Cell wall integrity modulates a PHYTOCHROME-INTERACTING FACTOR (PIF) – HOOKLESS1 (HLS1) signalling module controlling apical hook formation in Arabidopsis.

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Significance statement

Cell wall integrity modulates apical hook development through poorly understood mechanisms. We show here that, in Arabidopsis, repression of hook formation by either mutations in pectin biosynthesis or by isoxaben treatment is at least partially mediated by the downregulation of a gibberellin-controlled signalling module that comprises PIF4/5 and HLS1. Our results indicate that the signals derived from changes in the cell wall can
modulate hormone-mediated pathways to control asymmetric growth during plant development.

**Summary**

Etiolated seedlings of dicots form an apical hook to protect the meristems during soil emergence. Hook formation is the result of differential growth on both sides of the hypocotyl apex and is tightly controlled by environmental cues and hormones, among which auxin and gibberellins (GAs) are the main contributors. Cell expansion is tightly regulated by the cell wall, but whether and how feedback from this structure contributes to hook development is still unclear. Here we show that etiolated seedlings of the *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 FACTOR 4* and 5 (*PIF4/5*), positive regulators of hook formation, and accumulate reduced levels of the active gibberellin GA$_4$. Treatment of wild-type seedlings with the cellulose inhibitor isoxaben (isx) also prevents hook development and represses *HLS1* expression and PIF4 accumulation. Moreover, isx stabilizes the DELLA protein REPRESSOR OF ga1-3 (RGA), which inhibits *HLS1* expression and hook formation. Exogenous GAs or *HLS1* overexpression partially restore hook development in isx-treated seedlings. Notably, agar concentration in the medium restores, both in *qua2* and isx-treated seedlings, hook development and WT-like levels of PIFs and HLS1. We propose that turgor-dependent signals link changes in cell wall integrity to the PIF4/5-HLS1 signalling module to repress differential cell elongation during hook formation.
Introduction

Apical hook formation in etiolated seedlings depends on the differential cell elongation on the opposite sides of the hypocotyl apex, causing the shoot to bend by 180° (Guzman and Ecker, 1990; Abbas et al., 2013). Like most plant developmental processes, hook formation is largely controlled by the hormone 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 thaliana, hook development is positively controlled by the master regulator HOOKLESS1 (HLS1) (Guzman and Ecker, 1990; Guzman 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 development 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).

Increasing evidence indicates that the hormone-mediated signalling pathways that modulate hook formation are modulated by the cell wall (Aryal et al., 2020; Baral et al., 2021; Jonsson et al., 2021). Primary cell walls are mainly composed of cellulose, hemicelluloses, and pectin, which determine their mechanical properties, like resistance to tensile stress, thus regulating cell shape and organ morphogenesis (Cosgrove, 2005). A correlation between pectin composition, auxin responses and differential cell elongation during hook development was recently reported (Jonsson et al., 2021). Cells in the inner side of the hook, where auxin maxima occur, display a higher degree of methylesterification (DM) of homogalacturonan (HG), a major pectic polysaccharide, which
correlates with a reduction in cell elongation (Jonsson et al., 2021). Notably, plants overexpressing a pectin methylesterase inhibitor show a uniformly high pectin DM and fail to establish a proper auxin response gradient, resulting in defective hook formation (Jonsson et al., 2021). These observations suggest that pectin composition and/or architecture directly modulates the signalling pathways that control differential cell expansion in the hypocotyl. In addition, alterations in other cell wall structural components, including cellulose (Sinclair et al., 2017; Baral et al., 2021) and xyloglucan (Aryal et al., 2020) also impair apical hook development, suggesting that changes in different wall structural components converge into common responses that restrict differential cell elongation, preventing hook formation. However, the exact mechanisms linking cell wall composition to the signalling events that regulate hook development are not fully elucidated.

Plants have evolved mechanisms to monitor cell wall integrity (CWI) and, in case of alterations, mount compensatory responses that reinforce the cell wall to ensure its functional integrity and that can restrict cell expansion (Ellis et al., 2002; Engelsdorf et al., 2018). CWI can be experimentally altered by lesions in genes involved in the biosynthesis of cell wall structural polysaccharides or by chemicals that interfere with it, like isoxaben (isx), an inhibitor of cellulose deposition (Vaahteras et al., 2019). 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 impaired in HG biosynthesis, namely QUASIMODO1 (QUA1), encoding a putative glycosyltransferase (Bouton et al., 2002), and QUA2/TUMOROUS SHOOT DEVELOPMENT 2 (TSD2), encoding a Golgi-localized pectin methyltransferase (Krupkova et al., 2007; Mouille et al., 2007; Du et al., 2020), have shorter hypocotyls and defects in hypocotyl epidermis cell elongation and adhesion (Krupkova et al., 2007; Mouille et al., 2007; Raggi et al., 2015). 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, i.e., co-treatments with osmotic (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). Whether and how these responses can also affect developmental processes regulated by differential cell elongation, as in the case of apical hook formation, is currently
unclear. Here we report that loss of CWI represses a signalling module that comprises PIF4/5 and HLS1, resulting in a defective apical hook, and that these effects are suppressed by high agar concentration of the growth medium. Our results suggest that turgor-dependent responses to altered CWI directly modulate signalling events that control differential cell expansion during hook development.

Results

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

Apical hook formation was initially examined in a panel of *Arabidopsis* mutants impaired in different cell wall polysaccharides to determine the relative impact of changes in specific cell wall components on this process. Under our experimental conditions, four-day-old etiolated wild-type (WT) seedlings displayed a completely closed hook (Figure 1a-b), which, in contrast, was completely open in qua2 as well as in two other mutants affected in pectin composition, gae1 gae6 and murus1 (mur1) (Fig 1a-b). The gae1 gae6 double mutant carries mutations in two 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).

We also examined procaste1 (prc1) and korrigan1 (kor1), carrying mutations in a cellulose synthase subunit and in an endo-1,4-β-glucanase, respectively, and therefore impaired in cellulose deposition in primary cell walls (Desnos et al., 1996; Nicol et al., 1998). prc1 showed only a mild defect in hook formation, whereas kor1 was comparable to the wild type (Figure 1a-b). No significant defects in hook development were observed in mur4 and mur7 seedlings (Figure 1a-b), both impaired in the biosynthesis of arabinose (Reiter et al., 1997; Burget et al., 2003), a constituent of RG-I, RG-II, hemicelluloses and glycoproteins such as arabinogalactan proteins (Kaczmarska et al., 2022). Taken together, these results suggest that mutations in genes regulating HG biosynthesis have a major impact on hook formation, compared to defects in other wall components.

Turgor pressure mediates the activation of several responses triggered by altered CWI (Hamann et al., 2009; Engelsdorf et al., 2018). To verify if turgor-dependent responses
also mediate the effects of altered pectin composition on hook formation and to determine what phases of this process are specifically affected by the mutations, a kinematic analysis of hook development was performed in WT, qua2, gae1gae6 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]. WT seedlings grown on LA displayed a typical (Abbas et al., 2013) formation, maintenance and opening phase (Figure 2a-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 (Figure 2a-c). Moreover, the maintenance phase was deeply compromised in all mutants, leading to hook opening right after the maximum curvature was achieved (Figure 2a-c).

When WT seedlings were grown on HA, formation and maintenance of the hook were largely unaffected, though the opening phase was accelerated (Figure 2a-c). Notably, growth on HA partially restored hook formation in all mutant lines (Figure 2a-c), leading to a statistically significant increase of the maximum angle of curvature (Figure S1). In addition, HA also rescued the maintenance phase in mur1 seedlings (Figure 2c). Hook development could also be restored by sorbitol, an osmolyte previously shown to suppress responses induced by cell wall damage (Hamann et al., 2009; Engelsdorf et al., 2018) (Figure S2). Taken together, these results suggest that turgor-dependent responses are responsible for the impaired hook development observed in seedlings with altered pectin composition.

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

Hook formation is largely dependent on the differential elongation rate of epidermal cell on the two sides of the hypocotyl (Lehman et al., 1996). 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 qua2 etiolated seedlings, resulting in a defective hook. Individual cell elongation rates were therefore measured in the apical portion of the hook of WT and qua2 seedlings grown in the dark in LA and HA condition. As expected, cell expansion rate in WT seedlings was lower in the inner side than in the outer side of the hypocotyl, either in LA or HA condition (Figure 3a-b). In contrast, qua2 seedlings showed a significant reduction in the expansion rate in the outer side of the hook when grown on LA, but not on HA (Figure 3a-b).
As differential cell expansion is dependent on the establishment of an auxin gradient at the two sides of the apex (Abbas et al., 2013), the distribution of auxin signalling was evaluated in WT and qua2 seedlings expressing the auxin response marker 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 (Figure 3c). In contrast, reporter expression was equally distributed on both sides of the hypocotyl of qua2 seedlings grown in LA (Figure 3c). This alteration was fully restored when the mutant was grown on HA (Figure 3c). Taken together, our results indicate that turgor-dependent responses to altered HG hinder proper asymmetric auxin signalling and differential cell expansion during hook formation.

**Loss of pectin integrity represses HLS1 and PIF expression and GA accumulation in dark-grown seedlings.**

HLS1 combines upstream stimuli important for hook formation (Guzman 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 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 expression of the genes encoding these proteins. Indeed, transcript levels for both HLS1 and PIF4 were significantly reduced in etiolated qua2, mur1 or gae1 gae6 seedlings grown in LA, in comparison to the wild type (Figure 4a-b). Expression of both genes in the mutants was restored by HA, which indeed increased HLS1 transcript levels also in the WT (Figure 4a). The expression of PIF5, previously shown to positively regulate hook maintenance (Khanna et al., 2007), was also reduced in qua2 seedlings grown on LA, but not on HA (Figure 4c).

Hook formation and HLS1 expression are both positively regulated by GAs (An et al., 2012). To evaluate if loss of pectin integrity affects the levels of these hormones in a turgor-dependent manner, levels of GA4, the major active GA in Arabidopsis seedlings (Yamaguchi, 2006), were quantified in WT and qua2 etiolated seedlings grown in the dark on LA or HA medium. Under LA conditions, GA4 levels were significantly lower in the mutant, compared to the wild type (Figure 5a). In contrast, GA4 levels in qua2 seedlings grown on HA were like those observed in the wild type grown on LA (Figure 5a). Unexpectedly, GA4 levels were significantly reduced in WT seedlings grown on HA (Figure 5a). Consistent with the observed reduction in GA4 accumulation, transcript levels for
GA20ox1 and GA3ox1, required for GA₄ biosynthesis (Hedden and Phillips, 2000), were reduced in qua2 and, to a lesser extent, gae1gae6 and mur1 etiolated seedlings grown on LA (Figure 5b-c). Notably, transcript levels of GA20ox1 and GA3ox1 significantly increased in both WT and mutant seedlings grown in HA (Figure 5b-c).

To evaluate if reduced levels of GAs might contribute to the altered hook formation observed in pectin-related mutants, apical hook angle was measured in WT, qua2, gae1gae6 and mur1 seedlings grown on LA in the absence or presence of exogenous GAs. Indeed, exogenous GAs restored almost WT-like hook formation in all mutants (Figure 5d). Taken together, these results suggest that responses triggered by loss of pectin integrity, and dependent on turgor pressure, lead to a reduction of the levels of active GAs in etiolated seedlings, possibly via the repression of GA biosynthetic genes, and that such reduced GA levels might contribute to repress downstream signalling events that promote hook formation.

Isoxaben inhibits hook formation and represses HLS1 and PIF4/5 expression in a turgor-dependent manner.

To investigate if turgor-dependent repression of the PIFs/HLS1 signalling module is a specific response to altered pectin composition or a general response to loss of CWI, a pharmacological approach was adopted, growing etiolated WT seedlings in the presence of increasing concentrations of isx. Under LA conditions, isx compromised hook curvature at concentrations equal to or higher than 2.5 nM (Figure 6a). 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 (Figure 6a). As in pectin mutants, isx repressed the expression of GA3ox1 and GA20ox1, and this repression was alleviated by HA (Figure S3a-b). Moreover, expression of the GA2-oxidase gene GA2ox2, involved in GA₄ catabolism (Hedden and Phillips, 2000), increased in response to isx in LA, but not in HA conditions (Figure S3c). Isx also reduced transcript levels of HLS1, PIF4 and PIF5 under LA, but not HA conditions (Figure 6b-c; Figure S4a).

To further investigate the role of HLS1 down-regulation in the repression of hook development induced by isx, hook curvature was measured in transgenic hls1 seedlings overexpressing myc-HLS1 (Shen et al., 2016). The overexpression of HLS1 fully restored hook development in the presence of 2.5 nM isx (Figure 6d), though at higher doses hook formation was still compromised (Figure 6d). These results suggest that reduced HLS1 expression contributes to the defective hook formation caused by loss of CWI.
To determine if PIFs protein levels are significantly affected by isx, transgenic lines expressing a HA-tagged version of PIF4 or PIF5 (de Wit et al., 2016; Zhang et al., 2017) were grown in the dark on LA or HA medium. A significant reduction of PIF4-HA abundance was observed in seedlings grown on LA in the presence of isx, whereas the impact of isx was much less evident when seedlings were grown on HA and was significant only at the highest dose (Figure 6e-f). PIF5-HA levels were also repressed by high isx doses, when seedlings were grown in LA, but not in HA conditions (Figure S4b-c). As with pectin mutants (Figure 5d), exogenous GAs partially restored hook formation in seedlings treated with isx (Figure 6g), further supporting the hypothesis that suppression of GA-mediated signalling might contribute to the defective hook observed in response to loss of CWI. Since PIF4 stability and HLS1 expression are negatively regulated by DELLA proteins (An et al., 2012; Li et al., 2016), we examined the effects of isx on hook curvature in a pentuple mutant (della) for all DELLA genes (Feng et al., 2008). Even though hook curvature was still significantly reduced, the *della* line was less sensitive to isx (Figure 6h).

We then evaluated the effects of isx treatment on DELLA protein levels in a transgenic line expressing GFP-RGA (Silverstone et al., 2001). GFP-RGA levels significantly increased in response to isx in seedlings grown in LA, but not in HA conditions (Figure 6i-j). Taken together, these results indicate that, as in the case of mutations that impair pectin integrity, isx treatments repress GA-dependent signalling events that control PIF4/5 and HLS1 expression and hook curvature and suggest a common mechanism underlying the effects of loss of CWI caused by alterations in different cell wall components on hook development. Moreover, inhibition of hook formation in plants with altered CWI is accompanied by a stabilisation of DELLA proteins, which might be a consequence of reduced GA levels.

**Jasmonates are not involved in defective hook formation caused by altered cell wall integrity.**

Isx induces the accumulation of jasmonates in *Arabidopsis* seedlings (Engelsdorf et al., 2018). In etiolated seedlings, exogenous jasmonic acid (JA) antagonises apical hook development (Song et al., 2014; Zhang et al., 2014). We reasoned that the altered apical hook development 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 three jasmonates, compared to the wild type (Figure 7a). Under HA conditions, the concentration of JA in WT seedlings was unaltered, while JA-Ile and $\Sigma$ 11-/12-OHJA levels were moderately increased (Figure 7a). Growth on HA medium significantly reduced JA and JA-Ile levels in qua2, while $\Sigma$ 11-/12-OHJA concentration in the mutant was slightly increased (Figure 7a).

To assess whether high jasmonates levels are causing hook development alteration of qua2, qua2 was crossed with lines defective for JASMONATE RESISTANT 1 (JAR1), required for the synthesis of JA-Ile (Wasternack and Hause, 2013), or CORONATINE INSENSITIVE1 (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, hook impairment was slightly exacerbated (Figure 7b), while the qua2 jar1 double mutant did not show differences in hook angle, compared to qua2 (Figure 7c). Consistently, jar1 and coi1 single mutants treated with isx displayed hook defects comparable to those observed in the wild type (Figure 7d). 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 loss of CWI triggers turgor-dependent responses that suppress GA accumulation and GA-mediated downstream signalling events, including PIF and HLS1 expression that positively regulate hook formation (Figure 8). These effects are independent of jasmonate-mediated signalling, and likely disrupt auxin response asymmetry, differential cell elongation and proper hook formation.

**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 (Guzman and Ecker, 1990; Abbas *et al.*, 2013). Cell elongation occurs through the controlled expansion of the cell wall, which results from the interplay between turgor pressure and cell wall elasticity and extensibility (Ray *et al.*, 1972). It is 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 signalling pathways controlling hook development, little is known of how cell wall-derived signals 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 pectin integrity in qua2 seedlings compromises, in a turgor-dependent manner, asymmetric auxin maxima formation and differential cell elongation. Additionally, we show that turgor-mediated responses triggered by altered CWI downregulate a hook-promoting signalling events that are positively regulated by GAs and include PIF4/5 accumulation and HLS1 expression (Figure 8). These results suggest that turgor pressure links CWI to a GA-dependent signalling pathway to modulate hook formation and maintenance.

Cell wall assembly and remodelling 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 et al., 2021); therefore, the structural and functional integrity of the wall must be constantly monitored and fine-tuned to allow normal growth and development under unstressed conditions while preventing mechanical failure under adverse conditions (Rui and Dinneny, 2020). Increasing evidence points to a role of turgor-mediated responses in triggering several effects of loss of CWI on plant growth and development (Verger et al., 2018; Engelsdorf 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 increasing medium agar concentrations has been previously exploited to decrease turgor pressure and restore growth in plants with perturbed cell walls (Verger et al., 2018; Engelsdorf et al., 2018; Bacete et al., 2022). We have shown here that both sorbitol and HA restore hook development in plants with altered pectin composition (Figure 2; Figure S2). Indeed, analysis of cell growth rate showed that the impaired hook formation phase observed in qua2 is accompanied by a reduction of cell elongation rate in the outer cell layer (Figure 3a-b). Moreover, WT-like
growth rate was restored when qua2 seedlings were grown in HA condition (Figure 3a-b), further supporting the hypothesis that the defect in hook development observed in this mutant 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 (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 pectin composition might lead to compensatory mechanisms, possibly 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.

The observation that isx also impairs hook formation under LA, but not HA conditions (Figure 6a) indicates that loss of CWI caused not only by pectin alterations, but also by defects in cellulose deposition trigger turgor-dependent signals that hinder differential cell expansion. However, the exact nature of the signals linking altered CWI to turgor-dependent suppression of cell expansion 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). Further investigation will provide insights in the role of specific components of the CWI maintenance system in modulating differential cell expansion during hook formation.

**Loss of CWI represses a signalling module that promotes apical hook development.**

Differential elongation during hook development requires the formation of an auxin gradient, reaching a maximum in the inner side of the hook where it reduces 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 DM of HG with auxin redistribution (Jonsson et al., 2021). However, the mechanisms linking changes in cell wall properties and the signalling pathways that modulate differential cell expansion
are poorly understood. Our results suggest that CWI represses a signalling module, comprising PIF4/5 and HLS1 (Figure 4 and 6), 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 accumulation of AUXIN RESPONSE FACTOR 2 (ARF2) (Li et al., 2004), which negatively regulates hook formation and transcriptional control of auxin transporters downstream of xyloglucan defects (Aryal et al., 2020). Our results indicate that mutants with altered pectin composition and seedlings treated with isx show a reduction of HLS1 and PIF4/5 transcript levels (Figure 4 and 6), and that isx significantly reduces PIF4/5 protein levels in WT plants (Figure 6). The downregulation of HLS1 and PIFs might contribute to the disruption of asymmetric auxin maxima and of differential cell expansion observed in qua2 and might also contribute to the hook defect caused by altered cellulose deposition, as HLS1 overexpression confers a partial resistance to the inhibitory effect of isx (Figure 6d), pointing to a common regulation of hook formation in response to changes in different cell wall components.

Mechanical stress arising from turgor pressure changes might 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 (Figure 7a), confirming that cell wall stress-induced JA production is mediated by turgor pressure changes. However, JA signalling 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 (Figure 7b-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 levels, as 1) GA4 levels are reduced in qua2 seedlings (Figure 5a); 2) both pectin-related mutants and isx-treated plants show altered expression of genes involved in the homeostasis of GAs (Figure 5b-c and Figure S3a-c); 3) exogenous GAs restore hook formation in pectin mutants and in isx-treated WT seedlings (Figure 5d and 6g); 4) isx stabilizes the DELLA protein RGA (Figure 6i-j), whose nuclear accumulation is negatively regulated by GAs (Silverstone et al., 2001), and 5) lack of all five Arabidopsis DELLA proteins reduces the impact of isx on hook formation (Figure 6h). Notably, growth of seedlings on HA, which restores normal hook formation in both qua2 and isx-treated seedlings (Figure 2 and 6a), also restores GA4 accumulation in qua2 seedling (Figure 5a), and increases HLS1, PIF4/5 and GA biosynthetic gene expression in
both qua2 and isx-treated seedlings (Figure 4b-c, Figure 5b-c, Figure 6b-c, Figure S3a-c). Furthermore, HA conditions prevent the reduction of PIF4/5 protein levels and the increase of RGA levels in isx-treated WT seedlings (Figure 6e-f and i-j; Figure S4b-c). These results suggest a causal link between altered CWI, reduction of GA levels and suppression of GA-mediated signalling required for proper auxin signalling and differential cell expansion during hook formation and maintenance. Future work will provide further insights in the relative role of turgor-mediated responses to loss of CWI and of biochemical signals derived from specific cell wall structural components in modulating the signalling pathways that promote hook formation and other plant developmental processes that rely on differential cell elongation.

In conclusion, our results indicate that turgor-dependent responses link changes in CWI to the downregulation of a regulatory module, comprising GAs, PIFs and HLS1, that promotes asymmetric cell elongation and hypocotyl curvature during hook formation (Figure 8). 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 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 signalling cascades that modulate differential cell growth during plant developmental programs.

**Experimental Procedures**

**Plant lines**

The qua2-1 mutant was a gift of Gregory Mouille (INRA Centre de Versailles-Grignon), coi1-1 and jar1-1 mutant 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, gae1-1 gea6-1 double mutant, the pentuple della mutant (gai-t6, rga-i2, rgl1-1, rgl2-1 and rgl3-1) and the transgenic line expressing pRGA:RGA-GFP were obtained by the Nottingham Arabidopsis Stock Centre. Transgenic lines PIF4p:PIF4-HA pif4-301 and PIF5p:PIF5-HA pif5-3 were a gift of Christian Fankhauser (University of Lausanne, Center for Integrative Genomics). The 35S::Myc-HLS1/hls1-1 was a gift by Shangwei Zhong (Peking University).
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 experiments were performed on seedlings of the segregating progeny that were insensitive to JA in terms of root elongation.

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 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 an homozygous qua2-1 line. In both cases, double qua2-1 homozygous lines were isolated based on the presence of cell adhesion defects in the hypocotyl, and homozygosity of the transgene was confirmed based on signal fluorescence in 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 sterilised with absolute ethanol (v/v), air dried and sown on solid medium containing 2.2 g L\(^{-1}\) Murashige-Skoog (MS) salts (Duchefa), 1% (w/v) Suc, 0.8% or 2.5% (w/v) plant agar (Duchefa), pH 5.6. Plates were wrapped in aluminium foil and stratified at +4°C for 2-3 days. Isx (Merck) was dissolved in 0.01% dimethylsulphoxide (DMSO) and supplemented to growth medium at indicated concentrations. For etiolated growth, after stratification, germination was induced by exposure to white light for 4-6 hours, plates were wrapped in aluminium foils and placed in a growth chamber for indicated days. For hook angle analysis with sorbitol supplementation, seeds were sown on a sterilised 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 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 infra-red light (850 nm). Seedlings were photographed every hour using a
RASPBERRY PI camera. Apical hook angles were measured using Image J software (http://imagej.nih.gov/ij/).
For time-lapse imaging of cell expansion, WT myr-YFP and qua2 myr-YFP seedlings were imaged using a Zeiss LSM800 confocal microscope equipped with 10x/0.45 Plan-apo dry objective. Z-stacks were acquired without averaging with a 0.5-micron cubic voxel size. Dark grown seedlings were placed on an agar gel block on a microscopy slide and imaged at three-hour intervals. Between 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). 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 side of the hook were measured from each of 9 individual seedlings (135 cells). The data was statistically analysed by two-tailed Student’s t-test.

Gene expression analysis
To analyse gene expression, uppermost part of seedling hypocotyls, including the apical hook, were isolated using a razor blade, frozen in liquid nitrogen and homogenised with an MM301 Ball Mill (Retsch) mixer ill for about 1 min at 25Hz. Total RNA was extracted with NucleoZOL reagent (Macherey-Nagel) according to the manufacturer’s instructions. 1μg of total RNA was retrotranscribed with Improm II Reverse Transcriptase (Promega). cDNA was mixed with iTaq Universal SYBR Green Supermix (Bio-Rad) and primer pairs specific for genes of interest, loaded onto 96-well plates and samples were amplified using a CFX96 Real-time System (Bio-Rad). Gene expression, relative to UBIQUITIN5 (UBQ5), was calculated according to the ΔΔCT method.

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, 10% (v/v) β-mercaptoethanol]. Samples were heated for 5 min at 95°C and centrifuged for 1 min at 15,000g at room temperature. Proteins (20 µL of each sample) were separated by 8% acrylamide SDS-PAGE and transferred to a nitrocellulose membrane using the Trans-Blot Turbo transfer kit (Bio-Rad). 5% (w/v) milk dissolved in phosphate-buffered saline with 0.05% (v/v) Tween 20 was used
for blocking for 1.5 h at room temperature and antibody dilutions. For the detection of HA, a 1:1000 dilution of the (F-7) sc-7392 antibody (Santacruz) was used. For the detection of GFP, a 1:1000 dilution of primary antibody (Chromotek) was used. As a secondary antibody, a 1:2000 dilution of a HRP-conjugated anti-mouse immunoglobulin (Cell Signaling) was used. Anti-actin polyclonal primary antibody (Agrisera) was used as loading control, with HRP-conjugated anti-rabbit immunoglobulin (1:2000; Cell Signaling) as a secondary antibody. The chemiluminescent signal of HRP conjugated to secondary antibodies was detected with ECL Western Blotting Substrate (Promega) using a ChemiDoc XRS+ system (Biorad).

**Confocal laser-scanning microscopy**

For DR5::VENUS detection, three-day-old etiolated seedlings were placed between a microscopy slide and a cover slip. Images were made using a Zeiss LSM 880 laser scanning confocal microscope. Images were acquired using the Zen black software, with a 40X (C-Apochromat 40x/1.2 W Korr FCS M27) objective. Z-stacks were acquired without averaging with the image size 1024x1024 px and 0.345-micron pixel size and a Z-step size of 1μm. PI excitation was performed at 561 nm wavelength and the emission was collected in the range of 562-600 nm. VENUS excitation was performed at 514 nm wavelength and the emission was collected in the range of 518-560 nm. The laser reflection was filtered by a beam splitter.

**Hormone quantification**

Three-day-old dark-grown seedlings were homogenised with mortar and pestle in liquid nitrogen and reweighted into three replicates for gibberellin analysis and three replicates for jasmonates analysis (approximately 10 mg per sample).

Extraction and analysis of endogenous GA₄ were performed according to the modified method described in Urbanová *et al.*, (2013). Briefly, tissue samples of about 10 mg FW were ground to a fine consistency using 2.8-mm zirconium oxide beads (Retsch GmbH & Co. KG, Haan, Germany) and a MM 400 vibration mill at a frequency of 27 Hz for 3 min (Retsch GmbH & Co. KG, Haan, Germany) with 1 mL of ice-cold 80% acetonitrile containing 5% formic acid as extraction solution. The samples were then extracted overnight at 4°C using a benchtop laboratory rotator Stuart SB3 (Bibby Scientific Ltd., Staffordshire, UK) after adding internal standard ([²H₂]GA₄, OlChemlm, Czech Republic). The homogenates were centrifuged at 42030xg and 4°C for 10 min, and the corresponding
supernatants were further purified using mixed-mode SPE cartridges (Waters, Milford, MA, USA). The samples were finally analysed with a UHPLC-MS/MS system consisting of the Acquity I-class UPLC® chromatograph (Waters, Milford, MA, USA) coupled to the Xevo TQ-XS triple quadrupole mass spectrometer (Micromass, Manchester, UK). Masslynx 4.2 software (Waters, Milford, MA, USA) was used to analyse the acquired data.

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% aqueous methanol with the addition of isotopically labelled internal standards ([6H6]-JA and [6H2]-(-)-JA-Ile, purchased from OlChemIm, Czech Republic) and the resulting extracts were purified on Oasis® HLB SPE columns (1 cc/30 mg, Waters). 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

AT1G78240 (**QUA2**); AT4G37580 (**HLS1**); AT2G43010 (**PIF4**); AT3G59060 (**PIF5**); AT1G15550 (**GA3ox1**); AT4G25420 (**GA20ox1**); AT1G30040 (**GA20ox2**); 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**).

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Conflicts of interest

The authors declare that they have no conflict of interest with this work.

Supplementary materials

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

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 solid medium without sorbitol and, 24 h after germination, were transferred to solid medium containing increasing sorbitol concentrations (white boxes, 0 mM; yellow boxes, 125 mM; red boxes, 250 mM). After three 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.

Figure S3. Effects of isoxaben on the expression of genes involved in GA metabolism. Expression levels of GA3ox1 (a), GA20ox1 (b) and GA2ox2 (c) were determined in WT seedlings grown in the dark for three days 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 qPCR using UBQ5 as reference gene. Bars indicate mean relative expression levels ± SD (n≥3), compared to WT seedlings grown in LA in the absence of isx. Asterisks indicate statistically significant differences with WT in LA conditions, according to Student’s t-test (*, p≤0.05; **, p≤0.01).

Figure S4. Effects of isoxaben on PIF5 expression. (a), WT seedlings were grown in the dark in the presence of DMSO (mock) or 2.5 nM isoxaben (isx) on medium containing 0.8% (LA) or 2.5% (HA) agar (w/v). PIF5 transcript levels were determined by qPCR using UBQ5 as reference. Bars indicate mean relative expression, compared to WT seedlings grown in LA in the absence of isx, ± SD of at least three independent biological replicates. Asterisks indicate statistically significant differences with WT in LA conditions, according to Student’s t-test (*, p≤0.05). (b), Transgenic lines expressing PIF5-HA under the control of its native promoter (ProPIF5:PIF5-3×HA) were germinated and grown in the dark for three days in LA or HA medium supplemented with DMSO (mock), 2.5 nM isx or 5 nM isx. PIF5-HA levels were detected by immunoblot analysis using an antibody against HA. An antibody against Actin (ACT) was used as loading control. (c), Intensity of the PIF5-HA signal in (b), normalized for the ACT signal, was quantified and expressed as relative
levels compared to mock-treated seedlings under LA condition. (c). Bars indicate means of n≥3 independent biological replicates ± SD. Asterisks indicate statistically significant differences between mock-treated and isx-treated seedlings in the same agar concentrations, according to Student’s t test (*P < 0.05).

Table S1. Primers used for qRT-PCR analysis and genotyping.

References


is prompted by turgor-driven mechanical compression. *Science Advances, 7*, eabf0356.


**Figure legends**

Figure 1. Development of apical hook in Arabidopsis cell wall mutants. (a) Representative pictures of wild-type (WT) Columbia-0 (Col), WT Wassilewskija (Ws), qua2, mur1, mur4, mur7, gae1gae6, prc1 (in Col-0 background) and kor1 (in Ws background) four-day-old dark-grown seedlings. Scale bar, 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>20). Letters indicate statistically significant differences (P < 0.05) according to one-way ANOVA followed by post-hoc Tukey's HSD.

Figure 2. Kinematic analysis of apical hook formation in pectin mutants grown on low and high agar. Wild-type (WT, blue and grey lines) and qua2 (a), mur1 (b) or gae1gae6 (c) mutant (orange and yellow lines) seedlings were grown in the dark on medium containing either 0.8% (w/v) (LA, blue and orange lines) or 2.5% (w/v) agar (HA, gray and yellow lines). The hook angle was measured at the indicated times. Error bars represent mean angle ± SE (n≥15).

Figure 3. Effects of agar concentration on cell elongation and auxin response during apical hook formation in qua2 seedlings. (a) Heatmaps of growth rate of individual cells in the apical portion of the hypocotyl upon three-hour time lapse in wild-type (WT) and qua2 seedlings grown in the dark on medium containing 0.8% (LA) or 2.5% (LA) (w/v) agar. (b) Quantification of growth rate of individual cells in the outer (dark gray) and inner (light gray) side of the hypocotyl of seedlings grown as in (a). Data are average of three independent biological replicates ± SD. For each experiment, 15 cells from both inner and outer side 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. Scale bars, 50 μm.

Figure 4. HLS1 and PIF4/5 expression in pectin mutants. Total RNA was extracted
from three-day-old wild-type (WT), qua2, mur1, and gae1gae6 seedlings grown in the dark on medium containing 0.8% (LA) or 2.5% (HA) agar (w/v). Relative transcript levels of HLS1 (a), PIF4 (b) and PIF5 (c) were measured by qPCR, using UBQ5 as reference. Data are means of n≥3 independent biological replicates ± SD. Asterisks indicate statistical significance according to Student's t test (*P < 0.05; **P < 0.01).

Figure 5. Defects in GA accumulation and expression of biosynthetic genes in pectin mutants. (a) Levels of the bioactive GA4 in three-day-old WT (white bars) and qua2 (black bars) seedlings grown in the dark on medium containing 0.8% (w/v) or 2.5% (w/v) agar (LA and HA, respectively). Bars represent the mean of three independent biological replicates ± SD. Asterisks indicate statistically significant differences between WT and qua2, according to Student's t-test (*, p≤0.05; **, p≤0.01; ***, p≤0.001). (b, c) Expression of GA3ox1 (b) and GA20ox1 (c) in WT, qua2, mur1 and gae1gae6 seedlings grown as in (a). Transcript levels were determined by qPCR using UBQ5 as reference. Bars indicate mean relative expression, compared to WT seedlings grown in LA, ± SD of at least three independent biological replicates. Asterisks indicate statistically significant differences with WT in LA conditions, according to Student’s t-test (*, p≤0.05; **, p≤0.01). (d) Apical hook angles of three-day-old WT, qua2, mur1 and gae1gae6 seedlings grown in the dark on medium supplemented with ethanol (mock, white boxes) or 50 μM GA4 (GA, yellow boxes). Box plots indicate the 1st and 3rd quartiles split by median; whiskers show range. Letters indicate statistically significant differences, according to two-way ANOVA followed by post-hoc Tukey’s HSD (p< 0.05).

Figure 6. Isoxaben inhibits apical hook formation and GA-mediated signalling in a turgor-dependent manner. (a) Quantification of apical hook angles of three-day-old wild-type (WT) seedlings grown in the dark on medium 0.8% (LA) or 2.5% (HA) agar (w/v) and supplemented with isoxaben (isx) at the indicated doses. (b, c) Transcript levels of HLS1 (b) and PIF4 (c) in three-day-old WT seedlings grown as in (a). Transcript levels were determined by qPCR using UBQ5 as reference gene. Bars indicate mean relative expression levels, compared to WT seedlings grown in LA in the absence of isx, ± SD of at least three independent biological replicates. Asterisks indicate statistically significant differences with WT in LA conditions, according to Student’s t-test (*, p≤0.05; **, p≤0.01). (d) Quantification of apical hook angles of wild-type (WT) and 35S:Myc-HLS1/hls1-1 three-day-old seedlings grown in the dark in the presence of the indicated concentrations of isx.
(WT, white boxes; 35S:Myc-HLS1/hls1-1, yellow boxes). (e, f) Transgenic lines expressing PIF4-HA under the control of its native promoter (ProPIF4:PIF4-3xHA) 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 loading control. Bars in (f) indicate mean relative normalized intensity ± SD of the PIF4-HA signal, compared to mock-treated seedlings under LA condition (n≥3 independent biological replicates). Asterisks indicate statistically significant differences between mock and isx-treated seedlings at the same agar concentration, according to Student’s t test (*P < 0.05; **P < 0.01). (g) Apical hook angles of three-day-old dark-grown WT seedlings treated with DMSO or 2.5 nM isoxaben (isx) in the presence or absence of 50 μM GAs. (h) Apical hook angles of wild-type Ler (WT, white boxes) and della (yellow boxes) three-day-old seedlings grown in the dark in the presence of isx at the indicated doses. (i, j) Transgenic lines expressing GFP-RGA under the control of their native promoters were germinated and grown in the dark for three days on LA or HA medium supplemented with DMSO (mock) or isx at the indicated doses. GFP-RGA levels were detected by immunoblot analysis with an antibody against GFP. Intensity of the GFP-RGA band normalized for the loading control ACT and expressed as relative levels compared to mock-treated seedlings under LA condition. Bars in (j) indicate mean relative levels ± SD (n≥3 independent biological replicates). Letters in (a), (d), (g) and (h) indicate statistically significant differences according to two-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>20).

Figure 7. Inhibition of apical hook formation in response to altered cell wall integrity is independent of jasmonate signalling. (a) Levels of JA, JA-Ile, Σ 11-/12-OHJA in three-day-old wild-type (WT, white bars) and qua2 (black bars) seedlings grown in the dark on medium containing 0.8% (LA) or 2.5% (HA) agar (w/v). Bars represent means of three independent biological replicates ± SD. Asterisks indicate significant differences relative to WT, according to Student’s t-test (*p≤0.05, **p≤0.01, ***p≤0.001). (b-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 wild type (WT Columbia), jar1 and coi1 three-day-old seedlings grown in the dark in the presence of isoxaben (isx) at the indicated doses (mock, white boxes; 1.25 nM isx, yellow boxes; 2.5 nM isx, red boxes; 5 nM isx, blue boxes). Box plots in (b-d) indicate the 1st and 3rd quartiles split by median, and whiskers
show range. Letters indicate statistically significant differences (p<0.05) according to two-way ANOVA followed by post-hoc Tukey's HSD.

**Figure 8. Proposed model of the effects of loss of cell wall integrity on apical hook formation.** Perturbation of cell wall integrity (CWI), either caused by mutations in pectin composition or by isoxaben, activates turgor-dependent responses that repress accumulation of active gibberellins (GAs), leading to stabilization of DELLA proteins and reduction of PIF4/PIF5 (PIFs) 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.
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**Diagram 1:**
- GAs → PIFs → DELLAs → HLS1
- Asymmetric auxin maxima
- Differential cell elongation
- CWI maintenance

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**Diagram 2:**
- Turgor-mediated signals
- GAs
- PIFs → DELLAs → HLS1
- Asymmetric auxin maxima
- Differential cell elongation
- CW DAMAGE
- Low $\psi_w^{\text{(out)}}$