Mutations in the Pectin Methyltransferase QUASIMODO2 Influence Cellulose Biosynthesis and Wall Integrity in Arabidopsis

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Pectins are abundant in the cell walls of dicotyledonous plants, but how they interact with other wall polymers and influence wall integrity and cell growth has remained mysterious. Here, we verified that QUASIMODO2 (QUA2) is a pectin methyltransferase and determined that QUA2 is required for normal pectin biosynthesis. To gain further insight into how pectin affects wall assembly and integrity maintenance, we investigated cellulose biosynthesis, cellulose organization, cortical microtubules, and wall integrity signaling in two mutant alleles of Arabidopsis (Arabidopsis thaliana) qua2, qua2 and tds2. In both mutants, crystalline cellulose content is reduced, cellulose synthase particles move more slowly, and cellulose organization is aberrant. NMR analysis shows higher mobility of cellulose and matrix polysaccharides in the mutants. Microtubules in mutant hypocotyls have aberrant organization and depolymerize more readily upon treatment with oryzalin or external force. The expression of genes related to wall integrity, wall biosynthesis, and microtubule stability is dysregulated in both mutants. These data provide insights into how homogalacturonan is methylesterified upon its synthesis, the mechanisms by which pectin functionally interacts with cellulose, and how these interactions are translated into intracellular regulation to maintain the structural integrity of the cell wall during plant growth and development.

INTRODUCTION

The primary cell walls of plants are mainly composed of cellulose, hemicelluloses, and pectins, which interact to form structural networks and tune wall mechanical strength, thus regulating cell shape determination and organ morphogenesis (Cosgrove, 2005). Cellulose is synthesized by cellulose synthase complexes (CSCs) at the plasma membrane (Paredes et al., 2006), and its β-1,4-glucan chains are extruded into the apoplast and coalesce into microfibrils, the patterned deposition of which can be guided by cortical microtubules (Baskin et al., 2004). Xyloglucan, the major hemicellulose in the primary walls in eudicots, is thought to interact with cellulose microfibrils at “biomechanical hotspots” that control wall expansibility (Park and Cosgrove, 2012) and to bind to the surface of cellulose (Zheng et al., 2018). Pectins are complex acidic polysaccharides that are rich in galacturonic acid (GalA) and include homogalacturonan (HG), rhamnogalacturonan I (RG-I), and RG-II domains as well as xylogalacturonan and apio galacturonan in some species (Atmodjo et al., 2013). HG is an unbranched homopolymer of α-1,4-linked GalA (Atmodjo et al., 2013). After synthesis in the Golgi, HG is delivered to the apoplast in a highly methylsterified form and is demethylsterified in the wall by pectin methyltransferases (PMEs), releasing protons and methanol (Driouich et al., 2012). The patterns of this demethylsterification determine whether HG will form Ca2+-mediated cross-links and/or become susceptible to degradation by pectinases. Pectins function to control wall thickness, cell adhesion, and tissue integrity during plant development (Caffall and Mohnen, 2009). Pectin-modifying genes can thus influence wall and tissue mechanics; for example, Arabidopsis (Arabidopsis thaliana) plants overexpressing the pectin-degrading gene POLYGALACTURONASE INVOLVED IN EXPANSION2 or lacking PME35 have stems with altered mechanical robustness, supporting a function for pectins in regulating tissue mechanisms during development (Hongo et al., 2012; Xiao et al., 2017).
In Arabidopsis, multiple glycosyltransferases, methyltransferases, and acetyltransferases are required for pectin biosynthesis (Atmodjo et al., 2013). Several proteins involved in HG biosynthesis have been identified and characterized. GLUCURONATE 4-EPIMERASE1 (GAE1) functions in pectin biosynthesis by converting UDP-D-glucuronic acid to UDP-D-galacturonic acid and also influences cell wall integrity maintenance (Bethke et al., 2016). Arabidopsis GALACTURONOSYLTRANSFERASE1 (GAUT1) displays HG:GalA transferase activity and can form a complex with the homologous GAUT7 to synthesize high-molecular-weight polymeric HG via a "two-phase" mechanism (Sterling et al., 2006; Atmodjo et al., 2011; Amos et al., 2018). In grasses and woody dicot plants, GAUT4 possesses HG:GalA transferase activity, and knockdown of GAUT4 expression promotes plant growth and enhances biomass degradability (Biswal et al., 2018). In grasses and woody dicot plants, GAUT4 possesses HG:GalA transferase activity, and knockdown of GAUT4 expression promotes plant growth and enhances biomass degradability (Biswal et al., 2018). GAUT11, another confirmed HG α-1,4 GalA transferase, catalyzes HG elongation and likely functions in RG-I production in seed mucilage (Voiniciuc et al., 2018). QUASIMODO1 (QUA1/GAUT8) encodes a putative glycosyltransferase and is likely required for HG biosynthesis, since qua1 mutant plants have reduced uronic acid content in their walls and display cell adhesion defects (Bouton et al., 2002; Orfila et al., 2005; Durand et al., 2009; Verger et al., 2018). Either during or soon after polymerization, GalA residues in HG can be acetylated at O2 or O3 positions, and the carboxyl groups of most GalA residues in HG are methylsterified by methyltransferases (Atmodjo et al., 2013), but the proteins that perform these modifications have not yet been positively identified.

Several candidate HG methyltransferase genes have been identified in Arabidopsis. Arabidopsis QUA2, also named TUMOROUS SHOOT DEVELOPMENT2 (TS2D), encodes an annotated pectin methyltransferase, although methyltransferase activity has not previously been biochemically demonstrated for its gene product (Krupková et al., 2007; Mouille et al., 2007). HG-derived GalA from extracted pectin in the qua2 mutant is reduced by 50%, and both qua2 and tsd2 mutant seedlings have shorter hypocotyls with cell-cell adhesion defects in hypocotyl epidermal cells (Krupková et al., 2007; Mouille et al., 2007). Mutants for another allele of QUA2, osu1, are more sensitive to imbalanced carbon/nitrogen conditions, suggesting that QUA2 is also a key modulator of carbon and nitrogen nutrient balance during plant development (Gao et al., 2008). In addition, mutants of a rice (Oryza sativa) putative ortholog of AtQUA2 show altered wall composition and developmental defects (Qu et al., 2016; Xu et al., 2017). OsQUA2 shares high sequence similarity with AtQUA2, and mutation of OsQUA2 results in reduced HG methylesterification in culm-sieve element and root cell walls, affecting Suc transport and root development, respectively (Qu et al., 2016; Xu et al., 2017). QUA3, another putative HG methyltransferase, regulates cell wall biosynthesis in Arabidopsis suspension-cultured cells (Miao et al., 2011). Two more Arabidopsis genes, COTTON GOLGI RELATED2 (CGR2) and CGR3, encode putative HG methyltransferases, and cgr2 cgr3 double mutants display cell wall, photosynthesis, and growth defects (Kim et al., 2015; Weraduwage et al., 2016). Although cgr2 cgr3 double mutants show altered patterns of HG methylesterification during immunolabeling experiments and overexpression lines for either gene show increased general methyltransferase activity, these proteins were not shown to have specific HG methyltransferase activity (Kim et al., 2015).

HG has long been implicated in cell-cell adhesion in eudicots. For example, the cell adhesion defect in the Arabidopsis friable1 (fri1) mutant, in which a putative O-fucosyltransferase is mutated, has been attributed to alterations in pectin structure (Neumetzler et al., 2014).
indicating that results in expression changes in wall integrity-related genes, includes the sensing of pectin levels, con (Neumetzler et al., 2012). Thus, wall integrity signaling that in- and xyloglucan have led to a revision of the cellulose-xyloglucan or modi- Studies focusing on interactions between cellulose micro- and Fry, 2008; Cornuault et al., 2014). Despite these advances, the been posited to cross-link covalently with hemicelluloses (Popper which might template further orderly deposition of nascent cellu- cellulose hydrogels were synthesized in pectin solutions revealed intimate interactions between subsets of pectins and cellulose microfibrils in vitro (Lopez-Sanchez et al., 2016). Chemical genetic screening has uncovered a drug, cobtorin, that disrupts cellulose-microtubule coalignment and can be counteracted by over-expressing pectin-modifying genes or pectinase treatment, im- plying that pectins might be involved in the deposition of cellulose microfibrils in alignment with cortical microtubules (Yoneda et al., 2010). Aside from pectin interactions with cellulose, acidic pectins can also interact with structural glycoproteins such as the ex- tensins AtEXT3 and AtEXT4 to form pectate-extensin complexes, which might template further orderly deposition of nascent cell wall during cytokinesis (Cannon et al., 2008). Pectins have also been posited to cross-link covalently with hemicelluloses (Popper and Fry, 2008; Cornault et al., 2014). Despite these advances, the exploration of how pectins affect cellulose biosynthesis and orga- nization during cell wall assembly is still in its early stages.

In this study, we determined the molecular function of QUA2, which encodes an HG methyltransferase that is critical for normal HG biosynthesis. Then, we used two allelic Arabidopsis mutants with defects in pectin biosynthesis, qua2 and txd2, to investigate the molecular mechanisms that functionally link pectin and cel- lulose in the cell wall and the effects of pectin deficiency on cellulose, the cytoskeleton, and wall and tissue integrity. The resulting data reveal functional interactions between pectin and cellulose during cell wall assembly and provide new evidence at the genetic, molecular, and cellular levels of the links between pectins, cellulose, the microtubule cytoskeleton, and wall integrity sensing during plant cell growth and organ morphogenesis.

RESULTS
Purified QUA2 Shows Methyltransferase Activity on Polyalgalacturonic Acid In Vitro

Although HG methyltransferase activity has long been detected in crude membrane fractions from plants, no purified protein has been demonstrated to possess this enzymatic activity in vitro (Goubet and Mohnen, 1999; Ibar and Orellana, 2007; Miao et al., 2011; Kim et al., 2015). The QUA2 protein is predicted to contain 684 amino acids and to be anchored in the Golgi membrane by a single transmembrane domain near its N-terminus. Its C-terminus is predicted to reside in the Golgi lumen and harbors a large catalytic region, which contains a putative S-adenosylmethionine (SAM)-dependent methyltransferase domain (Figure 1A). To de- termine the enzymatic activity of QUA2, a sequence including the methyltransferase domain of QUA2 without the transmembrane domain (amino acids 150 to 684) was cloned and expressed in Escherichia coli. After amylase affinity chromatography and size exclusion chromatography, a truncated QUA2 protein fused with maltose binding protein (MBP) and a His tag (MBP-QUA2) was purified to homogeneity as verified by SDS-PAGE (Figure 1B) and immunodetection with an anti-His antibody (Figure 1C).

In general, SAM-dependent methyltransferases use SAM as a methyl donor and DNA, proteins, or other molecules as methyl acceptors to generate S-adenosylhomocysteine (SAH) and methylated products (Fontecave et al., 2004). In HG methylation, methyl groups from donor SAM molecules are thought to be transferred to C-6 carboxyls of GaLA residues, resulting in the accumulation of SAH. Therefore, the concentration of SAH pro- duced will be proportional to methyltransferase activity. Purified MBP or MBP-QUA2 was incubated with SAM and poly-galacturonic acid (PGA; the fully demethylated form of HG) for 2 h. After the completion of the reactions, the generated SAH was converted into ADP, which was subsequently converted into ATP and measured by luciferase activity (Hisao et al., 2016). In comparison with the MBP-only control, the reaction including both MBP-QUA2 and PGA yielded significant methyltransferase activity (Figure 1D). When PGA was omitted, accumulated SAH remained at background levels (Figure 1D). The methyltransferase assay was also performed for varying durations or supplied with different concentrations of MBP-QUA2 or PGA. The generated SAH increased with increasing incubation time or concentration of reaction components (Figures 1E to 1G), indicating that the methyltransferase activity of QUA2 is time- and concentration- dependent. The substrate specificity of MBP-QUA2 was inves- tigated using the polysaccharides PGA, alginate, and starch. As shown in Figure 1H, PGA supported significantly higher methyltransferase activity than the other two polysaccharides. To further investigate whether PGA acts as the methyl acceptor during the reaction, reaction products using different polysaccharides as substrates were recovered after varying incu- bation times and used for immunoblotting with JIM5 (recognizing low-methylesterified HG) and JIM7 (recognizing
Figure 1. QUA2 Displays Methyltransferase Activity on PGA In Vitro.

(A) Schematic of predicted protein structure of QUA2. The black box represents the transmembrane domain (TMD). The dark gray box indicates the catalytic domain, which is in the SAM-dependent methyltransferase_29 superfamily. The N and C termini of QUA2 putatively face the cytosol and Golgi lumen, respectively.

(B) SDS-PAGE and Coomassie blue staining of purified MBP and MBP-fused QUA2 proteins. MBP and MBP-QUA2 are predicted to migrate at ~40 and ~100 kD, respectively.

(C) Immunoblot of purified MBP and MBP-fused QUA2 proteins using an anti-polyhistidine antibody.

(D) Methyltransferase assay with purified proteins. For each 50-μL reaction, 1 μg of purified MBP or MBP-QUA2 protein was incubated with 50 μg of PGA in the presence of 10 μM SAM, 2 mM Mg2+, and 0.25 M Suc. Enzymatic activity is proportional to the accumulated reaction product SAH, which is converted into ADP, then to ATP, and finally monitored by luciferase activity. The change in luminescence (in relative light units [RLU]) is the net luminescence over background, which is calculated as the raw luminescence minus the background luminescence (no-enzyme control).

(E) to (H) Methyltransferase activity of purified MBP-QUA2 is time- and concentration-dependent. Reactions were set up as described in (D), except for the indicated protein concentrations (F) or PGA concentrations (G). After incubation at 30°C for 2 h except in (E), the reactions were stopped by the addition of 0.5% trifluoroacetic acid and analyzed for SAH content.

(H) Substrate specificity test of QUA2. Methyltransferase assays were performed when supplied with 50 μg of the indicated polysaccharide. In the presence of PGA, the SAH generated is more than sevenfold and 16-fold higher than when alginate and starch were supplied, respectively.

(I) Immunoblot of the product of the methyltransferase reaction of MBP-QUA2 using monoclonal antibodies JIM5 (recognizing low-methylesterified HG) and JIM7 (recognizing methylesterified HG). Products reacted in the presence of the indicated substrate for the indicated time were adsorbed to a nitrocellulose membrane for immunoblotting. Control dots were processed in the absence of primary antibody.

Each column in (D) to (H) represents the average of three replicates, and error bars show SE. **, P < 0.001, Student’s t test.
methylesterified HG) antibodies. The products using PGA as substrate showed binding with JIM5 and JIM7, although binding intensity with JIM7 was weaker than with JIM5. JIM5 binding to the PGA reaction products diminished slightly with increasing reaction time (Figure 1). Taken together, these results demonstrate that QUA2 is a functional HG methyltransferase in vitro.

Shorter Hypocotyls and Cell-Cell Adhesion Defects Are Evident in qua2 and tsd2 Mutants

To investigate the biological function of QUA2, we examined the phenotypes of two QUA2 mutants, qua2 and tsd2 mutants contain point mutations in the QUA2 (At1g78240) gene that result in stop codons (Supplemental Figure 1). Both mutants independently been shown to have shorter hypocotyls and cell adhesion defects in epidermal cells of hypocotyls (Krupková et al., 2007; Mouille et al., 2007). To confirm these hypocotyl phenotypes simultaneously, seeds of the Arabidopsis wild-type Columbia-0 (Col) ecotype controls and qua2 and tsd2 mutants were sown on Murashige and Skoog (MS) plates without Suc and grown in the dark. qua2 and tsd2 etiolated seedlings had shorter hypocotyls than Col controls from 2 to 6 d of growth, and tsd2 hypocotyls were slightly shorter than qua2 hypocotyls (Figures 2A and 2B). Six-day-old etiolated Col seedlings had an average hypocotyl length of 1.59 ± 0.17 (μm) cm, whereas 6-d-old etiolated qua2 and tsd2 mutants had average hypocotyl lengths of 0.59 ± 0.12 and 0.51 ± 0.09 cm, respectively (n ≥ 30 seedlings per genotype; Figures 2A and 2B). Similar to previous reports (Krupková et al., 2007; Mouille et al., 2007), epidermal cells in 3-d-old etiolated qua2 and tsd2 hypocotyls were often detached from other cells (Figure 2C); in addition, pairs of guard cells in 6-d-old light-grown cotyledons were also sometimes separated from one another as observed after propidium iodide staining (Figure 2D), although these defects were less evident in true leaves. Abnormal cell separation was not evident in cotyledons, roots, and hypocotyls (Supplemental Figures 2A to 2C). In adult plants, GUS staining was detected in cotyledons, roots, and parenchyma cells of stems (Supplemental Figure 2H). Together, these data indicate that QUA2 is expressed across a wide range of tissues and might function in the growth of multiple organs during plant development.

Uronic Acid Levels Are Reduced, but Degree of HG Methylesterification Is Not, in qua2 and tsd2 Mutants

As the above results show, QUA2 is an HG methyltransferase that is expected to function during the biosynthesis of pectic HG. However, previous studies reported an ~50% reduction in extractable HG, rather than total HG, in the qua2 mutant (Mouille et al., 2007) and no significant reduction in total uronic acid in the tsd2 mutant (Krupková et al., 2007), with no significant reduction in the degree of HG methylesterification in either mutant. To simultaneously compare both qua2 and tsd2 mutants with Col controls in the same set of experiments, we measured total uronic acid content and monosaccharide composition in 6-d-old etiolated seedlings. Total uronic acid content in qua2 and tsd2 mutants was significantly lower than in Col controls on a mol% basis (Figure 3A). To estimate the degree of pectin methylesterification, we measured methanol release upon saponification from cell walls of 6-d-old dark-grown seedlings. The amounts of released methanol did not differ significantly in qua2 or tsd2 samples from that released from Col samples (Figure 3B). Normalizing by the amount of HG in the mutants, the degree of HG methylesterification did not differ significantly from that in Col controls (Figure 3C). The lower uronic acid content in qua2 and tsd2 plants is consistent with results from sugar monosaccharide composition analysis, in which cell wall material from both mutants displayed significantly less GaLA on a mol% basis, with arabinose content being significantly higher in the mutants (Figure 3D). Together, these results suggest that mutations in QUA2 might result in the synthesis of an unstable or incomplete form of HG that is rapidly degraded, resulting in lower HG levels in...
the wall, but that QUA2 is not solely responsible for HG methylesterification.

Cellulose Biosynthesis Is Inhibited in qua2 and tsd2 Mutants

To investigate whether pectin deficiency in qua2 and tsd2 mutants affects cellulose biosynthesis, we first measured crystalline cellulose content according to the Updegraff method (Updegraff, 1969) from alcohol-insoluble residue (AIR) of 6-d-old etiolated seedlings. Average cellulose content was $139 \pm 4 \mu g/mg$ AIR in Col, whereas it was roughly $100 \mu g/mg$ AIR in both qua2 and tsd2 mutants, representing a reduction in cellulose content of $\sim 30\%$ (Figure 3E). Cellulose content was also decreased by $\sim 10\%$ in rosettes leaves of 4-week-old qua2 and tsd2 plants relative to Col controls (Figure 3F). Based on the finding of reduced cellulose content in qua2 and tsd2 mutants, we next investigated whether cellulose biosynthesis was affected in the mutants. Transgenic lines expressing GFP-CESA3, a marker for primary wall CSCs (Desprez et al., 2007), were produced in the qua2 and tsd2 mutant backgrounds and in the Col background as a control (Xiao et al., 2016). To monitor CSC dynamics in mutants, time-lapse images of GFP-CESA3 over 5 min at 5-s intervals were recorded in epidermal cells at the tops of 3-d-old etiolated hypocotyls and GFP-CESA3 particle movement was analyzed and quantified. Fewer linear

![Figure 2](https://academic.oup.com/plcell/article-32/11/3576/6099446)

**Figure 2.** qua2 and tsd2 Mutants Have Shorter Hypocotyls and Show Cell Adhesion Defects.

(A) Six-day-old etiolated Arabidopsis Col, qua2, and tsd2 seedlings. Bar = 1 cm.

(B) Quantification of hypocotyl length in dark-grown seedlings 2 to 6 d after sowing (DAS; $n = 30$ seedlings per genotype per time point). qua2 and tsd2 hypocotyls were much shorter than Col controls, and tsd2 hypocotyls were shorter than qua2 hypocotyls. Error bars represent SE. *, $P < 0.05$ and **, $P < 0.001$, Student’s t test.

(C) Hypocotyls from 3-d-old dark-grown Col, qua2, and tsd2 seedlings. Bar = 100 $\mu m$.

(D) Images of stomata present on the abaxial side of cotyledons of 6-d-old light-grown Col, qua2, and tsd2 seedlings. Cells were labeled with 100 $\mu g/mL$ propidium iodide and imaged by spinning-disk confocal microscopy. Bar = 10 $\mu m$. 

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tracks of CSCs were present in time-averaged projections in both qua2 and tsd2 mutants compared with the well-organized CSC arrays evident in Col cells, and the average speed of GFP-CESA3 particles was 223.8 ± 0.4 (ss) nm/min (n = 965 particles, 11 cells from 10 seedlings) in qua2 hypocotyls and 224.4 ± 0.3 nm/min (n = 1441 particles, 17 cells from 9 seedlings) in tsd2 hypocotyls, which were significantly slower than the average speed of 284.9 ± 0.4 nm/min (n = 1016 particles, 14 cells from 7 seedlings) in Col controls (Figure 4A; Supplemental Movie). The slower GFP-CESA3 particles in both mutants were also evident in kymographs, which showed steeper (slower) slopes and more unidirectional movement from CSC tracks as opposed to shallower (faster) slopes and bidirectional movement in Col controls (Figure 4B).

This finding was confirmed when particle-tracking software was used to calculate particle speeds for many hundreds of GFP-CESA3 particles for each genotype (Figure 4C). GFP-CESA3 particle density from z-series images in qua2 and tsd2 mutants was 0.82 ± 0.11 and 0.90 ± 0.12 particles/μm², respectively, which were not significantly different from a density of 0.88 ± 0.11 particles/μm² in Col controls (Figures 4D and 4E). Therefore, disruption of pectin results in both reduced GFP-CESA3 particle speed and reduced cellulose content in the qua2 and tsd2 mutants, strongly indicating that cellulose synthesis is defective in the mutants.
Cellulose Organization and Morphology Are Disrupted in qua2 and tsd2 Hypocotyl Cells

The change in cellulose biosynthesis we detected in the qua2 and tsd2 mutants prompted us to further explore how HG deficiency might affect cellulose microfibril arrangement in mutant walls. As a nondestructive method, sum frequency generation (SFG) can selectively detect the coherence of crystalline cellulose without interference from an amorphous polymer matrix (Makarem et al., 2019) and is sensitive to the mesoscale ordering of cellulose microfibrils over depths of hundreds of nanometers in plant cell walls (Lee et al., 2014; Makarem et al., 2017). Given that 6-d-old etiolated seedlings contain most of the deposited cellulose that is produced in etiolated seedlings, we observed SFG spectra in 6-d-old etiolated mutant and Col hypocotyls. The data displayed similar peak features along the lengths of the hypocotyls (Figure 5A), with sharp CH peaks centered at 2944 cm$^{-1}$ and broad OH peaks centered at 3320 cm$^{-1}$, as well as shoulder peaks at 3450 cm$^{-1}$. Peak intensity at 2944 cm$^{-1}$ can be used to estimate cellulose content (Barnette et al., 2012; Makarem et al., 2017). The change in cellulose biosynthesis we detected in the mutants, consistent with the above results from Updegraff assays. SFG intensity at 2944 cm$^{-1}$ from middle and basal hypocotyls in all three genotypes was slightly higher than that from top regions (Figure 5A), suggesting that more cellulose had been deposited in lower regions, which is consistent with reported measures of wall synthesis and thicknesses in etiolated hypocotyls of Arabidopsis (Refrégier et al., 2004; Derbyshire et al., 2007). The relative orientation of cellulose microfibrils can be quantified by comparing OH:CH peak area ratio in the experimental geometry, in which the laser incidence plane is aligned either parallel (longitudinal) or transverse to the hypocotyl elongation axis (Karle et al., 2014a, 2014b; Chen et al., 2017). The lower ratio of (OH:CH$_{longitudinal}$) to (OH:CH$_{transverse}$) in qua2 and tsd2 mutants (Figure 5B) indicated that cellulose microfibrils are aligned in a more random manner in the mutant cell walls than in Col controls.

To further examine cell wall organization in qua2 and tsd2 mutants, we observed wall structure in the innermost (newly deposited) layers of the wall in epidermal cells from the hypocotyls of 3-d-old etiolated seedlings by atomic force microscopy (AFM), which detects stiffer wall elements such as cellulose microfibrils and cross-linked matrix components, as well as by field emission scanning electron microscopy (FESEM) without or with treatment by pectate lyase to remove some matrix polysaccharides from the innermost face of the wall (Zhang et al., 2016). Compared with reported measures of wall synthesis and thicknesses in etiolated hypocotyls of Arabidopsis (Refrégier et al., 2004; Derbyshire et al., 2007). The relative orientation of cellulose microfibrils can be quantified by comparing OH:CH peak area ratio in the experimental geometry, in which the laser incidence plane is aligned either parallel (longitudinal) or transverse to the hypocotyl elongation axis (Karle et al., 2014a, 2014b; Chen et al., 2017). The lower ratio of (OH:CH$_{longitudinal}$) to (OH:CH$_{transverse}$) in qua2 and tsd2 mutants (Figure 5B) indicated that cellulose microfibrils are aligned in a more random manner in the mutant cell walls than in Col controls.

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Figure 5. Cellulose Is Disorganized in qua2 and tsd2 Etiolated Hypocotyls.

(A) SFG signal intensity from top, middle, and basal hypocotyl regions of 6-d-old etiolated Col, qua2, and tsd2 seedlings. a.u., Arbitrary units.
(B) Degree of anisotropic organization in fibrils of Col, qua2, and tsd2 genotypes (n = 6 location points from a bundle of 10 seedlings). Error bars represent SE. a.u., Arbitrary units.
(C) AFM images from top regions of 3-d-old etiolated Col, qua2, and tsd2 hypocotyls. Blue lines in inset images indicate cell long axis; white dashed arrows indicate scan direction. Bar = 200 nm.
(D) and (E) FESEM images from top regions of 3-d-old etiolated Col, qua2, and tsd2 hypocotyls without (D) and with (E) pectate lyase (PL) treatment. Purple arrows display the positions where fibrils were fragmented. Bars = 100 nm.
(F) Diagram of fibril patterning from FESEM images in (E).
(G) Quantification of fibril width from FESEM images (n ≥ 100 cellulose microfibrils from at least three images). The bottom whisker is from minimum to quartile 1, the bottom box is from quartile 1 to median, the top box is from median to quartile 3, and the top whisker is from quartile 3 to maximum. Error bars represent SD. **, P < 0.001, Student’s t test.
crooked fibrils in both mutants, with the differences being more obvious in tsd2 than in qua2 mutants (Figures 5D and 5E). Bright fragments on the wall surface in FESEM images in mutants without pectate lyase treatment (Figure 5D) were reminiscent of structures of Ca2+-cross-linked matrix polysaccharides in onion (Allium cepa) epidermal walls (Zhang et al., 2016), implying that these structures might represent fragments of demethylated HG that are overrepresented in qua2 and tsd2 mutants and are not integrated tightly into the wall matrix upon delivery to the apoplast. Long fibrils that might represent cellulose microfibrils appeared to be more fragmented in both mutants after pectate lyase treatment (Figure 5E), as depicted in the diagram in Figure 5F. Moreover, fibrils in FESEM micrographs from mutants were thinner than those in Col controls after treatment with pectate lyase (Figure 5G). In addition, we observed porous features in tsd2 walls (Figure 5E). The above data highlight the function of HG in supporting normal cellulose synthesis and orientation, as well as the assembly of properly integrated primary walls, in Arabidopsis.

Reduced Pectin Deposition in Mutants Promotes the Increased Molecular Mobility of Pectin and Cellulose

We also detected a substantial change in the molecular mobility of cell wall components in qua2 and tsd2 mutants using solid-state NMR experiments. Mobility is site-specifically monitored by comparing the relative intensity of a spectrum that only selects mobile molecules with a quantitative spectrum (Figure 6A; Phyo et al., 2017). For each peak, the intensity ratio between the two spectra is quantified, which represents the relative mobility of each carbon site (Figure 6B). Compared with the wild-type sample, both mutants showed higher mobility for pectin backbones (GalA C1 at 100 ppm and GalA C4/Rha C1 at 69 ppm), pectin side chains (Ara C1 at 108 ppm), surface cellulose (sC4 at 84 ppm), and interior cellulose (iC4 at 89 ppm). A few exceptions were evident at carbon sites with special properties; for example, the Rha C6 resonating at 18 ppm remained highly mobile in all three samples, as it is a CH3 group that rotates freely. The qua2 mutant generally had the highest intensity ratios, indicating that this mutant has the most dynamic molecules among the three samples. Enhanced pectin mobility should originate from a perturbed backbone and side chain structure and domain organization, which shortens 13C relaxation and enhances pectin intensity in the 13C direct polarization spectrum measured with short recycle delays, but it is unclear why cellulose also showed an increase of 5 to 10% in mobility in the qua2 mutant. Since cellulose is typically considered to be rigid, the change in cellulose intensity might be attributed to the enhanced spin exchange caused by its stronger interactions with more mobile pectin in the qua2 mutant. The tsd2 mutant displayed mobilities between the wild-type and qua2 samples.

Cortical Microtubule Orientation Is Disrupted in the Absence of Normal Wall Structure in qua2 and tsd2 Hypocotyl Cells

Given the functions of cortical microtubules in tissue mechanics and cellulose deposition, and the alteration of wall structure in qua2 and tsd2 mutants, we next investigated whether the disruption of wall biosynthesis in these mutants affects the patterning and dynamics of cortical microtubules. Transgenic lines expressing GFP-MAP4 (Marc et al., 1998) were generated in the Col, qua2, and tsd2 backgrounds. To observe the patterning of microtubules, z-stack images were collected from epidermal cells of top, middle, and basal hypocotyls in 3-d-old etiolated seedlings.

Figure 6. qua2 and tsd2 Mutants Have Increased Mobility for Both Cellulose and Pectin as Detected by NMR.

(A) 13C direct polarization (DP) spectra measured with short recycle delays of 2 s for probing mobile molecules (red) and 35 s for quantitative detection (black) at 293K. The relative intensities between these two spectra are indicated for resolved cellulose and pectin peaks, with the higher relative intensity representing higher mobility.

(B) Relative intensity ratios between the 2-s DP that selects the mobile molecules and quantitative DP spectra for wild-type (black), qua2 (blue), and tsd2 (red) cell walls.
Relative to the cell elongation axis, microtubules in the tops of Col hypocotyls were angled mostly from 40 to 70°, whereas microtubules in qua2 and tsd2 hypocotyl tops were more randomly distributed (Figures 7A and 7B). In comparison with the oblique microtubule angles in the middle regions of hypocotyls of Col controls that centered around 30° relative to the cell elongation axis, microtubules in the same regions of qua2 and tsd2 hypocotyls tended to be more transverse, with angles primarily between 50 and 90° (Figures 7A and 7B). In Col basal hypocotyls, most microtubules were longitudinal, whereas in qua2 and tsd2 mutants, some microtubules were oblique. We noticed that some microtubules in the qua2 background were wavier than in Col controls (Figure 7A). The aberrant orientation of microtubules in qua2 and tsd2 mutants implies that disruption of wall biosynthesis and cell adhesion defects affect cytoskeletal patterning in these genotypes, and/or vice versa.

**Mutations in QUA2 Result in Altered Expression of Wall Synthesis and Integrity-Sensing Genes**

To examine the effects of disruption of cell wall biosynthesis and organization in qua2 and tsd2 mutants at the transcriptional level, we measured transcript levels of genes related to pectin and cellulose biosynthesis by qPCR (Table 1). The expression of some pectin biosynthesis genes, including GAEL1, GAUT1, GAUT7, and GAUT12, was significantly upregulated, although GAUT8 and GAUT15 expression did not change, whereas genes encoding two pectin-interacting extensins, EXT3 and EXT4 (Cannon et al., 2008), were upregulated. FAD-LINKED OXIDOREDUCTASE (FADLOX), a gene involved in stress response and Ca2+ signaling (Verger et al., 2016), had higher transcript abundance in both mutants. The pectin modification gene PME35 (Hongo et al., 2012) had decreased expression. All three major CESAs that function in primary wall biosynthesis, CESAl, CESAn, and CESAs, along with COBRA and CELLULOSE SYNTHASE INTERACTIVE1 (CSI1), were significantly downregulated. The effects of mutation of QUA2 on transcriptional regulation of microtubule stability and wall integrity signaling were also investigated using qPCR (Table 1). Several microtubule-related genes, including MICROTUBULE-ASSOCIATED PROTEIN70-1 (MAP70-1), MAP70-5, CLASP, FRAGILE FIBER1 (FRA1), and KATANIN1 (KTN1; Korolev et al., 2005, 2007; Stoppin-Mellet et al., 2006; Ambrose et al., 2007), were significantly downregulated in both mutants, and MAP20 had lower expression in qua2 but not tsd2. However, the expression levels of MAP65-1 did not differ significantly among all three genotypes. Receptor kinases, including FERONIA (FER), HERKULES1 (HERK1), THESEUS (THE1), KINESIN13A (KIN-13A), FEI1, and FEI2 have been termed cell wall sensors (Hématy and Höfte, 2008; Xu et al., 2008; Guo et al., 2009). All of the above genes were significantly downregulated in both mutants, whereas all five WALL ASSOCIATED KINASE (WAK) genes (Kohorn and Kohorn, 2012) except WAK3 were downregulated in the tsd2 mutant but not in the qua2 mutant (Table 1). These data imply that cells respond to the disruptions in wall structure induced by mutations in QUA2 by reducing the synthesis of both pectin and cellulose, increasing the expression of wall-related structural proteins and dampering wall integrity signaling.
DISCUSSION

Previous studies have determined that pectic HG is methylated in the Golgi apparatus before being secreted into the cell wall (Goubet and Mohnen, 1999). Methyltransferase activity has been detected in Golgi-enriched fractions and isolated microsomes, and the catalytic domains of putative methyltransferases face the Golgi lumen (Goubet and Mohnen, 1999; Ibar and Orellana, 2007; Miao et al., 2011; Kim et al., 2015). A series of putative methyltransferases have been identified, including QUA2/TSD2, QUA3, CGR2, and CGR3 in Arabidopsis and their homologs in other species, but none of them has yet been characterized for enzymatic activity in vitro. In this study, we heterologously expressed QUA2 in E. coli and purified it to homogeneity. The in vitro methyltransferase activity of QUA2 was confirmed using demethylated HG (PGA) as a substrate and a sensitive and robust methods.
HG-derived GalA from cell walls of qua2 leaves was 50% lower than in wild-type controls (Mouille et al., 2007). Our data confirmed lower GalA levels in monosaccharide composition analyses and lower total uronic acid content in both mutant alleles, although the estimated degree of pectin methyltransferase activity did not show significant changes in etiolated seedlings of qua2 or tsd2 mutants (Figure 3). These data suggest at least three possible scenarios: first, mutations in QUA2 might inhibit HG biosynthesis without altering HG methyltransferase; second, HG in qua2 and tsd2 mutants might be secreted with a lower degree of methyltransferification and therefore be subject to faster degradation in the apoplast; and third, given that GAUTs and putative pectin methyltransferases (PMTs) can coexist in protein complexes, presumably to coordinate the synthesis of highly methyltransferified HG (Atmodjo et al., 2013), the loss of active QUA2 from HG-biosynthetic complexes that normally contain this protein might result in loss of function for those complexes, leading to reduced overall HG synthesis but an unchanged degree of methyltransferification in the remaining HG synthesized by other complexes.

In the plant cell wall, different structural components interact with each other to form a unified, multi-network architecture. Hence, it is likely that a defect in one constituent will affect the structure and assembly of other wall components. The extensive interaction of HG with cellulose detected by solid-state NMR highlights the potential for this interaction to help define the structure and function of plant cell walls (Dick-Pérez et al., 2011), although pectins do not competitively bind to cellulose with as much affinity as xyloglucan in vitro (Zykwinska et al., 2008). Pectic RG-I has also been shown to interact with cellulose (Zykwinska et al., 2005; Broxterman and Schols, 2018). Both pectin and cellulose play important roles in the assembly of cell walls during cell expansion, but it has remained unclear whether and how pectic polysaccharides might influence cellulose biosynthesis and microfibril deposition. Here, we used the pectin-deficient mutants qua2 and tsd2 to explore the effects of pectin deficiency on cellulose synthesis and wall organization using different microscopy techniques, including spinning-disk confocal microscopy, SFG, AFM, and FESEM. We found that cellulose synthesis was inhibited with reduced crystalline cellulose content, lower speed of CESA particles in the plasma membrane, and altered fibril organization in the walls in qua2 and tsd2 mutants compared with Col controls (Figures 4 and 5; Supplemental Movie). These observations provide evidence to support the idea that pectins influence the deposition of cellulose in the wall, which is also supported by the fact that CESA-overexpressing plants contain higher cellulose and pectin levels (Hu et al., 2018). Studies of physical interactions between wall components have largely focused on hemicellulose-cellulose interactions, while pectin-cellulose interactions have long been understudied. To help fill in this gap, we investigated wall dynamics using NMR in qua2 and tsd2 mutants and wild-type controls (Figure 6). The results showed that the partial reduction in pectin might facilitate stronger interactions between pectic polysaccharides and cellulose, since an increased molecular mobility was detected for both classes of wall polymers in both mutants. Future experiments investigating the detailed molecular interactions between pectin subtypes and cellulose in wild-type and qua2/tsd2 mutant walls should provide

### Table 1. Expression Levels of Wall and Microtubule-Related Genes

| Gene Name       | qua2  | tsd2  |
|-----------------|-------|-------|
| **Cellulose synthesis-related genes** |       |       |
| CESA1           | 0.58 ± 0.05** | 0.48 ± 0.01** |
| CESA3           | 0.69 ± 0.03*  | 0.51 ± 0.06*  |
| CESA6           | 0.50 ± 0.03*  | 0.43 ± 0.02** |
| KOR1            | 0.42 ± 0.02** | 0.33 ± 0.03** |
| COBRA           | 0.72 ± 0.04*  | 0.70 ± 0.02*  |
| SI1             | 0.54 ± 0.02** | 0.42 ± 0.01** |
| **Microtubule-related genes** |       |       |
| MAP20           | 0.59 ± 0.09*  | 0.86 ± 0.12  |
| MAP65-1         | 0.91 ± 0.02   | 0.87 ± 0.03  |
| MAP70-1         | 0.68 ± 0.04** | 0.58 ± 0.01**|
| MAP70-5         | 0.22 ± 0.01** | 0.26 ± 0.02**|
| CLASP           | 0.57 ± 0.05** | 0.41 ± 0.04**|
| FRA1            | 0.46 ± 0.01** | 0.31 ± 0.04**|
| KTN1            | 0.69 ± 0.04** | 0.62 ± 0.04**|
| **Cell wall integrity genes** |       |       |
| FER             | 0.65 ± 0.01** | 0.56 ± 0.03* |
| HERK1           | 0.36 ± 0.01** | 0.31 ± 0.02**|
| FEI1            | 0.68 ± 0.01** | 0.50 ± 0.07**|
| FEI2            | 0.37 ± 0.05** | 0.59 ± 0.03**|
| KIN13A          | 0.48 ± 0.02** | 0.40 ± 0.01**|
| THE1            | 0.41 ± 0.06*  | 0.25 ± 0.02**|
| WAK1            | 0.94 ± 0.01   | 0.18 ± 0.03* |
| WAK2            | 1.07 ± 0.31   | 0.42 ± 0.13* |
| WAK3            | 1.68 ± 0.24   | 0.94 ± 0.23  |
| WAK4            | 0.99 ± 0.20   | 0.20 ± 0.06* |
| WAK5            | 0.77 ± 0.29   | 0.15 ± 0.06* |
| **Pectin-related genes** |       |       |
| GAE1            | 0.71 ± 0.10*  | 0.53 ± 0.04* |
| GAUT1           | 0.68 ± 0.06*  | 0.65 ± 0.07* |
| GAUT7           | 0.68 ± 0.09*  | 0.63 ± 0.06* |
| GAUT8           | 1.08 ± 0.06   | 1.08 ± 0.03  |
| GAUT12          | 0.47 ± 0.07** | 0.19 ± 0.02**|
| GAUT15          | 1.04 ± 0.20   | 0.76 ± 0.03  |
| EXT3            | 1.66 ± 0.09** | 2.16 ± 0.07**|
| EXT4            | 5.79 ± 0.57** | 5.25 ± 0.49**|
| PME35           | 0.0065 ± 0.001** | 0.0036 ± 0.003**|

Detection of mRNA expression levels is shown from 3-d-old etiolated Col, qua2, and tsd2 seedlings by qPCR. EF1-a was amplified as an internal control. Expression levels in Col were normalized to 1, and the expression levels of target genes in qua2 and tsd2 seedlings were calculated relative to these values. Data present averages and so (n = 3 technical replicates). Asterisks indicate significant differences (*, P < 0.05 and **, P < 0.001, Student’s t test). See also the Supplemental Table.

method to test for methyltransferase activity (Hsiao et al., 2016). These data reveal the molecular function of QUA2 as a functional HG methyltransferase in vitro (Figure 1).

We also analyzed two mutant alleles of QUA2, qua2 and tsd2, which result from mutations at different positions in the QUA2 gene, and found that tsd2 mutants display more severe phenotypes such as shorter hypocotyls and smaller plants (Figure 2; Supplemental Figure 2). The tsd2 mutation disrupts the first exon of QUA2, possibly leading to more severe effects on protein function than the qua2 lesion, which resides in the seventh exon (Supplemental Figure 1). Previously, it was found that extractable
even more information about how pectin influences cellulose mobility in the wall, and vice versa.

By constituting a shape-constraining and anisotropic extra-cellular matrix, the cell wall regulates cellular and tissue mechanics during plant growth and development. Cellulose has been hypothesized to be the predominant mechanical actor in the cell wall, but matrix polysaccharides are emerging as important mechanical regulators as well. As the major hemicellulose in eudicot cell walls, xyloglucan has been demonstrated to influence cell wall mechanics and plant growth and to functionally interact with cellulose (Miedes et al., 2013; Xiao et al., 2016). Pectins have also been shown to influence plant mechanics during growth due to their unique physical and chemical properties (Peaucelle et al., 2011, 2015; Bou Daher et al., 2018). Compared with wild-type controls or the highly aligned and bundled fibrils in top hypocotyl regions of xxt1 xxt2 mutants (Xiao et al., 2016), the apparent structure of the cell wall in qua2 and tsd2 hypocotyls after pectate lyase application (Figure 5E) is in keeping with a posited function of HG in determining the extent of wall porosity (Baron-Epel et al., 1988). Defective HG in both qua2 and tsd2 also apparently enhances molecular mobility in the wall and promotes interactions of the remaining pectin with other wall components such as cellulose in order to maintain wall structural integrity (Figures 5 and 6). As the results show, tsd2 displays more severe phenotypes in general than qua2, including reduced hypocotyl length, dwarf adult plants, and adhesion defects. However, the NMR data in the qua2 mutant show the highest molecular mobility, implying strong interactions between pectin and cellulose. Given that cellulose exists as stiff fibrils in the wall, the enhanced mobility of cellulose in the mutants might result from its increased interaction with dynamic pectin molecules. It is evident that mutation of QUA2 leads to reduced HG and cellulose synthesis and remodeled polymer interactions. The above evidence underscores the functional interactions between pectin and cellulose and the effects of pectin defects on cellulose deposition and wall assembly.

Previous studies have shown that the defects in cellulose synthesis affect the alignment of cortical microtubules (Paredes et al., 2008). Additionally, microtubule patterning and stability are
aberrant in xxt1 xxt2 hypocotyls that lack detectable xyloglucan (Xiao et al., 2016). Pectin modification might trigger mechanical and growth symmetry breaking, subsequently resulting in cortical microtubule reorientation (Peaucelle et al., 2015). On the other hand, tubulin perturbation also affects pectin deposition during cell wall formation (Swamy et al., 2015). Additionally, cortical microtubules respond to mechanical signals concomitant with the alteration of microtubule orientation (Hamant et al., 2008). These findings imply close coordination between cell wall synthesis, wall mechanics, and microtubule behavior. Given the reduced pectin and cellulose contents in qua2 and tsd2 plants, we sought to investigate microtubule organization and dynamics in these mutants. In different regions of qua2 and tsd2 hypocotyls, compared with wild-type controls, microtubules display altered orientation (Figures 7A and 7B). Compared with the more longitudinal microtubules in middle regions in xxt1 xxt2 hypocotyls (Xiao and Anderson, 2016), the microtubules in qua2 and tsd2 hypocotyls were more transverse relative to the cell elongation axis, which might be due to cell swelling caused by defects in cell adhesion. Microtubules in qua2 and tsd2 hypocotyl cells were more sensitive to oryzalin treatment (Figure 7C). This suggests either that the drug gets to the microtubules more quickly in the qua2 and tsd2 hypocotyl cells or that the microtubule polymerization process itself is perturbed in qua2 and tsd2 mutants (Rajangam et al., 2008). Microtubules in qua2 and tsd2 hypocotyl cells were also more sensitive to mechanical stress (Figure 7D), underscoring the role of microtubules in mechanical responsiveness (Hamant et al., 2019). Accordingly, these data indicate a potential role for QUA2 in microtubule formation. A recent study reported an alteration of tension stress patterns due to mechanical discontinuity of the epidermis in a mutant of QUA1, which encodes a putative pectin synthetic protein, leading to the disruption of microtubule alignment with the direction of maximum tensile stress (Verger et al., 2018). It is possible that the alteration of microtubule alignment we observed in qua2 and tsd2 mutants is due to epidermal discontinuity in these mutants, which disrupts the transduction of tensile stress across the epidermis. However, we cannot rule out other causes of microtubule disruption triggered by pectin and cellulose deficiency in the mutants.

Plants respond to internal cues such as turgor pressure and growth factors, and external environmental cues such as light, temperature, and pathogen infection, through cell wall remodeling under the control of cell wall integrity signaling pathways. Pectin is involved in maintaining cell wall integrity (Bouton et al., 2002; Krupkova et al., 2007; Mouille et al., 2007; Neumetzler et al., 2012; Liu et al., 2014). However, it has not yet been clearly defined how pectin might interface with wall integrity-signaling pathways. To begin to answer the question of whether wall deficiency in the absence of QUA2/TSD2 function perturbs wall integrity signaling, we investigated the expression of cell wall integrity-related genes by qPCR: **FER**, **HERK1**, **FEI1**, **FEI2**, **KIN13A**, and **THE1** had lower expression levels in qua2 and tsd2 mutants (Table 1). **FER** is thought to sense cell wall integrity and mechanical stress on the plasma membrane (Cheung and Wu, 2011; Lindner et al., 2012). The extracellular domain of **FER** has been shown to interact with pectin in vitro, suggesting that pectin might function as a ligand of **FER** (Feng et al., 2018). Although functional interaction between **FER** and pectin in vivo remains to be determined, **FER** might perceive wall integrity signals through its interaction with pectin. Arabidopsis WAK receptors can bind to pectin in cell walls and are required for cell expansion during plant development (Kohorn and Kohorn, 2012). In tsd2, the expression levels of several WAK genes (Table 1) were reduced, although there was no significant change in WAK expression in the qua2 mutant. A previous study showed that mechanical stimuli can induce extensin gene expression (Tiré et al., 1994). In our data, the expression of **EXT3** and **EXT4** was increased in qua2 and tsd2 mutants, implying a potential alteration of extensin-pectin interactions (Cannon et al., 2008). Taken together, many genes related to wall integrity sensing are downregulated in qua2 and tsd2 mutants, indicating that stimuli resulting from changes in wall structure and mechanics might activate signaling involved in cell wall integrity maintenance, resulting in feedback-mediated downregulation of the cognate genes.

It has remained elusive why reduction in pectin content sometimes causes cell adhesion defects. In qua2 and tsd2 mutants, cell adhesion defects might be directly attributable to lower HG levels, whereas another mutant with cell adhesion defects, **frb1**, does not display a significant decrease in pectin content, although mutation of **FRB1** affects pectin methylesterification (Neumetzler et al., 2012). However, a reduced degree of pectin methylesterification has not been reported to cause cell adhesion phenotypes in Arabidopsis double mutants for the putative methyltransferases **CGR2** and **CGR3** (Kim et al., 2015). The suppressor mutation **esmeralda1** rescues adhesion defects in **qua1**, **qua2**, and **frb1** but does not restore pectin content, suggesting that cell adhesion defects might result from altered pectin-related feedback signaling from the state of the pectin in the cell wall (Verger et al., 2016). This implies that pectin modulates cell adhesion via a complicated mechanism potentially involving the physical adhesion between pectin molecules, pectin interactions with other wall components, pectin metabolism, and/or related signaling pathways. In addition to pectins, other wall components such as hemicelluloses might also function in cell adhesion (Ordaz-Ortiz et al., 2009).

Considering different cellular signaling pathways, it is worthwhile to ask whether there is a change in pectin structure and/or cross-linking in qua2 and tsd2 mutants that triggers calcium-mediated signal transduction. Ca$$^{2+}$$ can cross-link demethylated HG molecules into macromolecular networks, increasing wall stiffness (Vincken et al., 2003), which implies that changes in Ca$$^{2+}$$ concentration might disrupt the integrity of pectin networks to further influence wall mechanical properties. Ca$$^{2+}$$ ions can also act as second messengers, functioning in the response of plants to environmental cues (Riveras et al., 2015; Wilkins et al., 2016). In **qua1** mutants, the concentration of Ca$$^{2+}$$ was dramatically increased in the cytosol, indicating the role of QUA1 in regulating cytoplasmic Ca$$^{2+}$$ signaling (Zheng et al., 2017). **FADLOX** expression has been shown to respond to Ca$$^{2+}$$-dependent protein kinases, which are Ca$$^{2+}$$ sensors (Boudsocq et al., 2010). In addition, Ca$$^{2+}$$ is involved in the interactions of pectins with cellulose (Lopez-Sanchez et al., 2017). In qua2 and tsd2 mutants, wall synthesis was inhibited and **FADLOX** expression was induced, suggesting that alteration in wall structure might activate Ca$$^{2+}$$ signaling in response to disruption of wall integrity, although this idea needs to be explored further.
In summary, we propose a schematic for how pectin deficiency in qua2 and tsd2 mutants might affect pectin HG biosynthesis, cellulose biosynthesis, wall integrity, and microtubule stability (Figure 8). In qua2 and tsd2 mutants, the absence of a pectin methyltransferase, QUA2, hampers the biosynthesis of pectic HG in the Golgi and decreases its accumulation in the wall, and the consequent alteration of wall structure influences the activity of plasma membrane-localized CSCs. Loss of wall integrity destabilizes cortical microtubules, further decreasing the aligned deposition of cellulose in the wall. As pectin is reduced in mutants, the orientation of cellulose microfibrils that interact with pectins is changed, concomitant with increased molecular mobility of both cellulose and pectin. The experimental application of pectate lyase, which removes matrix polysaccharides, reveals thinner, more easily fragmented fibrils in the mutants, suggesting that cellulose structure might be fundamentally altered, as supported by our SFG data. Finally, the disruption of wall structure likely triggers changes in wall integrity signaling to respond to the deficiency of pectin and/or cellulose biosynthesis during plant developmental processes.

METHODS

Plant Materials and Growth Conditions

Arabidopsis (Arabidopsis thaliana) ecotype Col as well as qua2 (qua2-1) and tsd2 (tsd2-1) mutants, a gift from Tanya Falbel (University of Wisconsin), were used in this study. Transgenic plants expressing QUA2, hampers the biosynthesis of pectic HG in Arabidopsis (Arabidopsis thaliana) ecotype Col as well as qua2 (qua2-1) and tsd2 (tsd2-1) mutants, a gift from Tanya Falbel (University of Wisconsin), were used in this study. Transgenic plants expressing QUA2, GUS, and GFP-CESA3 (Desprez et al., 2007), GFP-MAP4 (Marc et al., 1998), and QUA2pro-GUS, respectively, were generated using the Agrobacterium tumefaciens tumorifaciens-based floral dipping method (Clough and Bent, 1998) with A. tumefaciens strain GV3101. Positive transformants were screened on MS plates containing 2.2 g/L MS salts (Caisson Laboratories), 0.6 g/L MES (Research Organics), 1% (w/v) Suc, 0.8% (w/v) agar-agar (Research Organics), and 25 mg/mL hygromycin (Omega Scientific), pH 5.6. Seedlings were transplanted from MS plates to soil for growth under a photoperiod of 16 h of white fluorescent light (4100K, 150 to 200 μmol m⁻² s⁻¹) for 12 h in a 22°C growth chamber.

Protein Expression and Purification

The catalytic domain of QUA2 (amino acids 150 to 684) was subcloned into the pMBP-parallelo2 by PCR. The construct containing the QUA2 catalytic domain with an N-terminal 6xHis-MBP tag was transformed and expressed in Escherichia coli strain BL21 (DE3) cells. After induction with 0.2 mM isopropyl β-D-thiogalactopyranoside overnight at 16°C, the cells were pelleted at 4000g for 10 min at 4°C. The pellets were lysed in a buffer containing 25 mM HEPES, pH 7.5, 150 mM NaCl, 0.5 mM Tris (2-carboxyethyl)phosphine-HCl, 250 mM Suc, and 1 mM phenylmethylsulfonyl fluoride by French press. The lysate was then centrifuged at 25,000g for 30 min at 4°C. The supernatants were loaded onto amylose resin using gravity columns at 4°C, which was subsequently washed three times with a buffer containing 25 mM HEPES, pH 7.5, 150 mM NaCl, 0.5 mM Tris (2-carboxyethyl)phosphine-HCl, 250 mM Suc, and 2 mM DTT. The peak fractions were pooled and snap-frozen in liquid nitrogen for storage.

The purified MBP (negative control) and MBP-QUA2 proteins were resolved on 12% SDS-PAGE gels and then stained with Coomassie blue or immunolabeled with anti-polyhistidine. For immunoblot analysis, separated proteins were transferred onto nitrocellulose membranes (GE Healthcare) in a Bio-Rad Mini-Transfer Cell at 100 V for 60 min. After blocking in Tris-buffered saline (TBS) with Tween 20 (25 mM Tris base, 150 mM NaCl, and 0.1% [v/v] Tween 20, pH 7.5) containing 5% (w/v) nonfat milk for 30 min, the membrane was incubated overnight with a monoclonal anti-polyhistidine primary antibody (Sigma-Aldrich, catalog no. H1029, 1:5000). Then the membrane was washed three times in TBS with Tween 20 and incubated with alkaline phosphatase-conjugated goat anti-mouse secondary antibody (Sigma-Aldrich, catalog no. A9316, 1:5000) for 30 min. After thorough washing, the membrane was developed in 5-bromo-4-chloro-3’-indoly1 phosphate p-toluidine salt/nitroblue tetrazolium chloride substrate solution (Amresco).

Enzymatic Activity Assay

The methyltransferase assay was performed by incubating the purified MBP-QUA2 catalytic domain or MBP alone (negative control) in the buffer containing 50 mM HEPES, pH 7.5, 2 mM MgCl₂, 0.25 M Suc, 10 μM SAM (Promega), and 50 μg of PGA (Sigma-Aldrich, catalog no. 81325) at 30°C for 2 h. The PGA substrate was dissolved in 37.5 mM sodium acetate and the pH was adjusted to 7.5. After completion of the reaction, the reaction product, SAH, was monitored using the MTase-Glo Methyltransferase Assay kit (Promega) according to the manufacturer’s directions. Briefly, 20 μL of reaction products was transferred onto a Corning 96-well white plate and mixed with 5 μL of 5× MTase-Glo reagent, which converts SAH into ADP. After the addition of 25 μL of MTase-Glo Detection Solution, the generated ADP was converted into ATP, which was monitored by a luminescent reaction. Luminescence was recorded using a plate-reading luminometer (Synergy H1 microplate reader, BioTek). The change in luminescence represents background-subtracted luminescence. For time- and concentration-dependent enzyme assays, the indicated reaction times, protein, or PGA concentration were applied. Upon reaction completion, 5 μL of 0.5% (v/v) trifluoroacetic acid (Sigma-Aldrich, catalog no. T6608) was mixed with 20 μL of reaction product to stop the reaction. To test the substrate specificity of QUA2, alginate (Sigma-Aldrich, catalog no. A1112) and starch (Sigma-Aldrich, catalog no. S9765), two other polysaccharides, were individually added to reactions.

Immuo Dot Blot Analysis

Immunodot blotting was performed as previously described with minor modifications (Pérez-Pérez et al., 2019). Aliquots of 3 μL of reaction products were spotted onto nitrocellulose membranes (GE Healthcare) and allowed to air-dry for 1 h. The membrane was blocked with TBS containing 1.5% (w/v) nonfat milk. Subsequently, the membrane was incubated with a primary antibody (Agrisera [JIM5, catalog no. AS184194; JIM7, catalog no. AS184195], 1:100) in blocking solution for 2 h at room temperature. After washing three times in TBS, the membrane was incubated with peroxidase-conjugated goat anti-rat secondary antibody (SeraCare, catalog no. 5220-0364, 1:1000) for 1 h. After washing three times in TBS, the blot was developed using Western ECL Substrate Solution (Bio-Rad, catalog no. 170-5060). The experiment was replicated three times.

Measurements of Hypocotyl Length and Width, Rosette Diameter, and Plant Height

Seeds were surface-sterilized in 30% (v/v) bleach + 0.1% (w/v) SDS, washed four times with sterile water, stored at 4°C for 3 d, and sown on MS plates without Suc. After exposure to light for 2 to 4 h at stimulate seed germination, seedlings were grown in the dark for 2 to 6 d at 22°C. Plates with etiolated seedlings were scanned using an HP Scanjet 8300 scanner at 600 dots per inch, and hypocotyl length was measured using

Pectin and Cellulose in Wall Integrity
ImageJ. Hypocotyl width was recorded on a Zeiss Observer SD spinning-disk confocal microscope with a 20× objective and quantified in ImageJ. Adult plants were imaged using a D5100 DSLR digital camera (Nikon). Rosette diameters, petiole length, and leaf area were measured in ImageJ, and plant height was measured using a ruler.

**Preparation of AIR**

Seedlings growing in the dark for 6 d were harvested and ground into a fine powder in liquid nitrogen with a mortar and pestle. The powder was suspended in 30 mL of 1:1 chloroform:methanol (v/v) with shaking for 1 h at room temperature. After precipitation by centrifugation at 4500g for 10 min at room temperature, the residue was washed four times in 70% (v/v) ethanol by resuspension and centrifugation at 4500g for 10 min at room temperature. The final AIR was suspended and centrifuged at 4500g for 10 min at room temperature in 100% acetone and air-dried in a chemical fume hood at room temperature (Fry, 1988).

**Measurements of Uronic Acids and Methanol Release**

For measurements of total uronic acids (Filisetti-Gozzi and Carpita, 1991), AIR was extracted from 6-d-old dark-grown seedlings. Samples with 1 mg of AIR were suspended in 141 μL of water in a glass tube. Fourteen microliters of 4 M sulfamic acid (Thermo Fisher Scientific)-potassium sulfamate (pH 1.6) was added and mixed thoroughly. Then 845 μL of concentrated H2SO4 containing 75 mM sodium tetraborate (Sigma-Aldrich) was added, and the solution was mixed vigorously by vortexing for 10 s. The solution was heated in a boiling water bath at 100°C for 5 min. After cooling down samples in a room temperature water bath, an aliquot of each sample was diluted 50-fold by adding 20 μL of sample to 980 μL of 85% (v/v) H2SO4. After thorough mixing, initial A258 was recorded in a plastic cuvette using a NanoDrop 2000C spectrophotometer. An aliquot of 28 μL of 0.15% (w/v) m-hydroxydiphenyl (Sigma-Aldrich) in 0.5% (w/v) NaOH was added to the diluted sample and mixed by inversion, and the mix was allowed to incubate at room temperature for 5 min before A258 was measured. GalA (Sigma-Aldrich) was used as a standard to calculate concentrations.

To measure released methylesters (Müller et al., 2013), ~1 mg of AIR was weighed out for each experiment. After washing in 1 mL of water and centrifugation at 21,130g for 10 min at room temperature, 400 μL of 0.5 M NaOH was added to samples to incubate at room temperature for 1 h to release methylesters, and then 200 μL of 1 M HCl was added and mixed by vortexing for neutralization. Samples were centrifuged at 2400g for 10 min at room temperature, and 125 μL of supernatant was added to 125 μL of 20 mM HEPES buffer (pH 7.5). Samples were oxidized in 250 μL of HEPES buffer containing 0.03 units of alcohol oxidase (Sigma-Aldrich) with 20 mM HEPES buffer (pH 7.5). After thorough mixing, initial A340 was recorded in a plastic cuvette using a NanoDrop 2000C spectrophotometer. An aliquot of 28 μL of 0.15% (w/v) m-hydroxydiphenyl (Sigma-Aldrich) in 0.5% (w/v) NaOH was added to the diluted sample and mixed by inversion, and the mix was allowed to incubate at room temperature for 10 min before A340 was measured. GalA (Sigma-Aldrich) was used as a standard to calculate concentrations.

**Cellulose Content Measurements**

Tissue from 6-d-old dark-grown seedlings and 4-week-old rosette leaves from Arabidopsis Col, qua2, and tsd2 genotypes were used to measure crystalline cellulose content following a procedure described by Xiao et al. (2016) based on the Updegraff method (Updegraff, 1969).

**SFG Imaging**

Etiolated 6-d-old seedlings of Arabidopsis Col, qua2, and tsd2 genotypes were harvested and immersed in deuterium oxide overnight in the dark for water exchange before SFG analysis. Ten to 15 seedlings were grouped and aligned closely into a bundle on a glass cover slip. After adding deuterium oxide, another glass cover slip was added on the top to seal the sample and keep the seedlings wet. Three regions from the top, middle, and base of each set of hypocotyls were analyzed, and two SFG spectra at each location were collected in longitudinal and transverse directions relative to the hypocotyl growth axis. Due to spatial variability across the samples, SFG spectra from nine locations of each hypocotyl region were collected and averaged for each region. The hydroxyl/alkyl (OH/CH) area ratio was calculated by dividing the OH peak area (3150 to 3700 cm⁻¹) with the CH peak area (2700 to 3050 cm⁻¹) for each SFG spectrum. In the SFG system, two high-intensity femtosecond laser pulses with wavelengths at 800 nm and tunable IR region (2.5 to 10 μm) were synchronized and overlapped on the samples. The 85-fs, 800-nm output pulses were generated at 2 kHz from a chirped pulse amplification system (Coherent, Libra) with a set of Ti:sapphire oscillator/amplifiers using an 80-kHz laser running at 20 fs and pumped by a frequency-doubled Nd:YLF diode-pumped laser producing 250-nm, 527-nm pulses at 2 kHz. By splitting the 800-nm output, 75% of the output was used to generate a tunable infrared (IR) line through an OPG/OPA system (Coherent, Opera Solo) equipped with a nonlinear difference frequency generation (NDFG) stage, and the remaining 25% of the pulses passed through a pair of angle-tuned Fabry-Pérot etalons (TeoOptics) for spectral narrowing. Both the p-polarized IR and the s-polarized visible laser were overlapped at the sample surface at an incidence angle of 45° with respect to the surface plane. The s-polarized SFG signal was selected and detected in the transmission geometry. The SFG signal was filtered with a 775-nm short-pass filter, dispersed with a volume-phase holographic grating (Andor, Holospec), and then detected with a back-illuminated deep-depletion charge-coupled device DU420A-BEX2-DD camera (Andor). The probe size of the SFG system was estimated to be ~120 μm along the laser incidence plane direction and ~80 μm perpendicular to the incidence plane. The probe depth was measured to be ~20 to 25 μm from the top external surface of the sample. SFG spectra were obtained by stitching
multiple NDFG spectra together because each NDFG setting only provides an IR profile with 150 to 200 cm\(^{-1}\) bandwidth. SFG spectra for cellulose need to be collected from 2700 to 3700 cm\(^{-1}\). After stitching, each SFG spectrum was normalized by the incident IR profile with the same frequency region (Lee et al., 2016).

**AFM**

AFM sample preparation and imaging followed Xiao et al. (2016) with minor modifications. Seedlings were grown in the dark at 22°C for 3 d before harvesting hypocotyls. Tissues were ground in liquid nitrogen and rinsed in 20 mM HEPES (pH 7.0) with 0.1% (v/v) Tween 20 at least three times with spinning in between rinses until the supernatant was clear. A volume of fresh buffer equaling 150 µL was added to the pellet and vortexed. The tubes were spun down, supernatant was removed, and 100 µL of fresh buffer was added. Tubes were put on a rocker for 30 min to wash and disperse the pellet thoroughly. A final spin was done to remove the supernatant, and 50 µL of 20 mM HEPES, pH 7.5, buffer with no detergent was added to the pellet and mixed. Drops of 5 µL of suspension were placed on glass slides and allowed to dry almost completely to aid in sticking the cuticle sides of cells to the glass. Slides were gently rinsed with 20 mM HEPES buffer to remove unbound material.

AFM imaging was performed on a Dimension Icon system with a NanoScope V controller (Bruker) using the PeakForce QNM program under fluid conditions. A ScanAsyst Fluid+ tip was calibrated using software to determine deflection sensitivity and spring constant. The AFM system is contained inside a vibration isolation hood (Technical Manufacturing) to reduce noise interference and allow for high-resolution images of biological materials. PeakForce QNM scanning parameters were controlled by the ScanAsyst software for most settings to achieve the best resolution for both topography and peak force error images, except for the scan rate, which was set to 0.8 Hz, and the peak force, which was set to 800 pN. Images were imaged at a size of 2 x 2 µm with 512 x 512 pixels. Images were processed in Nanoscope Analysis 1.8v software (Bruker) and exported as Tiffs.

**FESEM**

FESEM experiments were performed in apical hypocotyls from 3-d-old dark-grown seedlings without or with 10 µg/mL pectate lyase treatment (Megazyme E-PLYCJ, lot 130901a) following the protocol described by Xiao et al. (2016), except that the critical point dryer used in this experiment was a manually operated BAL-TEC CPD030.

**13C Labeling and Solid-State NMR Analysis**

Never-dried primary cell walls were prepared as described (Wang et al., 2015). Arabidopsis seedlings were grown in the dark for 14 d using a liquid perfusion system contained inside a vibration isolation hood (Technical Manufacturing). After dissection, the pellets were added to the pellet and mixed. Drops of 5 µL of suspension were placed on glass slides and allowed to dry almost completely to aid in sticking the cuticle sides of cells to the glass. Slides were gently rinsed with 20 mM HEPES buffer to remove unbound material.

AFM imaging was performed on a Dimension Icon system with a NanoScope V controller (Bruker) using the PeakForce QNM program under fluid conditions. A ScanAsyst Fluid+ tip was calibrated using software to determine deflection sensitivity and spring constant. The AFM system is contained inside a vibration isolation hood (Technical Manufacturing) to reduce noise interference and allow for high-resolution images of biological materials. PeakForce QNM scanning parameters were controlled by the ScanAsyst software for most settings to achieve the best resolution for both topography and peak force error images, except for the scan rate, which was set to 0.8 Hz, and the peak force, which was set to 800 pN. Images were imaged at a size of 2 x 2 µm with 512 x 512 pixels. Images were processed in Nanoscope Analysis 1.8v software (Bruker) and exported as Tiffs.

**Drug Treatments**

To analyze hypocotyl elongation after growth on oryzalin (Chem Service, N-12729), oryzalin was added onto MS plates at different concentrations, seeds were sown on the plates, and hypocotyl length and width from 6-d-old dark-grown seedlings were measured and imaged in ImageJ. Drug treatments for live-cell imaging were identical to those described previously (Xiao et al., 2016). Three-day-old dark-grown seedlings were submerged in 1 mL of liquid half-strength MS medium containing 10 µM oryzalin dissolved in DMSO (Sigma-Aldrich) and incubated in darkness for 10 to 240 min before imaging with spinning-disk confocal microscopy.

**Gene Expression Detection by qPCR**

Tissue from 3-d-old dark-grown Col, qua2, and tsd2 seedlings was used for total RNA extraction with a Plant RNA Kit (Omega Bio-Tek). Genomic DNA in samples was removed with RNase-free DNase I (NEB). RNA concentration was measured by spectrophotometer (NanoDrop 2000C), and first-strand cDNA was synthesized using qScript cDNA SuperMix (Quanta Biosciences). Gene expression analysis was performed using SYBR Green FastMix (Quanta Biosciences) with cDNA and gene-specific primers on a StepOne Plus Real-Time PCR machine (Applied Biosystems). The transcript levels of target genes relative to EF1 as a reference gene were calculated using the ΔΔCT method. Primer sequences are listed in the Supplemental Table.

**GUS Activity Analysis**

In addition to measurement of mRNA expression in different tissues by qPCR, the spatial expression pattern of QUAI2 was examined by histochemical analysis. Twenty independent transgenic lines expressing QUAI2pro:GUS were obtained (primer sequences are listed in the Supplemental Table), and T2 plants were used for GUS staining analysis. Tissues were immersed in buffer containing 50 mM sodium phosphate, pH 7.2, 0.2% (v/v) Triton X-100, and 2 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid and cyclohexylammonium salt and incubated at 37°C in the dark for 4 to 16 h, which was dependent on the tissue age. Young dark-grown and light-grown seedlings were stained for 4 h, and flowers, siliques, and stems of 5-week-old QUAI2pro:GUS adult plants were stained for 16 h. Pigment was removed from tissue with 70% (v/v) ethanol, and images of GUS staining were recorded using a Zeiss Discovery V12 fluorescence dissecting microscope.
Statistical Analyses

All experiments were repeated at least twice. Data are represented as averages ± so or se, as specified. Analyses were performed using Student’s t test (two-tailed) or one-way ANOVA with Tukey’s test to evaluate the significant difference, and the P value thresholds are shown as P < 0.05 or P < 0.001. Statistical results are available in Supplemental File 1.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: CES1 (At1g32410), CES3 (At5g05170), CES6 (At1g64740), CLASP (At2g20190), COBRA (At5g60920), CSI1 (At2g22125), EF1-a (At1g50390), EXT3 (At1g2310), EXT4 (At1g78930), FEI1 (At1g31420), FEI2 (At2g35620), FER (At3g51550), FADLOX (At1g26380), FEI1 (At1g47820), GAE1 (At4g30440), GAUT1 (At3g61130), GAUT7 (At3g59010), QUAI2/TS2 (At1g78240), THE1 (At1g54380), WAK2 (At1g21250), WAK2 (At1g21270), WAK3 (At1g21240), WAK4 (At1g21210), and WAK5 (At1g21230).

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: CES1 (At1g32410), CES3 (At5g05170), CES6 (At1g64740), CLASP (At2g20190), COBRA (At5g60920), CSI1 (At2g22125), EF1-a (At1g50390), EXT3 (At1g2310), EXT4 (At1g78930), FEI1 (At1g31420), FEI2 (At2g35620), FER (At3g51550), FADLOX (At1g26380), FEI1 (At1g47820), GAE1 (At4g30440), GAUT1 (At3g61130), GAUT7 (At3g59010), QUAI2/TS2 (At1g78240), THE1 (At1g54380), WAK2 (At1g21250), WAK2 (At1g21270), WAK3 (At1g21240), WAK4 (At1g21210), and WAK5 (At1g21230).

Supplemental Data

Supplemental Figure 1. Related to Figure 2. Schematic gene structure of QUASIMODO2.

Supplemental Figure 2. Related to Figure 2. qua2 and tsd2 plants are smaller and shorter than wild type.

Supplemental Figure 3. Related to Figure 2. QUAI2_pro:GUS expression patterns in different plant tissues.

Supplemental Figure 4. Related to Figure 7. Hypocotyls and microtubules of qua2 and tsd2 mutants are more sensitive to oryzalin treatment.

Supplemental Figure 5. Related to Figure 7. Tissue integrity in hypocotyls of qua2 and tsd2 mutants is sensitive to oryzalin treatment.

Supplemental Figure 6. Related to Figure 7. Cortical microtubules in qua2 and tsd2 mutants are more sensitive to 10 mM oryzalin treatment for 30 min to 240 min.

Supplemental Figure 7. Related to Figure 7. Cortical microtubules of qua2 and tsd2 are more sensitive to mechanical stress.

Supplemental Table. Primers used in this study.

Supplemental File. Detailed results of statistical analyses.

Supplemental Movie. GFP-CESA3 particle motility in 3-d-old dark grown Col, qua2, and tsd2 seedlings.

ACKNOWLEDGMENTS

We thank Liza Wilson and Sydney Duncombe for technical assistance with AFM experiments, Tanya Falbel for providing seeds of qua2 and tsd2 mutants, Richard Cyr for providing the GFP-MAP4 expression construct, and Nicole R. Brown for helpful comments on the article. With the exceptions below, this work was supported as part of The Center for Lignocellulose Structure and Formation, an Energy Frontier Research Center funded by the U.S. Department of Energy, Office of Science, Basic Energy Sciences (grant DE-SC0001090 to S.K., D.J.C., and C.T.A.).

Analysis of QUA2 protein activity was supported by Fundamental Research Funds for the Central Universities (grants YJ20173 and SCU2019D013 to C.X.), and the China Postdoctoral Science Foundation (grant 2018M643465 to J.D.).

AUTHOR CONTRIBUTIONS

J.D., C.X., S.O., S.H.K., T.W., D.J.C., and C.T.A. designed the research; J.D., C.X., A.K., L.W., S.H., W.J.B., S.N.K., Y.Z., Y.R., and M.R. performed research; J.D., C.X., S.H.K., T.W., D.J.C., and C.T.A. analyzed data; J.D., C.X., T.W., and C.T.A. wrote the article.

Received March 30, 2020; revised July 27, 2020; accepted August 31, 2020; published September 3, 2020.

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