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REVIEW

Tuning of pectin methylesterification: consequences for cell wall biomechanics and development

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Abstract

Main conclusion   **Recent publications have increased our knowledge of how pectin composition and the degree of homogalacturonan methylesterification impact the biochemical and biomechanical properties of plant cell walls, plant development, and plants' interactions with their abiotic and biotic environments. Experimental observations have shown that the relationships between the DM, the pattern of de-methylesterificaton, its effect on cell wall elasticity, other biomechanical parameters, and growth are not straightforward. Working towards a detailed understanding of these relationships at single cell resolution is one of the big tasks of pectin research.**

Pectins are highly complex polysaccharides abundant in plant primary cell walls. New analytical and microscopy techniques are revealing the composition and mechanical properties of the cell wall and increasing our knowledge on the topic. Progress in plant physiological research supports a link between cell wall pectin modifications and plant development and interactions with the environment.

Homogalacturonan pectins, which are major components of the primary cell wall, have a potential for modifications such as methylesterification, as well as an ability to form cross-linked structures with divalent cations. This contributes to changing the mechanical properties of the cell wall. This review aims to give a comprehensive overview of the pectin component homogalacturonan, including its synthesis, modification, regulation and role in the plant cell wall.

Pectin as biochemical components of the plant cell wall

Cell walls play diverse functions including protection and interaction with the environment. The cell wall is also responsible for the plant cell's capacity to develop turgor pressure, which is necessary for cell growth and ultimately for supporting the entire plant body structure.

Plant cell walls

The plant cell wall is a highly dynamic structure, continually being modified throughout development and in reaction to diverse environmental conditions. The genesis of each cell wall is the division process: during cell division, the primary cell wall grows from the cell plate to the nearest wall (Bordenave and Goldberg 1993). Subsequently, the surface area of this primary cell wall greatly increases at a special interface, the middle lamella, between the two newly formed, adjacent, primary cell walls. Finally, once growth has ceased, secondary cell walls are deposited onto the primary cell walls from the

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plasma membrane side. The secondary cell wall composition depends on the tissue and function of the cell, but their pectin content is lower than that of primary cell walls (Caffall and Mohnen 2009). Current models describe the primary cell wall as a complex highly cross-linked polymer framework (Fry 2004; Somerville et al. 2004; Cosgrove 2005; Cosgrove and Jarvis 2012). The secondary cell wall is generally less dynamic and malleable and may remain relatively static once deposited.

Most higher plants contain over 30 % pectic polysaccharides in their primary cell walls. Within the primary wall, pectin forms a hydrated gel phase in which the network of cellulose microfibrils and other cell wall components are embedded. Individual pectin polymers interact covalently and non-covalently with other cell wall components and other pectin molecules, contributing to cell wall structure and function.

Pectins are a group of complex polysaccharides whose defining features are 1,4-linked α-D-galacturonic acid residues. Pectins are classified into homogalacturonan (HG), the substituted galacturonan xylogalacturonan (XGA), and the two rhamnogalacturonans RG-I and RG-II (Ridley et al. 2001). HG is a polymer that contains only α-1,4-linked-d-galacturonic acid (Ridley et al. 2001). XGA has a HG backbone that it is substituted with single β-1,3-Xyl residues which can in turn be linked to an additional xylose residue. In contrast, RG-I has a backbone structure of [α-D-GalpA-1,2-α-L-Rhap-1,4]$_n$ repeating units; RG-I has been found to be largely unbranched in *Arabidopsis* seed mucilage (Western et al. 2001), but is frequently branched in other anatomical contexts with side chains of arabinan, galactan or arabinogalactan attached at the C-4 positions of backbone sugars (Lau et al. 1985). RG-II has a highly complex and conserved structure with a small HG backbone of only a few residues, and four well-defined side chains with a total of 12 different glycosyl residues in over 20 different linkages (Caffall and Mohnen 2009). RG-II is ubiquitous in the plant cell wall and represents up to 8 % of *Arabidopsis* plant cell wall polymers (Zablackis et al. 1995).

The different pectic polysaccharides can be covalently linked to each other, forming a massive pectin network. The known extent of this network has been expanded by several publications: HG can be covalently cross-linked to the pectic polysaccharides RG-I and RG-II (Willats et al. 2001), and also to the hemicellulose xyloglucan (XG) (Nakamura et al. 2002). In addition, HG can be covalently linked to arabinogalactan protein; it was recently demonstrated in *Arabidopsis* that a rhamnosyl residue in the arabinogalactan domain of the arabinogalactan protein ARABINOXYLAN PECTIN ARABINOGALACTAN PROTEIN1 (APAP1) was linked to RG-I and HG (Tan et al. 2013). Mutants lacking this protein and the corresponding wall structure showed an increased extractability of pectic and

hemicellulosic polysaccharides, which indicates that the pectin–protein linkage plays an important structural role (Tan et al. 2013). The ratio of HG, XGA, RG-I, and RG-II differs between species, tissues, and developmental states, but HG is typically the most abundant pectin polymer and will remain our focus in this review.

HG biosynthesis and secretion

HG is synthesized in the Golgi apparatus and secreted into the cell wall. HG biosynthesis and processing are summarized in Fig. 1. In general, nucleotide sugars are transported from the cytosol to the Golgi, where glycosyl transferases assemble monosaccharides into polysaccharides. In reality, this process is complex; for example, it has been shown that a large number of glycosyl transferases are required to synthesize the pectic polysaccharides (Ridley et al. 2001; Caffall and Mohnen 2009; Rautengarten et al. 2014).

According to the current model of HG synthesis, S-adenosyl methionine and UDP-GalA, precursors for pectin synthesis, are imported into the Golgi apparatus by specific transporters (Wolf et al. 2009a). Subsequently, a complex of HG galacturonosyltransferase (GAUT) and pectin methyl transferase (PMT) facilitates polymerization and methylesterification of HGs (Wolf et al. 2009a; Wang et al. 2013a). To date, GAUT1 is the only galacturonic acid transferase whose identity has been confirmed and characterized biochemically (Sterling et al. 2001). Two polypeptides of GAUT1 bind to one polypeptide of GAUT7, and this heterocomplex is responsible for retaining GAUT1 in the Golgi membranes (Atmodjo et al. 2011). Two further HG biosynthetic proteins are QUASIMODO1 (QUA1), which encodes the GalA transferase GAUT8 (Bouton 2002; Leboeuf et al. 2005), and QUA2 (also known as TUMOROUS SHOOT DEVELOPMENT2, TSD2), which has been proposed to be a pectin methyltransferase, (Krupková et al. 2007; Mouille et al. 2007). Due to their defects in HG biosynthesis, *qua* mutants have a decreased HG content. The length of the individual HG domains in the *qua-2* mutant remains similar to that of WT. However, the number of HG domains is reduced, which enhances the flexibility of the pectin polymer, with consequences on morphology that are discussed below (Ralet et al. 2008).

Little is known about the secretion process of HG pectin. Nevertheless, HG-specific antibody labeling revealed a non-uniform distribution of this pectin component in pea parenchyma cells (Willats et al. 2001) as well as *Nicotiana tabacum* and *Solanum chacoense* pollen tubes (Bosch and Hepler 2005; Parre and Geitmann 2005). This supports the existence of a mechanism for localized HG secretion from the Golgi to the cell wall, or redistribution and concentration of HG in specific cell wall regions in muro after secretion. An example of directional pectin secretion is *Arabidopsis* seed
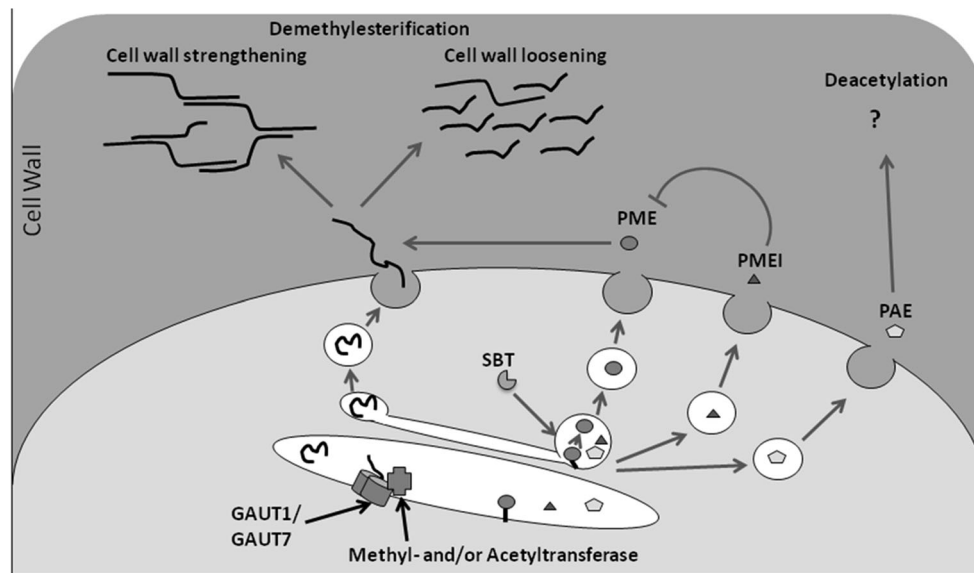
Fig. 1 A complex of synthetic enzymes including Galacturonosyl-transferase-1 (GAUT1) and -7 (GAUT-7) participates in the synthesis of the homogalacturonan (HG) pectin component (symbolized by *black lines* in the figure) in the Golgi apparatus. During synthesis, methylester groups and acetyl groups are added onto the HG by putative Methyl- or Acetyl-transferase. Subsequently, the HG pectin is delivered to the cell wall by the Trans Golgi Network inside secreted vesicles. Pectin Methyl Esterase (PME) enzymes and their inhibitors (PMEI) are also secreted into the cell wall. Unlike Group 1 PMEs, Group 2 PMEs, which include a pro-domain, require cleavage by Subtilisin-like serine protease (SBT) in the golgi for secretion to the cell wall. PMEIs may inhibit specific PMEs in muro. When not inhibited by PMEIs, the active PMEs will demethylesterify the HG pectin. This demethylesterification can lead to cell wall strengthening or loosening, likely depending on the pattern of demethylesterification. HG pectin can also be acetylated as well as deacetylated by pectin acetyl esterases (PAEs). Little is known about this process and its significance

coat mucilage, which is mainly composed of HG. The HG is deposited in seed coat cells specifically at the outward facing cell walls, through polarized secretion (Haughn and Western 2012). The trans-golgi network-localized ECHIDNA/YIP4a and 4b protein complex is required for the correctly localized secretion of cell wall polysaccharides, including HG, in the *Arabidopsis* seed coat (Gendre et al. 2013). The *echidna* mutant shows a highly reduced accumulation of pectineus mucilage in the cell walls of the seed coat, but mucilage can be detected in the vacuole, again highlighting the importance of a strict cellular control of the secretion mechanisms (McFarlane et al. 2013).

HG methylesterification and ion cross-links

HG GalA residues can be methylesterified at their C-6 carboxyl groups, or acetylated at their O-2 or O-3 (Ridley et al. 2001). The pattern and degree of these modifications is of central importance to the biochemical and biomechanical properties of the cell wall, and is therefore tightly regulated in a tissue-specific manner during development. Immunolabeling of un-esterified and highly methylesterified HG indicates that it is secreted in a methylesterified state into the cell wall (Zhang and Staehelin 1992; Staehelin and Moore 1995; Ha et al. 1997; Sterling et al. 2001; Mouille et al. 2007; Driouich et al. 2012) with as much as 80 % of the GalA residues being methylesterified before secretion (Mohnen 2008).

HG may then be de-methylesterified within the wall. De-methylesterified HG may encounter two general fates: (1) the formation of a stable structure with other HG molecules if at least ten consecutive GalA residues are de-esterified (Liners et al. 1989), or (2) degradation by polygalacturonases. The former, stable gel formation, will be our main focus. HG GalA residues are negatively charged and can ionically interact with divalent cations, mostly Ca^{2+}, in a structure often referred to as the 'egg-box'. Studies using antibodies against highly methylesterified HG (JIM7 and LM 20) or HG with a lower degree of methylesterification (DM; JIM5 and LM19) show that methylesterification patterns can vary on a very small spatial scale within tissues (Peaucelle et al. 2008; Lehner et al. 2010; Chebli et al. 2012). The non-homogeneous distribution of HG of various methylesterification degrees/patterns is thus a key factor contributing to spatial differences in the properties of the cell wall, even within a single cell (Zerzour et al. 2009). Within tissues, the distribution of stable pectin gels may also be spatially regulated, thus influencing mechanical properties on a tissue level, as well as growth.

In addition to HG, two pectin RG-II molecules can crosslink through ionic interaction by forming boron bridges (O'Neill et al. 2004). Contrary to the formation of Ca^{2+}-bridges between HG strands, which are the consequence of linear de-methylesterification *in muro*, boron bridges are

integrated when the RG-II molecule is synthesized and secreted (Chormova et al. 2014a, b). Disruption of the RG-II-boron complex causes a lack of wall expansibility in plants resulting in dwarfed stature, compromised cell adhesion, and defects in reproductive tissue function (Iwai et al. 2002, 2006). More recently, defects in boron transporters and subsequent decreases of cross-linked RG-II have been linked to developmental defects in *Zea mays* tassels (Durbak 2014; Chatterjee 2014). These data reinforce the important role of pectic polysaccharides in cell wall structure and plant growth and development.

HG acetylation

Pectin acetylation is not as well studied when compared to methylesterification (Whitcombe et al. 1995; Ishii 1997). Acetyl esters can be found on a number of cell wall polysaccharides including the pectin components (Manabe et al. 2011). The Golgi has been suggested to be the site for O-acetylation, with Acetyl-CoA as the putative donor substrate (Pauly and Scheller 2000). In support of this hypothesis, xyloglucan and putative xylan O-acetyltrans-ferases were shown to be located in the Golgi (Xiong et al. 2013; Gille et al. 2011). In vitro assays using potato stem cell suspension cultured with [^{14}C]acetyl-CoA led to the formation of radiolabeled polysaccharides including HG (Pauly and Scheller 2000), supporting the role of acetyl-CoA as a donor substrate for O-acetylation.

Reduced wall acetylation2 (RWA2) was the first protein shown to be involved in cell wall acetylation in planta (Manabe et al. 2011) and the *rwa2* mutant of *Arabidopsis* has a 20 % decrease in cell wall O-Acetylation. RWA2 is involved in acetylation of both pectic and non-pectic polysaccharides and is suggested to be an O-acetyl-transferase, but its bio-chemical activity is yet to be characterized. In a quadruple *rwa* mutant covering four redundant family members, there is a 63 % decrease in overall O-Acetylation. The decrease is associated with severe dwarfism, absence of inter-fascicular fibers and abnormal xylem cells (Manabe et al. 2011). This phenotype points to a key, perhaps understudied role, for pectin acetylation in proper growth and development.

Pectin acetyl esterases (PAEs) catalyze the cleavage of the ester bond between a glycosyl carbon and an acetyl group, thus releasing acetate from a modified pectin polysaccharide (Gou et al. 2012). The first plant PAEs were isolated from Orange (*Citrus sinensis*) (Williamson 1991; Christensen et al. 1996) and mung bean (*Vigna radiata*) (Bordenave et al. 1995). However, the functional characterization of PAEs has remained rather scarce. Tobacco plants (*Nicotiana tabacum*) overexpressing a poplar *PAE* (*PtPAE1*) are impaired in the cellular elongation of floral organs as well as in the germi-nation and growth of pollen tubes, inferring male sterility (Gou et al. 2012). Mutations in *AtPAE8* and *AtPAE9* led to a

20 % increase of acetate in the cell wall predominantly from a fraction rich in RGI (de Souza et al. 2014). The *pae8* mutant also shows a slight, but significant, increase in acetate content in the low molecular weight fraction most likely corre-sponding to HG. The additive phenotype of the double mutant *pae8/pae9* suggests that PAE8 has partial specificity for acetyl-esterified HG substrates (de Souza et al. 2014). The decrease of acetylation seen in *rwa* and the increase in *pae8* and *pae9* describe a complex relationship between growth and pectin acetylation: both display defects in elongation, which complicates their interpretation. Nevertheless, this remains an important puzzle to solve.

Pectin methylesterases (PMEs) and their inhibitors (PMEIs)

Pectin methylesterases (PMEs) catalyze the reaction by which methylesters are removed from an HG chain resulting in a free carboxyl group and the release of a proton and methanol (Wolf et al. 2009a). PMEs are antagonistically regulated in the cell wall by proteinaceous PME Inhibitors (PMEIs). PMEs are classified into two groups by their pri-mary structures with the distinguishing feature being the presence or absence of an N-terminal PRO region (Louvet et al. 2006; Wolf et al. 2009b; Wang et al. 2013b). The group one/type II PMEs have only a short or absent N-terminal PRO region, whereas the group two/type I PMEs possess one to three long PRO domains (Micheli 2001; Sénéchal et al. 2014b). Several lines of evidence indicate that the PRO region is cleaved off from some or all PMEs before they are secreted into the cell wall. Cell-wall-extracted PMEs from mung bean hypocotyls do not have the PRO regions (Bor-denave and Goldberg 1993), and only the mature form of AtPME3, i.e., the form without the PRO domain, was found in enriched cell wall samples (Guénin et al. 2011). Interest-ingly, the PRO region is highly similar in structure to specific domains of PMEIs. Mathematical modeling predicted that the major contribution of the PRO-containing group 2 PME family proteins to overall PME activity in different *Lepidium sativum* seed compartments is in de-methylesterification rather than in PMEI-like activity, supporting the hypothesis that the PRO region is generally cleaved off during protein maturation (Scheler et al. 2014).

Genome and EST sequencing projects have shown that *PMEs* belong to large multigene families in all plant species sequenced to date. In poplar (*Populus trichocarpa*), 89 ORFs have been annotated as putative full length *PMEs* compared to 41 ORFs in rice (*Oryza sativa*) (Coutinho et al. 2003; Wang et al. 2013b) Coutinho et al. (2003) listed 66 ORFs in *Ara-bidopsis* annotated as *PME* corresponding to 6.81 % of all carbohydrate-active enzymes in the species (Coutinho et al. 2003). Scheler et al. (2014) identified 67 putative PMEs in *Arabidopsis*, with 22 falling into group 1, and 45 into group 2.

The smaller family size in rice relative to that of *Arabidopsis* and poplar is consistent with the finding that HG is less abundant in monocots than in dicots (Pelloux et al. 2007).

There are almost as many *PMEI* genes as *PME* genes in sequenced dicot species such as *Arabidopsis* and *Populus*. Database analysis has shown that 69 genes encode putative PMEIs/invertase inhibitors in *Arabidopsis* (Scheler et al. 2014). Monocots have fewer *PMEI*s, consistent with remarks above; 35 genes encoding putative *PMEI*s were found in rice (Wang et al. 2013b). The mode of action of PMEI is still unclear, but several studies have found that individual PMEIs can interact in a 1:1 ratio with a number of PMEs in a pH-dependent manner, sometimes over species boundaries. For example, a pepper PMEI can inhibit *Arabidopsis* PMEs (An et al. 2008), and *Arabidopsis* and kiwi PMEIs inhibit tomato PMEs (Hothorn et al. 2004). The three-dimensional structure of *Arabidopsis* and kiwi PMEIs interacting with purified PMEs from tomatoes has been resolved, revealing that PMEI prevents access for the substrate by covering the PME catalytic cleft (Hothorn et al. 2004; Di Matteo et al. 2005).

Regulation of PMEs and PMEIs at the posttranscriptional and posttranslational level

Given the strong influence of pectin on plant development detailed above, it is of vital importance for the plant to spatially and developmentally control pectin-modifying protein abundance and activity of enzymes such as PME and PMEI. Expression of a large number of genes belonging to the *PME* and *PMEI* families has been detected in microarrays at all analyzed developmental stages in *Arabidopsis* [eFP-browser, http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi (Winter et al. 2007)]. Thus, given the presence of large numbers of mRNAs for both PMEs and PMEIs, it is likely that the main part of PME activity regulation happens on the posttranscriptional or posttranslational level. Candidate mechanisms include RNA processing, protein maturation and protein stability, interactions of the active PMEs with PMEIs, and modulation of enzyme activities in muro. We will briefly discuss these possibilities in order.

Alternative splicing may regulate the organ specificity of the functional forms of two PMEIs in wheat. Mature and fully spliced transcripts that are translated into functional proteins were only found in anthers, but transcripts with retained introns were present in other tissues as well (Rocchi et al. 2012).

In addition to regulation at the transcript level, group 2 PMEs can be regulated posttranslationally through their maturation process. The PRO region of PMEs mediates retention of unprocessed group 2 PMEs in the Golgi (Wolf et al. 2009b). Subtilisin serine proteases (SBTs) are strong candidates for the enzymes that cleave off the PRO region

in the Golgi have not been identified with certainty. The *Arabidopsis* SBT AtS1P was co-precipitated with the PME VDP1 from a microsome fraction, indicating interaction with unprocessed PMEs in the Golgi (Wolf et al. 2009b). It has furthermore been shown in *Arabidopsis* that AtSBT3.5 is not only co-expressed with PME 17, but also processes the PME for release of the mature form into the apoplast by cleaving off the PRO region at a specific motif (Sénéchal et al. 2014a). Because the PRO region is cleaved before secretion, it might be degraded or it could have a separate function in the cell, such as helping the correct folding of the PME, targeting of the PME to the cell wall, or inhibiting PME activity before secretion (Micheli 2001).

PME protein stability and degradation as regulatory factors of PME activity are just beginning to be explored. FLYING SAUCER1 1 (FLY1), a transmembrane RING E3 ubiquitin ligase, was recently proposed to regulate the DM of pectin by recycling pectin methylesterase enzymes in the endomembrane system of seed coat epidermal cells (Voiniciuc et al. 2013). FLY1 is orthologous to yeast E3 ligase involved in the quality control of membrane proteins in yeast. In *Arabidopsis*, the *fly1* mutant causes a lower DM in the pectin-enriched seed mucilage (Voiniciuc et al. 2013). It is evident that the processing of PME proteins may have a significant effect on their activity, and thus the DM of the cell wall pectin.

PME activity is highly dependent on pH. The demethylesterification patterns produced by PMEs depend on the pH of the cell wall environment, the pH optimum of the individual enzymes, and the degree of pectinification of the substrate (Catoire et al. 1998; Denès et al. 2000). The use of newly developed technologies for live imaging of extracellular pH might shed new light on the potential distribution of pH micro-domains in the wall of a given cell or within a given tissue (Gjetting et al. 2012). In addition, the type and concentration of cell wall cations can affect the activity of PMEs (Schmohl et al. 2000). Trivalent cations are more effective than bivalent cations which, in turn, are more effective than monovalent cations in increasing PME activity (Schmohl et al. 2000). The concentration of these cations is thought to modify the affinity of PMEs for their substrate (Moustacas et al. 1991). It is conceivable that localized micro-domains within the cell wall could differentially regulate PME and PMEI activity in spatial and temporal contexts.

Pectins in relation to plant development and plant interactions with the environment

The cell wall is a mediator of intrinsic processes such as growth, shape, and structural stability but also of interactions with extrinsic factors such as the environment and

pests. It is a physical actor in these processes, and as such its physical properties become highly relevant. In recent years, methods used in the engineering sciences have been adapted for plant research to better characterize the biomechanical and biochemical properties of whole plant organs, tissues, and cell walls (Burgert and Keplinger 2013; Milani et al. 2013; Moulia 2013). Several cell wall properties influencing plant development, morphology, and interaction with the environment are strongly linked with pectin composition and modification. These include cell wall elasticity, hydration, porosity, and adhesion (Braybrook et al. 2012). In the following sections, we will attempt to describe how pectin and cell wall mechanical properties are related to cell elongation, stem structural integrity, tissue integrity, seed hydration, abiotic and biotic interactions.

There is mounting evidence that the mechanical properties of the primary cell wall matrix, primarily comprising pectins, are key in regulating developmental processes in plants. Pectin gels are capable of large changes in hydration and stiffness, which can alter the behavior of cells and tissues. As an example, increasing stiffness of cell wall's pectin gel may result in decreased cell growth, or if stiffened enough in cell–cell separation by gel fracture. Alternatively, softening of a gel might lead to increases in growth or alteration in hydration effecting physiological processes such as seed germination. In the following examples, we will see that the story is not straightforward, but generally pectin softening is permissive of growth and hydration and stiffening restrictive of growth, required for strength and permissive of cell separation.

The softening and stiffening of a pectin-based cell wall matrix is largely achieved by alterations in pectin chemistry, here we will focus on HG pectin. Newly delivered HG, as mentioned earlier, is highly methylesterified (Zhang and Staehelin 1992; Staehelin and Moore 1995; Ha et al. 1997; Sterling et al. 2001; Mouille et al. 2007; Driouich et al. 2012). As high DM leads to a softer, more pliant gel in vitro and in planta, it may be assumed that new pectin is soft. The activity of PMEs on pectin can result in two major mechanical outcomes: further cell wall softening or cell wall stiffening. This apparently disparate set of outcomes results from the fate of low DM pectin: low DM pectin is a target for polygalacturonases which cleave it further and likely intensify gel softness; low DM pectin is also ready to cross-link with Ca^{2+} to increase gel rigidity (Vincent and Williams 2009). It is likely that the fate of low DM pectin is controlled by the presence/absence of polygalacturonases and/or calcium in a cell-specific manner. In line with this, the effect of PMEIs on cell wall matrix mechanics may also differ. In cases of cell adhesion and separation, we see the effect that PG can have on the mechanical outcome of PME activity. In most cases of

growth and strength, as we will see below, the activity of PMEs leads to stiffening and PMEIs to softening. However, there are several cases of the opposite being true, which complicate our understanding of cell wall mechanics and growth.

Cell elongation growth processes

The strength of the cell wall counteracts the cell's turgor pressure, and any growth process depends on tight spatial and temporal regulation of one of these two opposing forces (Schopfer 2006). At constant turgor pressure, cells grow in volume when the cell wall polymer network is loosened and extensibility increased through restructuring or dissolution of load-bearing bonds. The primary cell wall is a viscoelastic material that displays elastic properties below a stress threshold, which depends on its composition and state, but deforms like a viscous material when the threshold is crossed (Hayot et al. 2012). This can lead to the whole cell expanding isotropically, or more commonly in a tissue context, to anisotropic expansion or even bulging when only small domains in the cell wall deform (Schopfer 2006). Thus, in organs that grow particularly fast such as hypocotyls and stems, extensive cell wall modifications would be expected.

Several studies indicate a role for the DM in cell expansion and elongation. These studies are summarized in Table 1. In light-grown hypocotyls, low DM reduced cell elongation in GA-deficient mutants [ga (Derbyshire et al. 2007)]. Their shortened hypocotyl phenotype and low DM could be rescued by the application of exogenous GA (Derbyshire et al. 2007). Similarly in Arabidopsis, knocking out the ubiquitously expressed PME3 led to an increase in hypocotyl length in dark-grown hypocotyls (Guénin et al. 2011). Interestingly, not all evidence in dark-grown hypocotyls is congruent: Fourier Transform Infrared spectroscopy [FTIR, (Alonso-Simón et al. 2011)] analysis showed that their rapid growth was associated with a decrease of detectable pectin ester bonds suggested to be methylesters (Pelletier et al. 2010), and the transcription of a number of pectin-modifying enzymes, including PMEs and PMEIs, was upregulated during the growth phase (Pelletier et al. 2010). Overexpression of PMEI4 in dark-grown hypocotyls led to an increase in ester bonds in the cell wall pectin fraction, and delayed the growth acceleration (Pelletier et al. 2010).

The apparent contradiction that low DM in the cell wall has been documented to lead to opposite consequences, namely an increase (Pelletier et al. 2010) or decrease (Derbyshire et al. 2007; Guénin et al. 2011) in hypocotyl elongation, may result from several possible scenarios: differences in the pattern of de-methylesterification may yield different mechanical outcomes, and as mentioned

Table 1 Overview of studies on the role of the degree of pectin de-methylesterification in *Arabidopsis* development

Publication	Genotype	Treatment	Reference sample	DM	Phenotype	Increased DM and/or decreased PME activity associated with…	Comment
Hypocotyl							
Derbyshire et al. (2007)	ga1-3	Light-grown	WT	Decreased	Reduced cell elongation and hypocotyl length	Increased elongation	DM determined by FTIR
	ga1-3	Light-grown, exogenous GA	No GA	increased	Cell elongation and hypocotyl length rescued		DM determined by FTIR
Guénin et al. (2011)	pme3-1	Dark-grown	WT	Increased	Increased hypocotyl length, increased number of adventitious roots, decreased rosette diameter, decreased inflorescence stem length	Increased elongation in some organs, decreased in others	DM determined by FTIR and GC
Pelletier et al. (2010)	WT	Dark-grown, fast growth phase	WT, slow growth phase	Decreased	NA	Slow growth phase	DM determined by FTIR
	35S::PMEI4	Dark-grown	WT	Increased	Delayed onset of growth acceleration phase by 5 h	Dose-dependent delay of growth acceleration	DM determined by FTIR
	pPMEI4::PMEI4	Dark-grown	WT	Increased	Delayed onset of growth acceleration phase by 3 h	Increased acceleration	DM determined by FTIR
Meristem							
Peaucelle et al. (2008, 2011a)	35S::ALCR ALCA::PMEI3	Alcohol induction	Non-induced	Increased	Suppression of lateral organ formation, decreased cell wall elasticity in subepidermal layers, no difference in stem length	Suppression of lateral organ formation	DM determined by immunolabeling. Cell wall elasticity determined by AFM. phyllotaxis and were frequently dramatically enlarged
	35S::ALCR ALCA::PME5	Alcohol induction	Non-induced	Decreased	Increased cell wall elasticity in subepidermal layers	Disturbed phyllotaxis	DM determined by immunolabeling. Cell wall elasticity determined by AFM
	WT	Application of beads with active PME	Application of beads with deactivated PME	NR	Bulges turning into flower primordia at application site	Suppression of primordia formation	DM not determined
	at3g05620 (PME)	NA	WT	NR	No visible meristem phenotype	NA	NA
	at3g27980 (PME)	NA	WT	NR	No visible meristem phenotype	NA	NA
	pme5	NA	WT	NR	No visible meristem phenotype	NA	NA
Braybrook and Peaucelle (2013)	pin1	Exogenous IAA	pin1 without IAA	Decreased	Increased cell wall elasticity	Suppression of primordia formation	DM determined by immunolabeling. Cell wall elasticity determined by AFM. IAA cannot rescue the pin1-like meristem phenotype of plants overexpressing PMEI3

Table 1 continued

Publication	Genotype	Treatment	Reference sample	DM	Phenotype	Increased DM and/or decreased PME activity associated with…	Comment
Pollen tubes							
Jiang et al. (2005)	vanguard 1 (vgd1) (PME)	NA	WT	NR	Slower pollen tube growth, increased rate of pollen tube bursting	Faster elongation, decreased pollen tube stability	DM not determined, decreased PME activity in pollen assessed by enzyme assay
Tian et al. (2006)	atppme1	NA	WT	NR	Slower and aberrant pollen tube growth	Faster elongation	DM not determined, decreased PME activity in pollen assessed by enzyme assay
Röckel et al. (2008)	35S::AtPMEI2, transiently expressed in N. benthamiana	NA	N. benthamiana WT	NR	Faster pollen tube elongation	Faster elongation	DM not determined
	35S::AtPMEI1, transiently expressed in N. benthamiana	NA	N. benthamiana WT	NR	Mildly slower pollen tube elongation	Faster elongation	DM not determined
Dardelle et al. (2010)	WT	Tip of elongating pollen tube	WT, non-tip area of pollen tube	Increased	NA	Faster elongation	DM determined by immunolabeling and GC–MS.
Leroux et al. (2015)	pme48	NA	WT	Increased	Decreased germination, increased diameter and bursting of pollen tubes	Decreased germination, decreased pollen tube stability	DM determined by FTIR and immunolabeling
	pme49	Addition of Ca^{2+} to germination medium	No Ca^{2+}	NA	Pollen germination and tube growth rescued	NA	NA

NR not reported, PME pectin methylesterase, PMEI pectin methylesterase inhibitor, WT wildtype

earlier the presence of calcium and polygalacturonases may alter the fate of low DM pectin.

Changes in cell wall elasticity, brought about by changes in DM, have been shown to play a role in apical meristems (Peaucelle et al. 2008). Here, low DM led to cell wall relaxation in the meristem, which initiated organogenesis extemporally (Peaucelle et al. 2011a; Braybrook and Peaucelle 2013). This de-methylesterification could be induced through application of auxin, an accumulation of which is required for organ initiation. In line with this, auxin induction of initiation depended on the de-methylesterification process (Braybrook and Peaucelle 2013). Furthermore, in the *Arabidopsis* meristem, the transcription factor BELLRINGER (BLR) represses expression of *PME5* (Peaucelle et al. 2011b). In the *blr-6* mutant, ectopic expression of *PME5* in the meristem leads to altered phyllotaxis, which is rescued in a *blr/pme5* double mutant. The meristem is thus another example of a system in which a low DM leads to a decrease in cell wall stiffness, again pointing to the complexity of the connection between the DM and cell wall biomechanical properties.

Pectin modification also plays a key role in tip growth of plant cells, but here we return to evidence for high DM equaling softer walls. Pollen tubes elongate extremely fast through tip growth and PMEs have been shown to be involved, with a lower DM leading to slower elongation. *VANGUARD1* (*VGD1*) encodes a pollen-specific PME required for the modification of the cell wall necessary for pollen tube growth (Jiang et al. 2005). PME activity and pollen tube growth were reduced in the *vgd1* mutants, leading to an increase in male infertility. The closely related *At2g47030* complemented the *vgd1* mutant in contrast to the more distantly related *At3g62170* (Jiang et al. 2005), which suggests different functions or biochemical activities of these isoforms. The pollen-specific *AtPPME1* is involved in the determination of the shape and elongation of the pollen tube (Tian et al. 2006; Röckel et al. 2008), and can be inhibited by the equally pollen-specific AtPMEI2 in vitro (Röckel et al. 2008). The localisation of YFP fusion proteins in the pollen tube indicated that while AtPPME1 was present along the full length of the tube, AtPMEI2 was only detected in the apex, at the site of elongation (Röckel et al. 2008). This supports the hypothesis that PMEs localized at the pollen tube's apex are specifically inhibited, and that the apex therefore has a higher DM, which is conducive to elongation.

The inverse situation occurs in the mature part of the pollen tube, which has less inhibition of PME activity and a lower DM. The de-methylesterification could lead to reduced extensibility through the formation of Ca^{2+} cross-links. The lower DM in the mature part of the pollen tube was confirmed in immunocytochemical assays (Dardelle et al. 2010). Supporting this hypothesis, the overexpression of *AtPPME1* in tobacco pollen tubes led to a decrease in elongation, while the PME inhibitor AtPMEI2 increased the elongation rate (Röckel et al. 2008). It has recently been suggested that one of the biomechanical contributions of cell wall pectin to growth might be through its ability to undergo strain stiffening (Kierzkowski et al. 2012) as observed in vitro in pectin gels (Schuster et al. 2012). Strain stiffening is a rheological property of biological gels by which the stiffness of the gel increases under increasing strain, so that increasing force is required for increasing deformation. Strain stiffening in plant cell walls may limit growth to specific parts of tissues or cells, and could play a role in localized tip growth occurring during pollen tube elongation.

In addition to pollen tube elongation, PMEs play a role in pollen grain germination, as has recently been shown for PME48 (Leroux et al. 2015). *pme48* mutant pollen grains showed a decrease in PME activity, slower imbibition of water and delayed or impaired germination both in vitro and in vivo. In the grains that did germinate, an increased diameter and increased proportion of bursting of the pollen tubes was observed in liquid germination media compared to the WT (Leroux et al. 2015). These pollen tube phenotypes could be rescued by the addition of Ca^{2+} to the germination medium (Leroux et al. 2015).

Stem morphology

Arabidopsis stems, such as hypocotyls, grow by fast cell elongation in internode regions, and a number of cell-wall-modifying and pectin-related enzymes are differentially expressed at different stages during the maturation process (Hall and Ellis 2013). *Arabidopsis* PECTIN METHYLESTERASE 35 (PME35) was shown to act on primary cell wall pectins in the stem (Hongo et al. 2012). The *pme35* mutant has defects in de-methylesterification in cortex cells and inter-fascicular fibers, leading to a decrease in mechanical stem strength (Hongo et al. 2012). Stem morphology as well as stiffness is also affected by overexpression of *AtPMEI5* in *Arabidopsis* (Wolf et al. 2012; Müller et al. 2013a, b). These plants show stem twists and loops as well as fusions. Furthermore, in the *blr* mutant, the upregulation of PME5 expression led to reduced cell expansion and defects in internode elongation (Peaucelle et al. 2011a). A decrease in stem length was also observed after mutation of AtPME3 in the same plants that showed an increased hypocotyl elongation as described above (Guénin et al. 2011).

In addition to depending on the DM, stem growth can be affected by the relative pectin content of cell walls, as is the case in the *quasimodo* (*qua*) mutants. *qua1* (Bouton 2002; Leboeuf et al. 2005) and *qua2* (Krupková et al. 2007;

Mouille et al. 2007) have a decreased HG content, an increased flexibility of the pectin polymer dues to the increased ration of RG-I to HG domains, and altered stem growth (Ralet et al. 2008). In total, these findings point towards a further role of pectin modification in non-woody plants, regulating stem strength and structure.

In systems with more extensive wood formation, the DM is also important for strength. In hybrid aspen (*Populus tremula x tremuloides*), *PtPME1* was expressed in developing woody tissues, and its overexpression decreased the DM and inhibited both symplastic and intrusive growth of wood fiber cells (Siedlecka et al. 2008). Interestingly, in a very distinct model, it was shown that PMEs are likely to influence solid wood properties in *Eucalyptus*, shedding new light on putative additional functions of PMEs (Sexton et al. 2012).

Cell adhesion and separation

Pectins are the main components of the middle lamellae connecting the cell walls of adjacent plant cells. Therefore, pectins play a major role in cell adhesion, and pectin modification may be necessary for the partial cell separation occurring at the end of cell division (Lord and Mollet 2002). Pectin Ca^{2+} bridges are thought to play a major role in cell adhesion.

In the *Arabidopsis* root cap, cells detach from the root tip and are released into the surrounding medium in layers of interconnected cells (Vicré et al. 2005). Although a large number of cell wall mutants have been tested, only *qua1* and *qua2*, which have reduced amounts of HG in their cell walls, released individual cells instead of layers, indicating a reduced cell adhesion in the root cap (Durand et al. 2009). The *qua1* mutant also showed drastic seedling defects, with tissues dissolving and falling apart on the living seedling (Bouton 2002). This confirms the importance of HG in cell adhesion.

There is evidence for a contribution of pectin-modifying enzymes to cell adhesion. The *friable1* (*frb1*) mutant in *Arabidopsis* shows severe defects in cell adhesion, starting with the sloughing of outer cell layers and proceeding to the dissociation of tissues and organs, as well as developmental defects in embryos (Neumetzler et al. 2012). FRIABLE1 is a protein of unknown function, but localizes to the Golgi, and is hypothesized to be involved in secretory pathways. Seedlings were found to have a lower DM than WT as well as a significantly higher arabinose content. Interestingly, in addition to tissue dissolution, the *frb1* plants displayed organ fusions. Organ fusions, but not tissue dissociation, were also observed in *Arabidopsis* plants with reduced PME activity due to overexpression of *PMEI5* (Müller et al. 2013b).

The proteins Arabidopsis dehiscence zone polygalacturonase 1 (ADPG1) and ADPG2, which are closely related to one another, were shown to be crucial for seed pod opening in *Arabidopsis*, and showed strong expression in the abscission zones of floral organs (Ogawa et al. 2009). Leaf abscission was correlated with a reduced DM in the abscission zone of *Impatiens*, indicating that pectin de-methylesterification followed by polygalacturonase activity might also contribute to the separation of the cells necessary for the plant to shed its leaves as observed in pollen separation (Bowling and Vaughn 2011). The mesophyll of *pme3* mutants, which had an increased DM, could be treated with cell wall-degrading enzymes to form protoplast with a higher efficiency than the WT, indicating that the cell wall is more susceptible to degradation and the cells separate more easily from each other (Lionetti et al. 2015). The same phenotype can be observed in overexpressors of PMEI1 or PMEI2.

Seeds

Pectin gels can hold large amounts of water, and the hydration state of the wall and the strength of the pectic polysaccharide gel influence its biomechanical properties (Ha et al. 1997). An impressive example of this ability is the seed mucilage of species such as *Arabidopsis thaliana*, *Capsella bursa-pastoris*, *Lepidium sativum*, and many more (Western et al. 2001; Harpaz-Saad et al. 2012; Deng et al. 2013). On contact with water, specialized cells in the seed coats, which die during development, release cellulose fibrils as well as large quantities of pectins (Haughn and Chaudhury 2005; Haughn and Western 2012). The seed mucilage probably serves as a short-term water reservoir for the seed and as an adhesive, but is not necessary for successful seed germination in *Arabidopsis*, as mutant seeds with reduced or nonexistent mucilage can also complete germination (Western et al. 2000). Ruthenium red staining of seed mucilage shows very clearly that the addition of Ca^{2+} leads to a denser mucilage as the Ca^{2+} forms cross-links between de-methylesterified carboxyl groups (Voiniciuc et al. 2013; Dean et al. 2007). The addition of EDTA, which chelates calcium away from the HG strands, leads to less dense, more voluminous mucilage, as the reduced number of network cross-links leads to less counteraction to the swelling force (Voiniciuc et al. 2013; Dean et al. 2007). PMEI6 was shown to be essential for full seed mucilage release, as an Arabidopsis ecotype deficient in PMEI6 only released very small quantities of mucilage (Saez-Aguayo et al. 2013). A strongly increased mucilage release could be achieved by overexpressing PMEI6 (Saez-Aguayo et al. 2013). This effect was independent of the similar effect of a mutation in the subtilisin-like protease SBT1.7 on mucilage release (Rautengarten et al. 2008), as a double mutation *pmei6/sbt1.7* had an additive phenotype compared to the two independent

mutants (Saez-Aguayo et al. 2013), indicating different target PMEs. These experiments provide interesting insights into the role of seed mucilage in particular, but also into the properties of plant pectin gels in general.

In addition to its importance in determining the properties of seed mucilage and its release from the testa, pectin methylesterification also plays a role in embryo development and seed germination in *Arabidopsis* (Müller et al. 2013b; Levesque-Tremblay et al. 2015). At least 7 PMEs are expressed during seed coat and embryo development in *Arabidopsis* in a highly temporally controlled manner (Louvet et al. 2006). However, knockout analyses for most of these genes did not reveal obvious seed phenotype, most likely due to functional redundancy within the PME family. Nevertheless, a PME renamed highly methylesterified seeds (HMS) was recently isolated based on its high expression in the seed coat and the embryo during seed development (Levesque-Tremblay et al. 2015). The disruption of the HMS gene in the knockout *hms* mutant causes a decrease in overall PME activity and an increase in DM in developing seeds, which is associated with altered embryo morphology, reduced cell expansion and softening of the seed tissue (Levesque-Tremblay et al. 2015). HMS may thus be required for cell wall loosening in the embryo to facilitate cell expansion during the accumulation of storage reserves.

In addition to seed development, PME activity is also tightly controlled during the germination process. Seeds overexpressing *PMEI5* displayed lower PME activity in seeds during germination, and earlier onset and faster completion of germination compared to WT. This affected both stages of germination: testa rupture as well as endosperm rupture. PME activity in WT seeds peaked around testa rupture. The addition of ABA to the germination medium, which specifically delays endosperm rupture but does not affect testa rupture (Müller et al. 2006), led to a plateau phase of high PME activity, which only declined once the delayed endosperm rupture set (Müller et al. 2013b). High PME activity is thus associated with the ABA-induced delay in radicle elongation and endosperm weakening; two processes that take place prior to the completion of germination (Müller et al. 2006). In addition to the strong temporal regulation of PME and PMEI expression in seeds, PMEs and PMEIs have also been found to be differentially expressed in a tissue-specific manner during seed germination of the close Arabidopsis relative *Lepidium sativum* (Scheler et al. 2014). In support of a role for PME activity and the resulting changes in DM in the timing and process of testa rupture, changes in expression were temporally focused around testa rupture (Scheler et al. 2014).

The role of PMEs in seed germination is not limited to angiosperms: PME activity was also shown to be involved in the germination of a gymnosperm, the conifer yellow cypress (*Callitropsis nootkatensis*). PME activity increases in the megagametophyte and embryo in a tissue-specific manner during yellow cypress seed dormancy release and subsequent germination, with particularly high activity in the megagametophyte, where pectin is the predominant polysaccharide (Ren and Kermode 2000).

Abiotic interactions

As discussed above, the addition of divalent ions and chelators to the pectic mucilage gel of many seeds leads to the mucilage contracting and expanding, respectively (Voiniciuc et al. 2013; Dean et al. 2007), and to changes in its porosity and permeability. The same may, indeed, apply to the pectic gel in all plant cell walls. Porosity and permeability are crucial for any kind of transport or diffusion through the cell wall. The influence that the pectic polysaccharides have on cell wall porosity and density makes them strong candidates for cell wall components that are modified when drought or ice formation reduces the availability of free water in the plant cell. In support of this hypothesis, oilseed rape (*Brassica napus*) plants that underwent cold acclimation were found to have increased pectin levels in the cell wall, and higher PME activity as well as a lower DM and an increased tensile strength (Solecka et al. 2008).

Evidence for an accumulation of pectin during cold acclimation has also been found in other species. A promising *Allium fistulosum* epidermis testing system was recently developed which takes advantage of the large cell size and cold hardiness properties of this species for detailed investigations into cell changes during cold acclimation, freezing, and recovery from freezing (Tanino et al. 2013). Here, FTIR spectroscopy suggests that the pectin fraction of the cell wall increases when the plants are undergoing cold acclimation. This hypothesis is supported by findings in pea (*Pisum sativum*) plants, where a comparison between a frost-tolerant and a frost-sensitive genotype showed that during cold acclimation, the frost-tolerant plants—similar to the oilseed rape mentioned above—accumulated the pectic compounds HG, XG, and RG-I, while the frost-sensitive genotype accumulated xylans (Baldwin et al. 2014). However, contrary to oilseed rape, cold acclimation in the frost-tolerant pea plants led to a higher abundance of JIM7 epitopes, indicating a higher DM than in the frost-sensitive plants (Baldwin et al. 2014).

In addition to changes in the amount of pectin, changes in PME activity take place during cold acclimation. In accordance with the findings in rape seed described above, *Arabidopsis* plants exposed to cold stress showed an increase in PME activity. The expression and activity of *PME41* were shown to increase in reaction to chilling

stress as well as brassinosteroid application (Qu et al. 2011). Mutants deficient in brassinosteroid signaling did not show this increase, and only a reduced activity increase was observed in the mutant *pme41*. A lack of *PME41* also led to increased electrolyte leakage in response to cold stress. The increase in PME activity in the WT in response to chilling stress could lead to an increase in the formation of Ca^{2+} bridges, thereby decreasing cell wall porosity. If temperatures fall below 0 °C, the altered cell wall density could impede the spread of ice (Qu et al. 2011).

PMEs might also play a role in water flow in woody plant vasculature under non-water limiting conditions. During water transport in the xylem of *Fraxinus americana* trees and a number of other woody species, the water flow through the pit membrane is regulated through volume changes of a hydrogel in response to the presence or absence of cations. If ions are present, the size of microchannels in the pit membrane increases as the gel shrinks, decreasing the resistance to water flow (Zwieniecki et al. 2001). Deionised water can reverse this effect. This may suggest that the hydrogel contains pectins. It remains unclear, however, if pectin is actually present in the pit membrane (van Doorn et al. 2011).

Pectin content and modifications have also been observed in connection with exposure to drought. An increased sensitivity to drought has been observed in some pectin defective mutants such as *qua1* with their decreased HG content (Bouton 2002). Several PME isoforms are transcriptionally upregulated in crab apples (*Malus pumila*) under drought conditions after drought tolerance has been induced through application of DL-β-aminobutyric acid (Macarisin et al. 2009). The influence of pectin content and DM on drought sensitivity could be due to the effect of pectin on the water retention potential of the cell walls, and/or interactions of the pectin matrix with the cuticle, as a defective cuticle would lead to increased water loss through evaporation. PMEI5 overexpressors showed a defective cuticle, which allowed for the penetration of toluidine blue in patches on the leaves and stems (Müller et al. 2013b). This defect might be due to a role for pectin in the transport of cuticle wax components through the cell wall, or in their anchoring.

A further abiotic stress connected with changes in PME activity is heat. Heat acclimation of soybean seedlings induces an increase in PME activity (Wu et al. 2010). It can be reversed with the addition of a chelating agent during the recovery period after the heat shock, and can be reinstated by the addition of divalent cations (Wu et al. 2010). The addition of chelators increased electrolyte leakage, suggesting an increased cell wall porosity. The DM and availability of cations to form egg-box structures from demethylesterified pectin thus seem to be important for cellular integrity during heat stress.

In addition to the porosity of the pectin gel, the DM and the pattern of methylesterification influence the number of negative charges on HG. A low DM and therefore a high level of negatively charged galacturonate was hypothesized to play a role in increased susceptibility to aluminum toxicity and higher levels of aluminum in the roots of the maize (*Zea mays*) cultivar Lixis compared to more resistant cultivars (Eticha et al. 2005). In addition, the overall pectin content in root tips was higher in the susceptible cultivar (Eticha et al. 2005). A very similar observation was made in a comparison of different buckwheat (*Fagopyrum tataricum*) cultivars, where the most aluminum-susceptible cultivars showed a higher pectin content and increased PME activity as well as a decrease in DM in response to aluminum exposure (Yang et al. 2011). Analogous findings were made in rice (*Oryza sativa*) (Yang et al. 2008). Increased PME activity was also correlated with increased aluminum sensitivity in potato plants (*Solanum tuberosum*) (Schmohl et al. 2000). The phenomenon of higher aluminum sensitivity of cells with an increased pectin content and decreased DM could be reproduced in cell cultures, in which the pectin content had been controlled by the addition of salt to the medium and the DM altered by different lengths of exposure to PMEs (Schmohl et al. 2000). This response in rice seemed to be specific for aluminum and did not extend to other potentially toxic metals such as cadmium or copper (Yang et al. 2008).

A screening for mutants with compromised root growth in the presence of Zn^{2+} led to the isolation of *ozs2* carrying a mutation in the gene AtPME3 that prevents its efficient posttranslational processing and maturation (Weber et al. 2013). Interestingly, the hypersensitivity phenotype could be rescued by knocking out *OZS2* with RNAi, thus demonstrating that the defects were associated with the presence of the unprocessed PME3. Hypersensitivity could also be induced by overexpressing WT *PME3*, and could be alleviated when excess Ca^{2+} was supplied to the plants. The *ozs2* phenotype was hypothesized to cause hypersensitivity towards specific interference of Zn ions impacting cell wall-controlled growth processes (Weber et al. 2013).

Biotic interactions

PMEs play an important role in the biotic interactions of plants with pathogenic fungi and bacteria, as the cell wall forms a barrier that pathogens need to overcome to infect or spread an infection in a plant (Vorwerk et al. 2004; Cantu et al. 2008). Many pathogens secrete pectin-degrading enzymes that help break down the host's cell walls as a necessary part of the infection process (Espino et al. 2010).

In addition to the pectin-related enzymes introduced into the plant by the pathogen, the infection can cause changes

Table 2 Overview of studies on the role of the degree of pectin de-methylesterification in biotic interactions

Publication	Species	Genotype	Pathogen/herbivore	Reference sample	DM	Phenotype	Increased DM and/or decreased PME activity associated with…	Comment
Bacterial and fungal pathogens								
Raiola et al. (2011); Lionetti et al. (2007)	A. thaliana	pme3	B. cinerea, P. carotovorum	WT	Increased	Increased resistance	Increased resistance	DM determined by immunodot assays
		35S::PMEI1		WT	Increased	Increased resistance	Increased resistance	DM determined biochemically
		35S::PMEI2		WT	Increased	Increased resistance	Increased resistance	DM determined biochemically
Bethke et al. (2014)[a]	A. thaliana	pme22-1, pme35-1,-3, pme39-1,-2;-3, ppme1-1,-2,-3, pme 3-1,-2, pme17-1,-2, pme25-2, pme31-1,-2, pme44-1,-2	P. syringae pv maculicola ES4326	WT	NR	Decreased resistance	Increased resistance	DM not determined. All mutants had decreased baseline PME activity compared to WT, but equal or higher pathogen-induced PME activity. PME activity determined by gel diffusion assay
		pme12-1-2,-3, pme22-2, pme35-2, pme3-3, pme25-1	P. syringae pv maculicola ES4327	Wt		No difference in resistance	No effect on resistance	DM and PME activity not determined
Volpi et al. (2011)	T. durum	pUbi::AcPMEI	B. sorokiniana, F. graminearum	WT	Increased	Increased resistance	Increased resistance	DM determined by immunodot assays
An et al. (2008)	C. annuum	capmei1	X. campestris pv. vesicatoria	WT	NR	Decreased resistance	Increased resistance	DM not determined. CaPMEI1 inhibitor effect on orange peel PME determined in vitro by enzyme assay
	A. thaliana	35S::CaPMEI1	P. syringae pv. Tomato	WT	NR	Increased resistance	Increased resistance	DM not determined. CaPMEI1 inhibitor effect on orange peel PME determined in vitro by enzyme assay
	A. thaliana	35S::CaPMEI2	H. parasitica	WT	NR	No difference in resistance	No effect on resistance	DM not determined. CaPMEI1 inhibitor effect on orange peel PME determined in vitro by enzyme assay
Nematodes								
Hewezi et al. (2008)	A. thaliana	35S::PME3	H. schachtii	WT	NR	Decreased resistance	Increased resistance	DM not determined. Increased PME activity. PME activity determined by enzyme assay
		pme3		WT	NR	Increased resistance	Increased resistance	DM not determined. Increased PME activity. PME activity determined by enzyme assay

Table 2 continued

Publication	Species	Genotype	Pathogen/herbivore	Reference sample	DM	Phenotype	Increased DM and/or decreased PME activity associated with…	Comment
Virus								
Lionetti et al. (2014)	*N. tabacum*	35S::AcPMEI	Tobacco mosaic virus	WT	NR	Delayed systemic spread	Increased resistance	DM not determined. Decreased PME activity determined by gel diffusion assay
	A. thaliana	35S::PMEI2	Turnip vein clearing virus	WT	NR	Delayed systemic spread	Increased resistance	DM not determined. Decreased PME activity determined by gel diffusion assay
Herbivores								
Körner et al. (2009)	*N. attenuata*	napme1	*M.sexta*	WT	NR	Decreased resistance	Increased resistance	DM not determined. Decreased PME activity determined by gel diffusion assay

NR not reported, *PME* pectin methylesterase, *PMEI* pectin methylesterase inhibitor, *WT* wildtype

[a] Bethke et al. also tested several double and triple mutants that are not listed here

in the expression of pectin-modifying enzymes in the host plant, including PMEs and PMEIs. Table 2 summarizes findings on the relationship of PME activity, cell wall DM, and susceptibility to different biotic stress factors. The expression of a number of *Arabidopsis PME* and *PMEI* genes is altered in response to infection with both biotrophic and necrotrophic pathogens such as *Golovinomyces cichoracearum*, *Pseudomonas syringae*, *Blumeria graminis*, *Phytophthora infestans* and *Botrytis cinerea* (Lionetti et al. 2012). Interestingly, mutations in some of the upregulated PMEs can lead to an increased resistance to the pathogen, as was shown for *B.cinerea* and *P. carotovorum* infection of *Arabidopsis*. Infections with either pathogen leads to an upregulation of PME3, and the *pme3* mutant shows increased resistance and reduced PME activity (Raiola et al. 2011). Similarly, transgenic *Arabidopsis* (Lionetti et al. 2007) and durum wheat (Volpi et al. 2011) with a reduced PME activity due to overexpressing PMEIs had a higher DM in the cell walls, and showed increased resistance to both fungal and bacterial pathogens. The pepper (*Capsicum annuum*) CaPMEI1 is expressed in response to infection of pepper leaves with *Xanthomonas campestris* pv. *vesicatoria*, and mutants lacking that protein show an increased susceptibility to the pathogen (An et al. 2008). Heterologous overexpression of *CaPMEI1* in *Arabidopsis* conferred increased resistance to several biotic and abiotic stresses, indicating that the pepper PMEI could interact with *Arabidopsis* PMEs (An et al. 2008). All these studies have established a link between a decrease in PME activity and the resulting increased DM, and an increased level of resistance to the studied pathogens. Taken together with the observation that action of plant polygalacturonases expressed in ripening fruit on cell wall pectins was facilitated by a decrease in DM (Wakabayashi et al. 2003), it is possible that the increased resistance of the plants with a higher DM is due to the highly methylesterified pectin being less susceptible to being broken down by pathogen polygalacturonases.

However, in contrast to these findings, Bethke et al. showed that three out of four mutants lacking single PMEs that would be upregulated in response to an infection with a different pathogen, *Pseudomonas,* in the WT showed increased rather than decreased pathogen susceptibility (*pme3*, *pme17*, and *At1g11580*) (Bethke et al. 2014). The same was true for six out of 12 *pme* single mutants that have altered cell wall compositions compared to WT (*pme35*, *pme39*, *ppme1*, *pme17*, *pme31*, *pme44*), and to mutants deficient in more than one of these *PMEs* (Bethke et al. 2014). However, while baseline PME activity was reduced in many of the mutants, pathogen-induced PME activity in the majority of mutant lines including the higher order mutants was equal to WT or even increased, which could be related to compensation mechanism between

isoforms upon infection. Jasmonic acid was required to increase overall PME activity, while ethylene and salicylic acid had no influence (Bethke et al. 2014).

The role of the DM in the immune response continues beyond the initial infection process. Once the cell wall pectin starts to be broken down in a pathogen attack, its fragments can be recognized by wall-associated kinase1 (WAK1) and WAK2 and elicit an immune response. These transmembrane proteins have pectin-binding domains in the cell wall and kinase domains in the cytoplasm. Elicitor activities of pectic fragments differ based on their length and DM (Osorio et al. 2008; Ferrari et al. 2013; Kohorn et al. 2014). Fragments with a low DM showed increased elicitor activity through formation of Ca^{2+}-bridges and increased binding to WAK1 in *Arabidopsis* cell suspensions (Cabrera et al. 2008). *In planta* experiments in which a hyperactive allele of WAK1 was crossed into a *pme3* knockout mutant showed that the reduced DM of the mutant led to a suppression of the WAK hyperactivity phenotype, indicating WAK1 requires HG with a low DM for activation (Kohorn et al. 2014). When exogenous low DM OGs were supplied to the *pme3* plants expressing the hyperactive WAK1 allele, the WAK1 response was significantly stronger than in WT plants with the hyperactive WAK1 allele, possibly because of the decreased competition in the *pme3* mutants between the exogenous low DM OGs and the endogenous high DM pectins (Kohorn et al. 2014).

The PMEI-mediated control of pectin methylesterification is also likely to play a role in plant–virus interactions, although this topic has seen very little studies until recently. Tobacco plants overexpressing a PMEI from kiwi (*Actinidia chinensis*) showed increased resistance to the tobacco mosaic virus, characterized by a decrease of systemic movement of the virus and a decrease in symptoms (Lionetti et al. 2014), thus confirming earlier report of an interaction of PMEs with virus movement proteins (Chen and Citovsky 2003). An analogous effect was observed in *Arabidopsis* plants overexpressing AtPMEI-2, which showed increased systemic resistance to the turnip vein clearing virus (Lionetti et al. 2014).

In a distinct pathosystem, *Arabidopsis* PME3 was shown in a yeast-two hybrid screen to interact with the Cellulose Binding Protein (CBP) secreted by the sugar beet cyst nematode *Heterodera schachtii* during the infection process (Hewezi et al. 2008). In accordance with a role of CBP in facilitating cyst parasitism, overexpression of CBP in *Arabidopsis* led to a decrease of resistance to nematode infection, as well as to a small increase in PME activity. A similar level of increase in PME activity was observed in plants overexpressing PME, which was also associated with a decrease of resistance to the nematode, while the *pme3* mutant showed increased resistance (Hewezi et al. 2008). PME activity is thus a target of an infection-related pathogen-produced protein in this nematode–host interaction.

While it seems that PMEI upregulation and an increased overall DM are typical reactions to pathogen attack, PMEs are upregulated and the DM decreased in response to mechanical wounding by herbivores (von Dahl et al. 2006; Körner et al. 2009) as well as by artificial large-scale wounding of leaves (Dorokhov et al. 2012). When caterpillars of the moth *Manduca sexta* fed on *Nicotiana attenuata* plants, PMEs were upregulated in the attacked leaf, total PME activity increased, and the DM decreased (Körner et al. 2009). This response was brought about by the high pH of caterpillar oral secretions. In all cases where PME activity increases in response to a biotic interaction, the methanol released by PME activity can act as a signaling molecule to the plant that is being attacked, and potentially neighboring plants (von Dahl et al. 2006; Körner et al. 2009; Dorokhov et al. 2012; Dixit et al. 2013; Komarova et al. 2014). In the case of caterpillar feeding on *Nicotiana attenuata*, the release of methanol was significantly reduced in plants lacking a PME inducible by caterpillar oral secretions, and showed a lower expression of toxic defense proteins (Körner et al. 2009). It was also reported, however, that methanol treatment of *Nicotiana attenuata* reduced the level of defensive proteins when the methanol quantities were similar to those produced in response to herbivore attacks, and that larvae on methanol-treated plants gained more weight by feeding than did larvae on untreated plants (von Dahl et al. 2006). When PMEs were overexpressed in tobacco and methanol production thereby increased, the plants became more resistant to aphids, whiteflies, and two species of caterpillars (Dixit et al. 2013), possibly as a direct effect of methanol toxicity.

These opposite reactions in PME activity regulation to wounding and pathogen attack can contribute to an increased susceptibility of plants to pathogens following wounding, and changes in DM may play a large role in this effect. This has been illustrated in banana plants (*Musa*) (Ma et al. 2013). The pathogenic fungus *Fusarium oxysporum* enters banana plants through wounds in the roots, and has severe effects on the plants, ultimately leading to their death. Banana plants susceptible to the pathogen *Fusarium* showed a much larger increase in PME activity and subsequent decrease in the DM in response to wounding than did resistant banana plants (Ma et al. 2013). *Fusarium* secretes a number of pectinolytic enzymes, and it is likely that the resistance of the plants with the lower PME activity in response to wounding is caused by reduced lytic efficiency of those enzymes in cell walls with a higher DM.

Interplay of pectin modifications with overall cell wall composition

While it has been clear for some time that cell wall properties and composition may be changed through hormonal regulations in response to environmental conditions—a famous example being the auxin gradient in response to gravity that leads to unequal cell elongation on different sides of a hypocotyl—there is now also evidence that changes in cell wall properties might be able to trigger hormone signals that then lead to reactive cell wall modifications. Specifically, for pectins, changes in PME activity and the resulting changes in DM have been associated with changes in the brassinosteroid signaling pathway in *Arabidopsis*. In support of this association, several of the strong morphological effects of PMEI5 overexpression could be suppressed by a mutation in the brassinosteroid receptor protein (BRI) (Wolf et al. 2012).

The DM might also have an influence on the deposition of cellulose, which is one of the major fibrous load-bearing components of the cell wall (Yoneda et al. 2010). A chemical genetic screen showed that cobtorin {4-[(2-chlorophenyl)-methoxy]-1-nitrobenzene} interferes with cellulose microfibril deposition without disturbing the microtubule, although the microtubule network's orientation is generally considered to be the deciding factor in cellulose microfibril orientation (Yoneda et al. 2007) Cobtorin treatment also increased the DM and disturbed the distribution of methylesterified HG (Yoneda et al. 2010). The effect of cobtorin on cellulose deposition is suppressed when the pectin methylesterase AtPME1 or a putative polygalacturonase is overexpressed, supporting a link between pectin modifications and cellulose deposition. Increased levels of HG domains have also been reported in several cellular systems in which cellulose synthesis is disrupted (Shedletzky et al. 1990; Diaz-Cacho et al. 1999; Manfield et al. 2004). In those systems, the accumulation of HG may have an effect on cell wall biomechanics which compensates for the loss of cellulose in the cell wall. This is supported by the finding that treatment of *Arabidopsis* cell suspension with the cellulose synthesis-inhibiting compound isoxaben leads to an increase in the transcription of HG synthesis- and modification-related genes including *QUA1* and several *PMEs*, leading to an increase of HG and changes in DM. (Manfield et al. 2004). Ca^{2+}-mediated cross-linking of HG is thought to be part of the mechanism of compensation for the diminished load-bearing capacity of the disrupted cellulosic network. As our technical and methodological options improve and allow for more detailed analyses of the cell wall with higher spatial and temporal resolution, the interactions between the different cell wall polymers which are currently still most often studied separately from each other, and the effects of those interactions will become more accessible for investigations.

Conclusions and outlook

Pectin composition and the degree of HG methylesterification have a major impact on the biochemical and biomechanical properties of plant cell walls, which in turn are crucial for plant development as well as for plants' interactions with their abiotic and biotic environments. PMEs act on HG by either demethylesterifying residues in the GalA chain individually randomly, or linearly block-wise (Markovic and Kohn 1984). These two modes of action may have inverse consequences on the plant cell wall. When the PMEs act individually randomly, the de-esterification of HGs frees protons promoting the activity of polygalacturonases (Moustacas et al. 1991), but does not allow for the formation of Ca^{2+} bridges. The action of polygalacturonase can, in turn, break down the pectin and contribute to cell wall loosening. When the PMEs act linearly blockwise on HGs, long stretches of negatively charged carboxylate appear, which can interact with Ca^{2+} creating a denser gel structure that would be expected to lead to decreased porosity. Thus, PME activity can strengthen as well as loosen the cell wall. However, experimental observations of *pme* and *pmei* mutants have shown that the relationships between the DM, the pattern of de-methylesterification, its effect on cell wall elasticity, other biomechanical parameters, and growth are not straightforward. Working towards an understanding of the role of pectin and specifically HG methylesterification patterns on a molecular, biochemical and biomechanical level is one of the big tasks that PME biology is preparing to tackle. The current simple model will have to be reimagined and refined before we can begin to make detailed predictions of the effect of PME activity on plant development and environmental interactions. Further work should emphasize, at single cell resolution, how the fine-tuning of PME activity can affect pectin chemistry and regulate cell wall rheology to shape plant form.

An additional challenge to pectin research comes from the fact that pectin-modifying enzymes are very large families with significant functional redundancy, and little is known so far about the interactions between individual family members. It will be important to investigate individual PMEs, as well as to address the inhibitor target range of individual PMEIs and the putative polarized secretion of PMEs and PMEIs that could lead to their spatial separation in specific areas of the cell wall. A better understanding of these aspects will allow us to better pre-

dict the additional or contrary effect of a battery of PMEs and PMEIs expressed at the same time in the same tissues. It will also be important to take into account the different layers of regulation, in particular PME and PMEI post-translational regulation and protein stability after secretion into the cell wall.

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