Polygalacturonase activity promotes aberrant cell separation in the quasimodo2 mutant of Arabidopsis thaliana

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A B S T R A C T
In plants, cell adhesion relies on balancing the integrity of the pectin-rich middle lamella with wall loosening during tissue expansion. Mutation of QUASIMODO2 (QUA2), a pectin methyltransferase, causes defective hypocotyl elongation and cell adhesion in Arabidopsis thaliana hypocotyls. However, the molecular function of QUA2 in cell adhesion is obscured by complex genetic and environmental interactions. To dissect the role of QUA2 in cell adhesion, we investigated a qua2 loss-of-function mutant and a suppressor mutant with restored cell adhesion, qua2 esmeralda1, using a combination of imaging and biochemical techniques. We found that qua2 hypocotyls have reductions in middle lamella integrity, pectin methyl-esterase (PME) activity, pectin content and molecular mass, and immunodetected Ca2+-crosslinking at cell corners, but increased methyl-esterification and polygalacturonase (PG) activity, with qua2 esmd1 having wild-type like or intermediate phenotypes. Our findings suggest that excessive pectin degradation prevents pectin accumulation and the formation of a sufficiently Ca2+-crosslinked network to maintain cell adhesion in qua2 mutants. We propose that PME and PG activities balance tissue-level expansion and cell separation. Together, these data provide insight into the cause of cell adhesion defects in qua2 mutants and highlight the importance of harmonizing pectin modification and degradation during plant growth and development.

1. Introduction

Multicellularity is an evolutionary innovation which has provided benefits to organisms across kingdoms. For multicellularity to be possible, cells need to be able to communicate and coordinate their growth and development. In animals, this can be achieved by transmembrane proteins which directly connect two cells together. In plants, however, there is a cell wall surrounding each cell that prevents direct contact of between the plasma membranes of cells. The plant cell wall is important for maintaining the structure of the cell, regulating growth rate, and responding to mechanical and biochemical signals. Together with the middle lamella, a specialized layer between the walls of adjacent cells, the cell wall also maintains cell–cell adhesion, although the molecular determinants of cell–cell adhesion in plants are not yet fully understood (Bou Daher and Braybrook, 2015).

Plant cell walls are composed of cellulose, hemicellulose, pectins, and structural proteins. While all components of the cell wall are necessary for its function, pectins have been shown to be particularly important for cell–cell adhesion (Calcott et al., 2021). Pectins are acidic heteropolysaccharides that are the most abundant component of the primary cell walls of eudicots, such as Arabidopsis thaliana (Yang and Anderson, 2020). They regulate the mechanical properties of the cell wall, thereby influencing plant morphogenesis (Bou Daher et al., 2018; Safer, 2018). In addition to being abundant in the cell wall, pectins are also enriched in the middle lamella. The most abundant type of pectin is homogalacturonan (HG) which can be covalently linked to other pectic domains such as rhamnogalacturonan-I (RG-I) or rhamnogalacturonan-II (Atmodjo et al., 2013). HG is a linear polymer consisting of 1,4-linked α-D-galacturonic acid (GalA) residues and is polymerized in the Golgi by galacturonosyltransferase (GAUT) enzymes (Atmodjo et al., 2013, 2014).
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2011). During synthesis, HG is methyl-esterified at the C6 position by HG methyltransferases and is secreted to the apoplast with a high degree of methyl-esterification (DM). Apoplastic pectin methylesterases (PMEs) remove methyl groups from HG, producing negatively charged GalA residues (Hocq et al., 2017). Depending on the demethyl-esterification pattern, demethyl-esterified HG can undergo different fates (Cosgrove, 2016). Randomly demethyl-esterified HG can be hydrolyzed by polygalacturonases (PGs) or cleaved via β-elimination by pectate lyases (PLs), softening the pectin matrix. Alternatively, blockwise demethyl-esterified HG can form intermolecular Ca\(^{2+}\)-crosslinks, stiffening the pectin matrix.

The synthesis, modification, and degradation of pectin influences wall mechanics and cell adhesion in Arabidopsis. Mutations affecting RG-I and RG-II result in adhesion defects in cultured Nicotiana tissue (Iwai et al., 2002, 2001). In Arabidopsis, several genes implicated in the synthesis and modification of HG are required for normal cell adhesion. These include the glycosyltransferase QUA1, the putative O-fucosyltransferase PRIABLE1 (Neumetzler et al., 2012), a newly identified Golgi membrane protein, ELMO1 (Kohorn et al., 2021b), and the focus of this work, QUASIMODO2 (QUA2). Mutations in all of these genes have different effects on pectin synthesis or modification and can be rescued by the mutation of a putative O-fucosyltransferase gene, ESMERALDA1 (Kohorn et al., 2021b; Verger et al., 2016).

QUA2 is a recently validated HG methyltransferase (Du et al., 2020), the loss of which results in severe cell adhesion defects in which epidermal cells detach and peel away from the surface of the plant. Although the qu2a2 mutant has reduced pectin content overall, it exhibits a nearly normal DM (Du et al., 2020; Krupkova et al., 2007; Mouille et al., 2007). Interestingly, both qu2a and qu2a esmd1 walls have similarly diminished acid-extractable HG content compared to wild type controls (Verger et al., 2016), implying that pectin content alone does not determine wall integrity and cell adhesion during plant growth. Thus, QUA2 has undefined effects on pectin dynamics and cell adhesion, which we hypothesize to be related by HG modification status. Here, we analyzed mechanical, cellular, and molecular phenotypes of qu2a and qu2a esmd1 mutants to investigate how loss of QUA2 weakens cell adhesion. We found increases in PG activity in qu2a plants that help explain their cell adhesion defects. Based on these and other data, we propose a conceptual model where in the absence of QUA2, HG is rapidly or excessively degraded by PGs, resulting in reduced HG molecular mass and insufficient pectin Ca\(^{2+}\)-crosslinks to maintain cell adhesion.

2. Materials & methods

2.1. Plant materials and growth conditions

Arabidopsis (Arabidopsis thaliana) ecotype Columbia (Col) and qu2a-I (Mouille et al., 2007), and qu2a-1 esmd-1-1 (Verger et al., 2016) lines were used in this study. Seeds were sterilized and grown for 6 days on plates containing 1/2 MS media in dark conditions as previously described (Barnes and Anderson, 2018). For EGCG experiments, sterile 10 mM EGCG was added to autoclaved media.

Endo-PG (Megazyme E-PGALUSP) or PME (Sigma P5400) in 1/2 MS liquid were applied to 3-day-old etiolated seedlings according to the units defined by the manufacturer. Excess liquid was gently aspirated, leaving seedlings covered in a shallow layer of 1/2 MS with or without enzyme. Seedlings were then returned to darkness for 3 additional days before scanning.

Ethanol induction of etiolated Arabidopsis plants was performed by adding 20 μL of 100% ethanol directly to the bottom of the growth plate. For 6 day-induction, ethanol was added 1 day after sowing seeds and daily for 5 additional days. For 3 day-induction, ethanol was added 4 days after sowing and daily for 2 additional days. All seedlings were 6 days old at the time of imaging.

To test the effects of additional calcium or magnesium, seeds were germinated and grown in the dark for 6 days on 1/2 MS plates (containing 1.5 mM CaCl\(_2\) as part of the basal medium) or 1/2 MS plates supplemented with additional CaCl\(_2\) or MgCl\(_2\), with final concentrations of 7.5 mM CaCl\(_2\), 6 mM MgCl\(_2\), 15 mM CaCl\(_2\), or 13.5 mM MgCl\(_2\).

2.2. Hypocotyl measurements, separation assays, and staining

Seedlings were scanned (HP Scanjet 8300) and germinated hypocotyl lengths were measured in ImageJ. For hypocotyl breakage experiments, three 6-day-old etiolated seedlings were arranged in parallel and clamped with small rubber-capped alligator clips at the tops and bottoms of the hypocotyls in a constant force extensometer (Durachko and Cosgrove, 2009). A 4 g counterweight (0.4 N force) was lowered gently by hand to allow the samples to become taut before releasing it. In most cases, breakage occurred almost immediately after the counterweight was released. To image breakage patterns using fluorescence microscopy, hypocotyls were stained with 10 μg/mL Propidium Iodide (PI; Life Technologies) and imaged on a Zeiss Axio Observer microscope with a Yokogawa CSU-X1 spinning disk head and a 20X 0.5 NA air objective using a 561 nm excitation laser and 617/73 nm emission filter. Different hypocotyl regions were defined by dividing the hypocotyls equally into thirds, and the breakage location was recorded for each hypocotyl. To image cell adhesion defects in seedlings overexpressing pectin-modifying enzymes, hypocotyls were stained with 10 μg/mL Propidium Iodide and imaged on a Zeiss Axio Observer microscope with a Yokogawa CSU-X1 spinning disk head and a 10X 0.3 NA air objective using a 561 nm excitation laser and 617/73 nm emission filter.

2.3. Probe labeling and quantification

Immunolabeling of 6-day-old etiolated hypocotyl cross-sections was performed as described by Rui et al. (2017) with minor modifications. 0.5% Fast Green FCF (w/v; Electron Microscopy Sciences, Hatfield, PA) was used to locate embedded samples. For COS488 labeling, sections were labeled with a 1:2000 dilution of COS488 in 25 mM MES buffer pH 5.7 for 30 min. After (immuno)labeling, sections were counter-stained with 0.01% (w/v) Fluorescent Brightener 28 (FB28) for 10 min.

Z-stack images with a 1 μm z-step were collected as indicated above using a 20X 0.5 NA air objective with a 405 nm excitation laser and 450/ 50 nm emission filter for FB28, or a 488-nm excitation laser and a 525/ 50 nm emission filter for Alexa Fluor 488. This produced images of 512 × 512 pixels, with 0.65 μm/pixel. Quantification of labeling intensity was performed as previously described (Peaucelle et al., 2015).

2.4. FESEM and lesion quantification

Seedlings were dehydrated in an ethanol series, critical point dried (CPD-030), mounted on carbon tape, iridium coated (EMITECH K575X) for at least 10 s, and imaged on a Zeiss SIGMA VP-FESEM with a 10 kV beam current. Lesions were defined as gaps between cells at the transverse anticlinal wall. Lesion number was counted manually and quantified based on hypocotyl surface area. For whole hypocotyls, epidermal surface area was measured using ImageJ. Representative images of lesions across the length of the hypocotyl, when present, were chosen for use in the figures.

2.5. AIR preparation

Flash-frozen seedlings were lyophilized (Labconco), cryo-milled (Retsch) at 30 Hz for 5 min, and subjected to a series of 30 min extractions to remove proteins and lipids: 1.5 mL of 1.5% SDS (w/v) three times, 70% (w/v) ethanol three times, 1:1 chloroform:methanol three times, and acetone once before three water washes and lyophilization.
2.6. Uronic acid and methyl-ester content determination

For uronic acid assays, 141 µL of water, 14 µL of 4 M sulfuric acid–potassium sulfamate pH 1.6, and 845 µL of concentrated sulfuric acid containing 75 mM sodium tetaborate were added to 1 mg AIR. Samples were boiled for 5 min, cooled, and diluted ten-fold with 85% (v/v) sulfuric acid. Initial absorbance at 525 nm (A525) for each sample was recorded using a Nanodrop 2000c, then 28 µL of 0.15% m-hydroxydiphenyl (w/v) in 0.5% NaOH (w/v) was added to each cuvette and mixed. After 5 min, final A525 was measured. Initial A525 was recorded using a Nanodrop 2000c, then 28 µL of 0.15% m-hydroxydiphenyl (w/v) in 0.5% NaOH (w/v) was added to each cuvette and mixed. After 5 min, final A525 was measured. Initial A525 was estimated by dividing nmol methanol/mg AIR by the average nmol uronic acid/mg AIR for a given biological sample.

2.7. Pectin extraction and size-exclusion chromatography

CDTA-soluble pectin was extracted from 6-day-old etiolated seedling AIR, chromatographically separated, and assayed for uronic acids as previously described (Xiao et al., 2014).

2.8. PME, PG, and PL activity assays

Total PME activity assays were performed using the PECTOPLATE method (Lionetti, 2015) containing pectin from apple (50–75% esterification, Sigma, 93854) with 5 µg total protein in 20 µL buffer per well. Halo areas were fit to a standard curve using commercial PME (Sigma P5400).

Protein was extracted from 0.5 to 1 g of pooled 6-day-old etiolated seedlings for each genotype and assays were performed exactly as described in Xiao et al. (2014) for PG activity and Chen et al. (2021) for PL activity, with the exception that for the PL activity assay, absorbance was measured at 250 nm instead of 237 nm.

2.9. Statistical analyses

All experimental results were from at least three biological replicates and experiments. Data are shown as means ± SE. Significance was determined by Student’s t-tests, one-way ANOVAs with Tukey Tests, and Kruskal-Wallis tests with Dunn’s multiple comparison in Microsoft Excel (v14.7), R (v3.3.1), Past3 (Hammer et al., 2001) or Prism (v9.2.0).

2.10. Accession numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: QA2 (At1g79240), ESMD1 (At2g01480).

3. Results

3.1. qua2 hypocotyls exhibit aberrant breaking patterns

Previous analyses of qua2 and qua2 esmd1 mutants revealed aberrant growth patterns and pectin biochemistry (Du et al., 2020; Mouille et al., 2007; Verger et al., 2016). To compare these mutants directly, we first analyzed hypocotyl elongation and cell adhesion in etiolated seedlings. qua2 seedlings were stunted and had severe cell adhesion defects (lesions) that were absent in Col controls, and we unexpectedly found small lesions in qua2 esmd1 seedlings as well (Fig. 1A-C). Lesions were particularly noticeable in the bottoms of qua2 hypocotyls, suggesting developmental differences in wall integrity along the hypocotyl growth axis. To further explore wall integrity in hypocotyls, we imposed uniaxial stress in the direction of growth until material failure using a constant-force extensometer (Durachko and Cosgrove, 2009). We predicted uniaxial stress to be well tolerated in the bottom of hypocotyls where cell wall synthesis is complete and fully expanded cells have adapted to growth-derived stress, and poorly tolerated in actively growing portions near the top of the hypocotyl. In almost every instance, Col hypocotyls failed in the top region as predicted, whereas qua2 hypocotyls broke as often at the bottom as at the top, and qua2 esmd1 hypocotyls displayed intermediate breakage patterns (Fig. 1D). Imaging the broken ends revealed that Col seedlings broke straight across the hypocotyl (Fig. 1E). However, qua2 hypocotyls had intact cells at broken regions (Fig. 1E), indicating weakened middle lamellae between cells. qua2 esmd1 hypocotyls did not shear straight across the hypocotyl upon breakage but did break across cells as in Col hypocotyls (Fig. 1E). Together, these data indicate that qua2 hypocotyls have substantially weakened middle lamellae that are not restored to full strength by the esmd1 suppressor mutation.

3.2. PG activity is higher in qua2 seedlings and promotes lesion formation

Given that polygalacturonases (PGs) are responsible for cell separation events during normal plant development (Ogawa et al., 2009; Yang and Anderson, 2020), we hypothesized that increased PG activity might cause the loss of cell adhesion in qua2 seedlings. Consistent with this hypothesis, we found that PG activity was approximately five-fold higher on average in 6-day-old etiolated qua2 seedlings than in Col and qua2 esmd1 seedlings (Fig. 2A). Since pectate lyases (PLs) can also cleave HG, we also measured total PL activity in 6-day-old etiolated seedlings, but found no significant difference between genotypes (Supplemental Fig. S1). To assess how elevated PG activity influences pectin content, we measured uronic acid content in rapidly elongating 3-d-old seedlings and more fully elongated 6-d-old seedlings grown in the dark. No differences in uronic acid content were apparent in 3-d-old seedlings, indicating that the total level of pectin production was similar early in seedling growth for the three genotypes (Fig. 2B). However, in 6-day-old seedlings, uronic acid content was highest in Col, lower in qua2 esmd1, and lowest in qua2 (Fig. 2B), indicating that qua2 seedlings fail to accumulate pectin during hypocotyl elongation. Next, we extracted pectin from AIR with (1,2-cyclohexylenedinitrilo)tetraacetic acid (CDTA), a Ca2+ chelator, to solubilize Ca2+-crosslinked HG and complement previous mass analyses of acid-extracted pectin from Col and qua2 (Ralet et al., 2008), and analyzed pectin molecular mass via size-exclusion chromatography. qua2-derived CDTA-soluble pectin was enriched in lower molecular mass pectins (fractions 10–12) and peaked at a later (smaller) fraction than Col and qua2 esmd1 pectin (Fig. 2C). Thus, elevated PG activity is correlated with reduced pectin accumulation and molecular mass in qua2 seedlings.

To test whether PG activity by itself can affect cell adhesion in hypocotyls, we added exogenous fungal PG to growing etiolated seedlings and found that Col seedlings were insensitive to 10 U/mL PG, but that 100 U/mL PG inhibited hypocotyl elongation and caused the formation of some lesions (Fig. 3). qua2 hypocotyl elongation was insensitive to exogenous PG treatment, but both concentrations of PG treatment increased lesion formation (Fig. 3). A dosage-dependent effect of PG was observed for qua2 esmd1 hypocotyl elongation and cell adhesion (Fig. 3). These data imply that PG activity can cause lesion formation, even in the absence of the qua2 mutation, but that elevated PG activity in qua2 mutants causes a much higher degree of lesion formation.

We also asked whether overexpression of endogenous Arabidopsis PGs might cause cell adhesion defects, as has been observed for...
Fig. 1. *qua2* and *qua2 esmd1* mutants exhibit aberrant hypocotyl elongation, cell adhesion, and breaking patterns compared to Col. (A) 6-day-old Col, *qua2*, and *qua2 esmd1* etiolated seedlings. Scale bar = 1 cm. (B) Hypocotyl elongation curves for 2- to 6-day-old etiolated seedlings (n ≥ 40 seedlings per genotype, per day from 3 independent experiments). Error bars represent SE. Letters indicate significantly different groups (P < 0.05, one-way ANOVA and Tukey Test). (C) Contrast-enhanced maximum projections of epidermal cells of 6-day-old Col, *qua2*, and *qua2 esmd1* etiolated hypocotyls stained with PI. Yellow arrowheads indicate lesions. (D) Pie charts indicating the relative percentage of hypocotyl breakage events that occurred in a given region of the hypocotyl for 6-day-old etiolated seedlings (n ≥ 73 seedlings per genotype from 3 independent experiments). (E) Contrast-enhanced maximum projections of broken hypocotyl segments of 6-day-old etiolated seedlings stained with PI. (C,E) Scale bar = 50 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
overexpression of an apple PG (Atkinson et al., 2002). However, we did not observe lesions in AtPGX1OE (Xiao et al., 2014), AtPGX2AT (Xiao et al., 2017), AtPGX3OE (Rui et al., 2017) seedlings with modestly increased PG activity and hypocotyl elongation (Supplemental Fig. S2).

3.3. PME activity is reduced in qua2 seedlings, and inhibition of PME activity limits hypocotyl elongation but does not affect lesion formation

Given that PG activity is elevated in qua2 seedlings, but that qua2 and qua2 esmd1 plants contain similar amounts of acid-extractable HG (Verger et al., 2016), we asked whether changes in the metabolism and/or methyl-esterification status of HG might be affected by these mutations. We measured total PME activity and found that qua2 seedlings had 40% less PME activity than Col, with an intermediate level of PME activity being present in qua2 esmd1 seedlings (Fig. 4A, B). To investigate whether altered PME activity correlates with alterations in pectin methyl-esterification, we estimated the DM of 3-and 6-day-old seedling AIR as a molar percentage (mol methanol / mol uronic acids). No differences were found between genotypes in 3-day-old seedling AIR, but 6-day-old qua2 AIR had the highest DM, whereas the DM estimates of Col and qua2 esmd1 AIR were similar (Fig. 4C).

Increased DM of qua2 AIR runs counter to the function of QUA2 as a pectin methyltransferase (Du et al., 2020), indicating that apoplastic modifications such as PME-mediated demethyl-esterification can mask changes in the methyl-esterification of HG during its synthesis. To test whether exogenous PME application affects hypocotyl elongation and cell adhesion, we added commercial PME from orange peel (see Methods) to 3-day-old etiolated seedlings and assessed the effects 3 days later. Neither 10 U/mL nor 100 U/mL PME altered hypocotyl elongation or lesion frequency (Supplemental Fig. S3 B and S3C) in any genotype compared to mock controls. We then grew seedlings on media containing 100 μM epigallocatechin gallate (EGCG), which inhibits PME activity at physiologically relevant levels (Supplemental Fig. S4 A) (Lewis et al., 2008). All genotypes had shorter hypocotyls than untreated controls (Supplemental Fig. S4B) in the presence of EGCG, but lesion frequency was unchanged (Supplemental Fig. S4C and S4D).

When testing whether overexpressing PMEs in planta might also affect cell adhesion, we found that alcohol-inducible overexpression of AtPME5 stunted seedlings (Peaucelle et al., 2008) but did not promote lesion formation in 6-d-old etiolated seedlings after 3 or 6 d of induction (Fig. 4E-F). Constitutive overexpression of an Aspergillus nidulans PME in Arabidopsis was recently shown to limit plant growth to varying degrees (Reem et al., 2020). The two most severely stunted AnPME-expressing lines (PME2-1 and PME3-2; Reem et al., 2020) occasionally exhibited

Fig. 2. qua2 seedlings exhibit elevated PG activity, limited pectin accumulation, and reduced pectin molecular mass in 6-day-old seedlings. (A) Total PG activity from crude protein derived from 6-day-old etiolated Col, qua2, and qua2 esmd1 seedlings (n = 15 technical replicates from 3 biological samples per genotype). In this study, one unit of PG activity is defined as 1 μmol reducing ends generated per minute from one milligram of protein at 30 °C. Biological replicates are separated by shading, with replicate one being the lightest shade and three being the darkest. Error bars represent SE and letters indicate significantly different groups (P < 0.05, Kruskal-Wallis test and Dunn’s multiple comparison). (B) Uronic acid content of AIR derived from 3- and 6-day-old etiolated Col, qua2, and qua2 esmd1 seedlings (n = 15 total technical replicates from 3 biological samples per genotype and age). Error bars represent SE. Letters indicate significantly different groups (P < 0.05, one-way ANOVA and Tukey Test). (C) Normalized absorbance values at 520 nm (A520) relative to the sum of A520 for a given genotype for fractions of CDTA-solubilized wall material subjected to uronic acid detection assays after separation of CDTA-solubilized material by size-exclusion chromatography on a Superdex 75 5/150 GL column. Points represent the average normalized absorbance values of a given fraction from a total of six injections from three biological samples. The column was calibrated with protein standards: β-amylase (200 kD), Bovine Serum Albumin (66kD), carbonic anhydrase (29 kD), and cytochrome c (12.4 kD).
bulging cells and lesions in 6-d-old etiolated seedlings (Fig. 4 G-I), but to varying degrees in each seedling. Together, these data indicate that although qua2 seedlings have reduced PME activity, the application, inhibition, and overexpression of PME activity do not generally influence cell adhesion in Arabidopsis hypocotyls, with the exception of AnPME2 overexpression.

3.4. qua2 seedlings exhibit abnormal 2F4 and COS488 labeling of HG

To better understand any changes in pectin localization and/or abundance associated with cell adhesion defects, cross-sections were prepared from the bottoms of hypocotyls and labeled with LM20, an antibody that recognizes high DM HG (Verhertbruggen et al., 2009); LM19, an antibody that recognizes low DM HG (Verhertbruggen et al., 2009); 2F4, an antibody that recognizes Ca²⁺-crosslinked HG (Liners et al., 1989); or COS488, a chitosan oligosaccharide probe conjugated to Alexa Fluor 488 that binds to blockwise demethyl-esterified HG (Mravec et al., 2014). LM20 labeling was highest in the vasculature and outer epidermal walls in all genotypes (Supplemental Fig. S5). LM19 immunolabeling was present throughout cross-sections for all lines (Supplemental Fig. S6). However, 2F4 immunolabeling was conspicuously lacking in cell corners of qua2 seedlings compared to Col and qua2 esmd1 seedlings (Fig. 5A-B). COS488-labeling patterns mirrored the 2F4 labeling data (Supplemental Fig. S7).

Although quantitative comparisons of immunolabeling intensity across different genotypes are complicated by genotype-dependent variation in wall structure and composition, comparing relative immunolabeling intensity across different antibodies within a genotype can be useful for assessing wall composition. The relative proportions of LM20, LM19, and 2F4 labeling intensity were similar in Col and qua2 esmd1, but qua2 sections had relatively higher and lower proportions of LM19 and 2F4 labeling, respectively (Fig. 5C), indicating that Ca²⁺-crosslinked HG is less prominent in qua2 seedlings. To test whether the cell adhesion defect and reduced 2F4 labeling was caused by a lack of calcium in cell walls, we grew seedlings on medium containing extra calcium and quantified lesion frequency from field-emission scanning electron microscopy (FESEM) images. We found no consistent effect on hypocotyl elongation or lesion frequency in any genotype relative to controls (Supplemental Fig. S8). From these experiments, we conclude that qua2 hypocotyls have a lower proportion of 2F4 immunolabeled pectin than Col and qua2 esmd1, particularly at cell corners, and that this pectin epitope might be responsible in part for maintaining cell adhesion.

4. Discussion

In plants, the cell wall fixes cellular position throughout development. Although pectin has long been considered to be an adhesive molecule in plants (Iwai et al., 2002), cell adhesion can be restored in the qua2 esmd1 mutant without restoration of extractable HG content.
and reduction of water potential rescues cell adhesion defects in gaut8qua1-1 and qua2 mutants (Verger et al., 2018). These findings have called into question how QUA2 contributes to cell adhesion in Arabidopsis.

Here, we investigated the qua2 and qua2 esmd1 mutants and have integrated our data into a set of molecular mechanisms that might explain how QUA2 impacts pectin biochemistry and cell adhesion. In Col seedlings, pectin is synthesized in the Golgi by GAUTs and is methyl-esterified by QUA2 and other pectin methyltransferases. Once deposited in the apoplast, PMEs reduce the DM of HG to allow Ca<sup>2+</sup>-crosslinking and PG-mediated degradation of HG molecules required for coordinated hypocotyl elongation (Fig. 6A). In qua2 seedlings, HG is likely synthesized with a low DM. In the apoplast, normal levels of PME and PG act on low-DM HG and produce aberrant pectin molecules. Pectin sensing mechanisms are active in cell wall mutants (Wolf et al., 2012), and HG-derived oligosaccharides are important for skotomorphogenesis of etiolated seedlings (Sinclair et al., 2017). Some receptor-like kinases (RLKs) can bind pectins and/or their degradation products and

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participate in cell wall integrity sensing and signaling (Fig. 6) (Feng et al., 2018; Kohorn and Kohorn, 2012). Although the esmd1 mutation rescues cell adhesion defects in a manner that is not dependent on the presence of five wall-associated kinases, there might be other as-yet uncharacterized wall-associated proteins that interact with ESMD1 (Kohorn et al., 2021a). Du et al. (2020) demonstrated that the expression levels of wall integrity related RLKs were altered in qua2 allelic mutants, indicating that wall integrity signaling is active in qua2 seedlings. We propose that the downregulation of PME activity and the upregulation of PG activity in qua2 seedlings is the product of a quality control mechanism triggered by wall integrity sensing of altered pectin signals, whereby reduced PME activity slows the rate of HG demethyl-
esterification and enhanced PG activity degrades aberrant pectin molecules (Fig. 6B). However, as the aberrant pectin is a result of QUA2 dysfunction, the cells are unable to properly compensate and synthesize normally methyl-esterified HG molecules. The repercussions of QUA2 dysfunction are apparent during the later stages of cell elongation and wall maturation, where excessive PG activity pre-maturely degrades demethyl-esterified HG that otherwise might participate in Ca\(^{2+}\)-crosslinking, thus resulting in reduced pectin content and molecular mass, poor Ca\(^{2+}\)-crosslinking, and weakened middle lamellae, thereby promoting cell separation (Fig. 6B). In qua2 esmd1 seedlings, mutation of ESMD1 partially restores qua2 phenotypes, which might be tied to ESMD1-dependent regulation of PG activity (Fig. 6B). Transcriptomic analyses of qua2 and qua2 esmd1 might provide further evidence related to known and unknown cell wall sensing and signaling pathways alike. Our findings indicate that in muro pectin modification underlies the cell adhesion defects of qua2 seedlings.

Considering HG modifications, we can draw inferences about the respective impacts of plant and fungal PMEs and PGS on cell elongation and adhesion. Literature suggests that plant PMEs and PGS work differently from fungal PMEs and PGS; for instance, some plant PMEs are processive, whereas fungal PMEs can randomly de-methylesterify HG (Kars et al., 2005; Kohn et al., 1983; Limberg et al., 2000). These differences might help explain why the application of a fungal PG caused cell adhesion defects, but overexpression of AtPGX genes did not. However, endogenous plant PGS can in some cases cause cell adhesion defects when overexpressed (Atkinson et al., 2002), suggesting that the biochemical mechanism of action for a given PG or PME might depend more on its specific structure than on its kingdom of origin. It is also possible that the total level of PG activity for a given overexpression line or enzyme application results in different phenotypes: for example, the activity conferred by our exogenous PG application was likely much higher than that resulting from PGX overexpression and was concentrated at the epidermis where lesions are most noticeable. The level of PG activity and the type of PG that is overexpressed could be key factors in determining whether aberrant cell separation occurs in qua2 or other mutant plants. Although PG activity was restored to WT levels in qua2 esmd1, the double mutant still showed intermediate cell adhesion phenotypes. The reason for this could be that PME activity was not fully restored to WT levels or that the specific ratio of PME:PG activity is required to maintain cell adhesion. This could also be the case for qua3 RNAi knockdowns, which have reduced DM and normal pectin content, but do not show cell adhesion defects (Miao et al., 2011). These mutants were grown in liquid cell culture, which might account for some of the differences in phenotypes in comparison to qua2 mutants, and are knockouts rather than knockdowns. PG activity has not been reported for qua3 mutants, so it is possible that the lack of adhesion defects, and the normal pectin content, are due to differences in PME, PG, and/or PL activity that leads to more HG cross-linking and/or less HG degradation.

Mutation of a putative O-fucosyltransferase, ESMD1 (Verger et al., 2016), partially rescues PME activity and the cell adhesion phenotype of...
qua2, and fully restores PG activity to wild-type levels. Thus, ESM1D might regulate in muro pectin dynamics in qua2, possibly within the putative sensing and signaling pathways discussed above. Mutation of another annotated putative O-fucosyltransferase, FRIABLE1, also causes cell adhesion defects in Arabidopsis (Neumetzler et al., 2012). However, FRIABLE1 was recently demonstrated to possess RG-I rhamnosyl-transferase activity required for synthesizing the RG-I backbone, rather than O-fucosyltransferase activity (Wachananawat et al., 2020). Thus, further studies are required to identify the activity of ESM1D and better understand its role in suppressing cell adhesion defects in the qua2 mutant. Taken together, we have demonstrated that PG activity contributes to the severe cell adhesion defects found in qua2 seedlings. In the future, our findings can be applied to investigate the mechanisms underlying how plants sense and respond to changes in their cell walls and make, or fail to make, compensatory adjustments in wall metabolism and overall plant physiology during growth.

CRediT authorship contribution statement

William J. Barnes: Conceptualization, Investigation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing.

Ellen Zelinsky: Investigation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing.

Charles T. Anderson: Conceptualization, Supervision, Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Charles T. Anderson reports financial support was provided by US Department of Energy.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tcsw.2021.100069.

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