Ultrastructural and molecular basis of cell-cell adhesion in plants

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Cover: Epidermal junctions in 4-day-old dark-grown *Arabidopsis thaliana qua1-1,* visualized by cryo-SEM (photo: Ö. Erguvan)

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Abstract

Cell-cell adhesion is a fundamental requirement for multicellularity in plants, ensuring that cells remain connected despite the mechanical stress of turgor pressure and growth. Although adhesion has traditionally been attributed to the pectin-rich middle lamella (ML), growing evidence suggests that this view is overly simplistic. In this thesis, I investigated structural, molecular and developmental aspects of adhesion in Arabidopsis thaliana. In Paper I, finite element modelling and advanced microscopy demonstrated that outer epidermal edges are hotspots for cell-separating tensile stress, where we uncovered supracellular reinforcement points; the supracellular outer epidermal wall (SOEW) and the outer epidermal edge filling (OEEF) that function as load-bearing ultrastructural features that prevent separation, In Paper II, I showed that sucrose metabolism is one of the regulators of cell-cell adhesion maintenance. Exogenous sucrose intensified adhesion defects in multiple mutans and surprisingly induced “micro-cell adhesion defects” in wild type. These defects corelated with changes in tissue mechanics, radial expansion and cell shape anisotropy, in part likely linked to microtubules dynamics, identifying sugar metabolism as a regulator of cell adhesion. In Paper III, we investigated how cell wall integrity, particularly pectin biosynthesis, influences apical hook development. Mutants defective in homogalacturonan (qua2, gae1gae6, mur1), which display adhesion defects, failed to form a proper apical hook. In qua2, these defects correlated with loss of auxin asymmetry, reduced expression of HLS1 and PIF4 and altered GA metabolism, opening new avenues to explore how adhesion and wall integrity connect to hormonal signalling. Together, these studies open the door to new questions about how adhesion reinforcement points are established and maintained through regulatory and feedback mechanisms to ensure supracellular integrity in multicellular organisms.

Keywords: Adhesion, cell wall, ultrastructure, middle lamella, edges, cellulose, sucrose, cell wall integrity, apical hook

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Ultrastrukturell och molekylär grund för cell-celladhesion i växter

Abstract

Cell-cell-adhesion är ett grundläggande krav för flercellighet hos växter och säkerställer att celler förblir sammanbundna trots den mekaniska belastningen från turgortryck och tillväxt. Även om adhesion traditionellt har tillskrivits den pektinrika medellamellen (ML), tyder växande evidens på att denna syn är alltför förenklad. I denna avhandling har jag undersökt strukturella, molekylära och utvecklingsmässiga aspekter av adhesion i *Arabidopsis thaliana*. I Artikel I visade finita element-modellering och avancerad mikroskopi att de yttre epidermala kanterna är hotspots för cellseparerande dragspänning, där vi identifierade supracellulära förstärkningspunkter; den supracellulära yttre epidermalväggen (SOEW) och den yttre epidermala kantfyllnaden (OEEF), vilka fungerar som bärande ultrastrukturella element som motverkar separation. I Artikel II visade jag att sackarosmetabolism är en regulator av cell-cell-adhesion. Exogen sackaros förstärkte adhesionsdefekter i flera mutanter och inducerade även ”mikro-adhesionsdefekter” i vildtyp, vilka korrelerade med förändringar i vävnadsmekanik, radiell expansion och cellformanisotropi, delvis kopplat till mikrotubulidynamik. I Artikel III undersökte vi hur cellväggsintegritet, särskilt pektinbiosyntes, påverkar utvecklingen av den apikala kroken. Mutanter defekta i homogalakturonan (qua2, gae1gae6, mur1), som uppvisar adhesionsdefekter, misslyckades med att bilda en korrekt apikal krok, och i qua2 korrelerade dessa defekter med förlust av auxinasymmetri, minskat uttryck av HLS1 och PIF4 samt förändrad GA-metabolism, vilket öppnar nya möjligheter att utforska hur adhesion och cellväggsintegritet är kopplade till hormonell signalering. Tillsammans väcker dessa studier nya frågor om hur adhesionspunkter etableras och upprätthålls genom reglerande och återkopplande mekanismer för att säkerställa supracellulär integritet i flercelliga organismer.

Nycklord: Adhesion, cell wall, ultrastructure, middle lamella, edges, cellulose, sucrose, cell wall integrity, apical hook

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Dedication

To all the people who have been, are, and will be part of my life.

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. **Özer Erguvan**, Adrien Heymans, Asal Atakhani, Elsa Gascon, Richard S. Smith, Olivier Ali, Stéphane Verger* (2025). Outer epidermal edges mediate cell-cell adhesion for tissue integrity in plants. (Submitted)

II. **Özer Erguvan,** Adrien Heymans, Asal Atakhani, Camille Martin Desbouis, Lucija Lisica, Stéphane Verger* (2025). Sugar levels impact cell-cell adhesion in plants (Manuscript)

III. Riccardo Lorrai, **Özer Erguvan**, Sarra Raggi, Kristoffer Jonsson, Jitka Široka, Danuše Tarkowska, Ondřej Novák, Jayne Griffiths, Alexander M. Jones, Stéphane Verger, Stéphanie Robert, Simone Ferrari* (2024). Cell wall integrity modulates HOOKLESS1 and PHYTOCHROME INTERACTING FACTOR4 expression controlling apical hook formation. Plant Physiology, Volume 196, Issue 2, October 2024, Pages 1562–1578, https://doi.org/10.1093/plphys/kiae370

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The contribution of Özer Erguvan to the papers included in this thesis was as follows:

I. Performed most of the experiments and data analysis, contributed to the study design, develop a microscopy technique (Correlative Confocal and Atomic Force Microscopy), wrote the first draft of the manuscript, designed all figures, worked on subsequent versions of the manuscripts together with the supervisor, and finalized the formatting for publication.

II. Made the primary observation that wild-type plants develop micro-adhesion defects in the presence of sucrose, which led to the initiation of this project. Performed most of the experiments and data analysis, contributed to the study design, developed a microscopy technique (Correlative Confocal and cryo-Scanning Electron Microscopy), wrote the first draft of the manuscript, designed all figures, worked on the subsequent versions of the manuscript together with the supervisor.

III. Performed some experiments and data analysis, contributed to experimental planning and contributed to the first version of the manuscript.

Additional publications contributed by the author during the PhD studies that are not part of this thesis:

I. Abu Imran Baba, Lucija Lisica, Asal Atakhani, Bibek Aryal, Léa Bogdziewiez, **Özer Erguvan**, Adrien Heymans, Pawan Kumar Jewaria, Richard S. Smith, Rishikesh P. Bhalerao, Stéphane Verger* (2024). Rhamnogalacturonan-II dimerization deficiency impairs the coordination between growth and adhesion in plants. BioRxiv doi**:** https://doi.org/10.1101/2024.11.26.625362

II. Léa Bogdziewiez, Lucija Lisica, Abu Imran Baba, **Özer Erguvan**, Adrien Heymans, Asal Atakhani, Stéphane Verger* RRQuant: Ruthenium Red quantification of cell-cell adhesion defects in hypocotyls. (Under preparation)

III. Sara Raggi, Hemamshu Ratnakaram, Lorinda Devi Loitongbam, Adrien Heymans, **Özer Erguvan**, Asal Atakhani, Chenlu Liu, Marco Marconi, Sijia Liu, François Jobert, Siamsa M. Doyle, Elke Barbez, Krzysztof Wabnik, Stéphane Verger and Stéphanie Robert* The cuticle layer regulates tissue biomechanics via reactive oxygen species to facilitate anisotropic cell expansion. (Under preparation)

1. Introduction

Plants are fundamentally important to life on Earth. As they are capable of capturing solar energy and converting it into convert into chemical energy through photosynthesis, they sustain most terrestrial ecosystems (Field et al. 1998; Foley et al. 2011). In addition to this, they provide food, fiber and macromolecules for medicine that are economically and fundamentally important for humans. Beyond this, plants regulate atmospheric composition by fixing CO2 and releasing O2 thereby playing a central role in climate stability (Beerling & Berner 2005).

As one of the most striking features that distinguishes plants from other kingdoms is their sessile lifestyle, plants cannot move to avoid stress or find suitable resources; instead, they must adapt themselves in order to survive (Pires & Dolan 2012). Equally distinctive feature is the presence of a rigid but very dynamic cell wall, which not only provides structural support but also shapes how plants interact with their environment (Cosgrove 2005). While fungi also have cell walls and the ability to adapt themselves to the environment, the composition of these walls is significantly different; plant cell walls are rich in cellulose, hemicellulose, pectins, structural proteins and lignin (Somerville et al. 2004), whereas fungal walls are mostly made of chitin and glucan (Bowman & Free 2006).

The presence of a rigid but dynamic cell wall provides mechanical support, mediates adhesion between neighbouring cells allows growth and expansion and acts as a dynamic interface with the environment. The evolution of this extracellular matrix was crucial for land colonisation and the emergence of vascular plants. (Graham et al. 2000; Niklas 2000).

1.1 The plant cell wall and its structure

The plant cell wall is a complex and multifunctional extracellular matrix that establishes cell morphology, provides structural integrity and coordinates interaction not only within tissue but also with the external environment (Somerville et al. 2004; Cosgrove 2005). Unlike a passive barrier, it continuously undergoes remodelling to support growth, differentiation and stress response (Lampugnani et al. 2018).

In plants, most studies have focused on dicots, in which two main types are distinguished: the primary cell wall which is flexible and enriched in cellulose, hemicellulose, pectins and structural proteins (Carpita & Gibeaut 1993), and the secondary cell wall, which is relatively rigid and enriched in cellulose, xylans and lignins (Zhong & Ye 2015).

Even though the composition can be varied between species, tissue and developmental stages, the primary cell wall is made of three classes of polysaccharide: cellulose, hemicellulose and pectins together with proteins (Pauly & Keegstra 2010)(cite). Among these, pectins are especially important for cell-cell adhesion and will be discussed further in section 1.1.3.

1.1.1 Cellulose

Cellulose is the main biopolymer of the primary cell wall and is composed of linear β-(1→4)-glucan chains that assemble into crystalline microfibrils. These microfibrils form the structural framework of the cell wall and resist the internal turgor pressure by their load bearing capacity (Somerville 2006). In addition to this, cellulose microfibrils are the main determinant of wall anisotropy and play a central role in defining wall architecture (Baskin 2005; Cosgrove 2005).

In plants, cellulose is synthesized at the plasma membrane by cellulose synthases complexes (CSCs) typically arranged in rosette-shaped structures (Mueller & Brown 1980; McFarlane et al. 2014). Cortical microtubes (MTs) guide the movement of these complexes, thereby influencing directional cell expansion and plant growth (Paredez et al. 2006).

One of the most important features of cellulose organisation in the wall could be the outer epidermal wall (OEW), the outermost layer of primary cell walls in aerial organs (Kutschera 2008). This specialized structure is often considered as a tensile "skin", maintaining organ integrity while allowing irreversible expansion during growth (Zhang et al. 2016). Unlike inner walls, the OEW has a highly specialized architecture: cellulose microfibrils are organized into multiple lamellae with distinct orientations, which provides mechanical reinforcement and enable efficient mechanical stress distribution (Zhang et al. 2016). Such organization enables the epidermis to resist internal turgor pressure while allowing the organ growth.

1.1.2 Hemicellulose

Hemicelluloses are representing a diverse family of matrix polysaccharides that include xylan, mannans and xyloglucans. They are typically composed of β-(1→4)-linked sugars such as xylose, mannose and glucose, often substituted with side chains containing arabinose, galactose or other sugars. Their side chains and substitutions can be varied among plant species, organs and developmental stages, contributing to the structural and mechanical properties of the cell wall (Scheller & Ulvskov 2010). Hemicelluloses are synthesized by glycosyltransferases in the Golgi apparatus and then secreted in to the apoplast where they become integrated into the wall matrix (Reyes & Orellana 2008). Their interaction with cellulose adjusts wall mechanics and the extensibility of the wall (Cosgrove 2014).

1.1.3 Pectins

Pectins are structurally complex cell wall polysaccharides enriched in galacturonic acid, and they can account for up to one-third of the primary cell wall in dicot plants. Their synthesis takes places in the Golgi apparatus and then they are secreted to the cell wall (Micheli 2001; Pelloux et al. 2007). Based on structure and function, pectins can be classified three subgroups: homogalacturonan (HG); a linear α-1,4 linked D-galacturonic acid (GalA)

that can be methylesterified at the C6 position; rhamnogalacturonan I (RG-I) which has a repeating α-1,4 GalA-α-1,2-L-rhamnose backbone substituted with arabinan, galactan and arabinogalactan and rhamnogalacturonan II (RG-II) composed of at least eight α-1,4-GalA residues decorated with different sugars (Pelloux et al. 2007; Mohnen 2008).

HGs are secreted to the cell wall in a highly methylated form, but the degree and pattern of methyl-esterification of the HGs can then be modified by pectin methylesterases (PMEs) and their inhibitors (PMEIs), while acetylation is controlled by pectin acetylesterases (PAEs) (Atmodjo et al. 2013). De-methylesterified HGs can be further degraded by polygalacturonases (PGs) or lyases (PLs) leading to a softening of the wall and weakening of the middle lamella that can directly affect cell-cell adhesion strength (Leslie et al. 2007; Ballester & Ferrándiz 2017). On the other hand, the degree and pattern of HG methylesterification can determine the polymer's ability to form calcium mediated "egg-box" structures in which de-esterified GalA residues are crosslinking via Ca^{+2} bridges (Caffall & Mohnen 2009). This crosslinking is believed to be particularly important for mediating adhesion at the ML, where HGs are widely distributed (Wolf et al. 2009).

RG-I is another type of pectin characterised by a backbone of repeating disaccharide units: GalA and rhamnose. The rhamnose units works as "branching points" where different sugar side chains can attach (Ridley et al. 2001; Willats et al. 2001a). This branching makes RG-I very variable between plant species, tissues and developmental stages (Mohnen 2008).

RG-II is the most structurally complex form of pectin. It consists of an HG backbone with four distinct side chains (O'Neill et al. 2004). Despite its complexity, the structure of RG-II is remarkably conserved between plant lineages suggesting a critical role in wall function (Matsunaga et al. 2004). In addition to this, its ability to crosslink with borate makes it very important pectin class. Even though the amount of RG-II is significantly less than HG and RG-I it plays an important role in plant growth and cell wall mechanical properties (Kobayashi et al. 1996). For example, mutants impaired in RG-II exhibits severe growth defects, reduced fertility (Kobayashi et al. 1996; Baba et al. 2024).

1.2 Cell-Cell adhesion in plants

1.2.1 Adhesion as a key feature of multicellularity

Cell-cell adhesion is one of the most important features of multicellularity, as it enables cells to remain physically connected, which in turn allows functioning as a multicellular organism (Kirk 2005; Abedin & King 2010). In animals, cell-cell adhesion is mediated by plasma membrane proteins such as cadherins (Takeichi 1991; Harris & Tepass 2010). In contrast, in plants cell-cell adhesion is considered to be achieved primarily through the middle lamella (ML), a pectin-rich layer- particularly enriched in HG- that lies between adjacent cells (Daher & Braybrook 2015; Atakhani et al. 2022; Baba & Verger 2024). Proper cell-cell adhesion is particularly important in plants because of their high internal turgor pressure that constantly pushes cells apart. To withstand this, adhesion must be tightly controlled to maintain tissue and organ integrity and ultimately multicellularity (Knox 1992; Jarvis et al. 2003).

1.2.2 The classical view: the middle lamella as an adhesive layer

In plants, the widely accepted view is that pectins within the ML, - particularly HG- acts as a "glue" between adjacent cell to ensure cell-cell adhesion (Knox 1992; Jarvis et al. 2003). This accepted view mainly comes from the biochemical properties of HGs, which are the principal gel-forming polysaccharides in the cell wall due to their hydrophilic nature and the ability to form crosslinks (Ridley et al. 2001). Support for the adhesive role of pectins in plant cell adhesion was further reinforced by studies of Arabidopsis mutants such as *quasimodo1* (*qua1*) and *quasimodo2* (*qua2*). These mutants have reduced levels of HG in their cell walls and display severe adhesion defects (Bouton et al. 2002; Mouille et al. 2007). Such findings strongly supported the idea that HG in the ML is essential for maintaining cell-cell adhesion.

However, the idea that HG deficiency directly causes cell-cell adhesion defects has been challenged by other studies. For example, the Arabidopsis mutant *friable1* (*frb1*) does not show reduction in HG content but displays cell-cell adhesion defects (Neumetzler et al. 2012). Other lines, such as *pme3*, *irx8* mutants and *PGX1* overexpression line, show alterations in HG biosynthesis but do not exhibit cell-cell adhesion defects (Persson et al. 2007;

Guénin et al. 2011; Xiao et al. 2014). On top of this, it has been shown that the cell adhesion defects of *qua2* can be supressed by mutations in the gene *ESMRALDA1*, a putative O-fucosyltransferase, even without restoring of HG content. Characterization of this suppressor line suggested that cell-cell adhesion is not directly determined by pectin content but rather by pectin-related signalling, and led to the idea that cell-cell adhesion in plant is regulated by a feedback mechanism that sense the state of pectin (Verger et al. 2016). All these findings indicate that cell-cell adhesion cannot be explained solely by HG abundance in the ML, suggesting that other wall components or structural mechanism are also required.

1.2.3 More than pectins: Other players in cell-cell adhesion

In addition to the ML, other cell wall structures have been proposed as potential contributors to cell-cell adhesion in plants. One such candidate are the plasmodesmata, the intercellular channels that connect adjacent cells. These nanoscale pores are not just a passive bridge for molecular trafficking but are embedded within specialized wall microdomains that are reinforced compared to the surrounding wall (Sager & Lee 2014; Faulkner 2018). This could locally strengthen the wall and may generate focal adhesion point at the cell-cell interface (Orfila & Knox 2000). Another candidate structure is the cuticle, a lipid-rich outer layer of the epidermis. In addition to its classical role as protective hydrophobic barrier, the cuticle has been proposed to provide mechanical continuity across neighbouring epidermal cells which in turn might reinforce surface cohesion and contribute to cell-cell adhesion (Galletti et al. 2016; Ingram & Nawrath 2017).

It has also been proposed that adhesion reinforcement points exist at the edges of inner tissues tricellular junction where intercellular air spaces form. These points are thought help cells stay attached to each other while also controlling the airspace formation (Kollöffel & Linssen 1984; Parker et al. 2001; Ordaz-Ortiz et al. 2009). Microscopy studies have shown that cell walls at the tricellular junctions often have a district organization compared to adjacent wall regions, supporting the idea that these points play a role in maintaining cell-cell adhesion (Roland 1978; Willats et al. 2001a). However, none of the suggested structures mentioned above have been directly proven to contribute to maintaining cell-cell adhesion. Their proposed role is based

on correlations between their presence and observed adhesion states rather than direct mechanical testing.

1.2.4 Control of cell-cell adhesion in plants

For a long time, cell-cell adhesion in plants was seen as a passive process that happened automatically after cell division, when new walls and the ML are formed (Jarvis et al. 2003; Daher & Braybrook 2015). However, recent studies have revealed that adhesion is not just a by-product of wall formation, -it is an active process and plants carefully controls it (Verger et al. 2016).

As plants cells are under high internal turgor pressure that constantly pushes them apart, without properly controlled adhesion, the cells would separate, and tissues would lose their integrity. The fact that cell separation happens in some specific cases, such as organ abscission, lateral root emerges, or formation of air spaces shows that plants can actively regulate when and where adhesion can be loosed (Roberts et al. 2002). Similarly, because cell growth involves constant wall remodelling that could weaken adhesion and pull cells apart, there must be active mechanism that allow cells to expand while still maintaining proper adhesion. However, the nature of these mechanisms and how they operate in growing tissues remains poorly understood.

As discussed in Section 1.2.2, studies on HG-related mutants, especially suppression of the *qua2* cell adhesion phenotype by *esmd1*, strongly suggest that cell-cell adhesion maintenance during cell growth is not a passive consequence of wall chemistry. Instead, these findings point to adhesion as a genetically regulated process, where modification of HG and its interactions with other wall components a play central role. In other words, these findings propose a feedback mechanism in which cells can sense the state of pectins probably through cell wall integrity sensors and downstream signalling, thereby regulating adhesion to ensure tissue integrity.

In addition to pectins, cytoskeletal regulation has been implicated in cell-cell adhesion. For example, mutants for proteins of the ARP2/3 actin nucleation complex and its regulatory SCAR/WAVE complex display cell-cell adhesion defects in Arabidopsis (Le et al. 2003; Deeks & Hussey 2005; Tian et al. 2015). This suggests that actin-dependent process including trafficking

and secretion of wall components, may contribute to maintaining adhesion, although the precise mechanisms remain unclear.

Another important factor that contributes to how cell-cell adhesion is controlled is mechanosensing, the ability of cells to perceive and respond to mechanical cues. Since cell-separating tensions are one of the main threats to adhesion, meachanosensing is expected to play a critical role in maintaining it. A central player in this process is DEFECTIVE KERNEL1 (DEK1), a plant specific calpain-like protein that is important for epidermal integrity. *dek1* mutants display dramatic phenotypes, including cell adhesion defects, tissue disorganization and even embryo lethality (Tran et al. 2017). Thus, mechanosensing via DEK1 may represent a key pathway by which plants maintain adhesion while growing.

Finally, metabolic pathways may also play an important role in the regulation of cell-cell adhesion. One allele of *QUA2* was first identified in a screen for sucrose hypersensitivity, and subsequent work showed that other alleles as well as *QUA1* mutants also display oversensitivity to sucrose (Frank et al. 2002; Gao et al. 2008; Verger et al. 2016). This suggests a connection between sucrose and cell adhesion, likely through effects on wall composition and mechanics, highlighting sugar metabolism as a potential regulator of adhesion in plants.

Altogether, these previous studies suggest that the control of cell-cell adhesion in plants is more complex than simply pectins acting as a glue at the ML. Instead, adhesion emerges from a coordinated network that integrates wall chemistry, cytoskeletal organization, mechanosensing and metabolic regulation. This further supports that adhesion is an actively regulated process, tightly linked to growth and development, rather than a passive outcome of wall formation.

1.3 Cell wall integrity

The plant cell wall is not a just rigid barrier surrounding the cell membrane, it is a flexible and responsive structure. In order to grow properly, plants need to “check” the condition of their walls. This process is called cell wall integrity (CWI) maintenance (Engelsdorf & Hamann 2014; Hamant &

Haswell 2017). CWI maintenance was first proposed in yeast where specialized signalling pathways detect wall damage and activate compensatory response (Levin 2011). In plants, similar mechanisms have been proposed, although plants are more complex due to the diversity of wall components and the close relationship between wall structure, growth and environmental responses (Vaahtera et al. 2019).

A central idea of plant CWI is that the wall is under constant mechanical stress from the turgor pressure, and any disturbance such as change in wall composition due to the cell wall remodelling during growth, degradation due to pathogen attack or mechanical injury, must be sensed and compensated for (Hamann 2015). Plants achieve this through receptors that can sense changes in the wall and trigger downstream signalling which then adjust cell growth or strengthens the wall.

A key part of this mechanism involves receptors that interact with cell wall, among which the Catharanthus roseus Receptor-Like Kinase 1-Like (CrRLK1L) family plays a central role (Lindner et al. 2012). Members of this family are thought to link the physical state of the wall to intracellular signalling pathways that control growth and stress responses. Two of the well-studied examples are THESUS1 (THE1) and FERONIA (FER) (Hématy et al. 2007; Feng et al. 2018).

THE1 is a plasma membrane receptor-like kinase found in elongating cells. Its role was discovered through studies of cellulose deficient mutant for CESA6. Normally, the *CESA6* mutants show growth defects and ectopic lignification as part of a stress response. When *THE1* was mutated in these cellulose-deficient backgrounds, the defects became weaker, while overexpression of THE1 made them stronger, even though cellulose levels did not change. This shows that THE1 does not change cellulose content directly but controls how plants respond to cellulose deficiency. In other words, these findings indicate THE1 functions as a sensor of cell wall integrity (Hématy et al. 2007).

FER is another member of the CrRLK1L family that plays a central role in cell wall integrity. Unlike THE1, which mainly regulates responses to cellulose deficiency, FER has a much broader role. It helps coordinate

growth and stress responses by linking cell wall status with process such as hormone signalling and cell wall remodelling (Guo et al. 2009; Feng et al. 2018). FER is required for proper epidermal integrity and works as a hub receptor that integrates signals from multiple peptide ligands ALKALINIZATION FACTORs (RALFs) (Haruta et al. 2014; Li et al. 2015). One of FER's important function is regulating abscisic acid (ABA) signalling during drought stress, showing how wall sensing is directly linked to whole plant physiology (Yu et al. 2012). Furthermore, recent evidence indicates that FER plays a crucial role in maintaining cell integrity during growth, particularly in situations where high turgor pressure creates high mechanical stress on the wall. FER therefore seems to function to prevent cellular damage and preserve tissue integrity (Malivert et al. 2021). All in all, these findings show that FER ensures that changes in the mechanical state of the wall are matched with appropriate cellular and developmental response.

Taken together, current studies show that plants actively monitor the condition of their cell walls and adapt their response to maintain CWI. These CWI mechanisms do not simply repair damage but also coordinate growth, stress responses and hormonal signalling to be able to ensure that cell wall mechanism remain compatible with development and environmental challenges. Importantly, plants can perceive both the chemical and mechanical state of their walls, which is especially critical during rapid growth, when extensive wall remodelling combined with turgor-driven tension can threaten cellular integrity. Cell adhesion faces similar constraints: wall remodelling that allows growth may affect polysaccharide structure at important adhesion sites, while tissue tension tends to pull epidermal cells apart. This suggests that plants must have developed wall integrity and mechanical feedback to maintain adhesion while allowing fast growth (Baba & Verger 2024).

2. Objectives

The overall aim of my PhD was to deepen our understanding of the mechanisms mediating and regulating adhesion in plants. A major aspect of my PhD involved studying how the cell wall ultrastructure may contribute to adhesion, beyond the middle lamella. I was also interested in what regulates the state of cell adhesion and thus looked for and studied new regulator of cell adhesion. Finally, I took interest in how adhesion defects and pectin perturbations impact plant growth and development.

The specific goals of my project were:

I. To determine how the ultrastructure of the plant cell wall, rather than the middle lamella, contributes to the maintenance of cell-cell adhesion in dark-grown *Arabidopsis thaliana* (Paper I).

II. To identify molecular players involved in the maintenance of cell-cell adhesion in dark-grown *Arabidopsis thaliana* (Paper II).

III. To understand how cell wall integrity, particularly pectin biosynthesis contributes to apical hook formation in *Arabidopsis thaliana* (Paper III)

3. Result and Discussion

3.1 Beyond the middle lamella: do other ultrastructural features of the plant cell wall contribute to the maintenance of cell-cell adhesion?

At the beginning of my PhD studies, we hypothesised that, beyond the middle lamella, which is thought the be main player in cell-cell adhesion in plants (Knox 1992; Jarvis et al. 2003; Daher & Braybrook 2015; Atakhani et al. 2022; Baba & Verger 2024), the other features of the ultrastructure of the plant cell wall might also play a role in cell-cell adhesion. As discussed in the section 1.2.3, other ultrastructural features of the cell wall, such as plasmodesmata (Sager & Lee 2014; Faulkner 2018), the cuticle (Ingram & Nawrath 2017) and the putative reinforcement points at the tricellular junctions (Roland 1978; Willats et al. 2001b), have been proposed to play a role in maintaining adhesion. Adhesion is most critical in regions where mechanical tension threatens to pull neighbouring cells apart. This motivated us to ask three key questions: (i) What are the points in growing tissues where tension is highest? (ii) Are there ultrastructural features positioned at these high-tension sites to ensure proper adhesion? (iii) Can we experimentally test their contribution to maintaining cell-cell adhesion? In Paper I, we addressed these questions using Finite Element Modelling (FEM), cutting-edge microscopy techniques with well-characterised cell-cell adhesion defective mutant lines.

3.1.1 Outer epidermal edges are hotspots for cell-separating tensile stress

To investigate whether intercellular hotspots of tensile stress exist in plants, we first explored this hypothesis using FEM. To do that we first created a 2D model of a group of fully turgid cells adhering to one another representing an organ-like cross-section (**Paper I;** Fig. 1B). This model revealed an accumulation of stress in the outermost cell wall layer (**Paper I;** Fig. 1B′ and S1C and D) suggesting that adhesion at the outer epidermal surface layer needs to be reinforced in order to maintain proper cell-cell adhesion.

We then modelled a 2D mesh in which solid cells adhere to one another via a soft layer, called adhesive layer, and then we applied longitudinal

stretching to mimic growth driven stress (**Paper I;** Fig. 1C) as most of the plant tissues experience anisotropic stress when they grow and develop (Baskin & Jensen 2013; Robinson & Kuhlemeier 2018). In this model, we observed that high separating stress accumulated at the transverse adhesive interfaces between cells, which could threaten to pull the cells apart. In contrast, longitudinal interfaces experienced less stress, in a direction that does not threaten cell separation (**Paper I;** Fig. 1C′ and Figures S1E′–F′).

Finally, we created a model consisting of two pressurized cells adhering to each other via a middle lamella-like layer to investigate stress distribution (Paper II, Figure 1D). This model showed a very clear stress pattern where only the outer edges of the ML-like layer were under high separating tensile stress (**Paper I;** Fig. 1D″) whereas the rest of the ML-like layer was experiencing almost no stress (**Paper I;** Fig. 1D′ and S1 I-M).

All in all, our 2D models, spanning from organ level to cell wall level suggest that outer epidermal edges of transverse junctions are hotspots for cell-separating tensile stress. In turn, we can hypothesise that those hotspots could contain structures that reinforce adhesion to maintain tissue integrity.

3.1.2 Ultrastructural analysis of epidermal cell wall junctions reveals material continuity

As our 2D models suggest that outer epidermal cell wall junctions may harbour specific cell-cell adhesion reinforcement structures, we then used dark-grown *Arabidopsis thaliana* hypocotyls as a model system as its very high anisotropic growth in this tissue has been shown to lead to cell-cell adhesion defects in epidermis and making it an ideal system for us to use (Baskin & Jensen 2013; Robinson & Kuhlemeier 2018).

To test our model predictions, we selected cell-cell adhesion defective mutant lines namely *quasimodo2-1 (qua2-1)* (Mouille et al. 2007) and *actin related protein 2-1 (arp2-1)* (Le et al. 2003; Li et al. 2003; Mathur et al. 2003), which display strong and mild adhesion defects between epidermal adjacent cells, respectively. We first used propidium iodide (PI) staining and confocal microscopy to visualise the cell-cell adhesion phenotypes (**Paper I;** Fig. 2C-G) and we observed that most of the cell-cell adhesion defects

occurred at the anticlinal transverse junctions (Verger et al. 2018) where our model predicts specific adhesion reinforcement sites.

As the resolution of the confocal microscopy is limited (Schermelleh et al. 2010), we then turned to cryo-Scanning Electron Microscopy (cryo-SEM) to look at those junctions and examine the junctions in greater details. Interestingly, our cryo-SEM observations of *qua2-1 and arp2-1* revealed a partially ruptured layer of material spanning two adjacent cells in regions showing mild cell-cell adhesion defects in both mutant lines (**Paper I;** Fig. 2H-J).

Even though our cryo-SEM microscopy sample preparation method involves relatively few sample preparation steps, we wanted to confirm the cryo-SEM observations using a different technique to rule out the possibility of coating artefacts from cryo-SEM. To do that, we developed a correlative microscopy workflow where we combined confocal microscopy with Atomic Force Microscopy (AFM) (See **Paper I** method section). One of the biggest advantages of this workflow was the minimal sample preparation which reduces artefacts as well as the ability for the AFM cantilever to directly contact the sample surface, enabling us to confirm our cryo-SEM observations. This correlative microscopy workflow led to similar observations. We were able to observe a material layer continuity with AFM surface topology mapping where gaps are visible by confocal microscopy (**Paper I;** Fig. 2K-N and S2). When considered together, all these observations helped us to uncover a supracellular material continuity might play a role in maintaining cell-cell adhesion.

3.1.3 Thickening at outer epidermal cell edges can reduce cell-separating stress

As we observed supracellular material continuity between two adjacent cells, we then performed Transmission Electron Microscopy (TEM) on the *arp2-1* mutant line and wild-type Col-0 to investigate the ultrastructure of these junctions. We prepared longitudinal sections from both Col-0 and *arp2-1* (**Paper I;** Fig. 3A-C, G-I). We made two interesting observations. First, we identified a pseudo-triangle-shaped region between two adjacent cells, called the outer epidermal edge (OEE), and secondly, this region was thicker than the rest of the outer epidermal wall (**Paper I;** Fig. 3C, D, G, I). We quantified

local cell wall thickness in Col-0 (**Paper I;** Fig. 3H) and we found that the OEE was 2.6 times was thicker than the outer epidermal wall (**Paper I;** Fig. 3H). We then hypothesised that this local thickening, where our model also predicts specific adhesion reinforcement sites, could protect the junctions from separation tensile stress. To test this hypothesis, we used our 2D epidermal surface model (**Paper I;** Fig. 1C), and we ran the simulation with double the theoretical thickness compared to the cell domains. Surprisingly, the stress distribution pattern observed was the opposite of the first simulation: stress became high on the longitudinal interfaces and low on the anticlinal interfaces (**Paper I;** Fig. 1J). This suggests that thickened edges can reduce cell separating stress by reinforcing the cell wall at these locations. In other word, they redistribute mechanical forces and reducing the stress that could otherwise separate neighbouring cells.

3.1.4 Cellulose- and pectin-based wall continuity spans transverse junctions

To obtain further ultrastructural and compositional information about supracellular material continuity, we first examined TEM images of *arp2-1* and wild-type Col-0 and performed Airyscan confocal microscopy with two different cell wall stains using the *arp2-1* mutant line. When we examined the TEM images of Col-0, we first observed the characteristic cross-lamellate pattern of the cell wall (**Paper I;** Fig. 3G), in which successive layers of cellulose microfibrils are visible in a previously described pattern (Zhang et al. 2016). We observed that this wall structure also exits across cellular junctions, which to our knowledge has not been previously reported. Moreover, when we examined the *arp2-1* images we first observed that OEE was more clearly organized (**Paper I;** Fig. 3H) also the cuticle was clearly seen (**Paper I;** Fig. 3K). These TEM images allowed us to define some cell wall subdomains: the inner wall (IWs), expected to represent newly synthesized wall domains produced after cell division, which follows the plasma membranes of adjacent cells; the supracellular outer epidermal wall (SOEW), a continuous fibrous layer across outer epidermal surfaces of neighbouring cells that likely derives from the pre-existing mother cell's wall; and the outer epidermal edge filling (OEEF) a pseudo-triangular wall subdomain located at the outer edge of junctions between two adjacent cells, likely extending from the middle lamella as established during cell division.

We then tuned to Airyscan confocal microscopy to confirm the presence of cell wall material. To do this, we used the *arp2-1* mutant line and focused on mild cell-cell adhesion defects, using two cell wall stainings: propidium iodide (PI) which labels pectins and Direct Red 23 (DR23) which labels cellulose (Bidhendi et al. 2020). Our Airyscan confocal microscopy observations further confirmed the presence of cellulose-based lamellae across neighbouring cells (**Paper I;** Fig. 3N-Q).

3.1.5 Role of SOEW and OEEF in maintaining cell-cell adhesion

After confirming the composition of the structure, we asked whether SOEW and OEEF could play a role in maintaining cell-cell adhesion since we observed these structures in regions where our 2D models predicted stress hotspots (**Paper I;** Fig. 1) which we hypothesised could correspond to points requiring reinforcement. To address this, we again took advantage of 2D FEM simulation and created a 2D model in which two fully turgid cells adhere to each other via a ML. We also introduced three subdomains observed in our TEM images: IWs, SOEW and OEEF and applied stretching to investigate stress distribution on these subdomains (**Paper I;** Fig. 4A).

Then we tested a few different scenarios to understand their relationship and contribution to maintaining cell-cell adhesion. In the first scenario, we had a mesh where all the subdomains had uniform stiffness. In this case, the stress was high in the periclinal cell wall whereas it was low in the anticlinal wall (**Paper I;** Fig. 4B and S3B and D) confirming our initial simulation. This indicates that the pseudo-triangular, thickened geometry of the edge already strongly decreases stress at these junctions compared to the periclinal walls, and therefore the ML experiences minimal stress and contributes little to maintaining of cell-cell adhesion. Next, we tested their theoretical relative contribution by changing their relative stiffness. To do this, we made the ML domain softer, and we found that it did not affect the stress distribution (**Paper I;** Fig. S3 B, C and E). Then we softened the OEEF alone and we found high separating strain in the OEEF (**Paper I;** Fig. 4C′) while the SOEW compensated by becoming the main load bearing structure (**Paper I;** Fig. 4C). In the next scenario, we this time softened the SOEW alone and observed a decrease in stress only within the SOEW, whereas global stress and strain distribution remained same (**Paper I;** Fig. 4D and D′). In the final scenario, we softened both the SOEW and OEEF and observed the strongest

effect: both SOEW and OEEF had strong separating strain and SOEW could not function as a load-bearing structure (**Paper I;** Fig. 4E and E′). Overall, these scenarios support the key role of SOEW and OEEF in maintaining cell-cell adhesion and further suggest that cell separation may start from OEEF before SOEW raptures.

3.1.6 Initiation of cell separation

Through TEM analysis of the *arp2-1* mutant, we identified distinct stages of adhesion-defective intercellular junctions, beginning with nearly intact contacts to complete cell separation (**Paper I;** Fig. 4F-I).

In the first stage, we observed the earliest sign of wall failure: a small fracture began to nucleate at the base of OEEF, but the rest of the wall domains remained intact (**Paper 1**; Fig. 4F, F′). In the second stage, the OEEF was largely absent and replaced by a visible gap, leaving the two cells partially detached, although the SOEW still bridged the cell and maintained their overall connection (**Paper 1**; Fig. 4G, G′). In the third stage, the separation had advanced further, producing a broad opening at the cell junction. At this point the SOEW was still present as a continuous layer spanning across the cell (**Paper 1**; Fig. 4H, H′). In the final stage, complete detachment was evident: the SOEW itself had ruptured, leaving the cells fully separated, with only the IWs remaining intact (**Paper 1**; Fig. 4I, I′). All together these observations suggest that cell separation may start from the base of the OEEF, progress outward until the OEEF is lost and then continue until the SOEW ruptured, completing the detachment process which very well fits with the outputs of the multi-subdomain simulation.

3.1.7 Dual impairment of the SOEW and OEEF correlates with severe cell-cell adhesion defects

We next hypothesised that the severe cell-cell adhesion phenotype of *qua2-1* could be due to a combination of ML, OEEF and SOEW impairments as this mutant exhibit HG, cellulose and cuticle deficiencies (Du et al. 2020; Lorrai et al. 2021). A suppressor line of *quasimodo2*, *qua2-1 esmd1-1,* can supress the adhesion phenotype without restoring the pectin content and thus may only have ML and OEEF impairment like *arp2-1.* To test this, we again used TEM, focusing on the outer epidermal junction at a very early stage, when growth-driven stress is less intense and less likely to have already

pulled cells apart. It turned out that at this stage Col-0, *arp2-1* and *qua2-1 esmd1-1* outer epidermal junctions are almost identical to each other and appear intact (**Paper I;** Fig. 5A, B, D) whereas *qua2-1* junctions already had structural alterations (**Paper I;** Fig. 5C) suggesting that SOEW of *qua2-1* is indeed not well organized. To further confirm this, we used Direct Red 23 staining which labels cellulose (cite). In this analysis, we observed globular structures at the separated interface between cells on *qua2-1* (**Paper I;** Fig. 5G), whereas *arp2-1* and *qua2-1 esmd1-1* displayed fibrillar structures (**Paper I;** Fig. 5E, F, H), further indicating that the OEEF in *qua2-1* is not well organized.

Finally, we tested the contribution of the SOEW to maintaining cell-cell adhesion. To do so, we used our micro-extensometer coupled with a confocal microscope using the *arp2-1* line. This system allowed to us to observe how gaps form between two adjacent cells during the stretching. As expected, gaps began to appear between cells while the SOEW still was holding cells (**Paper I;** Fig. 5J, J′ and M, M′) and the cells fully separated once the SOECW ruptured (**Paper I;** Fig. 5K, K′ and N, N′). All in all, these observations show that SOEW can function as a load-bearing structure, and they align with both our TEM observations which indicates how cell separation may initiate and propagate and our simulation results.

Altogether, our results suggest that ultrastructural features of the plant cell wall beyond the ML contribute to cell-cell adhesion. FEM identified the outer epidermal edges of transverse junctions as hotspot for tensile stress, suggesting a need for reinforcement. Microscopy analyses uncovered a supracellular material continuity at these sites and allowed us to define three subdomains: IWs, SOEW, OEEF. Functional modelling and mutant analyses demonstrated that SOEW and OEEF act as reinforcement domains, with separation initiating at the OEEF and progressing until SOEW rupture. The severe adhesion defects of *qua2-1* correlated with dual impairment of these domains. Finally micro-extensometer assays revealed that SOEW can act as a load bearing structure spanning across adjacent cells. Together, these findings highlight SOEW and OEEF as critical reinforcement structures that protect cell junctions from high tensile stress and ensure tissue integrity.

3.2 What are the molecular players that mediate cell-cell adhesion in plants?

After exploring the structural aspect of cell-cell adhesion and its structural players, the next step in my PhD journey was to investigate the molecular players that regulate cell-cell. To explore this side of the plant cell-cell adhesion, we made a hypothesis based on the observation that sucrose intensifies cell-cell adhesion defects of the *qua2-1* mutant. We wondered whether this effect is a general phenomenon shared by other cell-cell adhesion defective mutants, and whether sugars play a more general role for cell adhesion regulation in plants.

3.2.1 Sucrose intensifies cell-cell adhesion defects

As it has been shown that pectin deficient mutants *quasimodo 1* (Bouton et al. 2002) and *quasimodo 2* (Mouille et al. 2007; Du et al. 2020) are hypersensitive to exogenous sucrose in the growth medium (Frank et al. 2002; Gao et al. 2008), we wondered whether this effect is a common effect on all cell-cell adhesion mutants or specific to these mutants. To explore this, we selected adhesion defective mutant lines affecting different biological pathways; *quasimodo2-1* Homogalacturonan biosynthesis (Mouille et al. 2007; Du et al. 2020); *actin related protein 2-1* cytoskeleton organization (Le et al. 2003; Li et al. 2003; Mathur et al. 2003)*; murus 1-2* rhamnogalacturonan II biosynthesis (Reiter et al. 1993; Bonin et al. 1997); and suppressor of *quasimodo2-1, quasimodo2-1 esmeralda1-1* (Verger et al. 2016).

To test this, we used our high-throughput Ruthenium Red Staining workflow (RRQuant, (Baba et al. 2024)) which enables rapid and quantitative assessment of adhesion defects across large numbers of seedlings. Using this setup, we found that exogenous sucrose consistently intensified cell-cell adhesion defects in all mutants, as shown by increased RR staining intensity compared with seedling grown without sucrose (**Paper II;** Fig. 1K′).

As Ruthenium Red can also penetrate tissue when the cells are dead, we wanted to be sure that high signal intensity that we found is related with enhanced cell-cell adhesion defects caused by sucrose in the growth medium. To verify this, we used Propidium iodide (PI) staining and confocal

microscopy. And we found that indeed high RR staining intensity was not caused by dead cells (**Paper II;** Fig.1M-O-Q-S-U).

With the RRQuant workflow we also quantified clear morphological changes across all genotypes. Both mutants and the wild-type Col-0 displayed an increase in hypocotyl width when grown in the presence of sucrose (**Paper II;** Fig1. K‴). However, hypocotyl elongation was reduced in all mutants except Col-0 (**Paper II;** Fig1. K″). All in all, these observations suggest that sucrose modulates cell-cell adhesion via a pathway distinct from those affected in the tested mutants.

3.2.2 Sucrose induces cell-cell adhesion defects on wild-type Col-0 without affecting outer epidermal continuity

Surprisingly, we also noticed cell-cell adhesion defects on the wild-type Col-0 samples grown in 1% sucrose (**Paper II;** Fig1. M) when we were investigating the effect of the sucrose on the mutant lines. However, we did not observe this type of small cell-cell adhesion defects on the wild-type Col-0 samples grown in without sucrose (**Paper II;** Fig. 1L). Therefore, we named these adhesion defects *micro cell adhesion defects* as these defects were much smaller than in adhesion mutants (**Paper II;** Fig. 1N-U), usually appearing at tricellular junctions and extending slightly along longitudinal rather than transverse interface.

To characterise this sucrose induced cell adhesion defect, we first developed a correlative confocal and cryo-Scanning Electron Microscopy (cryo-SEM) workflow (see **Paper II;** methodology section). Using the workflow, we observed that samples grown without sucrose displayed proper cell-cell adhesion between two adjacent cells on both confocal images (**Paper II;** Fig. 2A-B) and cryo-SEM images (**Paper II;** Fig. 2C-D). In contrast, samples grown in 1% sucrose showed a visible gap between two adjacent cells on the confocal images (**Paper II;** Fig. 2E-F), although no visible gap were detected on the cryo-SEM images (**Paper II;** Fig. 2G-H). These observations suggest that sucrose-induce micro cell adhesion defect might not impair the outer epidermal integrity.

To further confirmed this observation and be sure that it is not artefact of sample preparation, we used our recently developed correlative confocal and

Atomic Force Microscopy (AFM) workflow. Using the workflow, we were able to confirm the observation that we got from our correlative confocal and cryo-SEM (**Paper II;** Fig. 2I-J and L-M).

3.2.3 Sucrose-induced micro cell adhesion defects in dark-grown hypocotyls are mediated by metabolic pathways

The next question we asked was whether the underlying cause of the sucrose-induce micro cell adhesion defects was the metabolic or signalling role of sucrose, given its dual function as both a signalling molecule and a modulator of metabolic processes in plant growth and development (Ruan 2012; Yoon et al. 2021).

Before addressing above possibilities, we also tested two other possibilities. First, we wanted to understand whether the change in osmotic potential caused by sucrose in the growth medium could itself create micro cell adhesion defects. To test this, we grew wild-type Col-0 samples in a medium supplemented mannitol which had the same osmolarity that 1% sucrose had. We observed that mannitol failed to induce micro cell adhesion defects (**Paper II;** Fig. 3A). To further confirm this observation, we also used threefold higher mannitol concentration, and we again observed that mannitol cannot induce micro cell adhesion defects (**Paper II;** Fig. 3B). Next, we tested whether acidification of growth medium via active sucrose uptake could induce micro cell adhesion defects, as plants export protons into the apoplast, which can in turn acidify the growth medium (Palmgren 2001; Peiter 2003). As a next step, we grew wild-type plants in acidic mediums, adjusting the pH from 5.7 to 3.7 and we again observed no cell adhesion defects (**Paper II;** Fig. 3C-D), ruling out the acidification as a possible cause.

We then addressed our first question: whether the effect was driven by sucrose's signalling role or by its impact on metabolism. To do this, we grew wild-type samples in the presence of a nonmetabolized sucrose, turanose, at the same osmolarity as 1% sucrose. Once again, we did not observe any cell-cell adhesion defects (**Paper II;** Fig. 3E). In plants sucrose is normally broken down either via invertase (INV) into fructose and glucose or via sucrose synthase (SUS) into fructose and UDP-glucose respectively (Koch 2004). We therefore grew Col-0 samples either in glucose or fructose at the same osmolarity as 1% sucrose, and we found that both glucose and fructose

can induce micro cell adhesion defects to a similar extent as sucrose (**Paper II;** Fig. 3F-G). Taken together, these results indicate that induction of micro cell adhesion defects is linked to the metabolic effect of sugars rather than specific to sucrose.

3.2.4 Does sucrose alter epidermal cell shape anisotropy in dark-grown hypocotyls via a microtubule-dependent mechanism?

To further investigate the effect of sucrose on adhesion, we first study its impact on growth anisotropy. To do this, we acquired 3D spinning disk confocal images of full-length hypocotyls (**Paper II;** Fig. 4A-B) and measured cell length and width at the basal part of the hypocotyls (**Paper II;** Fig. 4A′-B′). We found that seedlings grown with sucrose had similar length to those grown without sucrose (**Paper II;** Fig. 4C). However, their cell width was significantly increased (**Paper II;** Fig. 4D) indicating that sucrose primarily affects radial expansion rather than longitudinal growth, thereby altering cell shape anisotropy.

As we found that sucrose alters epidermal cell shape anisotropy, we wanted to understand the underlaying mechanisms. To investigate this, we hypothesized that one of the underlying mechanisms might involve microtubules (MTs), as they guide cellulose microfibril depositions in the cell walls, thereby affecting the final cell shape (Baskin 2005; Paredez et al. 2006; Bringmann et al. 2012). We wanted to address this by using a microtubule reporter line *pPDF:mcitMBD* (Armezzani et al. 2018) and acquiring full-length 3D spinning disk confocal images using with and without sucrose conditions (**Paper II;** Fig. 4E-G). With this reporter line, we found no substantial changes in overall MT organization. However, in the 1% sucrose condition, some cells lacked MT signal (**Paper II;** Fig. 4G), whereas we did not notice this type of cells in samples grown without sucrose (**Paper II;** Fig. 4E′). To understand the status of those cells, we co-stained the sample with PI, and it turned out that those cells had undergone bursting (**Paper II;** Fig. 4H″).

Finally, we used the RRQuant tool to assess whether the global hypocotyl length and width were altered in the MT line. We found that MT reporter line had shorter hypocotyls and greater width than Col-0, matching Col-0 grown on 1% sucrose. Sucrose further reduced the length and increased width

(**Paper II;** Fig. 4I-J). These results suggest that sucrose effect on cell shape anisotropy could be acting via microtubules but is still unclear whether this can explain the effect on adhesion.

3.2.5 Sucrose modulates hypocotyl mechanical properties and alters cell anisotropy

As the cell wall and its mechanical properties is one of the key players (Atakhani et al. 2022) of cell-cell adhesion, we first investigated cell wall mechanical properties at the cellular level. To do this, we used Atomic Force Microscopy (AFM) and focused on the basal part of the hypocotyls where cells are fully developed (Gendreau et al. 1997; Bou Daher et al. 2018). Our AFM measurements revealed that the apparent stiffness of hypocotyl cells did not differ significantly in seedlings grown with and without sucrose, although there was a trend toward increased stiffness under 1% sucrose condition (**Paper II;** Fig. 5C). We then moved on to examine mechanical properties at the tissue level under the same conditions. To do so, we used extensometer-based tensile test which measures both stiffness and breaking strength. We found that both stiffness and breaking strength were significantly higher in the seedling grown under 1% sucrose condition compared to those grown without sucrose condition (**Paper II;** Fig. 5D-E). These results suggest that sucrose has only a limited effects on the epidermal cell wall mechanics, whereas its impact on overall tissue mechanics is pronounced.

Overall, we found that sucrose plays an active role in regulating cell-cell adhesion in plants. Using adhesion-defective mutants and the high-throughput assay RRQuant, we showed that sucrose consistently intensified adhesion defects, and surprisingly it also induced smaller “micro” adhesion defects in wild-type Col-0. Using two different correlative microscopy workflows, we confirmed that these defects occurred without loss of outer epidermal continuity which is likely corresponding to the SOEW described in Paper 1. We also demonstrated that micro-cell adhesion defects were linked to sucrose’s metabolic effect rather than its signalling role. Through mechanical analyses, we found that sucrose has a major effect at the tissue level while its impact at the cell level was minimal. Finally, using a MTs reporter line, we observed that sucrose appears to influence cell shape anisotropy via a microtubule-dependent mechanism. Altogether, our results

show that sucrose regulates cell adhesion through its metabolic activity by altering tissue mechanics and cell anisotropy potentially through a microtubule-dependent manner.

3.3 Do changes in cell wall integrity (CWI) especially in pectin biosynthesis affect apical hook formation in dark-grown *Arabidopsis*?

3.3.1 Pectin biosynthesis defects disrupt hook formation

As it has been shown that there is a feedback loop between auxin and cell wall composition that regulates apical hook formation (Aryal et al. 2020; Baral et al. 2021; Jonsson et al. 2021), we wondered whether all major cell wall polysaccharides could play role in this process. To test this, we used a panel of cell wall mutants; *qua2* affecting HG composition (Mouille et al. 2007; Du et al. 2020), the *gae1gae6* double mutant impaired in Homogalacturonan (HG) and possibly rhamnogalacturonan I (RG-I) biosynthesis (Mølhøj et al. 2004; Bethke et al. 2016); *murus1* defective rhamnogalacturonan-II (RGII), xyloglucans, and cell wall glycoproteins(Reiter et al. 1993; Bonin et al. 1997; Rayon et al. 1999; Freshour et al. 2003), *korrigan1* (*kor1*) (Nicol 1998) and *procuste1* cellulose deficient (Desnos et al. 1996), *mur4* and *mur7* impaired biosynthesis of arabinose (Reiter et al. 1997; Burget et al. 2003). We found that, three days after germination, wild-type seedlings formed fully closed hooks, whereas pectin-deficient mutants (*qua2-1*, *gae1gae6*, *mur1*) displayed completely open hooks. In contrast, mutants affecting other wall components (*kor1*, *mur4*, *mur7*) formed normal hooks, while *prc1* showed only a mild defect. These results indicate that defects in HG biosynthesis have the most pronounced impact on hook formation (**Paper III;** Fig. 1).

Then we wanted to understand if altered pectin composition triggers CWI response in a turgor-dependent manner. To do so, we performed kinematic analysis on the WT, *qua2, gae1gae6, mur1* seedlings under low (LA) and high agar (HA) conditions as high agar condition was previously shown to reduce epidermal tension (Verger et al. 2018). We found that seedlings with altered pectin composition; *qua2, gae1gae6, mur1* failed to fully close and maintain their apical hook under LA, despite normal initial formation.

Reducing turgor pressure either by growing on HA or adding sorbitol partially restored formation in all mutans and rescued hook maintenance in *mur1* indicating that lowering turgor can help overcome the defects caused by changes in pectins (**Paper III;** Fig. 2).

3.3.2 Pectin loss disrupts cell expansion and asymmetric auxin response in apical hook development

In order to understand whether the pectin integrity controls the differential elongation required for hook formation or turgor reduction restores that differential elongation in pectin mutant, we used again LA and HA conditions on *qua2* mutant. We found that WT maintained a higher expansion rate on the outer side under both conditions, whereas *qua2* showed reduced outer-side expansion only on LA, a defect rescued on HA (**Paper III;** Fig. 3A-B).

Because differential cell expansion relies on an auxin gradient between the two sides of the hook apex (Silk & Erickson 1978), we examined auxin response in WT and *qua2* seedlings using the DR5-VENUS-NLS reporter. WT showed strong signal in the inner epidermal cells regardless of agar concentration, whereas *qua2* grown on LA displayed equal signal on both sides. This asymmetry was restored in *qua2* under HA, indicating that turgor-dependent effects of altered HG disrupt proper auxin gradients and, consequently, differential cell expansion during hook formation (**Paper III;** Fig. 3C).

3.3.3 Disruption of pectin integrity suppresses HLS1 and PIF4 expression and modifies the regulation of genes associated with GA homeostasis

Next, we examined whether central regulator of hook formation, including *HOOKLESS1* (*HLS1*) (Guzmán & Ecker 1990), AUXIN RESPONSE FACTOR 2 (ARF2) (Li et al. 2004) and *PHYTOCHROME INTERACTING FACTOR4* (*PIF4*) (Zhang et al. 2018), are affected in *qua2*, as changes in their expression could explain the defective hook phenotype. Our results show that disruption of pectin integrity alters the central regulatory network controlling hook formation. In *qua2* seedlings, PIF4 transcript and protein levels, as well as HLS1 expression, were strongly reduced under LA condition, consistent with the defective hook phenotype. Strikingly, both

factors were restored to wild-type levels under HA, correlating with recovery of hook formation. Moreover, overexpression of HLS1 partially rescued the *qua2* hook phenotype, directly linking its reduced expression to the defect. These findings suggest that pectin integrity influences hook development through the regulation of PIF4 and HLS1, and that reduced turgor pressure can compensate for this defect by restoring their expression and function (**Paper III;** Fig. 4).

Because GAs promotes hook formation and HLS1 expression (An et al. 2012), we next asked whether defects in pectin integrity alter the expression of genes involved in GA metabolism and homeostasis. In *qua2* seedlings grown on LA, GA biosynthetic genes (*GA20ox1*, *GA3ox1*) were downregulated and the catabolic gene *GA2ox2* was upregulated, while expression was restored under HA. Exogenous GA or removal of DELLA repressors partially rescued hook formation, indicating that loss of pectin integrity disrupts GA metabolism and thereby impairs hook development (**Paper III;** Fig. 5).

3.3.4 Hook formation, along with HLS1, PIF4 and GA levels, was repressed by isx

Next, we investigated whether cellulose defects might also affect hook formation, since *prc1*, impaired in the cellulose synthase CEA6 (Desnos et al. 1996) shows partially defective hook formation (**Paper III;** Fig. 1). To test this, we treated WT seedlings with isoxaben (isx), a cellulose synthase inhibitor (Desprez et al. 2002). Under LA condition, isx caused a strong reduction in hook curvature, disrupted asymmetric auxin distribution, and reduced expansion on the outer side of the hook. These defects were largely rescued under HA condition. Thus, similar to pectin mutans, cellulose defects impair hook formation by disturbing auxin asymmetry and outer-side cell elongation (**Paper III;** Fig. 6)

Since loss of pectin integrity impaired hook formation by downregulating the GA-PIF4-HLS1 pathway, we next tested whether inhibition of cellulose biosynthesis by isx disrupts the same regulatory module. As in *qua2*, isx repressed HLS1 and PIF4 expression under LA but not HA, and HLS1 overexpression restored hook formation. Isx also downregulated GA biosynthetic genes and reduced GA levels under LA. Moreover, mutants with elevated GA levels were less sensitive to isx. These results indicate that

cellulose inhibition disrupts hook formation through the same GA-PIF4-HLS1 signalling pathway as pectin deficiency, revealing a shared mechanism linking cell wall integrity to hormone regulation (**Paper III;** Fig. 7 and 8)

Given the role of THE1 in response to cellulose perturbation (Bacete & Hamann 2020), we finally examined its involvement in isx-mediated hook formation. We found that loss-of-function *the1-1* and *the1-6* mutants were less sensitive to isx under both LA and HA conditions, whereas the gain-of-function *the1-4* mutant displayed increased sensitivity (**Paper III;** Fig. 9A). Moreover, both *the1-1* and *the1-6* fully restored hook development in *esmeralda1* seedlings (**Paper III;** Fig. 9B). These findings indicate that THE1-mediated responses contribute to the hook defects caused by altered cell wall integrity.

4. Conclusion and Future Perspectives

In Paper *I,* we questioned the textbook view that the middle lamella is the primary and perhaps only, structural determinant of cell-cell adhesion in plants. Using a combination of finite element modelling, high-resolution imaging, and correlative microscopy, we revealed that adhesion should instead be reinforced at specific "hotspots": outer epidermal edges of transverse junctions. Our modelling further showed that local thickening and specific wall subdomains, notably the supracellular outer epidermal wall (SOEW) ant the outer epidermal edge filling (OEEF), can act as load-bearing reinforcements, reducing the risk of cell separation.

Through TEM, cryo-SEM, Airyscan confocal microscopy, and AFM-based correlative workflows, we uncovered continuous cellulose- and pectin-based supracellular wall structures spanning neighbouring cell. These structures were not defined before in the context of cell-cell adhesion, yet they play a critical role in maintaining adhesion. On top of this, our ultrastructural analyses and simulations suggest that cell separation may initiate at the base of OEEF before propagating to the SOEW. Finally, live mechanical testing confirmed that the OECW can act as a true load-bearing structure supporting a revised, more complex model of cell-cell adhesion that extends far beyond the middle lamella. This work opens new avenues for understanding how cell-cell adhesion is maintained in plants. It also raises key questions, such as how plants reinforce these hotspots: is there a true cell-edge-specific-mechanism? and whether adhesion reinforcement occurs exclusively in the epidermis. Addressing these questions will be essential for gaining a complete understanding of how cell-cell adhesion is maintained in plants.

In Paper *II*, we investigated the molecular players of cell-cell adhesion by testing how sucrose influences adhesion-defective mutants and wild-type *Arabidopsis* dark-grown hypocotyls. We found that sucrose intensified adhesion defects in all the tested mutans and even induced subtle "micro" cell-cell adhesin defect in wild type which revealed the SOEW structure described in Paper I. These defects were not explained by osmotic effects or medium acidification could reproduced by glucose and fructose but not by the non-metabolized sugar turanose, indicating a metabolic rather than signalling origin.

Sucrose also increased tissue stiffness and promoted radial cell expansion, altering epidermal cell anisotropy and likely generating transverse stress that trigger separation at the tricellular junctions. Microtubule reporter imaging revealed occasional burst cells and suggested morphological sensitivity to sucrose, linking sugar metabolism to change in adhesion, mechanics and cell shape. However, the downstream mechanism by which sugar metabolism alters cell-cell adhesion, tissue mechanics, and cell anisotropy remain unknown. Nevertheless, this work provides an important first step toward dissecting the molecular pathways that control the maintenance of cell-cell adhesion in plants. To gain a more complete picture of how sucrose induces cell adhesion, further work should focus on characterizing changes inner tissue geometry, analysing cell wall thickness using TEM, and applying computational simulations to test whether such geometrical changes affect tension pattern.

In Paper *III,* we investigated how cell wall integrity controls apical hook formation in Arabidopsis. We found that defects in pectin biosynthesis, especially homogalacturonan (HG)- mutants namely *qua2, gae1gae6, and mur1* caused severe hook defects. In contrast mutations in other wall components had little or no effect. These defects were strongly turgor-dependent: under low agar pectin mutants failed to close or maintain hooks, but reducing turgor with high agar or sorbitol partially restored curvature. At the cellular level, pectin loss disrupted the normal epidermal expansion and auxin asymmetry required for hook development, both of which rescued under reduced turgor.

At the molecular level, pectin deficiency supressed the expression of PIF4 and HLS1 and altered GA metabolism, reducing biosynthesis and promoting catabolism. Restoring GA levels or removing DELLA repressors partially rescued the mutant phenotype, showing that pectin integrity regulates hook development via the GA-PIF4-HLS1 module. Inhibitor of cellulose biosynthesis by isoxaben produced similar defects, linking cellulose and pectin integrity to a common pathway. Finally, we showed the receptor-like kinase THE1 contributed to these responses. Together, our findings suggest that when cell wall integrity is compromised, turgor-dependent and THE1-mediated responses supress GA-dependent regulation of PIF4 and HLS1,

thereby disrupting auxin asymmetry, differential cell expansion and ultimately proper hook formation.

It is also important to note that pectin-related mutants examined here, *qua2, gae1gae6, and mur1*, all display cell adhesion defects. This raises the possibility that their hook formation phenotype arises not only from altered pectin content but also from impaired adhesion at the tissue level. Moreover, this work revealed that beyond what we already know about *qua2*, it also exhibits perturbations in auxin response, GA metabolism and in the regulation of key genes such as HLS1 and PIF4. Therefore, future studies of these pathways in the *qua2* mutant background could provide new insights into the molecular players underlying cell-cell adhesion maintenance and more broadly, into how adhesion contributes to plant development.

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Popular science summary

Plants are made of millions of tiny cells that must stick together to form tissues and organs. This is possible thanks to cell-cell adhesion. Proper adhesion is crucial because each plant cell is under high internal pressure, constantly pushing against its neighbours. If adhesion fails, tissues can tear apart, threatening the plant's growth and survival. For a long time, it was thought that adhesion mainly came from a supposedly sticky layer called the middle lamella, which is rich in pectins - one of the main building blocks of the plant cell wall. My thesis challenges this textbook view and suggests that adhesion is more complex, involving not just chemistry but also new structure and dynamic regulation.

Using advanced microscopy and computer modelling, we discovered that the outer edges where two epidermal cells meet, are hotspots for high cell-separating tensions, meaning they are the places where cells are most at risk of being pulled apart. At these critical points, plants have evolved special reinforcement structures in the cell wall that act as supporting structures, preventing separation and maintaining tissue integrity. These reinforcement points had not been described before, and they reveal a new layer of complexity in how plants preserve adhesion.

I also discovered that sugar metabolism plays a surprising role in regulating cell-cell adhesion in plants. Added sucrose not only worsened adhesion problems in mutants but even triggered tiny defects in wild-type plants. We called those 'micro-cell adhesion defects' because they appear as very small separations between neighbouring cells. This shows that cell-cell adhesion is not a static glue but an actively maintained process, with sugar metabolism identified as one of its key regulators.

Finally, we showed that cell wall integrity, which means the plant's ability to sense and respond to the status of its cell wall, and in particular pectin biosynthesis, is crucial for plant development. Mutants defective in pectin) failed to form a proper apical hook, the curved structure that protects young seedlings as they emerge from the soil. These defects were linked to disruptions in hormone balance, raising the possibility that their hook

formation problems may arise not only from altered pectin content but also from impaired adhesion at the tissue level.

Together, these findings show that plant cell adhesion is not a simple matter of sticky pectin in the middle lamella, but a dynamic and multifaceted process. Adhesion depends on structural reinforcement at cell edges, on active regulation by sugar metabolism, and on proper pectin biosynthesis that links wall status to developmental programs such as apical hook formation. This broader view highlights cell-cell adhesion as an actively maintained property that integrates mechanics, metabolism, and signalling to secure tissue integrity and guide plant growth.

Populärvetenskaplig sammanfattning

Växter består av miljontals små celler som måste hålla ihop för att bilda vävnader och organ. Detta är möjligt tack vare cell-cell-adhesion, en avgörande mekanism eftersom varje växtcell står under högt inre tryck och ständigt pressar mot sina grannar. Om adhesionen sviktar kan vävnader slitas isär, vilket hotar växtens tillväxt och överlevnad. Under lång tid trodde man att adhesion främst kom från ett klibbigt lager kallat medellamellen, som är rik på pektin – en av de viktigaste byggstenarna i växtcellväggen. Min avhandling utmanar denna läroboksbild och visar att adhesion är mer komplex, där inte bara kemi utan också struktur och reglering spelar en roll.

Med hjälp av avancerad mikroskopi och datorbaserad modellering upptäckte vi att de yttre kanterna av epidermisceller är hotspots för hög cellseparerande dragspänning, vilket betyder att det är just där cellerna riskerar att dras isär. På dessa kritiska punkter har växter utvecklat särskilda förstärkningsstrukturer i cellväggen som fungerar som bärande element, förhindrar separation och upprätthåller vävnadens integritet. Dessa förstärkningspunkter hade aldrig tidigare beskrivits och avslöjar en ny nivå av komplexitet i hur växter bevarar adhesion.

Jag visade också att sockeromsättningen har en oväntad roll i regleringen av cell-cell-adhesion i växter. Tillsatt sackaros förvärrade inte bara adhesionsproblem i mutanter utan orsakade även små defekter i vildtypsplantor, vilka vi kallade ”mikro-adhesionsdefekter” eftersom de uppträder som mycket små separationer mellan närliggande celler. Detta visar att cell-cell-adhesion inte är ett statiskt lim utan en aktivt upprätthållen process, där sockeromsättningen kan identifieras som en viktig regulator.

Slutligen visade vi att cellväggsintegritet, vilket innebär växtens förmåga att känna av och svara på cellväggens status, och i synnerhet pektinbiosyntes, är avgörande för växtutveckling. Mutanter med defekter i homogalakturonan (en viktig typ av pektin) misslyckades med att bilda en korrekt apikal krok, den böjda struktur som skyddar unga plantor när de växer upp ur jorden. Dessa defekter var kopplade till störningar i hormonbalansen och väcker möjligheten att problem med krokformationen inte bara beror på förändrat pektininnehåll utan också på försämrad adhesion på vävnadsnivå.

Tillsammans visar dessa resultat att celladhesion i växter inte enbart är en fråga om klibbigt pektin i medellamellen, utan en dynamisk och mångfacetterad process. Adhesionen beror på strukturell förstärkning vid cellkanter, på aktiv reglering av sockeromsättning och på korrekt pektinbiosyntes som kopplar cellväggens status till utvecklingsprogram som den apikala kroken. Detta bredare perspektiv framhäver cell-cell-adhesion som en aktivt upprätthållen egenskap som integrerar mekanik, metabolism och signalering för att säkerställa vävnadens integritet och styra växtens tillväxt.

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III

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Research Article

Cell wall integrity modulates *HOOKLESS1* and *PHYTOCHROME INTERACTING FACTOR4* expression controlling apical hook formation

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Abstract

Formation of the apical hook in etiolated dicot seedlings results from differential growth in the hypocotyl apex and is tightly controlled by environmental cues and hormones, among which auxin and gibberellins (GAs) play an important role. Cell expansion is tightly regulated by the cell wall, but whether and how feedback from this structure contributes to hook development are still unclear. Here, we show that etiolated seedlings of the Arabidopsis (*Arabidopsis thaliana*) *quasimodo2-1* (*qua2*) mutant, defective in pectin biosynthesis, display severe defects in apical hook formation and maintenance, accompanied by loss of asymmetric auxin maxima and differential cell expansion. Moreover, *qua2* seedlings show reduced expression of *HOOKLESS1* (*HLS1*) and *PHYTOCHROME INTERACTING FACTOR4* (*PIF4*), which are positive regulators of hook formation. Treatment of wild-type seedlings with the cellulose inhibitor isoxaben (isx) also prevents hook development and represses *HLS1* and *PIF4* expression. Exogenous GAs, loss of DELLA proteins, or *HLS1* overexpression partially restore hook development in *qua2* and isx-treated seedlings. Interestingly, increased agar concentration in the medium restores, both in *qua2* and isx-treated seedlings, hook formation, asymmetric auxin maxima, and *PIF4* and *HLS1* expression. Analyses of plants expressing a Förster resonance energy transfer-based GA sensor indicate that isx reduces accumulation of GAs in the apical hook region in a turgor-dependent manner. Lack of the cell wall integrity sensor THESEUS 1, which modulates turgor loss point, restores hook formation in *qua2* and isx-treated seedlings. We propose that turgor-dependent signals link changes in cell wall integrity to the PIF4-HLS1 signaling module to control differential cell elongation during hook formation.

Introduction

Etiolated seedlings of dicots form an apical hook to protect the meristems during soil emergence. Apical hook formation depends on the differential cell elongation on the opposite sides of the hypocotyl apex, causing the shoot to bend by 180° (Guzmán and Ecker 1990; Abbas et al. 2013). Like most plant developmental processes, hook formation is largely controlled by phytohormones including auxin (Abbas et al. 2013). Shortly after germination, the formation of an auxin response maximum restrains cell expansion on the concave side of the hook, leading to differential cell elongation and eventually shoot bending (Abbas et al. 2013). In Arabidopsis (*Arabidopsis thaliana*), hook formation is positively controlled by the master regulator HOOKLESS1 (HLS1; Guzmán and Ecker 1990; Lehman et al. 1996; Li et al. 2004; Zhang et al. 2018). HLS1 was reported to promote the asymmetric distribution of auxin between the concave and convex sides of the hypocotyl (Lehman et al. 1996) and to reduce the levels of AUXIN RESPONSE FACTOR 2 (ARF2), a repressor of auxin responses (Li et al. 2004). Both apical hook formation and *HLS1* expression are promoted by ethylene and gibberellins (Gas; Lehman et al. 1996; An et al. 2012) and negatively regulated by jasmonates (Song et al. 2014). Regulation of hook formation by GAs is mediated by the degradation of the key repressors DELLA proteins (Sun 2008). When GA levels are low, DELLAs promote the proteasome-mediated degradation of PHYTOCHROME INTERACTING FACTORS (PIFs; Li et al. 2016), a family of transcription factors that positively regulate the expression of *HLS1* (Zhang et al. 2018). In addition, DELLAs inhibit the activity of PIFs by sequestering their DNA recognition domain (de Lucas et al. 2008; Feng et al. 2008). On the other hand, jasmonates can repress hook formation by reducing *HLS1* expression (Zhang et al. 2014) and by repressing PIF function (Zhang et al. 2018). While hormonal signals

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coordinate hook development, their effects ultimately translate into changes in cellular properties, particularly the ability of the cell wall to yield to turgor pressure. Primary cell walls are complex and dynamic networks mainly composed of cellulose, hemicelluloses, and pectin (Cosgrove 2005). Increasing evidence indicates that changes in plant cell wall structural polysaccharides caused either by mutations in biosynthetic genes or by chemicals, like the cellulose inhibitor isoxaben (isx; Heim et al. 1990), impair cell wall integrity (CWI), leading to repression of cell expansion and induction of stress responses (Vaahtera et al. 2019). For instance, etiolated Arabidopsis seedlings with altered cellulose deposition display strongly reduced hypocotyl growth (Fagard et al. 2000) and accumulate high levels of jasmonates (Engelsdorf et al. 2018). Defects in pectin composition also restrict the growth of etiolated hypocotyls. Two Arabidopsis mutants defective for genes required for homogalacturonan (HG) biosynthesis, namely *QUASIMODO1* (*QUA1*), encoding a putative glycosyltransferase (Bouton et al. 2002), and *QUASIMODO2/TUMOROUS SHOOT DEVELOPMENT 2* (*QUA2/TSD2*), encoding a Golgi-localized pectin methyltransferase (Krupková et al. 2007; Mouille et al. 2007; Du et al. 2020), have defects in hypocotyl epidermis cell elongation and cell-to-cell adhesion (Krupková et al. 2007; Mouille et al. 2007; Raggi et al. 2015).

The molecular mechanisms regulating responses triggered by loss of CWI are only partly understood. Several responses triggered by cellulose alterations appear to be mediated by THESEUS 1 (THE1), a member of the *Catharanthus roseus* RLK1-like family of receptor-like kinases (Hématy et al. 2007; Engelsdorf et al. 2018). Perception of changes in pectin composition and activation of downstream responses are less characterized, though the FERONIA (FER) member of CrRLK1L family appears to be a possible sensor of pectin integrity (Feng et al. 2018; Lin et al. 2022). Turgor-sensitive processes appear to be relevant for the detection of CWI changes and the activation of downstream responses that restrict growth. For instance, several responses induced by isx are largely sensitive to osmotic manipulation by cotreatments with osmoticum (Hamann et al. 2009; Engelsdorf et al. 2018). Similarly, cell adhesion and elongation defects in *qua1* are suppressed by reducing external water potential via increased agar concentration in the growth medium (Verger et al. 2018).

Increasing evidence suggests that a feedback loop between auxin and cell wall composition regulates apical hook formation in Arabidopsis (Aryal et al. 2020; Baral et al. 2021; Jonsson et al. 2021). In particular, pectin composition seems to be associated to auxin response gradients and differential cell elongation during hook development (Jonsson et al. 2021). When auxin accumulates in the inner side of the hypocotyl, it promotes HG methylesterification, which correlates with a reduction in cell elongation (Jonsson et al. 2021). On the other hand, loss of asymmetric HG methylesterification in plants overexpressing a pectin methylesterase inhibitor alters the polar auxin transport machinery, disrupting the auxin gradient and resulting in a defective hook (Jonsson et al. 2021). In addition, alterations in other cell wall structural components, including cellulose (Sinclair et al. 2017; Baral et al. 2021) and xyloglucans (Aryal et al. 2020), also impair apical hook formation, suggesting that changes in various wall structural components converge into common responses that restrict differential cell elongation. However, the exact mechanisms linking CWI perception to the events that regulate hook development are not fully elucidated. Here, we report that loss of CWI represses a GA-modulated signaling module that comprises PIF4 and HLS1, resulting in a defective apical hook, and that these effects are suppressed by reduction of turgor pressure caused by low extracellular water potential. Our results suggest that turgor-dependent responses to altered CWI directly modulate signaling events that control differential cell expansion during hook formation.

Results

Defects in pectin biosynthesis impair hook formation and maintenance in a turgor-dependent manner

Apical hook formation was examined in a panel of Arabidopsis mutants impaired in different cell wall polysaccharides to determine the relative impact of changes in specific wall components on this process. Under our experimental conditions, 3 d after germination, etiolated wild-type (WT) seedlings displayed a completely closed hook (Fig. 1, A and B), which, in contrast, was completely open in *qua2-1* (henceforth, *qua2*) as well as in 2 other mutants affected in pectin composition, *gae1 gae6* and *murus1* (*mur1*; Fig. 1, A and B). The *gae1 gae6* double mutant carries mutations in 2 glucuronate 4-epimerases (GAEs) required for the biosynthesis of UDP-D-galacturonic acid (Mølhøj et al. 2004) and is defective in HG (like *qua2*) and, possibly, rhamnogalacturonan I (RG-I) biosynthesis (Bethke et al. 2016), while *mur1* is impaired in fucose biosynthesis (Bonin et al. 1997) and has therefore defective RG-II, xyloglucans, and cell wall glycoproteins (Reiter et al. 1993; Rayon et al. 1999; Freshour et al. 2003). In contrast, no significant difference in hook formation was observed in other cell wall mutants, namely *korrigan1* (*kor1*), impaired in primary cell wall cellulose deposition (Nicol et al. 1998), and *mur4* and *mur7* (Fig. 1, A and B), impaired in the biosynthesis of arabinose (Reiter et al. 1997; Burget et al. 2003), with the exception of *procuste1* (*prc1*; Desnos et al. 1996), that showed only a mild defect (Fig. 1, A and B). Taken together, these results suggest that mutations in genes involved in HG biosynthesis have a major impact on hook formation compared to genetic defects affecting other wall components.

Turgor pressure affects the activation of several responses triggered by loss of CWI (Hamann et al. 2009; Engelsdorf et al. 2018). To verify if turgor-dependent responses mediate the effects of altered pectin composition on hook formation and to determine what phases of this process are specifically affected, kinematic analysis was performed in WT, *qua2*, *gae1 gae6*, and *mur1* seedlings grown in the dark on medium containing 0.8% (w/v) or 2.5% (w/v) agar (henceforth indicated as low agar [LA] and high agar [HA], respectively). This method has been previously implemented to modulate turgor pressure in a controlled manner (Verger et al. 2018). WT seedlings grown on LA displayed typical hook development (Abbas et al. 2013), consisting in a formation phase, in which seedlings emerge from the seed and the hook angle reaches roughly 180° before 24 h after germination, followed by a maintenance phase, in which the hook is kept closed for about 48 h, and culminating in the opening phase, in which the hook opens reaching an angle of 0° (Fig. 2, A to C). In contrast, all mutants grown on LA showed a formation phase comparable, in length, to the WT, but were unable to form a fully closed hook (Fig. 2, A to C). Moreover, the maintenance phase was deeply compromised in all mutants, leading to hook opening right after the maximum curvature was achieved (Fig. 2, A to C).

When WT seedlings were grown on HA, formation and maintenance of the hook were largely unaffected, though the opening phase was accelerated (Fig. 2, A to C). Notably, growth on HA partially restored hook formation in all mutant lines (Fig. 2, A to C), leading to a significant increase in the maximum angle of curvature (Supplementary Fig. S1). In addition, HA also rescued the maintenance phase in *mur1* seedlings (Fig. 2B). Hook development could also be restored by sorbitol, an osmolyte previously shown

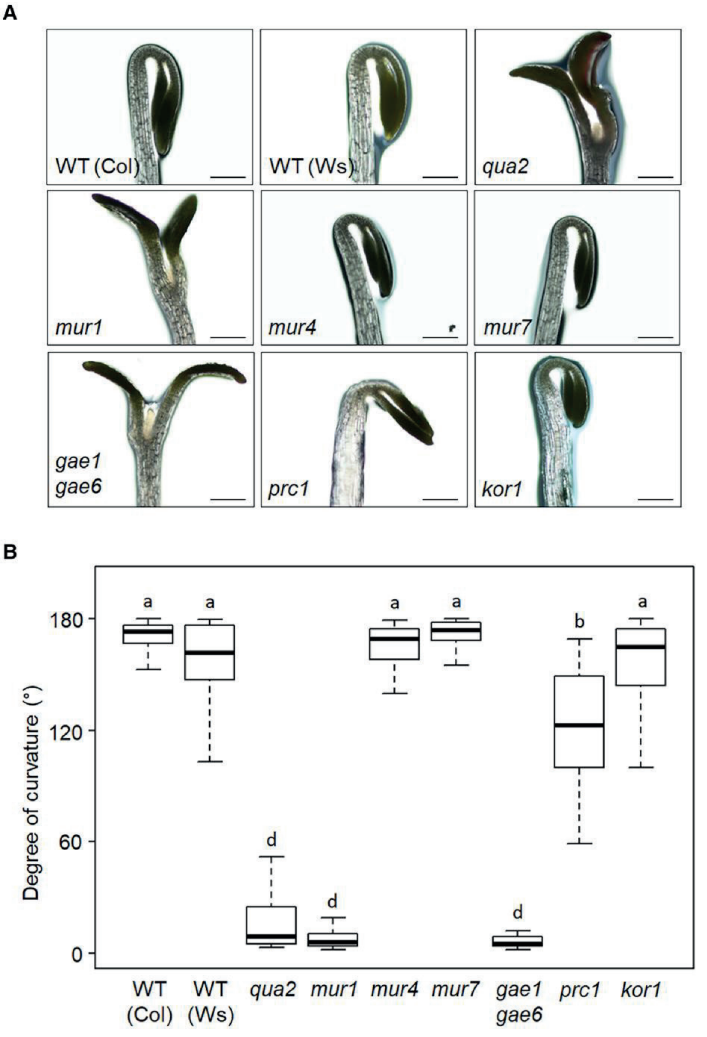

Figure 1. Apical hook formation in Arabidopsis cell wall mutants. **A)** Representative pictures of WT Col, WT Ws, *qua2*, *mur1*, *mur4*, *mur7*, *gae1 gae6*, *prc1* (in Col-0 background), and *kor1* (in Ws background) 3 d after germination. Scale bars in all panels, 0.5 mm. **B)** Quantification of apical hook angles of seedlings grown as in **A)**. Box plots indicate the 1st and 3rd quartiles split by median; whiskers show range ($n \geq 20$). Letters indicate statistically significant differences ($P < 0.05$) according to 1-way ANOVA followed by post hoc Tukey's HSD.

to suppress other responses induced by cell wall damage (Hamann et al. 2009; Engelsdorf et al. 2018; Supplementary Fig. S2). Taken together, these results indicate that the hook formation in seedlings with altered pectin composition is rescued under conditions that reduce turgor pressure.

Loss of pectin integrity disrupts differential cell expansion and asymmetric auxin response during apical hook development

Hook formation is thought to be largely dependent on the differential elongation rate of epidermal cell on the 2 sides of the hypocotyl (Silk and Erickson 1978). Defects in *QUA2* restrict cell expansion in the epidermis of adult leaves (Raggi et al. 2015), suggesting that alterations in cell expansion rates might also occur in the epidermis of the hypocotyl of etiolated seedlings with altered pectin composition, resulting in a defective hook. Individual cell elongation rates were therefore measured in the apical portion of the hook of WT and, as illustrative of loss of pectin integrity, *qua2* seedlings grown in the dark in LA and HA conditions. As expected, cell expansion rate in WT seedlings was lower on the inner side than on the outer side of the hypocotyl, either in LA or HA condition (Fig. 3, A and B). In contrast, *qua2* seedlings showed a significant reduction in the expansion rate

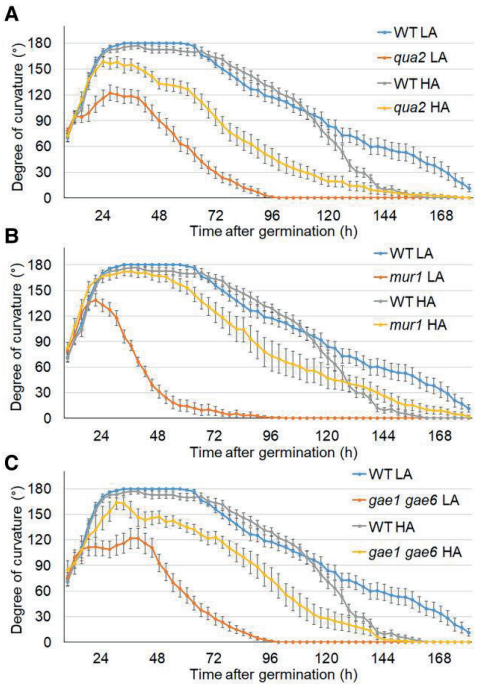

Figure 2. Kinematic analysis of apical hook formation in pectin mutants grown on LA and HA. WT and *qua2* **A)**, *mur1* **B)**, or *gae1 gae6* **C)** mutant seedlings were grown in the dark on medium containing either 0.8% (w/v) (LA) or 2.5% (w/v) agar (HA). The hook angle was measured at the indicated times. Error bars represent mean angle ± SE ($n \geq 15$).

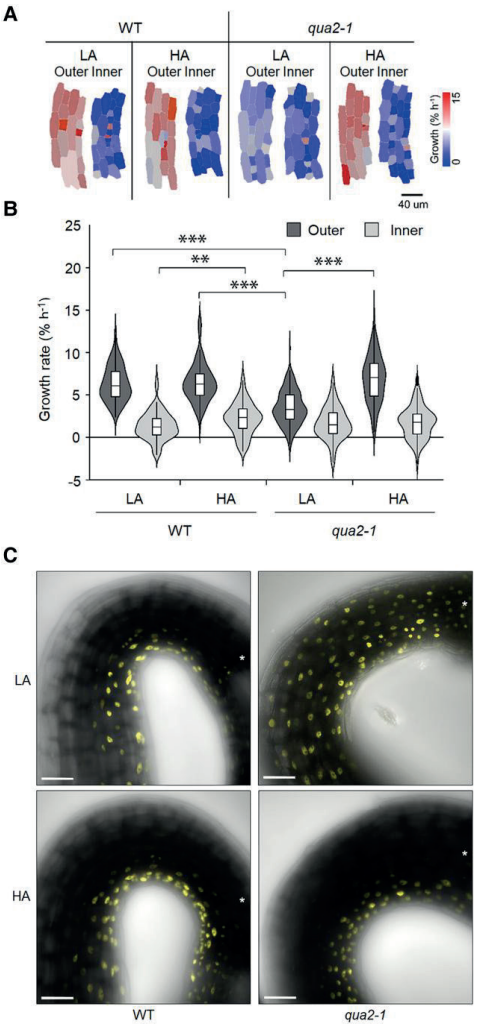

Figure 3. Effects of agar concentration on cell elongation and auxin response during apical hook formation in *qua2* seedlings. **A)** Heatmaps of the growth rate of individual cells in the apical portion of the hypocotyl upon a 3-h time lapse in WT and *qua2* seedlings grown in the dark on medium containing 0.8% (LA) or 2.5% (HA) (w/v) agar. **B)** Quantification of the growth rate of individual cells in the outer and inner sides of the hypocotyl of seedlings grown as in **A)**. Data are average of 3 independent biological replicates ± SD. In violin plots, the box limits represent the 1st and 3rd quartiles split by median, and whiskers show range. For each experiment, 15 cells from both the inner and outer sides of the hook were measured from each of 9 individual seedlings. Asterisks indicate statistical significance by Student's *t* test (**$P < 0.01$; ***$P < 0.001$). **C)** Representative confocal laser scanning microscopy images of WT and *qua2* seedlings expressing the DR5::Venus-NLS and grown in the dark on LA or HA. Asterisks in **C)** mark position of SAM. Scale bars in all panels, 50 μm.

in the outer side of the hook when grown on LA, but not on HA (Fig. 3, A and B).

As differential cell expansion is dependent on the establishment of an auxin gradient at the 2 sides of the apex (Abbas et al. 2013), the distribution of auxin response was evaluated in WT and *qua2* seedlings expressing the auxin response reporter *DR5*-VENUS-NLS (Heisler et al. 2005). WT seedlings displayed a strong fluorescent signal predominantly in the inner epidermal cells of the hook, and this pattern was not affected by the agar concentration in the medium (Fig. 3C). In contrast, reporter expression was equally distributed on both sides of the hypocotyl of *qua2* seedlings grown in LA (Fig. 3C). This alteration was fully restored when the mutant was grown on HA (Fig. 3C). Taken together, our results indicate that turgor-dependent responses to altered HG hinder proper asymmetric auxin signaling gradient and differential cell expansion during hook formation.

Loss of pectin integrity represses *HLS1* and *PIF4* expression and alters the expression of genes involved in GA homeostasis

HLS1 combines upstream stimuli important for hook formation (Guzmán and Ecker 1990), negatively regulating ARF2 levels (Li et al. 2004) and influencing auxin distribution (Lehman et al. 1996). Hook formation is also positively modulated by PIFs and, in particular, PIF4, which directly binds to the promoter of *HLS1* to activate its transcription (Zhang et al. 2018). We therefore evaluated if a defective pectin composition might affect the

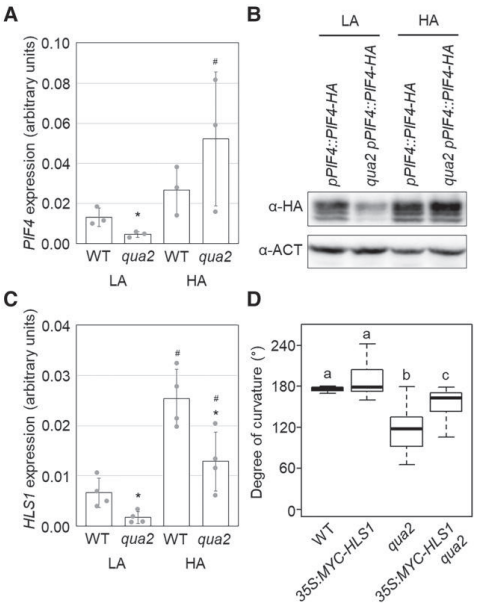

Figure 4. *HLS1* and *PIF4* expression in *qua2* mutant. Total RNA was extracted from WT and *qua2* seedlings 2 d after germination grown in the dark on medium containing 0.8% (LA) or 2.5% (HA) agar (w/v). **A)** Expression of *PIF4* was analyzed by RT-qPCR, using *UBQ5* as a reference. **B)** Transgenic lines expressing PIF4-HA under the control of its native promoter (ProPIF4:PIF4-3×HA) in *pif4*-101 or *qua2*-1 background were grown on LA or HA medium. PIF4-HA levels were detected by immunoblot analysis with an antibody against HA; an antibody against actin (ACT) was used as a loading control. **C)** Expression of *HLS1* was analyzed by RT-qPCR, using *UBQ5* as a reference. Bars (in **A** and **C**) indicate mean of at least 3 independent biological replicates ± SD. Asterisks indicate statistically significant differences with WT according to Student's *t* test (*$P < 0.05$), and number signs indicate statistically significant differences with LA between same genotype according to Student's *t* test (#$P < 0.05$). **D)** Quantification of apical hook angles of WT, 35S:Myc-HLS1/*hls1-1*, *qua2*, and *qua2* 35S:Myc-HLS1/*hls1-1* seedlings 2 d after germination grown in the dark. Box plots in **D)** indicate the 1st and 3rd quartiles split by median; whiskers show range ($n \geq 20$). Letters indicate statistically significant differences ($P < 0.05$) according to 1-way ANOVA followed by post hoc Tukey's HSD.

expression of the genes encoding these proteins. In *qua2* seedlings grown under LA conditions, *PIF4* transcript levels were sharply reduced, compared to the WT, but increased to levels comparable to the WT under HA conditions (Fig. 4A). Consistently, *qua2* seedlings transformed with a HA-tagged version of PIF4 under the control of its native promoter (Zhang et al. 2017) displayed, under LA conditions, reduced levels of protein that strongly increased and reached levels comparable to the wild type when seedlings were grown in HA (Fig. 4B). Transcript levels of *HLS1* were also significantly reduced in etiolated *qua2* seedlings grown in LA, in comparison to the wild type, and significantly increased in both genotypes under HA conditions (Fig. 4C). These results suggest that reduced expression of *HLS1* might impair proper hook formation in *qua2*, and that its increased expression under HA conditions might restore it. Consistently, 2-d-old *qua2* seedlings expressing a myc-tagged version of HLS1 under the control of the constitutive CaMV 35S promoter (Shen et al. 2016) and grown under LA conditions displayed significantly greater hook angle than untransformed mutant seedlings (Fig. 4D).

Hook formation and *HLS1* expression are both positively regulated by GAs (An et al. 2012). We therefore evaluated if loss of pectin integrity might affect the expression of genes involved in the homeostasis of these hormones. Etiolated *qua2* seedlings grown on LA showed reduced transcript levels for *GA20ox1* and *GA3ox1*, required for GA biosynthesis (Hedden and Phillips 2000; Fig. 5, A and B), and increased expression of *GA2ox2*, involved in GA catabolism (Hedden and Phillips 2000; Fig. 5C). In contrast, under HA conditions, expression of these genes in WT and *qua2* seedlings was comparable (Fig. 5, A to C). Furthermore, exogenous GAs restored almost WT-like hook formation in *qua2* mutants grown on LA (Fig. 5D). Consistently, loss of all Arabidopsis *DELLA* genes (Feng et al. 2008) in the *qua2* background partially restored hook formation (Fig. 5E). Taken together, these results suggest that responses triggered by loss of pectin integrity, and dependent on turgor pressure, repress the GA-dependent PIF4-HLS1 signaling module, hindering proper hook formation.

isx inhibits hook formation and represses *HLS1* and *PIF4* expression and GA accumulation in a turgor-dependent manner

Our results indicate that defects in pectin composition caused by the *qua2* mutation induce responses dependent on turgor pressure that suppress GA-dependent signaling events important for hook formation. As *prc1*, impaired in the cellulose synthase CESA6 (Desnos et al. 1996), shows a partially defective hook (Fig. 1), we hypothesized that also defects in cellulose might have the same effects. To verify this hypothesis, a pharmacological approach was adopted, growing etiolated WT seedlings in the presence of isx, which targets cellulose synthases, including CESA6 (Desprez et al. 2002). Under LA conditions, at 2 d after germination, seedlings grown in the presence of isx at concentrations equal to or higher than 2.5 nM showed strongly reduced hook curvature (Fig. 6, A and B). HA conditions restored hook formation in the presence of isx at a dose of 2.5 nM and, to a lesser extent, 5.0 nM (Fig. 6, A and B). Analysis of WT seedlings expressing *DR5*-VENUS-NLS showed that, as observed in *qua2*, isx disrupted asymmetric auxin distribution in LA, but not in HA conditions (Fig. 6C). Consistently, in the presence of isx, cells on the outer side of the hook region of the hypocotyl showed a significant decrease in expansion rate, compared to control seedlings, only in LA, but not in HA conditions (Fig. 6, D and E). Overall, these data suggest that, as observed in *qua2*, also defects in cellulose deposition impair hook formation, disturbing the formation of an auxin asymmetric distribution and repressing cell elongation on the outer side of the hook.

Furthermore, as in *qua2*, isx repressed the expression of *HLS1* under LA, but not HA conditions (Fig. 7A). Notably, overexpression of a myc-tagged version of HLS1 in *hls1-1* seedlings (Shen et al. 2016) was sufficient to restore a fully closed hook in the presence of 2.5 nM isx (Fig. 7B). Treatments with isx also reduced *PIF4* transcript accumulation under LA, but not HA conditions (Fig. 7C). Consistently, isx repressed accumulation of PIF4 protein in a dose-dependent manner, but this effect was reduced under HA conditions (Fig. 7D). As observed in *qua2* seedlings, the expression of the biosynthetic *GA3ox1* and *GA20ox1* was repressed in plants treated with isx only in LA conditions, whereas expression in mock- and isx-treated seedlings was comparable in HA conditions (Supplementary Fig. S3, A to C). Moreover, exogenous GAs partially restored hook formation in seedlings treated with isx

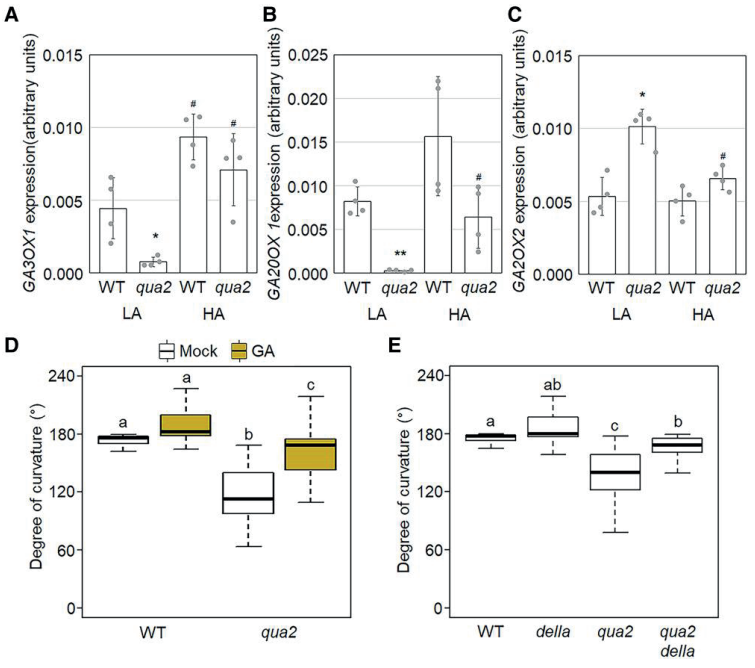

Figure 5. Defects in the expression of GA biosynthetic genes in *qua2* mutant. Expression of *GA3ox1* **A)**, *GA20ox1* **B)**, and *GA2ox2* **C)** in WT and *qua2* seedlings grown in LA and HA. Transcript levels were determined by RT-qPCR using *UBQ5* as a reference. Bars indicate mean of at least 3 independent biological replicates ± SD. Asterisks indicate statistically significant differences with WT according to Student's *t* test (*$P < 0.05$; **$P < 0.01$), and number signs indicate statistically significant differences with LA between same genotype according to Student's *t* test (#$P < 0.05$). **D)** Apical hook angles of WT and *qua2* seedlings 2 d after germination grown in the dark on medium supplemented with ethanol (mock) or 50 μM GA_4 (GA). **E)** Apical hook angles of WT Ler, *della*, *qua2*, and *qua2 della* sixtuple mutant seedlings 2 d after germination grown in the dark. Box plots in **D and E)** indicate the 1st and 3rd quartiles split by median; whiskers show range ($n \geq 20$). Letters indicate statistically significant differences, according to 2-way ANOVA followed by post hoc Tukey's HSD ($P < 0.05$).

(Fig. 8A). Hook formation in both the pentuple *della* mutant and in a *ga2ox* heptuple mutant, impaired in the GA catabolic GA2 oxidases GA2ox1/2/3/4/6/7/8 and therefore showing increased levels of active GAs in seedling hypocotyls (Griffiths et al. 2023), was less sensitive to isx (Fig. 8, B and C), suggesting that an alteration in GA homeostasis might contribute to the inhibition of hook formation in response to loss of CWI. To further investigate this hypothesis, we analyzed in vivo GA levels in response to isx using the Förster resonance energy transfer (FRET) biosensor Gibberellin Perception Sensor 2 (GPS2; Griffiths et al. 2023). Under LA conditions, GA levels in the hook region of the hypocotyl decreased in response to isx in a dose-dependent manner, while under HA conditions, GA levels appeared to be similar in control- and isx-treated seedlings (Fig. 8, D and E). These results indicate that, as in the case of *qua2*, isx downregulates GA-dependent signaling events that modulate *PIF4* and *HLS1* expression and control hook formation, suggesting a common mechanism underlying the effects of loss of CWI caused by alterations in different cell wall components on hook development.

As THE1 is a major player in the activation of responses triggered by altered cellulose deposition (Bacete and Hamann 2020), we evaluated if this protein is also important for the inhibition of apical hook formation mediated by isx. Indeed, hook curvature in 2 loss-of-function *the1-1* and *the1-6* mutants (Hématy et al. 2007; Merz et al. 2017) was less sensitive to isx both in LA and HA conditions (Fig. 9A). Conversely, the gain-of-function *the1-4* mutant (Merz et al. 2017) showed increased sensitivity to isx both in HA and LA media (Fig. 9A). Notably, both the *the1-1* and the *the1-6* mutations fully restored hook development in *qua2* seedlings (Fig. 9B). These results indicate that responses mediated by THE1 contribute to the defective hook development in plants with altered CWI.

Jasmonates are not involved in defective hook formation caused by altered CWI

isx induces the accumulation of jasmonates in Arabidopsis seedlings in a THE1-dependent manner (Engelsdorf et al. 2018). As exogenous jasmonic acid (JA) antagonizes apical hook formation in etiolated seedlings (Song et al. 2014; Zhang et al. 2014), we hypothesized that the hook defect observed in response to loss of CWI might be mediated by increased jasmonate levels. Levels of JA, jasmonyl-L-isoleucine (JA-Ile) and of the JA-derivative 11- and 12-hydroxyjasmonate (Σ 11-/12-OHJA, sum of unresolved 11- and 12-OHJA), were therefore quantified in dark-grown WT and *qua2* seedlings. Under LA conditions, mutant seedlings contained higher levels of all 3 jasmonates, compared to the wild type (Fig. 10A). Under HA conditions, the concentration of JA in WT seedlings was unaltered, while JA-Ile and Σ 11-/12-OHJA levels were moderately increased (Fig. 10A). Growth on HA medium

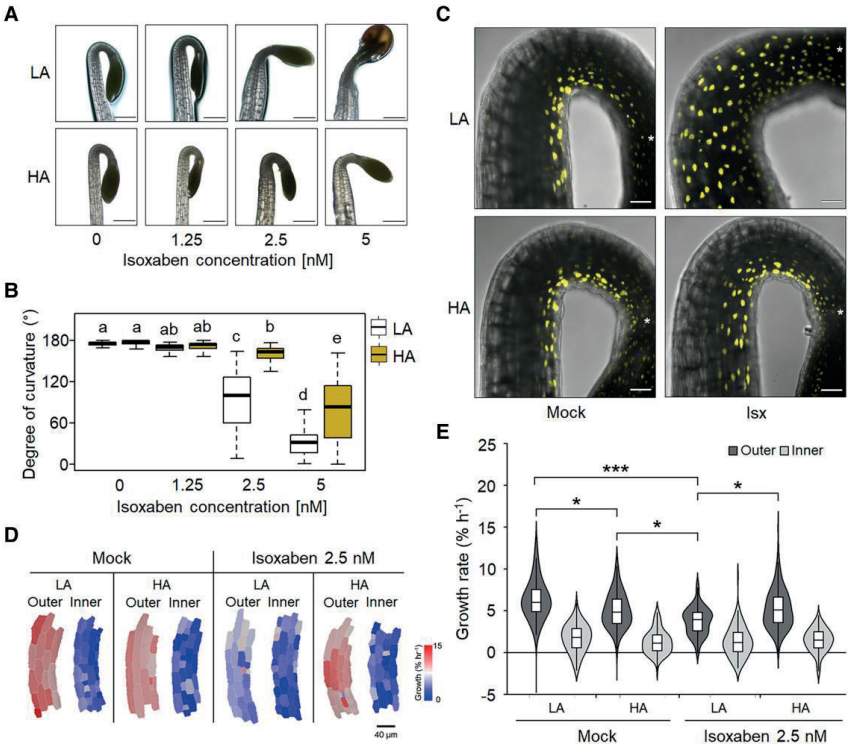

Figure 6. isx inhibits apical hook formation in a turgor-dependent manner. **A)** Representative pictures of WT seedlings 2 d after germination grown in the dark on medium 0.8% (LA) or 2.5% (HA) agar (w/v) and supplemented with isx at the indicated doses. Scale bars in all panels, 0.5 mm. **B)** Quantification of apical hook angles of WT seedlings grown as in **A)**. Box plots in **B)** indicate the 1st and 3rd quartiles split by median; whiskers show range ($n \geq 20$). Letters indicate statistically significant differences, according to 2-way ANOVA followed by post hoc Tukey's HSD ($P < 0.05$). **C)** Representative confocal laser scanning microscopy images of WT seedlings expressing the DR5::Venus-NLS grown in the dark with 2.5 nM isx in the dark on LA or HA. Asterisks in **C)** mark the position of SAM. Scale bars in all panels, 50 μm. **D)** Heatmaps of the growth rate of individual cells in the apical portion of the hypocotyl upon a 3-h time lapse in WT grown in the dark on medium containing 0.8% (LA) or 2.5% (HA) (w/v) agar supplemented with 2.5 nM isx. **E)** Quantification of the growth rate of individual cells in the outer and inner sides of the hypocotyl of seedlings grown as in **D)**. Data are average of 3 independent biological replicates ± SD. In violin plots, the box limits represent the 1st and 3rd quartiles split by median, and whiskers show range. For each experiment, 15 cells from both the inner and outer sides of the hook were measured from each of 9 individual seedlings. Asterisks indicate statistical significance by Student's *t* test (*$P < 0.05$; ***$P < 0.001$).

significantly reduced JA and JA-Ile levels in *qua2*, while Σ 11-/12-OHJA concentration in the mutant was slightly increased (Fig. 10A).

To assess whether high levels of jasmonates are responsible for the altered hook formation of *qua2*, this mutant was crossed with lines defective for *JASMONATE RESISTANT 1* (*JAR1*), required for the synthesis of JA-Ile (Wasternack and Hause 2013), or *CORONATINE INSENSITIVE 1* (*COI1*), a crucial component of the SCF COI1 E3 ubiquitin complex necessary for JA-Ile perception and transduction (Wasternack and Hause 2013). In *qua2 coi1* seedlings, 2 d after germination, hook impairment was slightly exacerbated (Fig. 10B), while the *qua2 jar1* double mutant did not show differences in hook angle, compared to *qua2* (Fig. 10C). Consistently, *jar1* and *coi1* single mutants treated with isx displayed hook defects comparable to those observed in the wild type (Fig. 10D). These results indicate that, despite loss of CWI triggers the accumulation of elevated levels of jasmonates in a turgor-dependent manner, these hormones do not contribute to the observed defects in hook formation.

Taken together, our results suggest that, in plants with altered CWI, turgor-dependent responses suppress, in a THE1-dependent manner, GA-mediated downstream signaling events controlling *PIF4* and *HLS1* expression. This leads to the disruption of auxin response asymmetry, differential cell elongation, and proper hook formation (Fig. 11).

Discussion

Cell wall alterations impair differential cell elongation during apical hook formation in a turgor-dependent manner

Differential cell elongation is widely used in plants to adapt growth and development to external and endogenous signals. This is exemplified by apical hook formation, which is largely dependent on the differential cell elongation on the opposite sides of the hypocotyl apex (Guzmán and Ecker 1990; Abbas et al. 2013). Cell elongation results from the interplay between turgor pressure and cell wall elasticity and extensibility (Ray et al. 1972). It is

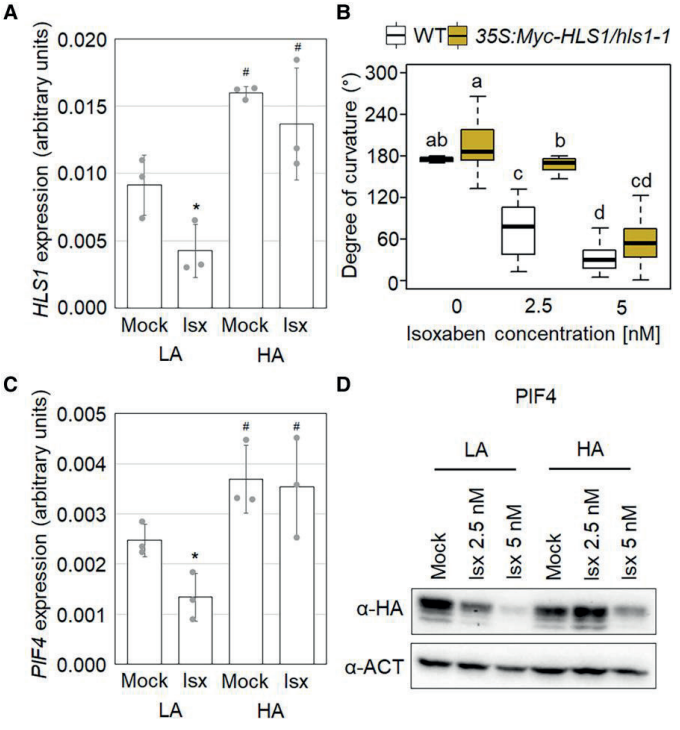

Figure 7. isx inhibits *PIF4* and *HLS1* expression in a turgor-dependent manner. **A)** Expression of *HLS1* in WT seedlings 2 d after germination grown in the dark with 2.5 nM isx in LA and HA. Transcript levels were determined by RT-qPCR using *UBQ5* as a reference. **B)** Quantification of apical hook angles of WT and 35S:Myc-HLS1/*hls1-1* seedlings 2 days after germination grown in the dark in the presence of the indicated concentrations of isx. **C)** Expression of *PIF4* in WT seedlings 2 d after germination grown in the dark with 2.5 nM isx in LA and HA. Transcript levels were determined by RT-qPCR using *UBQ5* as a reference. **D)** Transgenic lines expressing PIF4-HA under the control of its native promoter (ProPIF4:PIF4-3×HA) in *pif4*-101 background were grown on LA or HA medium supplemented with the indicated concentrations of isx. PIF4-HA levels were detected by immunoblot analysis with an antibody against HA; an antibody against actin (ACT) was used as a loading control. Bars in **A and C)** indicate mean of at least 3 independent biological replicates ± SD. Asterisks indicate statistically significant differences between mock- and isx-treated seedlings according to Student's *t* test ($^{*}P < 0.05$); number signs indicate statistically significant differences between similarly treated seedlings grown on LA or HA according to Student's *t* test ($^{\#}P < 0.05$). Letters in **B)** indicate statistically significant differences according to 2-way ANOVA followed by post hoc Tukey's HSD ($P < 0.05$). Box plots indicate the 1st and 3rd quartiles split by median; whiskers show range ($n \geq 20$).

therefore not surprising that cell wall composition has a major impact on hook formation, and that an extensive interplay occurs between cell walls and the hormonal networks controlling hook formation (Aryal et al. 2020; Jonsson et al. 2021). However, despite our considerable knowledge of the signaling pathways controlling hook development, little is known of how cell walls interact with these pathways to modulate differential cell expansion and hook bending. Here, we have shown that changes in CWI, either caused by mutations in genes affecting pectin composition or by interference with cellulose deposition triggered by isx, hinder hook formation in Arabidopsis seedlings in a turgor-dependent manner. Moreover, altered CWI compromises, again in a turgor-dependent manner, asymmetric auxin maxima formation and differential cell elongation in the hook region. Additionally, turgor-mediated responses triggered by altered CWI downregulate hook-promoting signaling events that are positively regulated by GAs and include PIF4 accumulation and *HLS1* expression (Fig. 11). These results suggest that turgor pressure links CWI to GA-dependent signaling to modulate hook formation and maintenance.

Cell wall assembly and remodeling must be finely controlled during growth processes to ensure proper cell expansion while maintaining mechanical integrity (Wolf et al. 2012). Moreover, alterations in CWI can occur in response to abiotic or biotic stress (Vaahtera et al. 2019; Lorrai and Ferrari 2021); therefore, the structural and functional integrity of the wall must be constantly monitored and fine-tuned to allow normal growth and development under physiological conditions while preventing mechanical failure under adverse conditions (Rui and Dinneny 2020). Increasing evidence points to the role of turgor-mediated responses in triggering several effects of loss of CWI on plant growth and development (Engelsdorf et al. 2018; Verger et al. 2018). Indeed, plant cells must sustain huge turgor pressures, and their connection with each other, which is mediated by the cell wall, allows the propagation of signals generated by turgor pressure and by differential growth (Jonsson et al. 2022). Plants with altered CWI may fail to counterbalance turgor pressure, causing mechanical stress and triggering downstream compensatory responses. Indeed, supplementation with osmolytes, like sorbitol, or

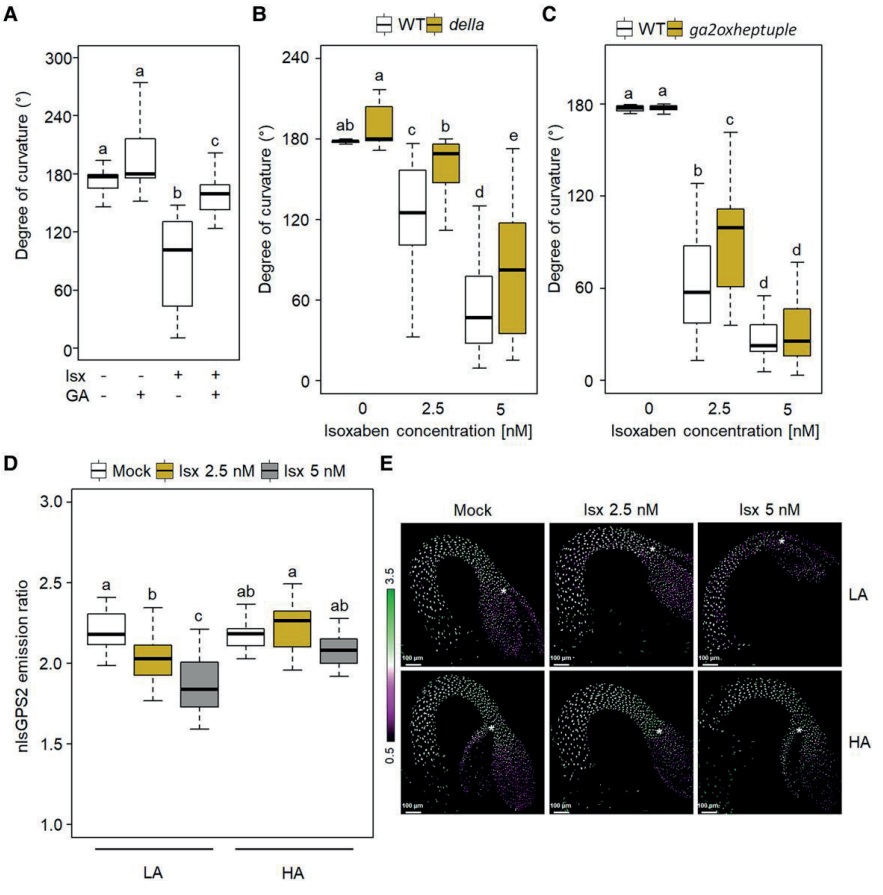

Figure 8. isx inhibits GA accumulation and signaling in a turgor-dependent manner. **A)** Apical hook angles of WT seedlings 2 d after germination grown in the dark and treated with DMSO or 2.5 nM isx in the presence or absence of 50 μM GAs. **B)** Apical hook angles of WT Ler and *della* seedlings 2 d after germination grown in the dark in the presence of isx at the indicated doses. **C)** Apical hook angles of WT Col-0 and *GA2oxheptuple* seedlings 2 d after germination grown in the dark in the presence of isx at the indicated doses. **D)** nlsGPS2 nuclear emission ratios from $n \geq 8$ hypocotyls of seedlings 1 d after germination grown in the dark in the presence of the indicated amount of isx on medium containing 0.8% (LA) or 2.5% (HA) agar (w/v). **E)** Representative images of nlsGPS2 emission ratios of the hypocotyls of seedlings grown as in **D)**. Letters in **A to D)** indicate statistically significant differences according to 2-way ANOVA followed by post hoc Tukey's HSD ($P < 0.05$). Box plots indicate the 1st and 3rd quartiles split by median; whiskers show range ($n \geq 20$) in **A to C)** ($n \geq 12$) in **D)**. Asterisks in **E)** mark position of SAM. Scale bars in all panels, 100 μm.

increasing medium agar concentrations have been previously exploited to decrease turgor pressure and restore growth in plants with perturbed cell walls (Engelsdorf et al. 2018; Verger et al. 2018; Bacete et al. 2022). We have found that both sorbitol and HA restore hook development in plants with altered pectin composition (Fig. 2; Supplementary Figs. S1 and S2). Analysis of cell growth rate showed that the impaired hook formation phase observed in *qua2* or in isx-treated seedlings is accompanied by a reduction of cell elongation rate in the outer cell layer and that WT-like growth rate was restored when seedlings were grown in HA condition (Figs. 3, A and B, and 6, D and E), further supporting the hypothesis that the compromised hook formation observed in plants with altered CWI is largely mediated by turgor-dependent mechanisms.

It has been proposed that loss of cell adhesion in plants with altered HG is a consequence of excessive tension in the epidermis caused by mechanical stress (Verger et al. 2018). Moreover, tension-mediated signals triggered by altered pectin composition might induce compensatory mechanisms that restrict cell expansion and therefore relieve mechanical stress. We have previously observed that the reduced cell expansion observed in *qua2* seedlings is at least partly mediated by an increased expression of *AtPRX71*, encoding a ROS-generating apoplastic peroxidase, which is also involved in H_2O_2 production in response to isx (Raggi et al. 2015). Notably, *AtPRX71* expression is also induced by hypoosmolarity (Rouet et al. 2006), a condition leading to excessive turgor pressure. This suggests that turgor-dependent responses triggered by altered CWI might lead to compensatory mechanisms, possibly

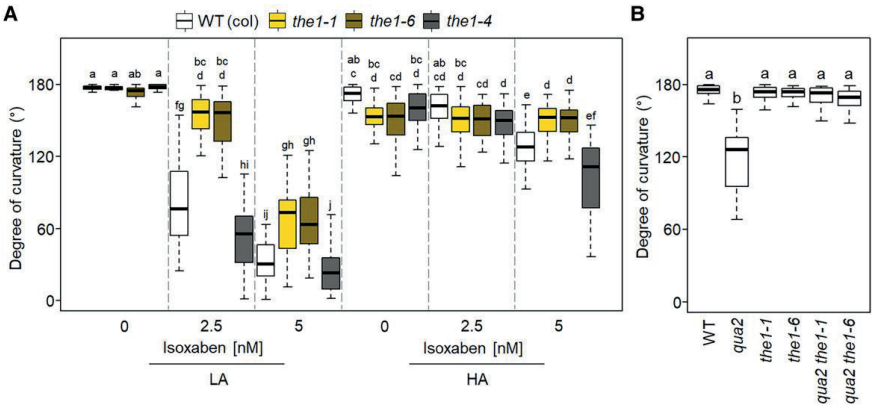

Figure 9. Apical hook inhibitions by isx supplementation or *qua2* mutation is dependent on THE1. **A)** Quantification of apical hook angles of WT (Col-0), *the1-1*, *the1-6*, and *the1-4* seedlings 2 d after germination grown in the dark on medium 0.8% (LA) or 2.5% (HA) agar (w/v) and supplemented with isx at the indicated doses. **B)** Quantification of apical hook angles of WT, *qua2*, *the1-1*, *the1-6*, *qua2 the1-1*, and *qua2 the1-6* seedlings 2 d after germination grown in the dark. Letters indicate statistically significant differences according to 2-way ANOVA followed by post hoc Tukey's HSD ($P < 0.05$). Box plots indicate the 1st and 3rd quartiles split by median; whiskers show range ($n \geq 20$).

including peroxidase-mediated cell wall crosslinking, that ultimately restrict cell expansion. Such mechanisms might take place also during apical hook formation, causing the turgor-dependent defect in differential cell expansion observed in *qua2* and in isx-treated seedlings.

The observation that both the *qua2* mutation and isx impair proper hook formation under LA but not HA conditions indicates that loss of CWI caused by alterations in either HG or cellulose trigger turgor-dependent signals that hinder differential cell expansion. However, the exact nature of these signals still needs to be clarified. It has been proposed that loss of CWI results in distortion or displacement of the plasma membrane relative to the cell wall that can be detected by a dedicated CWI maintenance mechanism (Engelsdorf et al. 2018). Our results suggest that THE1 plays an important role in mediating pectin- and isx-triggered inhibition of hook formation, possibly controlling the activation of responses that lead to reduced cell expansion. It has been recently proposed that THE1 might indirectly influence changes in cell wall stiffness in response to ISX/sorbitol cotreatments, possibly as a consequence of THE1 function in modulating responses to ISX (Bacete et al. 2022). Further investigation will provide insights into the role of specific components of the CWI maintenance system in modulating differential cell expansion during hook formation.

Loss of CWI represses a signaling module that promotes apical hook development

Differential elongation during hook development requires the formation of an auxin gradient, reaching a maximum on the inner side of the hook where it reduces the cell growth rate (Abbas et al. 2013). The cell wall is a key hub in this process, as a positive feedback loop mechanism couples cell wall stiffness, mediated by changes in the degree of methylesterification of HG, with auxin redistribution (Jonsson et al. 2021). However, the mechanisms linking changes in cell wall properties and the signaling pathways that modulate differential cell expansion are poorly understood. Our results suggest that loss of CWI represses a signaling module, comprising PIF4 and HLS1, that positively regulates auxin biosynthesis and distribution and ultimately hook formation (Lehman et al. 1996; Franklin et al. 2011; Zhang et al. 2018). HLS1 suppresses the accumulation of ARF2 (Li et al. 2004), which negatively regulates hook formation and transcriptional control of auxin transporters downstream of xyloglucan defects (Aryal et al. 2020). We observed that mutants with altered pectin composition and seedlings treated with isx show a reduction of *HLS1* and *PIF* transcript levels (Figs. 4, A and C, and 7, A and C) and of PIF4 protein levels (Figs. 4B and 7D). The downregulation of *HLS1* and *PIF4* might contribute to the disruption of asymmetric auxin maxima and differential cell expansion observed in *qua2* and might also contribute to the hook defect caused by altered cellulose deposition, as HLS1 overexpression confers partial resistance to the inhibitory effect of isx (Fig. 7B) and of *qua2* mutation (Fig. 4D). These observations point to a common regulation of hook formation in response to changes in different cell wall components.

Mechanical stress arising from turgor pressure changes can activate JA-mediated stress responses in plants with altered CWI (Engelsdorf et al. 2018). Recently, it has been proposed that JA-Ile accumulation in the roots of the *kor1* mutant is prompted by turgor-driven mechanical compression at the level of the cortex (Mielke et al. 2021). We found that *qua2* seedlings accumulate high levels of jasmonates, which decrease when the mutant is grown in HA conditions (Fig. 10A), confirming that cell wall stress-induced JA production is mediated by turgor pressure changes. However, JA signaling does not appear to be involved in the repression of hook development caused by loss of CWI neither in *qua2* nor in isx-treated seedlings (Fig. 10, B to D). On the other hand, our results suggest that hook defects in plants with an altered cell wall might be at least partially mediated by a reduction in GA accumulation, as (i) GA levels are reduced in isx-treated seedlings (Fig. 8, D and E) under LA conditions and are restored by HA; (ii) both *qua2* and isx-treated WT seedlings show altered expression of genes involved in the homeostasis of GAs (Fig. 5, A to C; Supplementary Fig. S3); (iii) exogenous GAs restore hook formation in *qua2* and in isx-treated WT seedlings (Figs. 5D and 8A); and (iv) lack of DELLA or GA2ox proteins that increase GA response

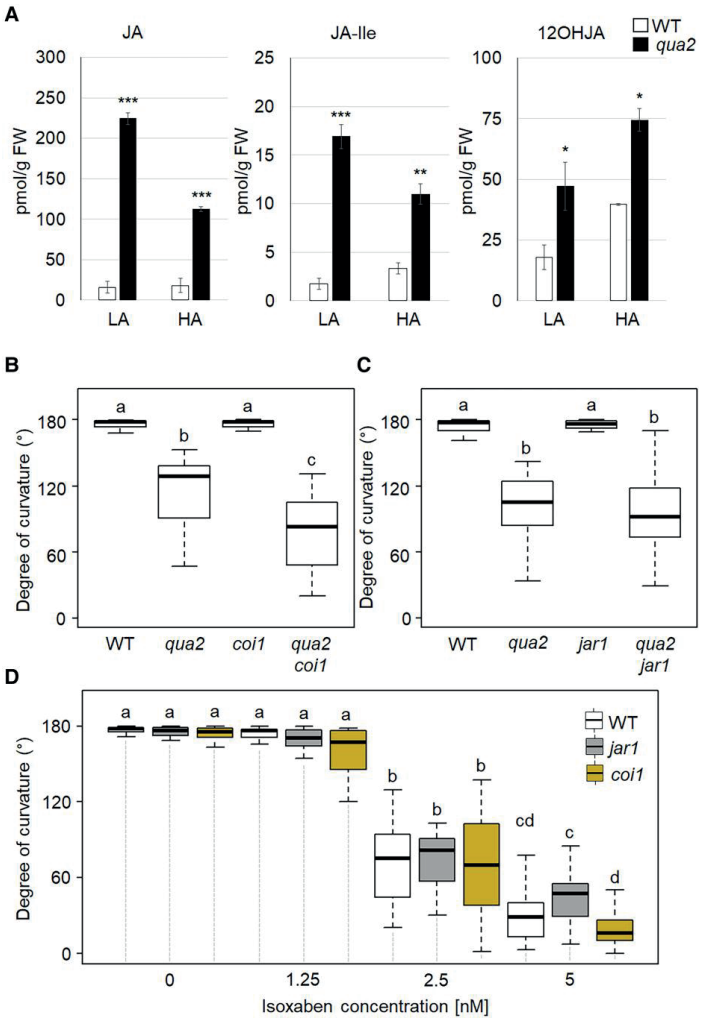

Figure 10. Inhibition of apical hook formation in response to altered CWI is independent of jasmonate signaling. **A)** Levels of JA, JA-Ile, and Σ 11-/12-OHJA in WT and *qua2* seedlings 2 d after germination grown in the dark on medium containing 0.8% (LA) or 2.5% (HA) agar (w/v). Bars represent means of 3 independent biological replicates ± SD. Asterisks indicate significant differences relative to WT, according to Student's *t* test (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$). **B and C)** Apical hook angles of WT, *qua2*, *coi1*, and *qua2 coi1* **B)** or *jar1* and *qua2 jar1* **C)** grown as in **A)**. **D)** Quantification of apical hook angles of WT (WT Columbia), *jar1*, and *coi1* seedlings 2 d after germination grown in the dark in the presence of isx at the indicated doses. Box plots in **B to D)** indicate the 1st and 3rd quartiles split by median, and whiskers show range ($n \geq 20$). Letters indicate statistically significant differences ($P < 0.05$) according to 2-way ANOVA followed by post hoc Tukey's HSD.

or levels, respectively, reduces the impact of isx on hook formation (Fig. 8, B and C). Notably, growth of seedlings on HA increases *HLS1*, *PIF4*, and GA biosynthetic gene expression in both *qua2* and isx-treated seedlings (Figs. 4, A and C, 5, A to C, and 7, A and C; Supplementary Fig. S3), and overexpression of HLS1 restores hook formation in *qua2* and in isx-treated seedlings (Figs. 4D and 7B). Furthermore, HA conditions prevent the reduction of PIF4 protein levels in *qua2* and in isx-treated seedlings (Figs. 4B and 7D). These results suggest a causal link between altered CWI, reduction of GA levels, and suppression of GA-mediated signaling required for proper auxin signaling and differential cell expansion during hook formation and maintenance.

In conclusion, our results indicate that turgor-dependent responses link changes in CWI to the downregulation of a regulatory module, comprising GAs, PIF4 (and, possibly, other PIFs), and HLS1, that promotes asymmetric cell elongation and hypocotyl curvature during hook formation (Fig. 11). However, it cannot be ruled out that additional mechanisms might contribute to compromise hook formation in plants with defective cell wall composition. Intriguingly, it was reported that short fragments

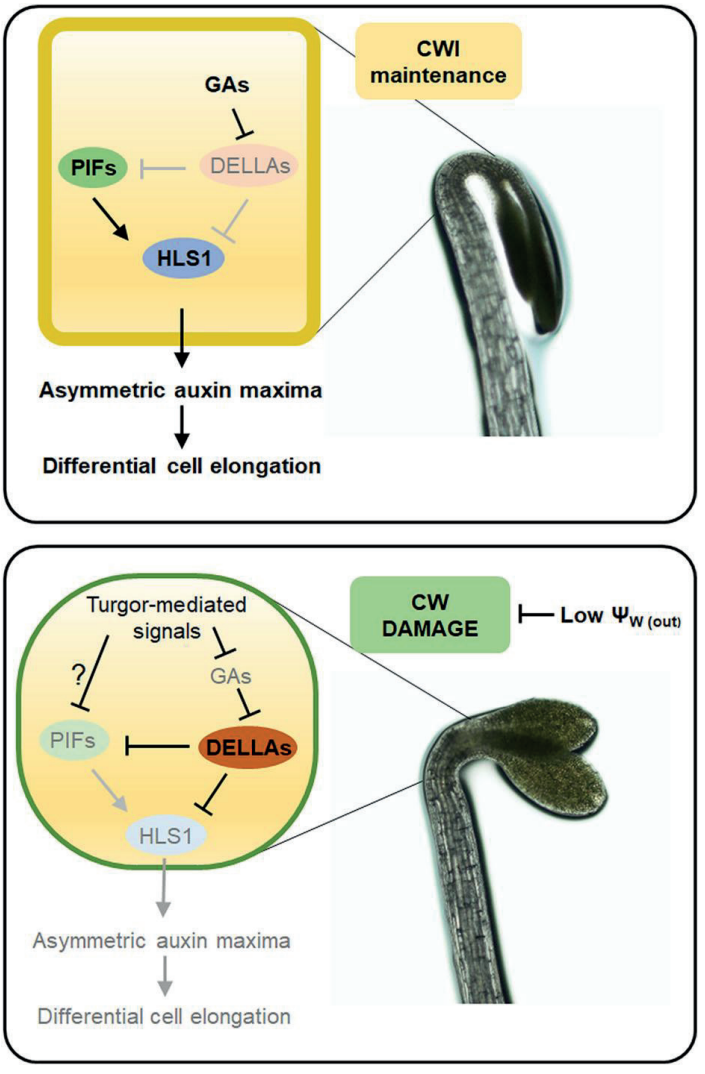

Figure 11. Proposed model of the effects of loss of CWI on apical hook formation. Perturbation of CWI, either caused by mutations in pectin composition or by isx, activates turgor-dependent responses that repress accumulation of active GAs, leading to stabilization of DELLA proteins and reduction of PIF4 and possibly other PIF protein levels. Increased DELLAs and reduced PIFs result in impaired *HLS1* expression, impairing proper formation of auxin response maxima and differential cell elongation and ultimately inhibiting apical hook development. The arrows indicate positive regulation, and blunt-ended bars indicate inhibition. Question mark indicates unidentified signaling elements. Elements in gray indicate reduction of levels or reduced downstream responses. Ψ_w, water potential; PIFs, PHYTOCHROME INTERACTING FACTORs; HLS1, HOOKLESS1; DELLAs, DELLA proteins; GAs, gibberellins; CW, cell wall; CWI, cell wall integrity.

of HG restore hook development in dark-grown mutants impaired in pectin composition (Sinclair et al. 2017), suggesting that, in WT plants, HG-derived fragments might act as signals that promote hook formation. Future research will help elucidate the mechanisms linking changes in the cell wall biochemical and physical properties occurring in response to internal and environmental cues to the signaling cascades that modulate differential cell growth during plant developmental programs.

Materials and methods

Plant lines

All experiments were performed using Arabidopsis (*A. thaliana*) lines. The *qua2-1* mutant was a kind gift of Gregory Mouille (INRA Centre de Versailles-Grignon); *coi1-1* and *jar1-1* mutants were a gift of Edward Farmer (Department of Plant Molecular Biology, University of Lausanne). The *mur1-1*, *mur4-1*, *mur7-1*,

prc1-1, *kor1-1*, and *gae1-1 gea6-1* mutants and the pentuple *della* mutant (*gai-t6*, *rga-t2*, *rgl1-1*, *rgl2-1*, and *rgl3-1*) were obtained by the Nottingham Arabidopsis Stock Centre. The transgenic *PIF4p:PIF4-HA pif4-301* (Zhang et al. 2017) line was a kind gift of Christian Fankhauser (University of Lausanne, Center for Integrative Genomics). The 35S::Myc-HLS1/*hls1-1* line was a gift by Shangwei Zhong (Peking University). The *the1-1*, *the1-4*, and *the1-6* mutants were a gift by Herman Höfte (INRA Centre de Versailles-Grignon). Generation of the *ga2oxheptuple* mutant (*ga2ox1/2/3/4/6/7/8*) is described in Griffiths et al. (2023).

The *qua2-1 coi1-1* and *qua2-1 jar1-1* double mutant lines were generated by crossing single mutants. Double homozygous lines were isolated based on the presence of cell adhesion defects in the hypocotyl and on primary root resistance to exogenous JA. *qua2-1 coi1-1* double homozygous mutants were crossed with a *qua2-1/qua2-1 coi1-1/COI1* sesquimutant, and homozygous individuals of the segregating progeny were selected based on their insensitivity to JA in terms of root elongation. The *qua2-1 the1-1* and *qua2-1 the1-6* double mutant lines were generated by crossing single mutants. Double mutants were screened for *qua2-1* homozygous mutation for the presence of cell adhesion defects in the hypocotyl, while PCR was used to identify *the1-1* and *the1-6* homozygous individuals. The *qua2-1* 35S::Myc-HLS1/*hls1-1*, *qua2-1* homozygous mutation was identified by the presence of cell adhesion defects while 35S::Myc-HLS1/*hls1-1* was isolated by PCR.

The *PIF4p:PIF4-HA pif4-301 qua2-1* line was generated by crossing. The *qua2* DR5-VENUS line was generated by crossing a WT line expressing DR5-VENUS (*pDR5rev::3XVENUS-N7*; Heisler et al. 2005) with *qua2-1*. The *qua2-1* myr-YFP line, expressing the myr-YFP plasma membrane marker line, was obtained by crossing a WT line carrying the pUBQ10::myr:YFP construct (Willis et al. 2016) with a homozygous *qua2-1* line. In all cases, double *qua2-1* homozygous individuals were isolated based on the presence of cell adhesion defects in the hypocotyl, and homozygosity of the transgene was confirmed based on the F3 generation.

All lines used in this work were in the Col-0 background, except for *kor1-1*, in Wassilewskija (Ws) background, and *della*, in Landsberg erecta (Ler) background.

Plant growth conditions

Seeds were surface sterilized with absolute ethanol (v/v), air dried, and sown on a solid medium containing 2.2 g/L MS salts (Duchefa), 1% (w/v) Suc, and 0.8% or 2.5% (w/v) plant agar (Duchefa), pH 5.6. Plates were wrapped in aluminum foil and stratified at +4 °C for 2 to 3 d. isx (Merck) was dissolved in 0.01% (v/v) DMSO and supplemented to a growth medium at indicated concentrations. For etiolated growth, after stratification, germination was induced by exposure to white light for 4 to 6 h, and plates were wrapped in aluminum foils and placed in a growth chamber for the indicated days. Images of the apical hook were acquired with an optical microscope using 5× magnification with light from below the sample at the indicated time after germination. For hook angle analysis with sorbitol supplementation, seeds were sown on a sterilized nylon mesh placed on agar medium plates without sorbitol and placed in the dark as described above. After 24 h, the nylon mesh was transferred under a green dim light to new plates containing sorbitol. All supplements were added in the indicated concentrations to autoclaved control media. For RNA and protein analysis, seedlings were harvested under dim green light and flash frozen in liquid nitrogen.

Kinematic analysis of apical hook development and cell elongation measurement

Seedlings were grown vertically on solid medium plates in the dark at 21 °C, illuminated with far infrared light (940 nm). Seedlings were photographed every hour using a Raspberry Pi camera (www.raspberrypi.com). Apical hook angles were measured using ImageJ software (http://imagej.nih.gov/ij/).

For time-lapse imaging of cell expansion, WT myr-YFP and *qua2-1* myr-YFP seedlings were imaged using a Zeiss LSM800 confocal microscope equipped with 10×/0.45 Plan-apo dry objective. Z-stacks were acquired without averaging with a 0. 62-micron cubic voxel size. YFP excitation was performed at 525 nm wavelength (laser intensity between 1% and 3.2%), and the emission was collected at 400 to 650 nm for (donor emission), gain between 620 and 650. Dark-grown seedlings were placed on an agar gel block on a microscopy slide and imaged at 3-h intervals. Between the acquisition of images, seedlings were placed vertically in a dark chamber to maintain skotomorphogenic conditions. Cell elongation was calculated using the software MorphographX (MGX). Using MGX, epidermal cell surface area from Z-stacks was extracted as described previously (Barbier de Reuille et al. 2015). The longitudinal expansion was calculated in MGX by overlaying Z-stacks with a fitted curved Bezier grid providing axial growth coordinates. For each condition and genotype, 15 cells from both the inner and the outer sides of the hook were measured from each of 9 individual seedlings (135 cells). The data were statistically analyzed by 2-tailed Student's *t* test.

Gene expression analysis

To analyze gene expression, the uppermost part of seedling hypocotyls, including the apical hook, was isolated using a razor blade, frozen in liquid nitrogen, and homogenized with an MM301 ball mill (Retsch, Germany) mixer ill for about 1 min at 25 Hz. Total RNA was extracted with NucleoZOL reagent (Macherey-Nagel, Germany) according to the manufacturer's instructions. One microgram of total RNA was retrotranscribed with ImProm II Reverse Transcriptase (Promega, USA). cDNA was mixed with iTaq Universal SYBR Green Supermix (Bio-Rad) and amplified using a CFX96 Real-time System (Bio-Rad, USA) using primer pairs specific for the genes of interest (Supplementary Table S1). Expression levels of each gene, relative to the *UBIQUITIN5* (*UBQ5*), were determined using a modification of the Pfaffl method (Pfaffl 2001) as previously described (Ferrari et al. 2006).

Protein extraction and immunoblot assays

Total proteins were extracted from etiolated seedlings ($n = 30$) grounded in liquid nitrogen and resuspended in 120 μL of extraction buffer (125 mM Tris, pH 6.8, 4% [w/v] SDS, 20% [v/v] glycerol, 0.02% [w/v] bromophenol blue, and 10% [v/v] β-mercaptoethanol). Samples were heated for 5 min at 95 °C and centrifuged for 1 min at 15,000 × *g* at room temperature. Proteins (20 μL of each sample) were separated by 8% (v/v) acrylamide SDS-PAGE and transferred to a nitrocellulose membrane using the Trans-Blot Turbo transfer kit (Bio-Rad, USA). Five percent (w/v) milk dissolved in phosphate-buffered saline with 0.05% (v/v) Tween 20 (Sigma) was used for blocking for 1.5 h at room temperature and antibody dilutions. For the detection of HA, a 1:1,000 dilution of the (F-7) sc-7392 antibody (Santa Cruz Biotechnology, USA) was used. As a secondary antibody, a 1:2,000 dilution of horseradish peroxidase (HRP)-conjugated anti-mouse immunoglobulin (Cell Signaling Technology, USA) was used. An anti-actin polyclonal primary antibody (Agrisera) was used as a loading

control, with HRP-conjugated anti-rabbit immunoglobulin (1:2,000; Cell Signaling) as a secondary antibody. The chemiluminescent signal of HRP conjugated to secondary antibodies was detected with ECL Western Blotting Substrate (Promega, USA) using a ChemiDoc XRS+ system (Bio-Rad, USA).

Confocal laser scanning microscopy

For DR5::VENUS detection, 2 d after germination, etiolated seedlings were placed between a microscopy slide and a cover slip. Images were acquired using a Zeiss LSM 880 laser scanning confocal microscope, using the Zen black software, with a 20× (C-Apochromat 20×/1.2 W Korr FCS M27) objective. Z-stacks were acquired without averaging with the image size 1,024 × 1,024 pixels and 0.345-micron pixel size and a Z-step size of 1 μm. VENUS excitation was performed at 514 nm wavelength (laser intensity 1%), and the emission was collected in the 518 to 560 nm range, gain 600. The laser reflection was filtered by a beam splitter.

For in vivo GA analysis, 1 d after germination, dark-grown seedlings were mounted in liquid 1/4× MS medium (1/4× MS salts, 0.025% [w/v] MES, pH5.7), covered with a coverslip, and the entire hypocotyl was imaged. Confocal images were acquired with a format of 1,024 × 1,024 pixels and a resolution of 12 bit on an upright Leica SP8-iPhox using a 20× dry objective. For FRET analysis, the same settings described in Rizza et al. (2017) were applied. The 3 fluorescence channels collected for FRET imaging were as follows: Cerulean donor excitation and emission or DxDm, Cerulean (CFP) donor excitation, Aphrodite (YFP) acceptor emission or DxAm, and Aphrodite acceptor excitation and emission or AxAm. CFP excitation was performed at 448 nm wavelength (laser intensity 5%), and the emission was collected at 460 to 500 nm for CFP (donor emission) and 525 to 560 nm for YFP (FRET emission), gain 110. For segmentation, YFP excitation was performed at 514 nm wavelength (laser intensity 3%) and the emission was collected at 525 to 560 nm, gain 110. Imaging processing and analysis were performed with FRETENATOR plugins (Rowe et al. 2022, 2023). The AxAm channel was used for segmentation. For segmentation, Otsu thresholds were used, a difference of Gaussian kernel size was determined empirically, and a minimum region of interest (ROI) size was set to 20. Distance from meristem was defined using FRETENATOR ROI labeler.

Jasmonate quantification

For hormone-level determination, dark-grown seedlings were harvested 2 d after germination, homogenized with mortar and pestle in liquid nitrogen, and reweighted into 3 replicates (approximately 10 mg per sample). Analysis of jasmonates was performed following a previously described protocol (Floková et al. 2014). Briefly, the samples were extracted in 1 mL of ice-cold 10% (v/v) aqueous methanol with the addition of isotopically labeled internal standards (JA-d_6 and JA-d_2-Ile, purchased from OlChemIm, Czech Republic), and the resulting extracts were purified on Oasis HLB SPE columns (1 cc/30 mg, Waters, Milford, MA, USA). The analyses were carried out using a 1290 Infinity liquid chromatography system coupled to an Agilent 6490 Triple Quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). The data were processed in MassHunter Quantitative B.09.00 software (Agilent Technologies, Santa Clara, CA, USA; Široká et al. 2022).

Accession numbers

The Arabidopsis Genome Initiative numbers for the genes mentioned in this article are as follows: AT1G78240 (*QUA2*); AT4G37580 (*HLS1*); AT2G43010 (*PIF4*); AT1G15550 (*GA3ox1*); AT4G25420 (*GA20ox1*); AT3G51160 (*MUR1*); AT1G30620 (*MUR4*); AT4G30440 (GAE1); AT3G23820 (*GAE6*); AT5G64740 (PRC1); AT5G49720 (*KOR1*); AT2G46370 (*JAR1*); AT2G39940 (*COI1*); AT2G01570 (*RGA*); AT1G14920 (*GAI*); AT1G66350 (*RGL1*); AT3G03450 (*RGL2*); AT5G17490 (*RGL3*); AT1G78440 (*GA2OX1*); AT1G30040 (*GA2OX2*); AT2G34555 (*GA2OX3*); AT1G47990 (*GA2OX4*); AT1G02400 (*GA2OX6*); AT1G50960 (*GA2OX7*); AT4G21200 (*GA2OX8*); and AT5G54380 (*THE1*).

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Author contributions

R.L. and S.F designed the project. R.L., Ö.E., K.J., D.T., Sa.R., J.G., and J.Š. performed experiments. R.L, S.F., S.V., S.R., A.M.J., and K.J. analyzed data and critically discussed results. S.F., S.V., S.R., K.J., O.N., and A.M.J. acquired the funding. R.L and S.F. wrote the manuscript together with contributions from all authors.

Supplementary data

The following materials are available in the online version of this article.

Supplementary Figure S1. Apical hook angle in pectin mutants grown on LA and HA.

Supplementary Figure S2. Osmotic support suppresses apical hook defects in pectin mutants.

Supplementary Figure S3. Effects of isx on the expression of genes involved in GA metabolism.

Supplementary Table S1. Primers used for RT-qPCR analysis and genotyping.

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Conflict of interest statement. None declared.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

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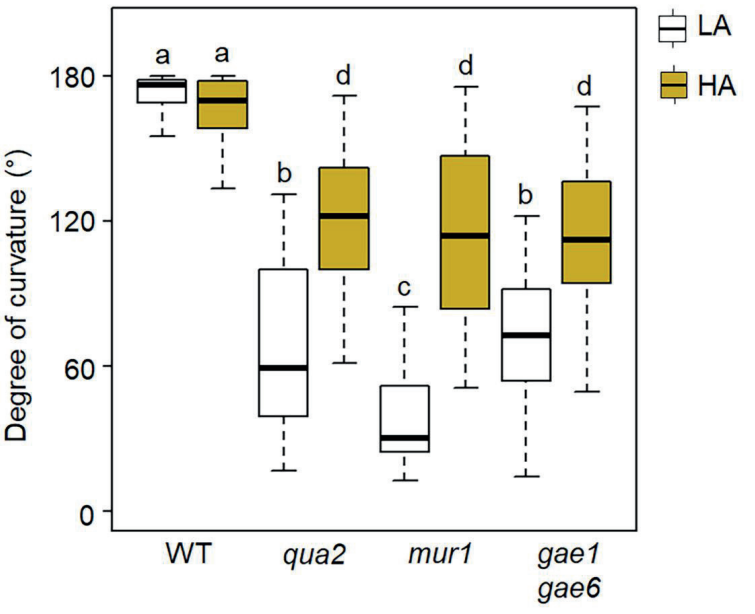
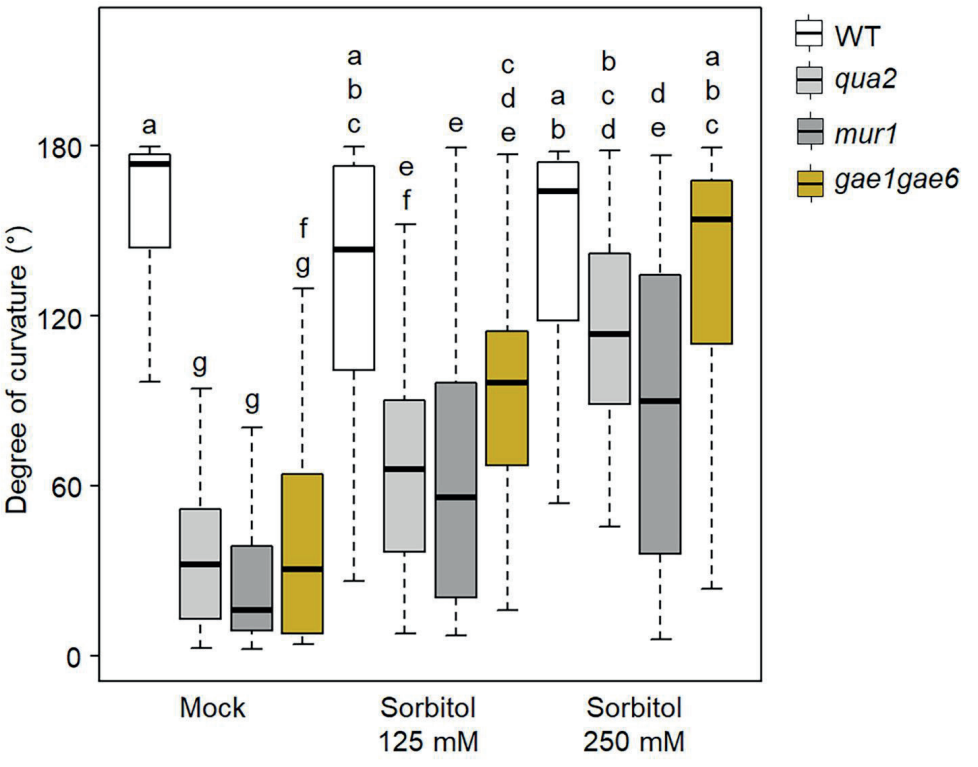
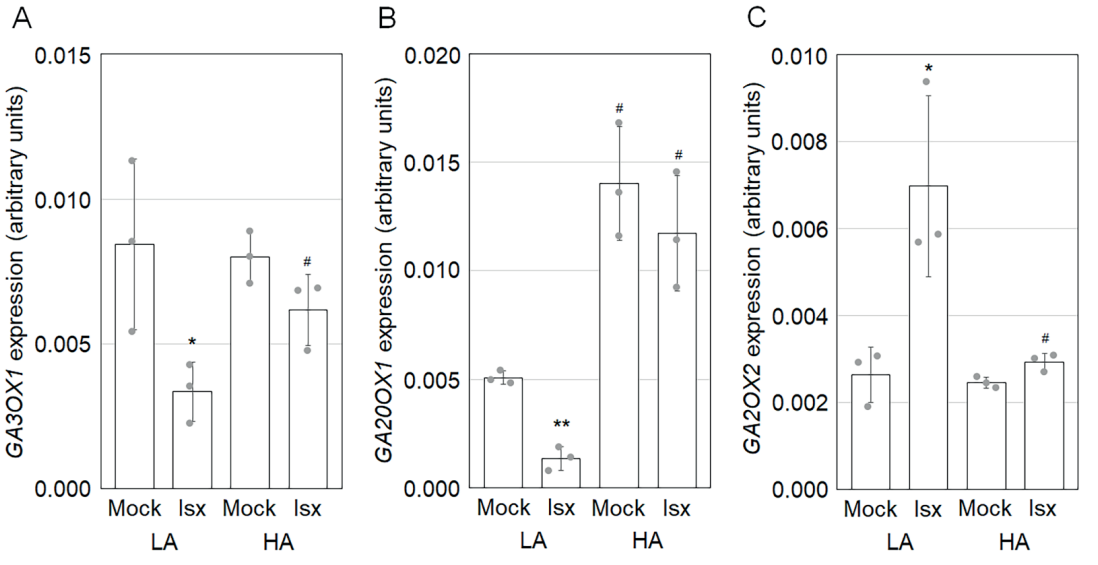


Supplementary Figure S1. Apical hook angle in pectin mutants grown on low and high agar. Apical hook angles of wild-type (WT), *qua2*, *mur1* and *gae1gae6* seedlings three days after germination grown in the dark on a medium containing either 0.8% (w/v) (LA, white boxes) or 2.5% (w/v) agar (HA, yellow boxes). Box plots indicate the 1st and 3rd quartiles split by median; whiskers show range (n≥20). Letters indicate statistically significant differences ($p<0.05$) according to two-way ANOVA followed by post-hoc Tukey's HSD.



Supplementary Figure S2. Osmotic support suppresses apical hook defects in pectin mutants. Wild-type (WT), *qua2*, *mur1* and *gae1 gae6* seeds were germinated in the dark on a solid medium without sorbitol and, 24 h after germination, were transferred to a solid medium containing increasing sorbitol concentrations as indicated (white boxes, WT; light grey boxes, *qua2*; grey boxes, *mur1*, yellow boxes, *gae1 gae6*). After four days from light exposure, the apical hook angle was measured. Box plots indicate the 1st and 3rd quartiles split by median; whiskers show range (n≥20). Letters indicate statistically significant differences ($p<0.05$) according to two-way ANOVA followed by post-hoc Tukey's HSD.



Supplementary Figure S3. Effects of isoxaben on the expression of genes involved in GA metabolism. Expression of *GA3ox1* (**A**), *GA20ox1* (**B**) and *GA2ox2* (**C**) in WT seedlings two days after germination grown in the dark on medium containing 0.8% (LA) or 2.5% (HA) agar (w/v) in the presence of DMSO (mock) or 2.5 nM isoxaben (isx). Transcript levels were determined by RT-qPCR using *UBQ5* as a reference. Bars indicate mean of at least three independent biological replicates ± SD. Asterisks indicate statistically significant differences between mock- and isx-treated seedlings according to Student's t-test (*, $p<0.05$; **, $P<0.01$); number signs indicate statistically significant differences between similarly treated seedlings grown on LA or HA according to Student's t-test (#, $P < 0.05$).

Gene	Sequence	
UBQ5	GGAAGAAGAAGACTTACACC	qPCR
	AGTCCACACTTACCACAGTA	qPCR
PIF4	TACCTCGATTTCCGGTTATGGATC	qPCR
	GTTGTTGACTTTGCTGTCCCGC	qPCR
GA3ox1	GCTTAAGTCTGCTCGGTCGG	qPCR
	AGTGCGATACGAGCGACG	qPCR
GA20ox1	AGCGAGAGGAAATCACTTGC	qPCR
	CGGCCCGGTTTTTAAGAGAC	qPCR
GA2ox2	TCCGACCCGAACTCATGACT	qPCR
	CGGCCCGGTTTTTAAGAGAC	qPCR
HLS1	GAATCCGACATTCACCTTCC	qPCR
	CATCCTCTAATCATGCCCACT	qPCR
THE1-1BA	ATTGAGCTTTTGGGTTTTCTT	genotyping
	TTGGAAAGTTATGTTTTGTGAGGAT	genotyping
THE1-6ECO	CAAAACAGAATCTTTGTTCCGAATT	genotyping
	AGGTGAGAAATGAAGACGGAT	genotyping
COI1BAM	TGAAAGCATAGGCACATATCTGAA	genotyping
	ATTCACCTACGTAACCCAGCAGGAT	genotyping
PIF4	CAGACGGTTGATCATCTG	genotyping
	CTCGATTTCCGGTTATGG	genotyping
pif4-101 oVCG61	TAGCATCTGAATTTCATAACCAATCTCGATACAC	genotyping
PIF4-HA	AGATACAGAGCCCGGTACAG	genotyping
	GAACCACCCAAAGAAGCGTA	genotyping

Supplementary Table S1. Primers used for RT-qPCR analysis and genotyping.