with variation in cell wall composition as shown with TEM after labeling with anti-glycan antibodies. Based on the AFM data, they numerically simulated an anticlinal wall with asymmetric mechanical properties across and along the wall. After stretching the wall, corresponding to pressurizing the cells, the authors observed deformations, reminiscent of lobes (Majda et al., 2017). Subsequent modeling attempts, however, were only able to simulate extremely small deformations, which entirely disappeared in the presence of periclinal (parallel to the surface) walls (Bidhendi et al., 2019). So, whether changes in cell wall elasticity alone can directly affect morphogenesis remains an open question in this case.

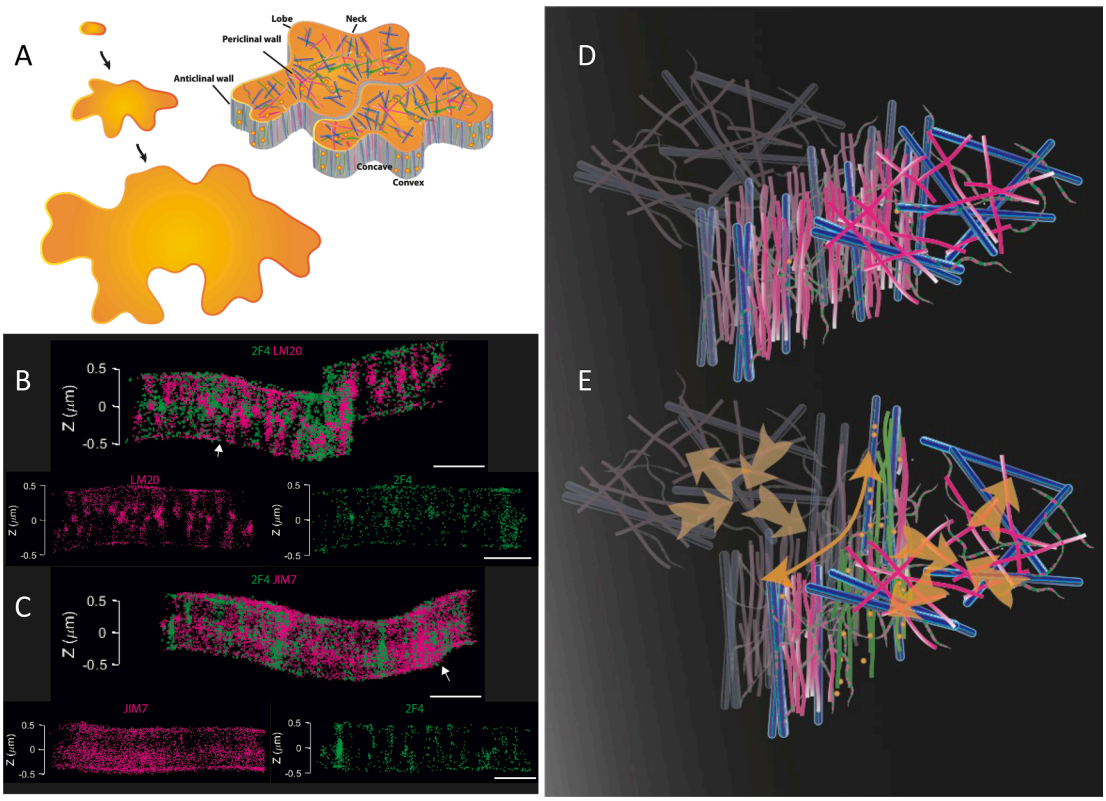

Fig. 6. Homogalacturonan phase transition driving cell wall expansion. (A) lobe formation in an Arabidopsis pavement cell and the localisation of cellulose (blue), methylesterified (pink) and de-methylesterified (green) HG nanofibrils in the anticlinal (normal to the cell surface) cell walls of the lobes. (B,C) Super-resolution images (see also supplemental movies) of anticlinal walls doubly labeled with (B) antibodies against methylesterified (LM20, pink) and de-methylesterified (2F4, green) HG or (C) against partially methylesterified (JIM7, pink) and de-methylesterified (2F4, green) HG. 2F4 and LM20 reveal rows of epitopes oriented perpendicularly to the surface. In contrast JIM7 does not reveal oriented structures. In (B) and (C) white arrows indicate the center of the wall segments showed below as single color images. Scale bars = 500 nm. (D,E) Representation of the architecture of cellulose and HG filaments in anticlinal and periclinal (parallel to the surface) outer walls of pavement cells. (D) In anticlinal walls, methylesterified HG filaments (pink) are oriented perpendicular to the surface and parallel to cellulose microfibrils (blue). Partially de-methylesterified HG (recognized by JIM7) do not show a preferential orientation nor do the HGs in the periclinal wall. (E) According to the expanding beam model, lobe formation is initiated by the de-methylesterification of HG on the future convex side of the anticlinal wall, this triggers a phase transition in the HG filaments leading to the expansion of the anticlinal cell wall. The expansion is expected to create stresses in the periclinal wall (orange arrows), due to its extension at the concave side and compression on the convex side of the lobe, which might be relieved by local cell wall remodeling. Redrawn from (Haas et al., 2020b). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

An alternative explanation for the elasticity-growth connection was provided by a study suggesting that pectin volume transitions not only facilitate wall compliance during turgor driven growth but may actually provide a driving force for cell wall expansion (Haas et al., 2020b). The authors developed a new 2-color, 3D direct Stochastic Optical Reconstruction Microscopy (dSTORM) super-resolution immunolocalisation method on tissue sections with an estimated lateral and axial resolution of ~ 40 and 70 nm respectively. Using a panel of anti-pectin antibodies, they observed in anticlinal cell walls that HGs did not have a random orientation as expected for an amorphous gel, but were oriented, like cellulose microfibrils, perpendicularly to the cell's outer surface (Fig. 6) (Haas et al., 2020b). No such organization was observed in periclinal walls. They also showed that HG was preferentially de-methylesterified on the convex site of the cell wall in the developing lobes. This led them to formulate the "expanding beam" model in which lobe formation is driven by the transition from dense methylesterified HG to less dense de-methylesterified HG. To understand this, it is important to compare the nature of the interactions between HG chains. Indeed, X-ray diffraction on HG chains that were uni-axially oriented *in vitro*, showed that both HG^{HM} and low methylesterified HG (HG^{LM}) can form polycrystalline fibers (Walkinshaw and Arnott, 1981a). HG^{HM} chains aggregate through hydrophobic interactions between methyl groups stabilized by hydrogen bonds between rare unesterified carboxyl groups (Walkinshaw and Arnott, 1981b). HG^{LM} chains instead, are kept together by Ca^{2+} cross-links (Fig. 3C) (Walkinshaw and Arnott, 1981a). The important observation was that HG^{HM} is more densely packed with a 1.4 × smaller surface occupied by HG chains than Ca^{2+}-crosslinked HG^{LM}. Finite element modeling showed that this 1.4 × increase in surface is sufficient to explain lobe formation (Haas et al., 2020b). Further evidence for the model was provided by scanning electron microscopy on anticlinal walls, which also showed that oriented filaments were present, and that enzymatic or chemical de-esterification increased the cross-sectional area occupied by polymer chains by a factor of around 1.4. Finally, homogenizing PME activity on both sides of the cell wall through the ectopic expression of either a PME or a PMEI prevented lobe formation. A later study showed that not all pectin epitopes were oriented in these anticlinal walls. Indeed, partially methylesterified HG detected by JIM7 antibodies, showed a more homogeneous distribution, although this qualitative assessment needs to be backed up with a quantification of the anisotropy within the three-dimensional point patterns (Fig. 6C) (Haas et al., 2020b). This revealed an unexpected dimension in pectin organization, where HG methylesterification patterns are correlated with the formation of higher order structures, relevant for macroscale growth and pattern formation.

In summary, according to the expanding beam model, the cell wall has an intrinsic growth capacity, which depends on three key

parameters that remain to be explored: pectin volume transition induced by selective de-methylesterification, HG orientation/fiber formation and regulated HG secretion. We will discuss the implications of this model in the following section.

4. Implications of the expanding beam model.

4.1. The regulation of PME activity during growth

The model requires that PME activity is under tight spatio-temporal control. The cell wall pH may be a critical regulator, given the strong pH-dependence of PME activity (Wolf et al., 2009). In this context it is interesting to note that in pollen tubes and root hairs that show oscillatory growth, the growth rate oscillates together with cytosolic Ca^{2+} levels, wall thickness and surface pH (Bascom et al., 2018; Monshausen et al., 2007, 2008; Schoenaers et al., 2018, 2017) (Fig. 7A). Oscillations in surface pH are expected to be paralleled by oscillations in the activity of cell wall modifying agents, most of which have defined pH optima. In this view, wall alkalinisation promotes PME activity, which creates negative charges on HG leading to its swelling, whereas acidification promotes HG condensation and expansin-mediated crosslink removal (Zhang et al., 2017). The pH oscillations reflect at least in part the periodic activity of plasma membrane H^+-ATPases, (Tan et al., 2017) and understanding the mechanism underlying this periodicity will be a major challenge for future research. Whereas temporal pH variations can be observed in tip growing cells, they have not yet been reported in diffusely growing cells. Evidence exist, however, for spatial pH gradients going from the plasma membrane into the wall. Indeed, Martinière et al. (Martinière et al., 2018) compared outputs of genetically-encoded pH sensors either secreted into the cell wall or retained close to the plasma membrane (GPI anchored sensor) in order to monitor the pH in different cell wall domains. They observed steep pH gradients from pH 6.4 close to the plasma membrane to a pH < 5 throughout the cell wall. Given the very efficient diffusion of protons in aqueous solution (Diffusion coefficient, $DH^+ = 9.3 \times 10^{-5}$ cm^2/s), such steep pH gradients over nm distances cannot be maintained solely by the proton pump. One interesting possibility is that uronic acid-containing polymers such as pectins (Moustacas et al., 1991) or arabinogalactan proteins (Lopez-Hernandez et al., 2020), play a local buffering role.

The periodicity in the construction of the cell wall is also reflected in the periodic architectures of many cell walls. This can be observed in both tip- and diffusely growing cells but also in secondary walls deposited after cessation of cell expansion. Fig. 7B-E shows examples of the periodic cell wall deposition patterns in tobacco pollen tubes (B) (Derksen et al., 2011), multilamellated outer wall of onion epidermis cells (C,D) (Zhang et al., 2016) and the periodic helicoidal structure of external epidermal cell walls of dark-grown mung bean hypocotyls (E) (Roland et al., 1982).

Another implication of the expanding beam model is that the effect of HG de-methylesterification as measured by AFM indentation on growth may be independent of its effect on cell wall elasticity (Peaucelle et al., 2011, 2015). The latter could simply reflect the drop in cell wall density associated with the cell wall volume increase. This would explain, at least in part, the puzzling correlation between cell wall elasticity and growth.

4.2. HG fibril formation

HG fibril formation *in vivo* is, in contrast to what was claimed in a recent opinion paper (Cosgrove and Anderson, 2020), not unprecedented. Indeed, pectin fibers and their crystallinity have been observed *in vitro* and *in planta* since the 1930 s using electron microscopy and X-

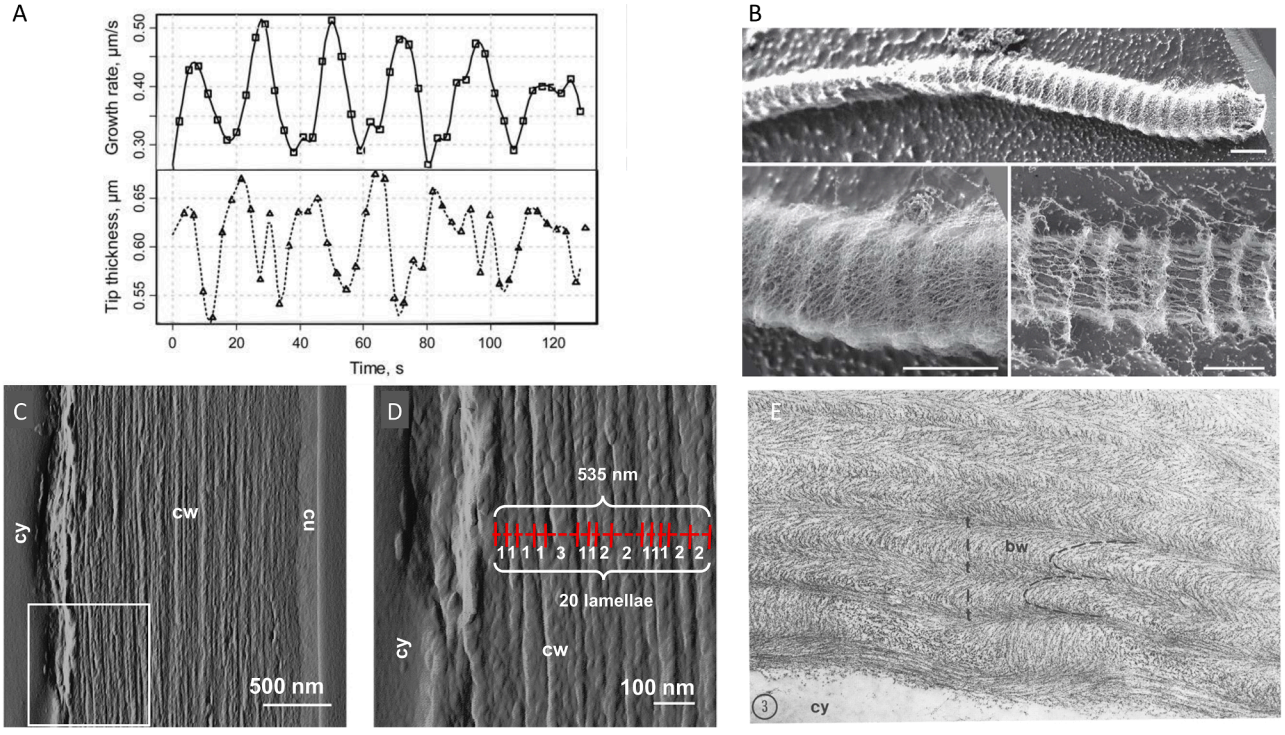

Fig. 7. Temporal and spatial periodicity in cell wall assembly and growth. (A) Growth rate and tip thickness oscillations in a lily pollen tube (adapted from (Mckenna et al., 2009)). (B) Scanning electron microscopy of tobacco pollen tubes fixed after EDTA extraction, showing periodic cell wall deposition patterns with a period of ~ 5 µm, which corresponds to the distance spanned during one growth rate period. Lower right panel was extracted for a longer time than the other two panels. Scale bars = 10 µm. (C,D) Periodic wall deposition patterns in onion epidermis cells. Cy: cytoplasm, cw: cell wall, cu: cuticle. (D) higher magnification of inset in (C) showing the dimensions of the wall layers. (E) Transmission electron microscopy on oblique sections through outer cell walls of dark-grown mung bean hypocotyl epidermis cells showing periodic helicoidal cell wall deposition patterns. Magnification x80000. cy: cytoplasm, bw: bow-shaped patterns. Adapted from (Derksen et al., 2011; Mckenna et al., 2009; Reis et al., 1985; Zhang et al., 2016).

ray diffraction (Palmer et al., 1947; Roelofsen and Kreger, 1951; Sterling, 1957; Van Iterson, 1933). Later studies suggested that phase transitions of pectic gels are an inherent property of the plant cell wall (Chen et al., 2007; Leppard and Colvin, 1971; Ramamoorthy and Leppard, 1977). Referring to a newly identified "non-cellulosic fibrillar polygalacturonic acid", they stated that their data supported a mosaic-like concept of the cell wall rather than the more widely assumed model of a more uniform reinforced gel (Leppard et al., 1971). The work was based on original observations of fine fibrous material stretching out from the cell wall into the extracellular medium in liquid plant cell culture (Leppard et al., 1971). These were seen either as individual linear strands or ordered into bundled rope-like structures and sheets with continuations with part of the cell wall that resembled the middle lamella. Furthermore, it appeared the same material could be found on the inner (cytoplasmic-facing) cell wall surface suggesting a structure that could be formed soon after deposition (Leppard et al., 1971). These structures were initially named "lignofibrils" due to their weak UV absorption properties and weak Phloroglucinol-HCl staining. Further chemical analyses, however, with IR spectroscopy being key, showed that the thin strands were composed of a GalA polymer (Leppard et al., 1971). These strands, termed fibrillar pectin, were easily resolved by transmission electron microscopy methods with a diameter in the order of 20 nm and were not confined to cultured cells but were additionally found in some sections of the root epidermis (Leppard and Colvin, 1972). With regards to the UV absorbing characteristics, this could be a consequence of bound tyrosine-rich proteins with candidates including polycationic extensins or extension-related factors and may go some way to explain why these fibres were reported to be largely resistant to pectinases (Leppard et al., 1971). Analogous structures, pectin fibrils with diameters in the range 20–30 nm, have been reported to extend into the lumen from the corners of poplar xylem fiber cells (Arend et al., 2008). The authors proposed a role for these hydrophilic fibers in water storage in the xylem. In common with the earlier work, these structures can be specific to species, cell type and even subcellular location. The existence of pectin fibrils raises the question how the HG chains can reach their uni-axial orientation. Is this through interaction with equally oriented cellulose, through a self-assembling nematic crystalline phase (Sanchez et al., 2012; Saw et al., 2018) or are they deposited in an oriented way? The latter, on first sight incongruent, possibility should not be discarded given the precedent of tubular exocytosis in neurons (Jullie et al., 2014) as well as the recent observation of extracellular vesicular-tubular structures that are involved in cell wall assembly (De Bellis et al., 2021).

4.3. Regulated matrix secretion

The "expanding beam" finite element model required a hypothetical feedback mechanism that down-regulates HG secretion as a function of the PME activity, since constitutive secretion would lead to unlimited cell expansion upon PME overexpression (Haas et al., 2020b). It will be interesting to investigate whether the cell wall matrix secretion rate depends on the pectin charge density and, if so, how such a feedback is regulated either directly or indirectly (e.g. through mechanosensing (Hamant and Haswell, 2017)).

5. What drives cell wall expansion: Cell wall tension, polymer insertion-expansion or both?

In the traditional creep-dependent growth models, cell wall tension, generated by the turgor pressure, is the motor for growth (Cosgrove, 2018). Cell and organ morphogenesis reflects local cell wall relaxation and the mechanical anisotropy of the cell walls, imposed by the orientation of cellulose microfibrils (Baskin, 2005) (Geitmann and Ortega, 2009). The models focus on the relaxation of the outer periclinal cell walls as a major growth regulator (Zhang et al., 2017) and less attention is paid to the anticlinal cell walls. In the expanding beam model, lobe formation in pavement cells is driven by the expansion of the anticlinal walls (Haas et al., 2020a), whereas periclinal walls, which do not have oriented HG filaments, grow isotropically. This process does not depend on cell wall tension but it generates tension in the periclinal cell walls, leading to compression of the wall in between the lobes (Fig. 6D,E). The cell wall tension might be resolved by a mechanism involving tension-driven remodeling of cell wall crosslinks (Zhang et al., 2017) at these locations. In this sense, the expanding beam model and tension-driven creep might be complementary mechanisms underlying cell expansion at least during lobe formation in pavement cells.

The creep model also implies that the mechanical properties are averaged over the width of the cell wall, with the mechanical anisotropy resulting from the orientations of the cellulose microfibrils in the different cell wall layers. On the other hand, cell growth reorientation has been related to instantaneous changes in the orientation of microtubule arrays and cellulose (Baskin, 2005; Lindeboom et al., 2013). This implies that the most recently deposited cell wall layers and not the accumulated cellulose orientation determine the growth direction, which somehow undermines the creep model. In the expanding beam model instead, growth is intrinsically related to the expansion of the most recently deposited HG, the orientation of which may depend on microtubules. In this way the growth direction can instantaneously respond to microtubule reorientation. The process, however, would generate tension between the expanding and older cell wall layers, which again might be resolved through a tension-induced creep mechanism

6. Conclusions and perspectives

In this review we discussed a number of examples showing that phase separation in polymer mixtures is widely exploited in the control of biological processes not only in the cytosol, nucleoplasm and at membrane interfaces, but also in the extracellular matrix of plant cells. We discussed recent data showing a role for phase separation in the spatial organization of the exine in pollen grains, the surface features of *Penium* cells, the regulated hydration of pollen grains and seed mucilage as well as the possible recruitment of a cell wall modifying enzyme in specific wall domains of seed coat cells. We also discussed how pH-induced changes in pectin assemblies and enzyme-regulated volume transitions in crystalline pectin fibers may contribute to cell wall expansion and how this may fit in the current turgor-driven growth models. These observations illustrate the importance of considering mesoscale emergent properties of polymer networks to understand polysaccharide structure–function relations. This will require more precise methods to study nano-to mesoscale architecture of intact cell walls. Solid state NMR on never dried cell walls has recently provided important insights into *in situ* polymer structure and interactions (Cosgrove, 2018; Simmons et al., 2016) but lacks information on the topology of these interactions within the cell wall. Super-resolution microscopy offers great potential in this area. The current state-of-the-art optical nanoscopes, however, lack the resolution required for observing associations between polymers, for instance to determine whether HG nanofilaments consist of pectin only or of cellulose coated with pectin (Haas et al., 2020b; Peaucelle et al., 2020). The next generation of nanoscopes, such as MINFLUX (Schmidt et al., 2021), in combination with novel nano-probes should provide near-molecular scale maps of cell wall polymers in intact cell walls in the near future. New fluorescent tools are needed to study phase transitions in living cells plus simplified *in vitro* and corresponding *in silico* models to investigate how polysaccharide structure affects the phase behavior of assemblies. Other areas for future research concern the enzymology of PMEs and other cell wall-modifying enzymes in model assemblies and if possible *in vivo*, the structural basis of the phase partitioning of cell wall enzymes and the control of the orientation of matrix polymers in the cell wall, the control of the cell wall pH and redox state, the secretion of cell wall polymers and the regulation of the periodicity in cell wall assembly

processes.

We are entering a new exciting era in plant cell wall research, which brings together glycochemistry, soft matter physics and cell biology to provide new insights in the control of the cell wall architecture, plant growth and most likely also abiotic stress resistance and immunity (Voxeur and Höfte, 2016). The understanding of the emergent properties of co-evolved polymer assemblies is also expected to provide inspiration for the development of new functional nanomaterials. Finally it will be interesting to see whether the concepts discussed in this review are also relevant for other, biochemically distinct, cell walls such as those of algae, bacteria, oomycetes or fungi.

Important questions for future research:

Use improved resolution nanoscopy and nano-probes to elucidate wall architecture
Distinguish pectin filaments from pectin-coated microfibrils
What controls the orientation of HG?
Synthesis and function of different HG domains and substitution patterns
In vivo PME activity: regulation, dependence on pH, Ca^{2+}, polymer network.
Regulation of (targeted) secretion at the proper time and place.
Mechanism of and causal relation between oscillations in growth, pH, ROS, secretion
Determinism of phase transitions in cell wall polymer assemblies *in vitro* and *in vivo*.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

The work was financed in part by Agence Nationale de Recherche (ANR) grants “Pectosign”, “Goodvibrations” and “Homeowall”. The IJPB benefits from the support of Saclay Plant Sciences-SPS (ANR-17-EUR-0007). We apologize to the authors of relevant publications, we haven’t been able to cite due to space constraints.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tcsw.2021.100054.

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