

## RESEARCH ARTICLE

# Cell wall damage attenuates root hair patterning and tissue morphogenesis mediated by the receptor kinase STRUBBELIG

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## ABSTRACT

Cell wall remodeling is essential for the control of growth and development as well as the regulation of stress responses. However, the underlying cell wall monitoring mechanisms remain poorly understood. Regulation of root hair fate and flower development in *Arabidopsis thaliana* requires signaling mediated by the atypical receptor kinase STRUBBELIG (SUB). Furthermore, SUB is involved in cell wall integrity signaling and regulates the cellular response to reduced levels of cellulose, a central component of the cell wall. Here, we show that continuous exposure to sub-lethal doses of the cellulose biosynthesis inhibitor isoxaben results in altered root hair patterning and floral morphogenesis. Genetically impairing cellulose biosynthesis also results in root hair patterning defects. We further show that isoxaben exerts its developmental effects through the attenuation of SUB signaling. Our evidence indicates that downregulation of SUB is a multi-step process and involves changes in SUB complex architecture at the plasma membrane, enhanced removal of SUB from the cell surface, and downregulation of *SUB* transcript levels. The results provide molecular insight into how the cell wall regulates cell fate and tissue morphogenesis.

**KEY WORDS:** *Arabidopsis*, Cellulose, Cell wall, Cell wall signaling, Isoxaben, Receptor kinase

## INTRODUCTION

Plant cells are encapsulated by a semi-rigid cell wall. As a consequence, cell wall remodeling represents a central pillar in the control of growth, development and the defense against abiotic and biotic stresses. In recent years, the mechanisms monitoring and modulating cell wall integrity in plants have received much attention (Bacete et al., 2018; Gigli-Bisceglia et al., 2020; Rui and Dinnyen, 2020). However, the molecular framework controlling signaling from the cell wall during development and stress responses is still poorly understood.

The plant cell wall is composed of carbohydrates, including cellulose, hemicellulose, pectin and phenolic compounds, but also

contains a large number of cell-wall-bound proteins (Höfte and Voxeur, 2017; Lampugnani et al., 2018). Cellulose synthesis is carried out by cellulose synthase (CESA) protein complexes (CSCs) at the plasma membrane. The CESA1, CESA3 and CESA6 subunits represent the core CSC subunits of the primary cell wall of expanding cells (Desprez et al., 2007; Persson et al., 2007). The herbicide isoxaben induces a rapid clearing of CESA complexes from the plasma membrane (Paredes et al., 2006). The reaction to cellulose biosynthesis inhibition (CBI) induced by isoxaben or by defects in CESA subunits represents a well-characterized compensatory cell wall damage (CWD) response (Gigli-Bisceglia et al., 2020; Vaahtera et al., 2019). Factors implicated as cell wall sensors mediating this response include the cell surface receptor kinase THESEUS 1 (THE1) (Hématy et al., 2007), a member of the *Catharanthus roseus* RECEPTOR-LIKE KINASE 1-LIKE (CrRLK1L) subfamily, and the leucine-rich repeat (LRR) receptor kinase MIK2 (LRR-KISS) (Van der Does et al., 2017), among others (Rui and Dinnyen, 2020).


The role of the cell wall in morphogenesis has long been appreciated. Cell wall remodeling is central to cellular growth (Cosgrove, 2018). In addition, the cell wall tightly connects neighboring cells. There is growing evidence that differential growth in physically coupled cells causes mechanical stresses that may in turn influence morphogenesis (Echevin et al., 2019; Uyttewaal et al., 2012; Whitewoods and Coen, 2017). Interestingly, the extracellular matrix of animal cells as well as the cellulose-containing cell wall of brown algae have been shown to influence cell fate (Berger et al., 1994; Hynes, 2009; Watt and Fujiwara, 2011), but a clear demonstration of a similar role for the plant cell wall is missing.

Cell fate regulation in *Arabidopsis* involves the atypical leucine-rich repeat receptor kinase STRUBBELIG (SUB). SUB, also known as SCRAMBLED (SCM), controls root hair specification (Fulton et al., 2009; Kwak et al., 2005) and regulates additional developmental processes, including floral morphogenesis and integument outgrowth (Chevalier et al., 2005; Lin et al., 2012). Present evidence indicates that SUB fulfills its role in these developmental processes in a complex with the transmembrane protein QUIRKY (QKY) (Fulton et al., 2009; Song et al., 2019; Trehin et al., 2013; Vaddepalli et al., 2014). Recent work has revealed that SUB also participates in the isoxaben-induced CWD response in young seedlings (Chaudhary et al., 2020). Interestingly, QKY was found to play only a minor role in this process. Moreover, SUB, THE1, and MIK2 appear to function in different CBI-induced CWD pathways (Chaudhary et al., 2020).

Here, we report that SUB activity is regulated by the cell wall. We show that exposing plants to sub-lethal doses of isoxaben results in *sub*-like morphological defects. We also show that cell wall alterations eventually cause altered SUB complex architecture, increased endocytosis of SUB, and reduced SUB transcript levels.

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Our data further reveal that ectopic upregulation of *SUB* expression counteracts the assayed morphological effects of cellulose deficiency.

## RESULTS

### Isoxaben reduces *SUB* expression levels

In light of the role of *SUB* in the CBI-induced CWD response (Chaudhary et al., 2020) we investigated whether isoxaben modulates *SUB* activity. To this end we made use of a well-

characterized line carrying the *sub-1* null allele and a fully complementing transgene encoding a SUB:EGFP translational fusion driven by its endogenous promoter (pSUB::SUB:EGFP) (Gao et al., 2019; Vaddepalli et al., 2011, 2014). We observed considerably weaker pSUB::SUB:EGFP reporter signal in liquid-grown seedlings exposed to 600 nM isoxaben in comparison with reporter signal in mock-treated seedlings (Fig. 1A,B). Signal reduction could be detected from around 5 h after the start of the

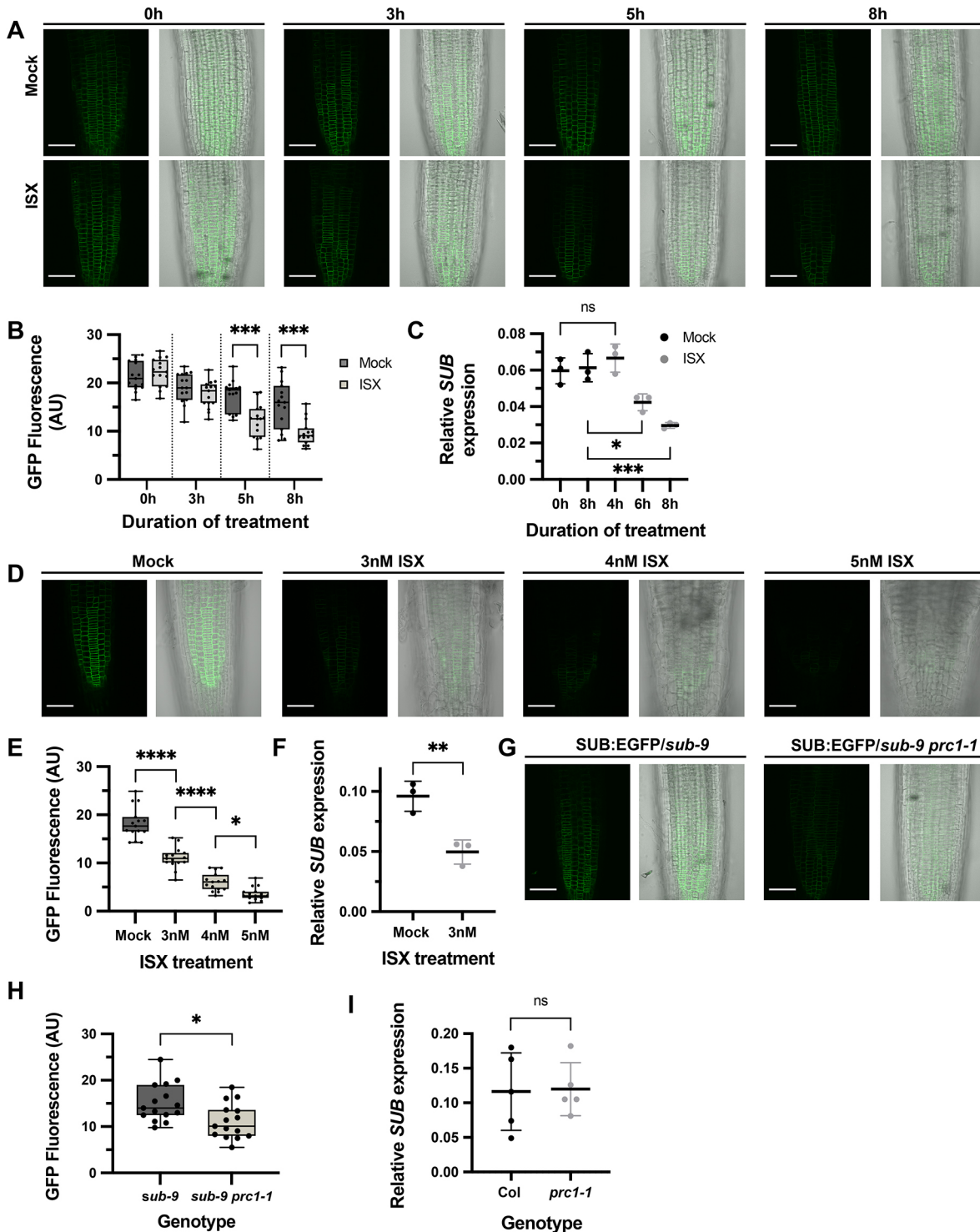


Fig. 1. See next page for legend.

**Fig. 1. Effect of isoxaben treatment on SUB expression.** (A) Signal intensity of a functional pSUB::SUB:EGFP reporter in *sub-1* upon exposure of liquid-grown seedlings to mock or 600 nM isoxaben. Note the reduction of signal in isoxaben-treated seedlings that starts at about 5 h of treatment. (B) Quantification of the data shown in A.  $n=15$ .  $***P<0.0006$ ; one-way ANOVA followed by post hoc Tukey's multiple comparison test. The experiment was performed three times with similar results. (C) Relative mRNA levels of *SUB* in 7-day-old seedlings exposed to mock or 600 nM isoxaben for 8 h. Expression was detected by qPCR. Data are mean $\pm$ s.d.,  $n=3$  biological replicates each with mean of three technical replicates.  $*P<0.02$ ;  $***P=0.0007$ ; one-way ANOVA followed by post hoc Tukey's multiple comparison test. The experiment was performed three times with similar results. (D) Signal intensity of a functional pSUB::SUB:EGFP reporter in *sub-1* upon exposure of plate-grown seedlings continuously exposed to mock or 3, 4 or 5 nM isoxaben. Note the reduced signal in isoxaben-treated seedlings. (E) Quantification of the data shown in D.  $n=15$ .  $****P<0.0001$ ; one-way ANOVA followed by post hoc Tukey's multiple comparison test. (F) Relative expression of *SUB* in 7-day-old seedlings grown on plates containing mock or 3 nM isoxaben. Expression was detected by qPCR. Data are mean $\pm$ s.d.,  $n=3$  biological replicates each with mean of three technical replicates.  $**P<0.009$ ; unpaired *t*-test with Welch's correction, two-tailed *P*-values. (G) Signal intensity of a functional pSUB::SUB:EGFP reporter in 7-day-old *sub-9* and *sub-9 prc1-1* plate-grown seedlings. Note the reduced signal in *sub-9 prc1-1* seedlings. (H) Quantification of the data shown in G.  $n\leq 15$ .  $*P=0.0101$ ; unpaired *t*-test with Welch's correction, two-tailed *P*-values. (I) Relative expression of *SUB* in 7-day-old seedlings grown on plates in Col and *prc1-1*. Expression was detected by qPCR. Data are mean $\pm$ s.d.,  $n=3$  biological replicates each with mean of three technical replicates. ns, not significant; unpaired *t*-test with Welch's correction, two-tailed *P*-values. Box and whiskers plots show the median values (middle bars) and the 25th to 75th percentiles (box). The whiskers show the smallest and largest values. A, D and G show confocal micrographs depicting optical sections through the roots of 7-day-old seedlings. Imaging parameters between the mock and isoxaben treatments, or between genotypes, were identical. Experiments in B, E and H were performed three times with similar results. Scale bars: 50  $\mu$ m.

treatment and was clearly evident after 8 h. Signal did not completely disappear but reached  $\sim 50\%$  of the intensity detected before the start of the isoxaben treatment. Importantly, reporter signal strength appeared unaltered in isoxaben-resistant *ixr2-1* seedlings carrying a mutation in the *CESA6* gene (Fig. S1) (Desprez et al., 2002; Heim et al., 1990a; Scheible et al., 2001). Furthermore, we detected significantly diminished endogenous *SUB* mRNA levels in seedlings treated with isoxaben for up to 8 h by quantitative real-time polymerase chain reaction (qPCR) in five out of six biological replicates (Fig. 1C). Transcript levels were noticeably reduced at the 6 h time point in three out of three biological replicates.

Next, we tested the effects of prolonged exposure of seedlings to isoxaben on SUB levels. *Arabidopsis* seedlings growing in the presence of isoxaben exhibited a response gamut ranging from near normal growth to essentially no growth in the narrow range of 1 to 10 nM isoxaben, with an  $I_{50}$  at 4.5 nM (Heim et al., 1989). Thus, we chose to analyze 7-day-old *sub-1* pSUB::SUB:EGFP seedlings that were continuously grown on agar plates containing 3 nM, 4 nM and 5 nM isoxaben. In comparison with mock-treated samples we observed a concentration-dependent decrease in reporter signal in isoxaben-treated seedlings (Fig. 1D,E). A reduction in SUB:EGFP abundance was confirmed by western blot analysis (Fig. S2). We then assessed whether endogenous *SUB* transcript levels were affected in plate-grown wild-type seedlings exposed to 3 nM isoxaben for 7 days. We detected a reduction of *SUB* transcript levels by  $\sim 50\%$  in comparison to untreated seedlings (Fig. 1F).

*PRC1* encodes the *CESA6* subunit of cellulose synthase and the predicted null allele *prc1-1* shows reduced cellulose levels (Fagard

et al., 2000). To assess whether SUB abundance is also diminished when cellulose biosynthesis is genetically perturbed, we generated a double mutant using the predicted null allele *sub-9* and *prc1-1*. The *sub-9 prc1-1* double mutant was made homozygous for the pSUB::SUB:EGFP reporter. We then compared the reporter signal in root tips of 7-day-old plate-grown seedlings of the strong *sub-9* mutant with that of the *sub-9 prc1-1* double mutant. We observed a noticeable reduction in reporter signal in *sub-9 prc1-1* in comparison with *sub-9* (Fig. 1G,H; Fig. S2). Next, we analyzed endogenous *SUB* transcript levels in wild-type and *prc1-1* seedlings by qPCR but we did not detect significant differences between the two genotypes (Fig. 1I).

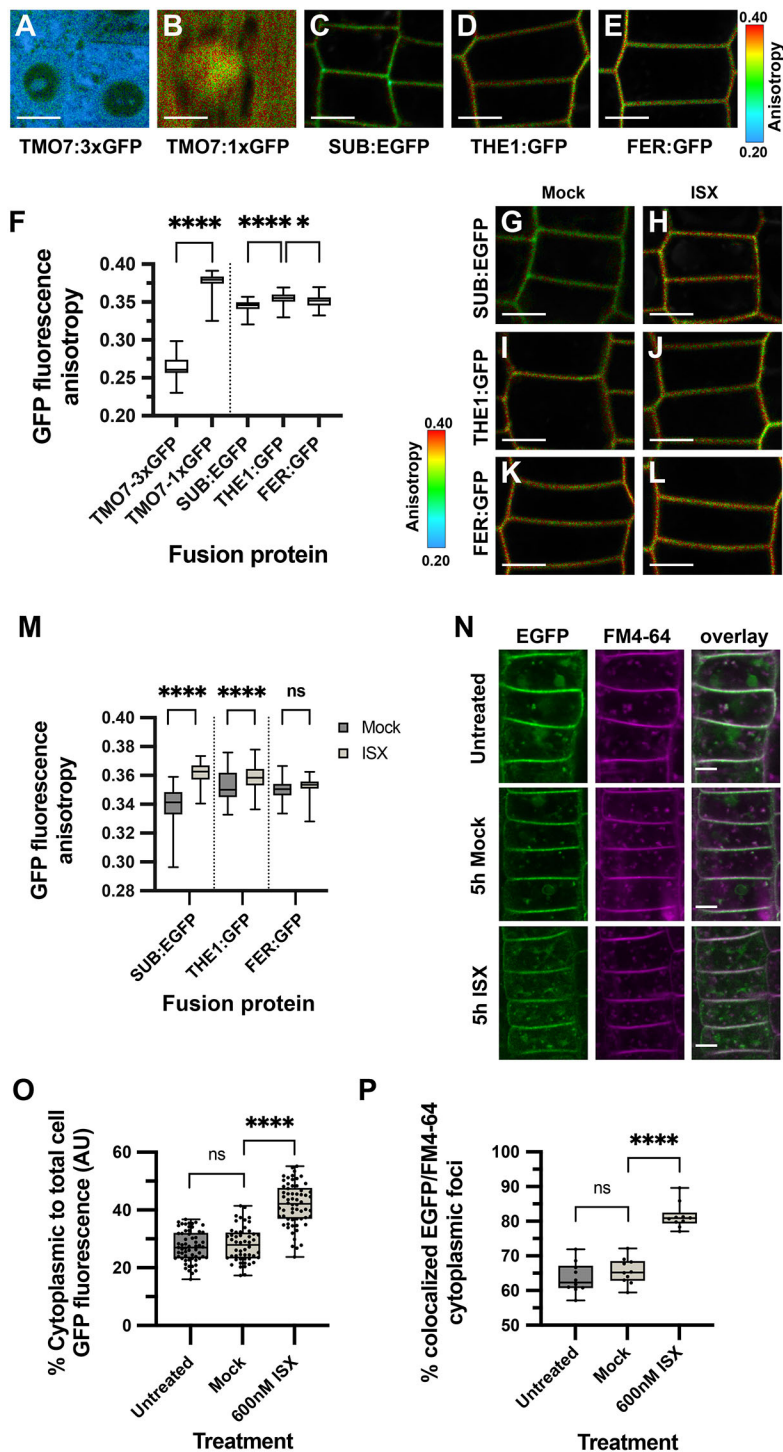
We then investigated whether downregulation of *SUB* by isoxaben involves *QKY* function. We generated pSUB::SUB:EGFP *qky-8* plants and analyzed the signal of the pSUB::SUB:EGFP reporter in the roots of the respective plate-grown seedlings. We observed an additive effect on reporter signal strength when comparing untreated seedlings with seedlings exposed to isoxaben (Fig. S3). The result indicates that isoxaben and *QKY* affect SUB abundance through parallel pathways in seedling roots.

### Cellulose biosynthesis inhibition affects SUB-complex architecture at the plasma membrane

The results mentioned above prompted us to investigate whether isoxaben influences the composition of SUB-containing protein complexes at the plasma membrane. Therefore, we assessed the steady-state fluorescence anisotropy of SUB:EGFP following mock or isoxaben treatment. Fluorescence anisotropy (*r*) describes the rotational freedom of a fluorescent molecule, such as GFP. Upon protein homo-oligomerization of GFP-based fusion proteins Förster resonance energy transfer (FRET) can occur (homo-FRET) resulting in a decrease in fluorescence anisotropy (Bader et al., 2011; Grossmann et al., 2018; Weidtkamp-Peters and Stahl, 2017). Monitoring changes in fluorescence anisotropy has been successfully applied in receptor kinase interaction studies involving for example CLV1 or BAK1 (Somssich et al., 2015; Stahl et al., 2013). As control, we used two lines carrying translational fusions of the TARGET OF MP5 (TMO7) transcription factor to one or three GFP moieties, respectively (Schlereth et al., 2010). We measured the fluorescence anisotropy values for the two fusion proteins in the nuclei of epidermal cells of the root meristem of plate-grown seedlings. We observed a fluorescence anisotropy of 0.38 for TMO7:1 $\times$ GFP and 0.26 for TMO7:3 $\times$ GFP (Fig. 2A,B,F). Free GFP in a plant cell has a steady-state anisotropy value of 0.33 (Somssich et al., 2015; Stahl et al., 2013). The higher anisotropy of TMO7:1 $\times$ GFP indicates that this fusion protein is more restricted in its rotational freedom than free GFP. The low value for TMO7:3 $\times$ GFP is indicative of homo-FRET between the three closely-linked GFP moieties.

We then determined the fluorescence anisotropy values for SUB:EGFP localized at the plasma membrane of epidermal cells of the root meristem. For comparison, we investigated two additional receptor kinases translationally fused to GFP and driven by their native promoters: THE1 and FERONIA (FER). THE1 and FER are both implied in monitoring the cell wall status (Cheung and Wu, 2011; Dünser et al., 2019; Feng et al., 2018; Hématy et al., 2007). In cells of untreated plate-grown seedlings we observed a fluorescence anisotropy value of 0.345 for SUB:EGFP, 0.355 for THE1:GFP and 0.352 for FER:GFP (Fig. 2C-F). The results could suggest that SUB:EGFP has a slightly higher rotational freedom in comparison to THE1:GFP and FER:GFP. Alternatively, differences in the linker sequences separating the kinase and GFP moieties of the three





**Fig. 2. Effect of isoxaben treatment on fluorescence anisotropy and sub-cellular localization of pSUB::SUB:EGFP.** (A,B) Fluorescence anisotropy of TMO7::3xGFP and TMO7::1xGFP in cytoplasm. Note the increased anisotropy in TMO7::1xGFP (B). (C-E) Fluorescence anisotropy at the plasma membrane of the functional reporters pSUB::SUB:EGFP in *sub-1*, pTHE1::THE1:GFP (*the1-1*) and pFER::FER:GFP (*fer-4*). A minimum of 25 photons per anisotropy event were counted. (F) Quantification of the data shown in A-E.  $150 \leq n \leq 169$ .  $*P < 0.04$ ;  $****P < 0.0001$ ; one-way ANOVA followed by Sidak's multiple comparison test. (G-L) Confocal micrographs depicting GFP-based fluorescence anisotropy in root epidermal cells of the meristematic region in 7-day-old seedlings after 4.5 h mock or 600 nM isoxaben treatment. Genotypes as in C-E. Imaging and fluorescence anisotropy calculation parameters were identical in C-E and G-L. A minimum of 25 photons per anisotropy event were counted. Note the increased anisotropy after isoxaben treatment in SUB:EGFP (G,H) and THE1::GFP (I,J). (M) Quantification of the data shown in G-L.  $121 \leq n \leq 185$ .  $****P < 0.0001$ ; one-way ANOVA followed by Sidak's multiple comparison test. (N) Fluorescence micrographs show optical sections of epidermal cells of root meristem of 7-day-old seedlings. Endocytic vesicles are marked by FM4-64. Note the increased SUB:EGFP signal in the cytoplasm as well as increased SUB:EGFP signal abundance in FM4-64 marked vesicles after isoxaben treatment. (O) Box and whisker plot depicting quantitative analysis of ratio of SUB:EGFP signal intensity in cytoplasm to total cells shown in N.  $52 \leq n \leq 61$  cells from 10 different roots.  $****P < 0.0001$ ; one way ANOVA followed by Sidak's multiple comparison test. (P) Box and whisker plot depicting the result of colocalization analysis of SUB:EGFP and FM4-64 shown in N.  $n = 10$  roots, each data point represents a mean of at least five cells per root.  $****P < 0.0001$ ; one way ANOVA followed by Sidak's multiple comparison test. ns, not significant. Experiments in F, M, O and P were performed three times with similar results. Box and whiskers plots show the median values (middle bars) and the 25th to 75th percentiles (box). The whiskers show the smallest and largest values. A-E show confocal micrographs depicting fluorescence anisotropy of GFP in root epidermal cells of the meristematic region in 7-day-old seedlings. Scale bars: 5  $\mu$ m.

fusion proteins could affect the fluorescence anisotropy values as they may influence the relative positions of the GFP moieties which in turn affects homo-FRET efficiency. We then analyzed the fluorescence anisotropy values of these reporters upon isoxaben treatment. Plate-grown seedlings were transferred onto Murashige and Skoog (MS) plates containing 600 nM isoxaben and incubated for 4.5 h. We observed a fluorescence anisotropy value for SUB:EGFP of 0.340 in mock-treated seedlings and a value of 0.361 upon isoxaben treatment (Fig. 2G-M). We scored fluorescence anisotropy values of 0.352 and 0.358 for THE1:GFP and 0.350 and 0.353 for FER:GFP, respectively. Thus, there is a noticeable difference in

fluorescence anisotropy values for SUB:EGFP in mock versus isoxaben-treated cells. By contrast, we detected only minor alterations in these values for THE1:GFP and FER:GFP. The results indicate that SUB:EGFP-containing protein complexes experience different architectures depending on the presence or absence of isoxaben-induced CWD.

#### Perturbation of cellulose biosynthesis promotes internalization of SUB

We then tested whether isoxaben treatment affects the subcellular distribution of the pSUB::SUB:EGFP reporter signal in epidermal

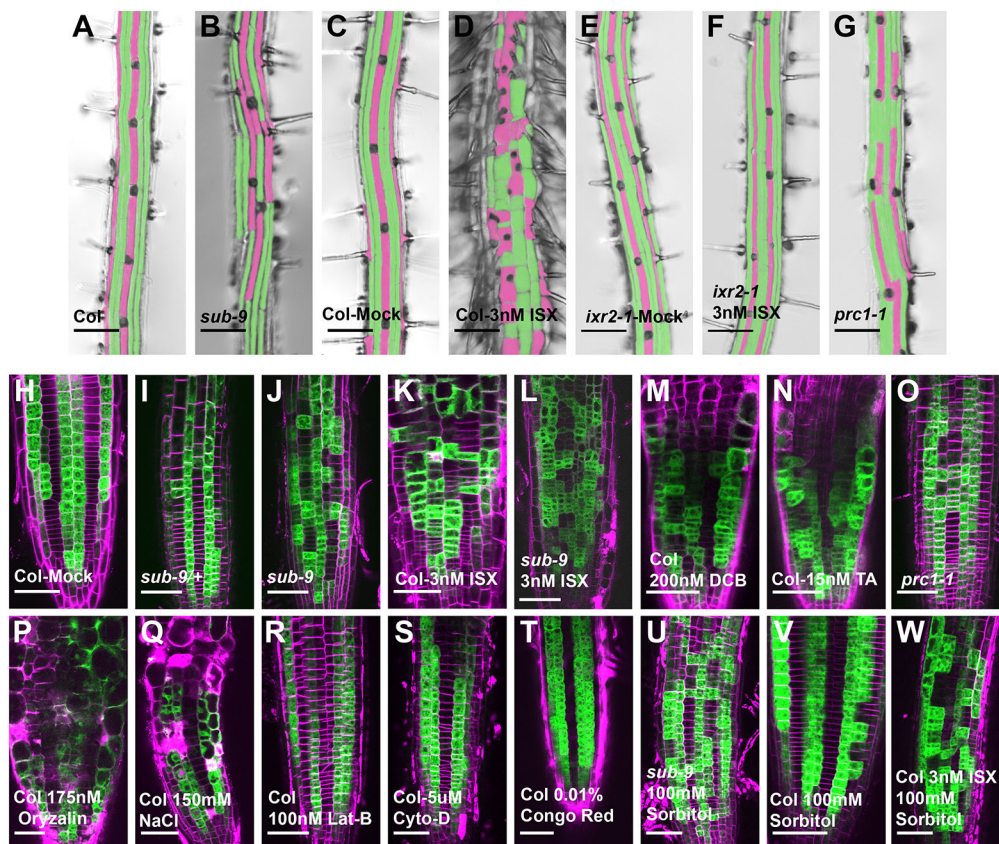
cells of the root meristem (Fig. 2N-P). In untreated seedlings SUB is known to undergo ubiquitylation and continuous internalization (Gao et al., 2019; Song et al., 2019). We found that the percentage of cytoplasmic SUB:EGFP foci increased upon isoxaben treatment in comparison with untreated or mock-treated cells (Fig. 2N,O). To assess whether the accumulation of cytoplasmic SUB:EGFP foci upon isoxaben treatment related to endocytosis we imaged cells of seedlings that were exposed to the different types of treatment upon a 5-min incubation with the endocytic tracer dye FM4-64. Applying a convenient criterion for colocalization (Gao et al., 2019; Ito et al., 2012), the internal SUB:EGFP and FM4-64 signals were considered colocalized when the distance between the centers of the two types of signals was below the limit of resolution of the objective (0.24  $\mu\text{m}$ ). In untreated or mock-treated seedlings we observed that 63.7% ( $n=322$ ) and 65.6% ( $n=536$ ), respectively, of all cytoplasmic SUB:EGFP foci were also marked by FM4-64, confirming the previous observation that SUB:EGFP undergoes recognizable internalization in the absence of any apparent stimulation (Gao et al., 2019). Upon isoxaben treatment, we noticed that 81.5% ( $n=633$ ) of all cytoplasmic SUB:EGFP foci were also marked by FM4-64. These data support the notion that isoxaben treatment eventually leads to increased endocytosis of SUB:EGFP.

### Exposing plants to sub-lethal doses of isoxaben induces root hair patterning defects

As isoxaben treatment results in the downregulation of *SUB* we investigated whether isoxaben treatment of wild-type plants results in a *sub*-like phenotype. In a first step we explored whether application of isoxaben induces root hair patterning defects in 7-day-old seedlings. We compared the number of hair (H) and

nonhair (N) cells in the N and H positions of the root epidermis, respectively, in untreated and treated wild-type seedlings to the respective numbers in untreated *sub-9* roots (Fig. 3; Table 1). In untreated plate-grown wild-type plants, we found that 97.1% of cells at the H position were hair cells whereas only 3.7% of cells at the N position were hair cells (Fig. 3A). In contrast, in mock-treated *sub-9* seedlings only 71.1% of cells at the H position were hair cells and 23.4% of cells at the N position were hair cells (Fig. 3B), confirming previous results (Kwak et al., 2005). Wild-type seedlings grown on 3 nM isoxaben plates for 7 days exhibited 68.0% hair cells in the H position and 25.1% hair cells in the N position (Fig. 3C,D). We also found that treating *sub-9* seedlings with 3 nM isoxaben plates resulted in similarly strong defects (Table 1), suggesting that application of isoxaben did not noticeably enhance the root hair patterning defect in *sub-9*. Root hair patterning appeared unaltered in mock or isoxaben-treated *ixr2-1* mutants (Fig. 3E,F). To test whether a genetic defect in cellulose biosynthesis results in aberrant root hair patterning we analyzed *prc1-1* mutants. We observed a mild but robust difference in root hair patterning in comparison with the wild type (Fig. 3G).

Next, we tested whether the altered root hair patterning is reflected at the molecular level (Fig. 3H-N; Table 2). We made use of Col-0 plants carrying a pGL2::GUS:GFP reporter. The *GLABRA2* (*GL2*) promoter drives expression in N cells but not in H cells of the root epidermis (Duckett et al., 1994), and the expression pattern of a reporter driven by the *GL2* promoter thus serves as a convenient and faithful proxy for root hair patterning (Gao et al., 2019; Kwak et al., 2005; Masucci et al., 1996). Seedlings were first grown on MS plates for 5 days and then transferred to mock plates or plates containing 1-3 nM isoxaben for 48 h. As expected, mock-treated wild-type seedlings showed the



**Fig. 3. Reduced cellulose biosynthesis results in altered root hair cell patterning.** (A-G)

Micrographs show epidermal root hair cell patterning in 7-day-old seedlings in the presence or absence of isoxaben. Pink and green colors mark root hair cells and non-root hair cells, respectively. Note the straight-line arrangement of hair cells. For a quantification, see Table 1. The experiment was performed three times with similar results. (H-W) Confocal micrographs depicting the epidermal cell layer of roots of 7-day-old seedlings showing pGL2::GUS:GFP reporter signal. The cell wall was counter-stained with propidium iodide, except for T. For a quantification of the aberrant effects see Table 2. The experiment was performed three times with similar results. Genotypes and/or treatments are indicated in the panels. Scale bars: 100  $\mu\text{m}$  (A-G); 50  $\mu\text{m}$  (H-W).



**Table 1. Distribution of root hair and nonhair cells in the root epidermis**

Genotype/Treatment	Roots*	H position <sup>‡</sup>			N position <sup>‡</sup>		
		Cells total	Hair (%)	Nonhair (%)	Cells total	Hair (%)	Nonhair (%)
Col-0/Mock	17	321	97.1±3.4	2.9±3.4	472	2.0±2.9	98.0±2.9
Col-0/3 nM ISX	17	442	68.0±5.0	32.0±5.0	601	25.1±6.1	74.9±6.1
<i>sub-9</i> /Mock	17	435	71.1±4.2	28.9±4.2	612	23.4±4.2	76.6±4.2
<i>sub-9</i> /3 nM ISX	20	535	69.7±8.8	30.3±8.8	539	29.1±7.7	70.9±7.7
<i>ixr2-1</i> /Mock	15	204	94.5±6.9	5.5±6.9	341	3.9±3.8	96.1±3.8
<i>ixr2-1</i> /3 nM ISX	15	262	94.9±5.2	5.1±5.2	346	5.7±5.0	94.3±5.0
Col-0	15	414	97.1±3.4	2.9±3.4	550	3.7±2.6	96.3±2.6
<i>prc1-1</i>	15	291	83.4±3.0	16.6±3.0	373	13.4±4.2	86.6±4.2

\*Total number of different roots analyzed.

<sup>‡</sup>Data are mean±s.d. Two-tailed Mann–Whitney tests revealed significant differences ( $P<0.0001$ ) for the following comparisons: Col/mock versus Col/3 nM isoxaben (ISX), Col/mock versus *sub-9*/mock, Col/mock versus *sub-9*/3 nM ISX, Col versus *prc1-1*, *sub-9*/mock versus *prc1-1*. Two-tailed Mann–Whitney tests failed to reveal significant differences ( $P>0.2$ ) for the following comparisons: Col versus Col/mock, *sub-9*/mock versus *sub-9*/3 nM ISX, *sub-9*/3 nM ISX versus Col/3 nM ISX, Col/mock versus *ixr2-1*/mock, Col/mock versus *ixr2-1*/3 nM ISX, *ixr2-1*/mock versus *ixr2-1*/3 nM ISX.

The experiment was repeated twice with similar results.

regular spatial expression pattern (Fig. 3H). Mock-treated *sub-9*/+ heterozygotic seedlings showed mild defects in reporter expression, revealing a weak haploinsufficiency of *SUB* and indicating that *SUB* levels are important to its function (Fig. 3I). Mock-treated *sub-9* homozygous seedlings clearly exhibited an altered expression pattern (Fig. 3J; Table 2). Upon exposing wild-type seedlings to 1–3 nM isoxaben we found a pronounced and concentration-dependent increase in defects in the expression pattern of the reporter (Fig. 3K; Table 2). The prominent misexpression resembled the expression pattern of the reporter in *sub-9* mutants (Fig. 3J,K; Table 2). Isoxaben-treated *sub-9* seedlings showed an effect that was comparable with the one shown by mock-treated *sub-9* seedlings (Fig. 3L; Table 2). We similarly treated wild-type seedlings with 200 nM 2,6-dichlorobenzonitrile (DCB) or 15 nM Thaxtomin A, two other cellulose biosynthesis inhibitors (Heim et al., 1990b; Scheible et al., 2003; Tateno et al., 2016). Both drugs induced *sub*-like expression pattern defects of the pGL2::GUS:GFP reporter (Fig. 3M,N; Table 2). Moreover, we observed that *prc1-1* mutants exhibited a mild but noticeable aberration in the expression pattern of the reporter (Fig. 3O). Thus, plants treated with three different cellulose inhibitors and a cellulose biosynthesis mutant all exhibit aberrant spatial expression of the pGL2::GUS:GFP reporter.

Cortical microtubules direct the movement of CSCs (Paredez et al., 2006). Salt stress leads to microtubule disassembly followed by the formation of a stress-tolerant microtubule network required for sustained cellulose synthesis during salt stress (Endler et al., 2015; Wang et al., 2011). In addition, regular cell wall deposition depends on the actin cytoskeleton (Crowell et al., 2009; Gutierrez et al., 2009; Sampathkumar et al., 2013). Thus, we explored whether drugs affecting the cytoskeleton had an effect on the pGL2::GUS:GFP expression pattern. We tested seedlings grown for 5 days on MS plates followed by exposure for 48 h to 175 nM oryzalin, 150 mM NaCl, 100 nM latrunculin B or 5  $\mu$ M Cytochalasin D, respectively. In all instances we did not notice an obvious effect on the pGL2::GUS:GFP expression pattern (Fig. 3P–S) although the applied drug concentrations affect the organization of microtubules and actin filaments (Baskin et al., 1994; Ketelaar et al., 2003; Staiger et al., 2009). We also tested whether application of Congo Red, a stain that allows the detection of cellulose microfibrils (Verbelen and Kerstens, 2000) and that possibly interferes with their organization, has an effect on the pGL2::GUS:GFP pattern. We did not observe a noticeable effect upon staining with Congo Red (Fig. 3T).

The isoxaben-induced CWD response is generally sensitive to turgor pressure (Hamann et al., 2009). We investigated whether the effect of isoxaben on root hair patterning follows the same pattern (Fig. 3U–W; Table 2). We thus analyzed pGL2::GUS:GFP reporter expression in sorbitol-treated *sub-9* or wild-type seedlings and wild-type seedlings simultaneously exposed to isoxaben and sorbitol. We found that application of sorbitol did not influence the reporter patterns in *sub-9* or wild-type seedlings (Fig. 3U,V). In addition, we found that reporter expression remained aberrant in wild-type seedlings exposed to isoxaben and sorbitol (Fig. 3W), suggesting that the isoxaben-induced effect on root hair patterning is not sensitive to alterations in turgor pressure. Thus, these data indicate that the CWD-sensitive mechanism controlling *SUB* expression levels is distinct from the regulation of other CBI-induced CWD responses, including callose accumulation, cell cycle gene expression or root cell shape changes (Engelsdorf et al., 2018; Gigli-Bisceglia et al., 2018; Hamann et al., 2009).

### Exposing plants to sub-lethal doses of isoxaben induces *sub*-like floral defects

To explore further the effect of isoxaben on tissue morphogenesis we tested whether isoxaben treatment also induces *sub*-like defects in flowers and ovules. We compared wild-type (*Ler*) and *sub-1* plants that were cultivated on soil in the presence of isoxaben. Plants were initially grown without any treatment. Just before bolting we began watering wild-type plants with 100–500 nM isoxaben and continued watering with isoxaben in 3-day intervals for 2 weeks. We then analyzed stage 3 floral meristems (stages according to Smyth et al., 1990; Fig. 4A–C). Floral meristems of *sub-1* mutants show aberrant cell division planes in the L2 layer (Chevalier et al., 2005; Fulton et al., 2009). Analysis of floral meristems of isoxaben-treated wild-type Col-0 plants revealed similar defects (Fig. 4B) the frequency of which increased with increasing concentrations of isoxaben (Table 3). Next, we compared stage 13 flowers of isoxaben-treated *Ler* plants with flowers from untreated *sub-1* plants (Fig. 4D–F). We noticed that flowers from isoxaben-treated plants exhibited a *sub*-like altered arrangement of petals. Finally, we analyzed the ovule phenotype of isoxaben-treated wild-type plants. We observed *sub*-like defects in integument outgrowth in late stage 3 or early stage 4 ovules (ovule stages according to Schneitz et al., 1995; Fig. 4G–I; Table 4). The frequency and severity of the integument defects also depended on the concentration of isoxaben (Table 4).

**Table 2. Position-dependent pattern of pGL2::GUS:GFP reporter expression in root epidermal cells upon a 48 h exposure to isoxaben**

Genotype	Treatment	H cells total*	H position <sup>‡,§,¶</sup>	N cells total*	N position <sup>‡</sup>
Col	–	633	3.0±2.8	741	96.4±3.6
<i>sub-9/+</i>	–	580	10.6±3.2	688	90.6±2.4
<i>sub-9</i>	–	548	33.6±9.2	615	75.3±5.4
Col	1 nM ISX	441	14.1±4.2	589	86.1±6.1
	2 nM ISX	375	26.7±9.4	459	78.3±3.2
	3 nM ISX	395	30.5±6.6	579	77.4±7.1
<i>sub-9</i>	3 nM ISX	519	37.8±4.6	504	74.8±4.5
Col	200 nM DCB	334	29.5±7.2	488	70.7±7.4
Col	15 nM TA	352	27.9±6.2	484	71.4±6.0
<i>prc1-1</i>	–	543	9.5±2.2	699	91.0±2.4
<i>sub-9</i>	100 mM sorbitol	506	31.5±8.7	619	74.0±7.6
Col	100 mM sorbitol	475	3.0±3.0	571	97.1±2.1
Col	3 nM ISX	453	30.0±6.4	573	75.9±7.0
	100 mM sorbitol				
SUB OE L1	–	423	7.2±4.8	558	77.4±4.4
	1 nM ISX	616	4.2±3.5	663	95.8±2.2
	2 nM ISX	327	1.2±2.2	396	92.5±3.4
	3 nM ISX	401	16.9±6.3	445	87.2±5.2
SUB OE O3	–	508	4.5±4.5	701	82.3±4.6
	1 nM ISX	638	3.7±3.4	706	94.7±2.6
	2 nM ISX	320	3.2±3.9	396	92.9±5.8
	3 nM ISX	359	11.3±5.1	475	86.9±5.1

For each genotype and treatment 10 different roots of 7-day-old seedlings were analyzed. The experiment was performed twice with similar results.

\*Total number of cells at H or N position scored.

‡Percentage of cells at H or N position expressing the pGL2::GUS:GFP reporter.

§Two-tailed Mann–Whitney tests revealed significant differences for the following comparisons: Col versus *sub-9/+*, Col versus *prc1-1* ( $P<0.002$ ); Col versus *sub-9*, Col versus Col/200 nM DCB, Col versus 10 nM TA, Col versus Col/1-3 nM isoxaben (ISX) ( $P<0.0001$ ); Col/1-3 nM ISX versus SUB OE L1/1-3 nM ISX (equivalent treatments were compared,  $P<0.0002$ ); Col/1-3 nM ISX versus SUB OE O3/1-3 nM ISX (equivalent treatments were compared,  $P<0.0001$ ).

¶Two-tailed Mann–Whitney tests failed to reveal significant differences for the following comparisons: *sub-9/+* versus *prc1-1*, *sub-9* versus *sub-9/3* nM ISX, *sub-9* versus Col/3 nM ISX, *sub-9* versus *sub-9/100* mM sorbitol, Col/3 nM ISX versus Col/3 nM ISX+100 mM sorbitol ( $P>0.4$ ).

### Ectopic expression of *SUB* attenuates the detrimental effects of isoxaben on root hair patterning and floral development

If isoxaben treatment results in a downregulation of *SUB* and a *sub*-like phenocopy, ectopic expression of *SUB* should counteract this outcome. We tested this hypothesis by analyzing the effects of isoxaben on two well-characterized lines carrying a pUBQ::SUB:mCherry transgene (lines L1 and O3) (Chaudhary et al., 2020) (Fig. S4). To this end we generated L1 and O3 lines homozygous for the pGL2::GUS:GFP construct. We then analyzed reporter signal in 7-day-old plate-grown seedlings that had been grown on regular MS plates for 5 days before being transferred to mock plates or plates containing 1 nM, 2 nM or 3 nM isoxaben for another 48 h before analysis (Fig. 5A–D; Table 2).

Ectopic expression of *SUB* in *p35S::SUB* plants results in aberrant pGL2::GUS expression and a mild defect in root hair patterning (Kwak and Schiefelbein, 2007). It is unclear why loss-of-function or overexpression of *SUB* results in root hair patterning defects but may relate to the proposed role of *SUB* acting as a scaffold in a multi-protein complex, the stoichiometry of which may be important (Chaudhary et al., 2020). Confirming this finding, we found that L1 and O3 showed an altered expression pattern of the pGL2::GUS:GFP reporter in roots of untreated seedlings, with more cells in the H position and fewer cells in the N position exhibiting reporter signal compared with wild type (Fig. 5A; Table 2). In the case of the isoxaben-treated L1 and O3 lines we detected significant differences to wild type. Exposing seedlings of L1 and O3 to 1 or 2 nM isoxaben resulted in pGL2::GUS:GFP patterns that resembled the pattern observed in untreated wild-type seedlings and that were less aberrant than the pGL2::GUS:GFP patterns observed in corresponding isoxaben-treated wild-type seedlings (Fig. 5B–D; Table 2). Moreover, the defects were weaker when compared with

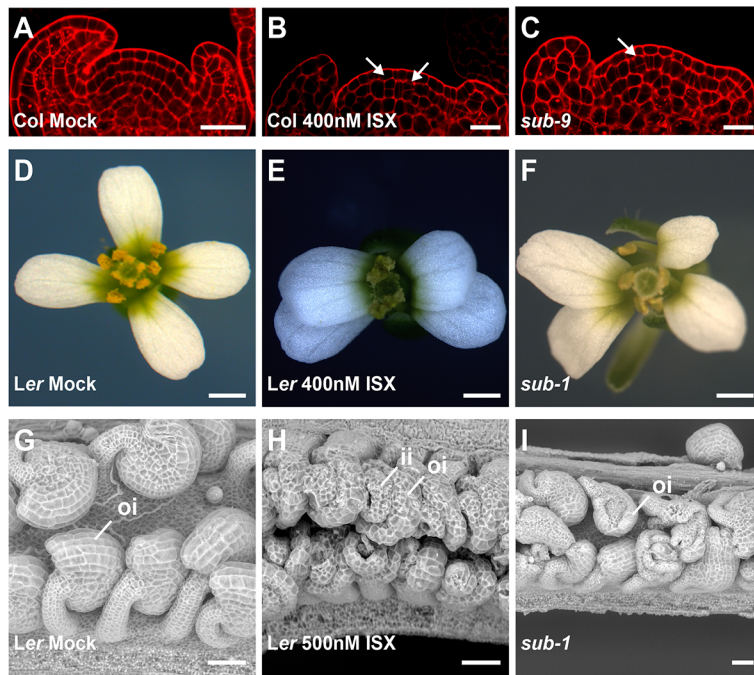
the aberrations exhibited by untreated L1 and O3. Exposing L1 or O3 seedlings to 3 nM isoxaben resulted in defects that were still less severe in comparison with wild-type plants treated with 3 nM isoxaben (Table 2).

Finally, we tested whether ectopic expression of *SUB* also alleviated the effects of isoxaben on floral development by cultivating lines L1 and O3 in the presence of different concentrations of isoxaben as described above. We found that the defects in floral meristem organization, petal arrangement and ovule development were noticeably reduced in both lines compared with wild type (Fig. 5E–H; Tables 3, 4).

### DISCUSSION

The extracellular matrix in animal cells not only provides structural support but also exerts additional functions, including developmental patterning, as in the control of epidermal stem cell fate (Hynes, 2009; Watt and Fujiwara, 2011). By contrast, the role of the plant cell wall in the regulation of cell fate is less well explored. The presented results strongly indicate that alterations in cell wall composition induced by the herbicide isoxaben or genetically in *prc1-1* mutants are associated with defects in root hair patterning in the root epidermis. Thus, they demonstrate a role of the plant cell wall in the control of root hair cell fate.

The combined data indicate a shared molecular framework underlying cell-wall-mediated regulation of root hair cell fate, floral morphogenesis and ovule development that involves the control of the atypical receptor kinase *SUB*, a well-known regulator of these developmental processes (Chevalier et al., 2005; Fulton et al., 2009; Kwak and Schiefelbein, 2007; Kwak et al., 2005). We found that exposure of seedlings to sub-lethal concentrations of isoxaben affects the architecture of *SUB*:EGFP-containing protein complexes at the plasma membrane, leads to increased internalization of *SUB*:



**Fig. 4. Effect of isoxaben treatment on floral and ovule morphogenesis.** (A-C) Confocal micrographs depicting mid-optical sections through stage 3 floral meristems stained with propidium iodide. Arrows indicate irregular periclinal cell divisions in L2. For a quantification of the effects see Table 3. (D-F) Morphology of mature stage 13 or 14 flowers. Compare E and F with D and note the aberrant arrangement of petals. Of those flowers examined, 87% (13/15) showed the phenotype in D, 82% (18/22) showed the phenotype in E and 71% (12/17) showed the phenotype in F. The experiment was repeated twice with similar results. (G-I) Electron scanning micrographs depicting mature stage 3 or 4 ovules. For a quantification of the effects see Table 4. Compare to H and I with G and note aberrant integuments. Genotypes and/or treatments are indicated in the panels and Table 4. Scale bars: 20  $\mu$ m (A-C); 0.5 mm (D-F); 50  $\mu$ m (G-I).

EGFP and causes a dose-dependent reduction in *SUB* transcript levels. Importantly, application of isoxaben did not enhance the root hair patterning defect in *sub-9* null mutants, indicating that isoxaben affects root hair cell fate mainly through downregulation of *SUB*. Moreover, ectopic expression of *SUB::mCherry* attenuated the detrimental effects of isoxaben on all the investigated developmental processes, providing further support for the notion that the isoxaben-induced decrease in *SUB* abundance is of biological relevance. Thus, the collective evidence suggests that the control of *SUB* levels by the cell wall is a central aspect of *SUB*-mediated signal transduction.

Our data expose a quantitative effect of cellulose reduction on *SUB* expression levels and concomitantly reveal that different levels of *SUB* are limiting for different *SUB*-dependent processes. We propose that the regulation of root hair patterning or floral morphogenesis depends on higher levels of *SUB*, whereas the CBI-induced compensatory CWD response in seedlings requires only a basal level of *SUB*. This is supported by the observation that exposure of seedlings to sub-lethal doses of isoxaben resulted in reduced *SUB*

transcript levels in seedlings and prominent defects in root hair patterning. Under such conditions the *SUB*-dependent CBI-induced CWD response remains operational (Chaudhary et al., 2020). Moreover, *QKY* is essential for *SUB*-mediated root hair patterning, floral morphogenesis and ovule development (Fulton et al., 2009; Song et al., 2019; Trehin et al., 2013; Vaddepalli et al., 2014). However, with the exception of lignin accumulation, *QKY* is not required for the *SUB*-mediated CBI-induced CWD response (Chaudhary et al., 2020), despite the fact that *SUB* abundance is noticeably reduced in *qky* seedlings (Song et al., 2019) (Fig. S3). Interestingly, we observed a reduction in p*SUB*::*SUB*:EGFP signal in *prc1-1* while *SUB* transcript levels were unaltered. The finding suggests that a decrease in functional *SUB* protein levels is sufficient to lead to mild defects in root hair patterning. Further evidence for a limiting role for *SUB* in this process is provided by the observation that *sub-9* heterozygotes exhibit a *prc1-1*-like root hair patterning phenotype (Fig. 3I,O; Table 2). Thus, we propose that the different functions of *SUB* relate to different levels of *SUB* and the presence of *SUB* complexes with diverse architectures at the plasma membrane.

The differential effects of *PRC1* and isoxaben on *SUB* transcript levels are consistent with the notion that the control of *SUB* includes at least two distinct processes that react to variations in the CWD signal: post-transcriptional regulation and control of *SUB* transcript levels. A comparably low reduction in cellulose content would originate a CWD signal that leads to increased *SUB* internalization. A more pronounced drop in cellulose content would elicit a stronger or different CWD signal that would further affect *SUB* transcript levels. The stronger effect of higher concentrations of isoxaben in comparison with *prc1-1*, which carries a mutation in the *CESA6* gene (Fagard et al., 2000), is explained by the observation that isoxaben affects several primary cell wall *CESA* subunits (Desprez et al., 2002; Heim et al., 1990a; Scheible et al., 2001). Moreover, *CESA6* function is buffered by redundantly acting *CESA6*-like genes (Desprez et al., 2007; Persson et al., 2007).

There is crosstalk between cell wall components (Cosgrove, 2018). For example, plants with a defect in hemicellulose production or in pectin methylation exhibit reduced cellulose

**Table 3. Number of periclinal cell divisions in L2 layer of stage 3 floral meristems of plants exposed to different concentrations of isoxaben**

Genotype	ISX [nM]*	FM <sup>‡</sup>	PCD <sup>§</sup>	Percentage <sup>¶</sup>
Col	0	29	5	17.2
<i>sub-9</i>	0	31	14	45.2
Col	200	14	4	28.6
	300	21	8	38.1
	400	25	15	60.0
SUB OE L1	0	8	2	25.0
	400	27	6	22.2

\*Isoxaben (ISX) concentration in water.

<sup>‡</sup>Number of floral meristems analyzed.

<sup>§</sup>Number of periclinal cell divisions observed.

<sup>¶</sup>Two-sided Chi-squared tests revealed significant differences ( $P < 0.02$ ) for the following comparisons: Col versus *sub-9*, Col versus Col/400 nM ISX, Col/400 nM ISX versus SUB OE L1/400 nM ISX.

The experiment was performed twice with similar results.



**Table 4. Comparison of integument defects of Col, *sub-9*, *sub-21* and Col plants exposed to different concentrations of isoxaben**

Genotype	ISX [nM]*	Ovules†	Defective ovules§,¶
Col	0	502	0.0
<i>sub-9</i>	0	629	44.2
<i>sub-21</i>	0	578	49.5
Col	200	547	13.5
	300	576	25.4
	400	563	34.3
	500	551	50.5
	SUB OE L1	0	511
200		593	2.4
300		527	4.0
400		573	8.7
500		581	14.1
SUB OE O3	0	570	0.0
	200	587	1.9
	300	508	3.3
	400	571	8.4
	500	541	16.1

For each genotype and treatment at least 15 different carpels from four different plants were analyzed.

\*Isoxaben (ISX) concentration in water.

†Total number of ovules scored.

§Percentage of ovules with *sub*-like integument defects.

¶Statistical significance  $P < 0.0001$ : Col versus *sub-9* or *sub-21*; Col versus Col/200-500 nM ISX; Col/200-500 nM ISX versus SUB OE L1/200-500 nM ISX or SUB OE O3/200-500 nM ISX. Equivalent treatments were compared. Significance was estimated by a two-sided Fisher's exact test. Similar values were obtained with a two-sided Chi-squared test. The experiment was performed three times with similar results.

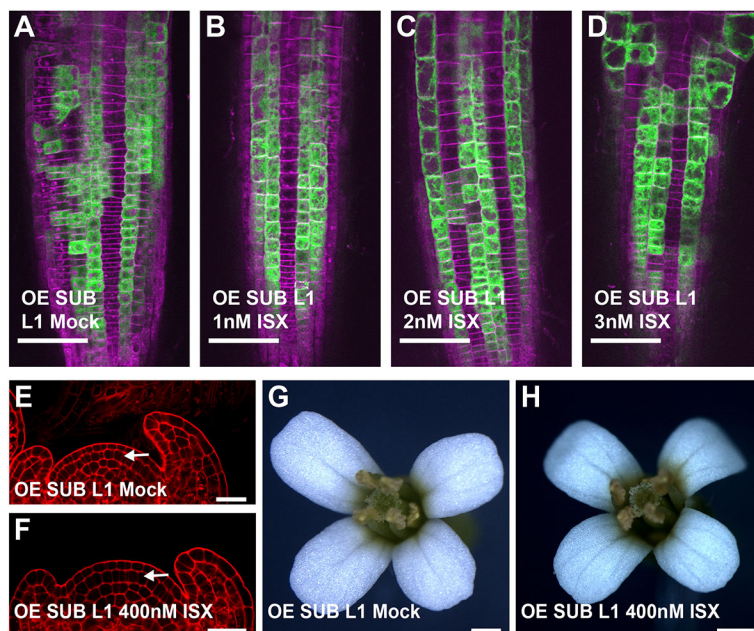
content (Du et al., 2020; Xiao et al., 2016). Indeed, we found that *SUB* transcript and SUB:EGFP protein levels were downregulated upon application of epigallocatechin gallate (EGCG), an inhibitor of pectin methylesterase activity (Lewis et al., 2008), to 7-day-old seedlings. Moreover, the treated seedlings exhibited root hair patterning defects (Fig. S5). The relative influence of altered pectin architecture and reduced cellulose content on these processes remains to be investigated.

The signal that controls SUB internalization and *SUB* transcript levels is currently unknown, but the cell wall represents an obvious possible source. However, movements of CSCs are guided by cortical microtubules (Paredes et al., 2006) and several components of CSCs interact with microtubules (Bringmann et al., 2012; Endler et al., 2015; Gu et al., 2010; Kesten et al., 2019; Liu et al., 2016; Li et al., 2012). Application of isoxaben results in the rapid internalization of CESA subunits (Paredes et al., 2006) and isoxaben-treated wild-type plants as well as several *cesa* mutants were shown to exhibit altered cortical microtubule alignment (Chu et al., 2007; Fisher and Cyr, 1998; Paredes et al., 2008). Thus, the signal regulating SUB abundance could also originate from the cytoskeleton. However, we think it unlikely as treatment of seedlings with pharmaceutical compounds affecting the microtubule or actin cytoskeleton did not result in noticeable aberrations in root hair patterning. It will be interesting to identify the cell wall-derived signal in future studies.

## MATERIALS AND METHODS

### Plant work and lines

*Arabidopsis* (L.) Heynh. var. Columbia (Col-0) and var. Landsberg (*erecta* mutant) (*Ler*) were used as wild-type strains. Plants were grown on soil as previously described (Fulton et al., 2009). Plate-grown seedlings were grown in long-day conditions (16 h light/8 h dark) on half-strength Murashige and Skoog (1/2 MS) agar plates supplemented with 0.3% sucrose. The following mutant alleles were used: *sub-1* and *qky-8* (*Ler*) and *sub-9* and *sub-21* (Col) (Chaudhary et al., 2020; Chevalier et al., 2005; Fulton et al., 2009), *prc1-1* (Col) (Fagard et al., 2000), *ixr2-1* (Col) (Desprez et al., 2002). The *sub-1*, *sub-9* and *sub-21* alleles all represent predicted null alleles that cause comparable defects in root hair patterning and ovule development. The lines carrying pSUB::SUB:GFP, pUBQ::SUB:mCherry (O3, L1, Col) and pGL2::GUS:GFP (Col) have been previously reported (Chaudhary et al., 2020; Gao et al., 2019; Vaddepalli et al., 2011, 2014). The pTHE1::THE1:GFP line was a gift from Herman Höfte (INRA, AgroParisTech, Versailles, France). The pFER::FER:GFP (Duan et al., 2010) as well as the TMO7:1×GFP and TMO7:3×GFP reporter lines (Schlereth et al., 2010) have been previously described. For the generation of the various multiple mutant lines see supplementary Materials and Methods for further details.



**Fig. 5. Overexpression of SUB:mCherry attenuates the isoxaben effects on root hair patterning and floral morphogenesis.**

(A-D) Confocal micrographs depicting optical sections through roots of 7-day-old seedlings showing the pGL2::GUS:GFP expression pattern. The cell wall was counter-stained with propidium iodide. Note the mild aberration in pGL2::GUS:GFP pattern in A (compare with Fig. 3H). Note the normal to mildly abnormal pGL2::GUS:GFP patterns in B-D (compare with Fig. 3I). For a quantification of the effects see Table 2. The remaining roots showed the occasional single cell displacement in the reporter expression as also sometimes seen in wild type (Table 2). The experiment was performed three times with similar results. (E,F) Confocal micrographs depicting mid-optical sections through stage 3 floral meristems stained with propidium iodide. For a quantification of the effects see Table 3. Arrows highlight regular L2 layers. (G,H) Morphology of stage 13 or 14 flowers. Note the normal petal arrangement. Of those flowers examined, 74% (14/19) showed the phenotype in G and 82% (18/22) showed the phenotype in H. The experiment was repeated twice with similar results. Genotypes and/or treatments are indicated in the panels. Scale bars: 50  $\mu$ m (A-D); 20  $\mu$ m (E,F); 0.5 mm (G,H).

### Chemical treatments

Isoxaben, DCB, Thaxtomin A, Latrunculin B, Cytochalasin D, oryzalin, sorbitol, Congo Red and EGCG were obtained from Sigma-Aldrich and used from stock solutions in DMSO (isoxaben: 100  $\mu$ M; DCB: 1 mM; Thaxtomin A: 100  $\mu$ M; Latrunculin B: 100  $\mu$ M; Cytochalasin D: 500  $\mu$ M; Oryzalin: 100  $\mu$ M; EGCG: 1 mM) or in water (sorbitol: 1 M; NaCl: 2.5 M; Congo Red: 1%). FM4-64 was purchased from Molecular Probes (2 mM stock solution in water). For FM4-64 staining seedlings were incubated in 4  $\mu$ M FM4-64 in liquid 1/2 MS medium for 5 min before imaging.

### PCR-based gene expression analysis

For qPCR of *SUB*, 35–40 seedlings per flask were grown in liquid culture under continuous light at 18°C for 7 days, followed by treatment with mock or 600 nM isoxaben for 8 h on plates (21°C, long-day conditions). RNA extraction and quality control were performed as previously described (Box et al., 2011). cDNA synthesis, qPCR and analysis were carried out essentially as previously described (Enugutti et al., 2012). Primers are listed in Table S1.

### Western blot analysis

We collected 300 mg of 7-day wild-type or mutant *Arabidopsis* seedlings with forceps, placed them in a 2 ml Eppendorf tube and froze them immediately using liquid nitrogen. After lysing the seedlings using a Tissue Lyser (Qiagen), two volumes of 2 $\times$  Laemmli buffer [65.8 mM Tris-HCl (pH 6.8), 2.1% (w/v) SDS, 26.3% (v/v) glycerol, 2.5% (v/v)  $\beta$ -mercaptoethanol, 0.01% bromophenol blue] were added to the homogenate. Samples were boiled at 70°C for 15 min, centrifuged at 12,000 rpm (10,625 g) for 20 min, and the supernatant was loaded on the gel. Proteins separated in SDS-10% PAGE were transferred to a PVDF membrane using the Trans-Blot Turbo Transfer System (Bio-Rad). Membranes were blocked with blocking buffer [2.5% milk powder in TBS-T (50 mM Tris-Cl, 150 mM NaCl, 0.1% Tween-20, pH 7.4)] and incubated with a GFP-antibody (3H9, Chromotek, 1:2500) at 4°C. Anti-rat horseradish peroxidase-conjugated antibody was used as a secondary antibody (AS10 1187, Agrisera, 1:5000). Blots were developed using chemiluminescence (Supersignal West Atto, Thermo Fisher Scientific) and stained afterwards with Ponceau S for assessing loading.

### Microscopy

Scanning electron microscopy and fixing and staining of floral meristems were essentially performed as previously described (Fulton et al., 2009; Gao et al., 2019; Schneitz et al., 1997). Confocal laser scanning microscopy was performed with an Olympus FV1000 set-up using an inverted IX81 stand and Fluoview software (FV10-ASW version 01.04.00.09, Olympus Europa) equipped with a water-corrected  $\times$ 40 objective (NA 0.9) at  $\times$ 3 digital zoom. For GFP fluorescence intensity measurements the mean gray values of GFP fluorescence signal intensity in the root epidermis of the pSUB::SUB:EGFP reporter was analyzed with ImageJ software (Schindelin et al., 2012). For each root, a region located 500  $\mu$ m above the root tip (excluding the root cap) was used for analysis. To obtain the ratio between signal in cytoplasm versus total fluorescence intensity per cell we measured signal intensity in two different regions of interests (ROIs). One ROI covered the cytoplasm and the other ROI included the cytoplasm and the outer cell boundary.

For SUB:EGFP subcellular localization upon drug treatments or colocalization with FM4-64, confocal laser scanning microscopy was performed on epidermal cells of root meristems located  $\sim$ 8–12 cells above the quiescent center using a Leica TCS SP8 X microscope equipped with GaAsP (HyD) detectors. The following objectives were used: a water-corrected  $\times$ 63 objective (NA 1.2), a  $\times$ 40 objective (NA 1.1), and a  $\times$ 20 immersion objective (NA 0.75). Scan speed was set at 400 Hz, line average at between 2 and 4, and the digital zoom at 4.5 (colocalization with FM4-64), 3 (drug treatments) or 1 (root hair patterning). EGFP fluorescence excitation was performed at 488 nm using a multi-line argon laser (3% intensity) and detected at 502–536 nm. FM4-64 fluorescence was excited using a 561 nm laser (1% intensity) and detected at 610–672 nm. For the direct comparisons of fluorescence intensities, laser, pinhole and gain

settings of the confocal microscope were kept identical when capturing the images from the seedlings of different treatments. For determination of colocalization, the distance from the center of each EGFP spot to the center of the nearest FM4-64 signal was measured by hand on single optical sections using ImageJ/Fiji software. If the distance between two puncta was below the resolution limit of the objective lens (0.24  $\mu$ m) the signals were considered to colocalize (Ito et al., 2012). *Arabidopsis* seedlings were covered with a 22 $\times$ 22 mm glass coverslip of 0.17 mm thickness (no. 1.5H, Paul Marienfeld). Images were adjusted for color and contrast using ImageJ/Fiji software.

Steady state fluorescence anisotropy was measured using a FV3000 confocal laser scanning microscope (Olympus Europa) equipped with a single photon counting device with picosecond time resolution (LSM upgrade kit, PicoQuant). GFP was excited at 485 nm with a linearly polarized, pulsed (40 MHz) diode laser (LDH-D-C-485, PicoQuant) using a 60 $\times$  water immersion objective (Olympus UPlanSApo, NA1.2). The emitted light was collected in the same objective and was separated into perpendicular and parallel polarization with respect to excitation polarization. GFP fluorescence was then detected using a PMA Hybrid 40 detector (PicoQuant) in a narrow range of its emission spectrum (BP520/35). A minimum 25 photons per pixel were counted with time correlated single photon counting (TCSPC) resolution of 25 ps of ROI size 512 px $\times$ 512 px with 0.103  $\mu$ M/px. Pixel-wise anisotropy was calculated with help of SymPhoTime 64 software (PicoQuant) using instrument correction factors L1 (0.035) and L2 (0.031) as well as the G-factor (1.018).

### Statistics

Statistical analysis was performed with PRISM9 software (GraphPad Software). All statistical tests including *P*-values are described in the respective figure legends.

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### Competing interests

The authors declare no competing or financial interests.

### Author contributions

Conceptualization: A.C., X.C., S.W., K.S.; Methodology: A.C., X.C., B.L., R.B., J.G., S.W.; Formal analysis: A.C., X.C., B.L., R.B., S.W., K.S.; Investigation: A.C., X.C., B.L., R.B., J.G., S.W.; Writing - original draft: K.S.; Writing - review & editing: A.C., R.B., S.W., K.S.; Visualization: A.C., K.S.; Project administration: K.S.; Funding acquisition: S.W., K.S.

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