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

Ajeet Chaudhary, Xia Chen, Barbara Leśniewska, Rodion Boikine, Jin Gao, Sebastian Wolf and Kay Schneitz

DOI: 10.1242/dev.199425

Editor: Yka Helariutta

Review timeline

Original submission:	12 January 2021
Editorial decision:	6 April 2021
First revision received:	27 May 2021
Accepted:	14 June 2021

Original submission

First decision letter

MS ID#: DEVELOP/2021/199425

MS TITLE: Cell wall-dependent control of root hair cell fate and floral morphogenesis in Arabidopsis thaliana

AUTHORS: Ajeet Chaudhary, Xia Chen, Barbara Leśniewska, Jin Gao, Sebastian Wolf, and Kay Schneitz

I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

We are aware that you may be experiencing disruption to the normal running of your lab that make experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

Chaudhary and colleagues show that the inhibition of cellulose biosynthesis through isoxaben (ISX) treatments leads to developmental phenotypes similar to those found in mutants in STRUBBELIG (SUB), encoding for an atypical plasma membrane receptor kinase involved in plant cell wall integrity signaling. The authors convincingly show that ISX leads to reduced SUB transcriptional and translational abundance in the root meristem, hence correlating the phenotypic outputs. Consistently, they show that ISX treatments result in reduced SUB oligomerization at the plasma membrane (measured by fluorescence anisotropy as a proxy for SUB protein complex formation) and increased SUB endocytosis. They also use cellulose-deficient mutants in CesA6 (prc1-1 and ixr2-1) to ascertain the impact of cellulose deficiency on regulating SUB levels, and partially confirm their ISX results. From these findings the authors conclude that cellulose deficiency regulates SUB abundance through a multistep process that first involves SUB depletion from the plasma membrane and successively reduces its transcription.

The next sections in the paper describe phenotypic similarities between ISX treatments and sub mutants. The sub phenotypes analyzed include previously described alterations in root hair patterning and floral organ development. For root hair patterning, the authors also analyze the localization of a transcriptional GL2 reporter, specific for non-hair (N) cells. The reporter indicates that GL2 expression is altered following ISX and to a certain degree in a sub mutant. From the correlative evidence provided in 2 different tissues, the authors claim that ISX exerts its developmental effects through SUB, and that the cell wall regulates cell fate and tissue morphogenesis. However, the data provided is not sufficiently robust to substantiate these claims.

Overall, the paper provides many interesting but fragmented results, leading to a storyline that may be difficult to follow. The general aims of the study are not clear and should be better introduced: do the authors aim to characterize SUB's role in cell wall integrity signaling and impact on root hair and floral development?; SUB's regulation following cellulose-deficiency?; SUB's role in cell fate determination (only examined for root hairs, not reproductive organs)? Similarly, the results are often presented without explanations on how they relate to the author's hypothesis, or are not analyzed adequately. In some cases, data presented in a main figure is not described in the text. To yield a coherent story, several figures should be properly re-assembled and results better introduced and described.

Comments for the author

MAJOR POINTS:
1. Known ISX targets are CesA proteins, whose relative mutants are ISX insensitive. If indeed ISX exerts its cell wall function through SUB signaling, then sub mutants should show (partial) insensitivity to ISX with respect to the WT. Is this the case in terms of root hair patterning (Fig.3) and floral organ morphogenesis (Fig4)? In other words, the authors should include sub mutants treated with ISX and compare their response to the one observed in the WT (for results presented in Fig3 and 4). As is, the phenotypes between ISX treatment and sub mutants remain correlative.

2. Claiming that the cell wall regulates cell fate is disproportionate from the data provided and would require extensive additional experiments such as meticulous spatiotemporal analysis of cell identities and trajectories that extend beyond the scope of this work. I recommend the authors to rephrase their claims about cell fate throughout the manuscript and replace them with more accepted cell wall roles in tissue patterning and morphogenesis.

3. T-tests are not appropriate to compare more than 2 samples nor for testing statistical differences in treatment responsiveness. Adequate statistical tests (eg. multiple comparison tests and linear models) should be used throughout the manuscript (Fig. 1B, 1C, 1E, 2F, 2Q, 2R, S1B, S2D, S3A, S3B, all tables)

4. Fig.3-5. Qualitative images should be supported by quantitative measurements of all (!) data sets analyzed and presented in graphs showing the relative statistical analyses (as done in Fig.1 and 2). It is unclear why the authors show the data in tables for only a subset of samples without

performing any statistical analysis to support their claims (missing statistics in Table1,3; inadequate statistical tests in Table2,4). Quantifications listed in Table1-4 should include all depicted genotypes/treatments in the main figures 3-5 (and not only a subset, and presented as graphs in relative figures). Also, grouping results according to hypothesis and experimental conditions should greatly improve clarity in the text (eg. Fig. 3A-E, 3F-G, 3H-N, 3O-Q, 3V-W). Fig. 3O-Q, it is unclear why are these drugs tested and why is the cytoskeleton relevant? The working hypothesis should be included in the result section, and data properly quantified. Also, what's the rationale of figures presented in 3R-T? They are not even described in the text. In the legends, the authors should provide how many cells were analysed per root in all relative figures/tables (and not only the total number of cells, i.e. root number should be the relevant biological replicate)

5. Fig.4 As stated above, quantifications of aberrant cell divisions in floral meristems/ petal angle arrangement/ ovule integument outgrowth, should be quantified and presented with appropriate statistical analysis including sub mutants treated with ISX to assess their responsiveness.

6. The logic of experiments in Fig.5. is clear and results suggest that indeed at least part of ISX effects on patterning and morphogenesis can be rescued by increasing SUB abundance. However, similarly to Fig.3 and 4, quantitative data and proper statistical analysis need improvement. The authors should also include control images for pUBQ::SUB:mCherry before and after ISX treatment to show that the effect is due to increased SUB abundance at the plasmamembrane. There is no legend for panels E-H. How do the authors explain that both absence and overexpression of SUB lead to GL2 misexpression and aberrant root hair patterning?

7. Some results are presented only in the Discussion (Fig. 3M, S3). Instead, they should be presented in the results section and introduced accordingly. Also, several discussion points should actually serve to introduce parts of the results (eg. cytoskeleton drugs).

OTHER POINTS
- The organization of Figures 1 and 2 is very asymmetric with many white spaces, suggesting something is missing (there are no 2G and 2H panels)
- legend Fig.1A-E: indicate genetic background of the SUB-GFP reporter
- The authors often refer to SUB activity when assaying SUB-GFP signals or transcript levels. To my knowledge, it is not yet known what SUB activity is, in terms of ligand binding, phosphorylation, etc. The authors should use more specific terms throughout the manuscript, such as 'abundance, levels, localization..' according to the context.
- Line 165: Is it known which membrane protein complexes contain SUB, and if SUB oligomerizes? Does it form complexes with THE and FER? An introduction would help understanding the need for these experiments
- 'to this end' is used excessively throughout the manuscript
- Line 317: 'Normal plates' should be rephrased to 'mock/control plates' or something similar, here and throughout the text
- Figure legend 5: title it should be SUB-mCherry instead of SUB-EGFP?
- Figure legend S3: It should be pGL2:EGFP instead of pGL2:SUB-EGFP
- It would be helpful to describe (or refer to) the mutations and phenotypic differences between sub-1 (in Ler) and sub-9 (in Col) alleles used, eg. are both full loss of function?. As above, the qualitative images provides should correspond to the quantified genotypes. This is often not the case: eg. Fig4G shows sub1 and WT (Ler), but the quantifications in Table4 are for sub-9 and wt (Col)

Reviewer 2

Advance summary and potential significance to field

Cell wall integrity sensing and detection mechanisms play an important role in plant growth and development.
In the last years this topic has received considerable attention. Schneitz and co-workers have already made important contributions to this filed and with this manuscript, they aim at further understanding how the atypical receptor kinase STRUBBELIG (SUB) functions in this pathway. They provide evidence that SUB mutants are more resistant to isoxaben and that SUB is required for

proper cell wall monitoring and thereby regulating cell fate and morphogenesis. In parallel, SUB levels are reduced in cellulose synthase mutant prc1-1. They propose that SUB is part of a complex that is regulated by cellulose biosynthesis and that a certain threshold of SUB level is required for normal morphogenesis. They reinforce this statement by providing data that shows that ectopic expression of SUB attenuates the detrimental effects of isobaxen. In general, this work reveals new insights into the function of SUB and how it might be intertwined in the CWI sensing pathway.

Comments for the author

In the current manuscript by Schneitz and co-workers, they further explore the role of SUB in plant morphogenesis. In particular, they focus on root hair patterning and flower morphogenesis.In general the manuscript is well written and rather easy to follow. Most of the data is presented in a clear way. I have only some minor suggestions that I think can be easily addressed by the authors.

- I think the title is a bit too vague and not really conveying the message of the manuscript. I think it can be a bit more precise, since the manuscript is focussed on SUB and potential links to the cell wall.

- Most of the quantifications of SUB levels are performed either via qRT-PCR or pixel intensities using a CLSM. Can the authors also provide a western blot showing the SUB protein levels in wt, prc1-1 and after the isoxaben treatments? I think this could provide useful additional data.

- With regard to the anisotropy measurements on the different receptor-GFP fusions, did the authors check whether the constructs have made use of comparable linkers? I could imagine that a more flexible linker could affect the rotational freedom of the GFP. In addition, I do not understand the value of showing the TMO7 fusions, since you are comparing plasma membrane localised proteins. If it is just to show that anisotropy measurements can tell something about potential multimerisation state, then a simple reference would suffice. Have the authors attempted to measure the anisotropy of SUB-GFP when it is internalised? It might be interesting to test whether they have similar values compared to when localised at the plasma membrane.

- Since the authors talk about the activity of SUB in the discussion, I would like to know what they mean with this. How do they know when SUB is active since it apparently has a non-functional kinase domain based on earlier work from the same lab. I think this needs to be revised in the manuscript since they do not provide any data that shows a way to differentiate activated SUB from inactive SUB.

First revision

Author response to reviewers' comments

Reviewer 1 Advance Summary and Potential Significance to Field:
Chaudhary and colleagues show that the inhibition of cellulose biosynthesis through isoxaben (ISX) treatments leads to developmental phenotypes similar to those found in mutants in STRUBBELIG (SUB), encoding for an atypical plasma membrane receptor kinase involved in plant cell wall integrity signaling. The authors convincingly show that ISX leads to reduced SUB transcriptional and translational abundance in the root meristem, hence correlating the phenotypic outputs. Consistently, they show that ISX treatments result in reduced SUB oligomerization at the plasma membrane (measured by fluorescence anisotropy as a proxy for SUB protein complex formation) and increased SUB endocytosis. They also use cellulose-deficient mutants in CesA6 (prc1-1 and ixr2-1) to ascertain the impact of cellulose deficiency on regulating SUB levels, and partially confirm their ISX results. From these findings the authors conclude that cellulose deficiency regulates SUB abundance through a multistep process that first involves SUB depletion from the plasma membrane and successively reduces its transcription.

The next sections in the paper describe phenotypic similarities between ISX treatments and sub

mutants. The sub phenotypes analyzed include previously described alterations in root hair patterning and floral organ development. For root hair patterning, the authors also analyze the localization of a transcriptional GL2 reporter, specific for non-hair (N) cells. The reporter indicates that GL2 expression is altered following ISX and to a certain degree in a sub mutant. From the correlative evidence provided in 2 different tissues, the authors claim that ISX exerts its developmental effects through SUB, and that the cell wall regulates cell fate and tissue morphogenesis. However, the data provided is not sufficiently robust to substantiate these claims.

Overall, the paper provides many interesting but fragmented results, leading to a storyline that may be difficult to follow. The general aims of the study are not clear and should be better introduced: do the authors aim to characterize SUB's role in cell wall integrity signaling and impact on root hair and floral development?; SUB's regulation following cellulose-deficiency?; SUB's role in cell fate determination (only examined for root hairs, not reproductive organs)? Similarly, the results are often presented without explanations on how they relate to the author's hypothesis, or are not analyzed adequately. In some cases, data presented in a main figure is not described in the text. To yield a coherent story, several figures should be properly re- assembled and results better introduced and described.
XX

Reviewer 1 Comments for the Author:

MAJOR POINTS:
1. Known ISX targets are CesA proteins, whose relative mutants are ISX insensitive. If indeed ISX exerts its cell wall function through SUB signaling, then sub mutants should show (partial) insensitivity to ISX with respect to the WT. Is this the case in terms of root hair patterning (Fig.3) and floral organ morphogenesis (Fig4)? In other words, the authors should include sub mutants treated with ISX and compare their response to the one observed in the WT (for results presented in Fig3 and 4). As is, the phenotypes between ISX treatment and sub mutants remain correlative.

We believe this might be a misunderstanding. Throughout the manuscript we never state that SUB represents the receptor for ISX. Indeed, as rightly pointed out by the referee, a mutation in such a receptor should result in insensitivity to ISX treatment. This is clearly not the case as *sub* mutants remain sensitive to ISX. What we do claim is that application of ISX alters the cell wall and that this leads to previously unrecognized problems in root hair patterning and floral morphogenesis. Thus, under normal conditions there is a cell wall-dependent mechanism that maintains root hair patterning and floral morphogenesis by controlling SUB levels. This mechanism is indirectly disrupted by treatment with ISX (or in a *prc1-1* mutant, or by EGCG treatment).

The ISX or EGCG-induced morphological aberrations resemble the known and well- described morphological aspects of the *sub* phenotype. Importantly, we also found that overexpression of *SUB* alleviates the effects of ISX. This central observation reveals that the control of *SUB* is the main access point of the ISX-dependent mechanism as far as root hair patterning and floral morphogenesis are concerned. However, the referee is correct to request an analysis of root hair patterning in ISX- treated *sub-9* seedlings to confirm that the effect of ISX on this process is solely achieved via the regulation of SUB. This has been done and the results included in Fig. 3 and Tables 1 and 2. We find no significant differences in root hair patterning defects between *sub-9* and *sub-9* treated with 3nM ISX, corroborating that downregulation of SUB is the central factor in the effect of ISX-induced cell wall alterations on root hair patterning.

Taken together, our findings show that downregulation of *SUB* and the *sub*-like phenotypes induced by the exposure to ISX do not simply correlate but provide evidence that there is a causal link between the two observations.

2. Claiming that the cell wall regulates cell fate is disproportionate from the data provided and would require extensive additional experiments such as meticulous spatiotemporal analysis of cell identities and trajectories that extend beyond the scope of this work. I recommend the authors to rephrase their claims about cell fate throughout the manuscript and replace them with more accepted cell wall roles in tissue patterning and morphogenesis.

Here, we disagree. We provide pharmacological as well as genetic evidence that interfering with cellulose biosynthesis (ISX/DCB/Thaxtomin A, *prc1-1*) or with the pectin component of the cell wall (EGCG is supposed to interfere with pectin methylesterase activity) results in clearly observable defects in the regulation of root hair fate. This is a new finding. It indicates that the cell wall contributes to the control of root hair fate. Our evidence further shows that the cell wall contributes to the maintenance of proper mRNA and protein levels of SUB, a well-known regulator of root hair fate, as these two parameters are affected in ISX-treated seedlings. In addition, EGCG treatment also affects *SUB* mRNA levels. Furthermore, our results reveal that the regulation of SUB constitutes a central aspect of the control of root hair patterning by the cell wall since the root hair patterning phenotype of *sub* null mutants is not exacerbated by application of sub-lethal doses of ISX. Moreover, overexpression of SUB alleviates the effects of ISX.

Although the molecular mechanism maintaining SUB expression remains to be elucidated, and we clearly state so in the Discussion, we argue that our evidence demonstrates that the cell wall participates in the control of root hair cell fate.
However, to accommodate the criticisms and suggestions by both referees we altered the title to make it more specific. In addition, the final sentence of the first paragraph of the Discussion now reads “Thus, they demonstrate a role of the plant cell wall in the control of root hair cell fate.”

3. T-tests are not appropriate to compare more than 2 samples nor for testing statistical differences in treatment responsiveness. Adequate statistical tests (eg. multiple comparison tests and linear models) should be used throughout the manuscript (Fig. 1B, 1C, 1E, 2F, 2Q, 2R, S1B, S2D, S3A, S3B, all tables)

This is a misunderstanding. In Figures 1B/C/E, 2F, 2Q and 2R (which are actually 2O and 2P due to labelling errors that were fixed in the revision), S1B, S2D, S3B, we use *one-way ANOVA followed by multiple comparisons tests. The statistical method is outlined in the respective figure legends.*

Regarding the statistics used in the tables see comment to issue #4.

4. Fig.3-5. Qualitative images should be supported by quantitative measurements of all (!) data sets analyzed and presented in graphs showing the relative statistical analyses (as done in Fig.1 and 2). It is unclear why the authors show the data in tables for only a subset of samples without performing any statistical analysis to support their claims (missing statistics in Table1,3; inadequate statistical tests in Table2,4). Quantifications listed in Table1-4 should include all depicted genotypes/treatments in the main figures 3-5 (and not only a subset, and presented as graphs in relative figures).

We obtained expert advice on the statistical analysis of our data. In the various experiments the dependent variables have two outcomes (e.g. H cell, yes or no; at least one periclinal cell division yes or no; defective ovules, yes or no) and thus we applied the recommended tests for each table. We also tested other approaches, such as ANOVA followed by multiple comparisons with Tukey’s tests (although we learned that ANOVA should not be used in such experimental designs), Chi-square tests or Fisher’s exact tests. In each case we obtained similar results.

Tables 1-4 provide the quantifications for the central observations made in Figures 3 to 5.

Fig. 3/Table 1: cellular morphology of root hair patterning in different genotypes/treatments.

We now include values for Col mock, *sub-9* +/- ISX, *ixr2-1* +/- ISX and *prc1-1*.

Fig. 3/Table2: pGL2::GUS:EGFP patterns in different genotypes/treatments.

We added values for *sub-9*/+, *sub-9* +/- ISX, Col + DCB, Col + TA, *prc1-1*, *sub-9* + sorbitol, Col + sorbitol, Col + ISX/sorbitol.

Since we did not observe any obvious effects on the spatial distribution of pGL2::GUS:EGFP pattern in seedlings treated with oryzalin, NaCl, Lat-B, Cyto-D and Congo Red, we feel that a statistical analysis is not essential in these cases.

Fig. 4/Table 3: quantification of periclinal cell divisions in L2 of floral meristem in different genotypes/treatments.
Fig. 4/Table 4: quantification of integument defects in different genotypes/treatments.

Percentage of flowers with petal defects was added to the figure legend of Fig. 4.

Fig. 5/Table 2: quantification of the pGL2::GUS:EGFP pattern in Col OE lines + ISX Fig 5 /Table 3: quantification of periclinal cell divisions in L2 of floral meristem in Col OE lines + ISX.

Table 4: quantification of integument defects in Col OE lines + ISX.

Percentage of flowers with petal defects was added to the figure legend of Fig. 5.

Also, grouping results according to hypothesis and experimental conditions should greatly improve clarity in the text (eg. Fig. 3A-E, 3F-G, 3H-N, 3O-Q, 3V-W).

We rephrased and reorganized the entire section in the manuscript that deals with Fig. 3. We reordered Fig. 3A-G. In addition, we rearranged the corresponding paragraph in the manuscript and reorganized Fig. 3H-W. All panels of Fig. 3 are mentioned in the text.

Fig. 3O-Q, it is unclear why are these drugs tested and why is the cytoskeleton relevant? The working hypothesis should be included in the result section, and data properly quantified.

The reasons to perform experiments with the mentioned drugs are now better introduced. We did not detect any defects so we believe quantification is not essential.

Also, what's the rationale of figures presented in 3R-T? They are not even described in the text.

See comments above.

In the legends, the authors should provide how many cells were analysed per root in all relative figures/tables (and not only the total number of cells, i.e. root number should be the relevant biological replicate)

As indicated in the figure legend, quantification of the relevant results is provided in Table 2, including the number of cells analyzed. In the revised version Table 2 was expanded to include additional quantifications.

5. Fig.4 As stated above, quantifications of aberrant cell divisions in floral meristems/ petal angle arrangement/ ovule integument outgrowth, should be quantified and presented with appropriate statistical analysis including sub mutants treated with ISX to assess their responsiveness.

The quantification of the aberrant cell divisions in FMs and integument defects are given in Tables 3 and 4 as mentioned in the text. We now also refer to these tables in the legend to Fig. 4. The petal phenotype is obvious but difficult to quantify. We now include the percentage of flowers with such defects in the legend to Fig. 4.

6. The logic of experiments in Fig.5. is clear and results suggest that indeed at least part of ISX effects on patterning and morphogenesis can be rescued by increasing SUB abundance. However, similarly to Fig.3 and 4, quantitative data and proper statistical analysis need improvement.

The quantitative data plus statistical analysis are given in Figure 5 and in Table 4. Regarding the statistics see comments to issue #4.

The authors should also include control images for pUBQ::SUB:mCherry before and after ISX treatment to show that the effect is due to increased SUB abundance at the plasmamembrane.

By using the pUBQ promoter we uncoupled *SUB* from its regular transcriptional control. The two independent overexpression lines show drastically increased combined *SUB* and *SUB:mCherry* expression (≥500 fold, Chaudhary et. al. 2020 PLoS Genetics 16:e1008433). We now show in Fig. S4 that SUB:mCherry signal is readily detectable before and after ISX treatment. A direct comparison between the pSUB::SUB:EGFP and pUBQ::SUB:mCherry reporters is practically impossible due to the different tags

There is no legend for panels E-H.

The legends have been added.

How do the authors explain that both absence and overexpression of SUB lead to GL2 misexpression and aberrant root hair patterning?

The observation that loss-of-function or overexpression of SUB causes root hair patterning is an old question that remains presently unexplored. We now mention this in the text and offer a possible explanation (SUB may act as a scaffold in a multi- protein complex, the stoichiometry of SUB may matter).

7. Some results are presented only in the Discussion (Fig. 3M, S3). Instead, they should be presented in the results section and introduced accordingly. Also, several discussion points should actually serve to introduce parts of the results (eg. cytoskeleton drugs).

The *sub-9*/+ result (previously Fig. 3M) is now mentioned in the corresponding Results section (new Fig. 3I). The cytoskeletal drugs and the Congo Red stain are now better introduced in the Results section as well. Given the strict length limit of DEVELOPMENT we choose to keep the EGCG results in the Supplement (Fig. S4).

OTHER POINTS

- The organization of Figures 1 and 2 is very asymmetric with many white spaces, suggesting something is missing (there are no 2G and 2H panels)

We have reorganized the two figures.

- legend Fig.1A-E: indicate genetic background of the SUB-GFP reporter

We added the genetic background.

- The authors often refer to SUB activity when assaying SUB-GFP signals or transcript levels. To my knowledge, it is not yet known what SUB activity is, in terms of ligand binding, phosphorylation, etc. The authors should use more specific terms throughout the manuscript, such as 'abundance, levels, localization..' according to the context.

We applied the term "*SUB* activity" in a genetic sense. In the revised version of the manuscript we keep this term in only two instances. All other occurrences were removed and more specific terms were added as suggested.

- Line 165: Is it known which membrane protein complexes contain SUB, and if SUB oligomerizes? Does it form complexes with THE and FER? An introduction would help understanding the need for these experiments

The only other known component of a SUB complex is QKY as outlined in the Introduction. THE-GFP and FER-GFP constitute two receptors that are implied in monitoring the cell wall status. Thus, they were analyzed to compare FA values between receptor kinases involved in CWI surveillance and essentially serve as controls. This has been clarified in the text.

- *'to this end' is used excessively throughout the manuscript*

"To this end" is now occurring only twice in the manuscript.

- *Line 317: 'Normal plates' should be rephrased to 'mock/control plates' or something similar, here and throughout the text*

Corrected.

- *Figure legend 5: title it should be SUB-mCherry instead of SUB-EGFP?*

Corrected.

- *Figure legend S3: It should be pGL2:EGFP instead of pGL2:SUB-EGFP*

Corrected.

- *It would be helpful to describe (or refer to) the mutations and phenotypic differences between sub-1 (in Ler) and sub-9 (in Col) alleles used, eg. are both full loss of function?. As above, the qualitative images provides should correspond to the quantified genotypes. This is often not the case: eg. Fig4G shows sub1 and WT (Ler), but the quantifications in Table4 are for sub-9 and wt (Col)*

The well-described *sub-1* (L*er*), *sub-9* (Col), and *sub-21* (Col) alleles are all predicted null alleles. They cause comparable defects in root hair patterning and ovule development. This was clarified in the Materials and Methods section and in the Results.

Reviewer 2 Advance Summary and Potential Significance to Field:
Cell wall integrity sensing and detection mechanisms play an important role in plant growth and development. In the last years this topic has received considerable attention. Schneitz and co-workers have already made important contributions to this filed and with this manuscript, they aim at further understanding how the atypical receptor kinase STRUBBELIG (SUB) functions in this pathway. They provide evidence that SUB mutants are more resistant to isoxaben and that SUB is required for proper cell wall monitoring and thereby regulating cell fate and morphogenesis. In parallel, SUB levels are reduced in cellulose synthase mutant prc1-1. They propose that SUB is part of a complex that is regulated by cellulose biosynthesis and that a certain threshold of SUB level is required for normal morphogenesis. They reinforce this statement by providing data that shows that ectopic expression of SUB attenuates the detrimental effects of isobaxen. In general, this work reveals new insights into the function of SUB and how it might be intertwined in the CWI sensing pathway.

Reviewer 2 Comments for the Author:
In the current manuscript by Schneitz and co-workers, they further explore the role of SUB in plant morphogenesis. In particular, they focus on root hair patterning and flower morphogenesis.In general the manuscript is well written and rather easy to follow. Most of the data is presented in a clear way. I have only some minor suggestions that I think can be easily addressed by the authors.

- *I think the title is a bit too vague and not really conveying the message of the manuscript. I think it can be a bit more precise, since the manuscript is focussed on SUB and potential links to the cell wall.*

We have changed the title to "Cell wall damage attenuates root hair patterning and tissue morphogenesis mediated by the receptor kinase STRUBBELIG".

- *Most of the quantifications of SUB levels are performed either via qRT-PCR or pixel intensities using a CLSM. Can the authors also provide a western blot showing the SUB protein levels in wt, prc1-1 and after the isoxaben treatments? I think this could provide useful additional data.*

The western blots and graphs depicting the quantifications have been added to (new Fig. S2). The results confirm the other data.

- *With regard to the anisotropy measurements on the different receptor-GFP fusions, did the authors check whether the constructs have made use of comparable linkers? I could imagine that a more flexible linker could affect the rotational freedom of the GFP.*

The comparisons of the FA values for each fusion protein +/- isoxaben constitute the central aspects of the FA measurements in Fig. 2. When comparing relative FA differences for a given fusion protein +/- changing conditions the linker is not relevant.

Regarding the comparisons of FA values between different fusion proteins it should be noted that the rotational aspect can be largely neglected for large GFP-based fusion proteins. However, the linkers could principally constitute an issue since they may influence how the GFP is positioned relative to the rest of the fusion protein and that positioning may affect homo-FRET. For example, there is less FRET if the dipole moments of two GFPs are not aligned in parallel. We found that the linkers of the three fusion proteins differ in length and composition with only SUB:EGFP having the standard polyalanine linker. Thus, we softened the statement about varying rotational freedoms and added a sentence mentioning this alternative possibility.

In addition, I do not understand the value of showing the TMO7 fusions, since you are comparing plasma membrane localised proteins. If it is just to show that anisotropy measurements can tell something about potential multimerisation state, then a simple reference would suffice.

The TMO7 experiment simply serves as a general positive control for the FA experiment. Since FA experiments are not yet broadly used in plant cell biology we explained the control experiment in a bit more detail.

Have the authors attempted to measure the anisotropy of SUB-GFP when it is internalised? It might be interesting to test whether they have similar values compared to when localised at the plasma membrane.

We have not performed this experiment, as it is beyond the scope of this manuscript. It is certainly part of future research.

- *Since the authors talk about the activity of SUB in the discussion, I would like to know what they mean with this. How do they know when SUB is active since it apparently has a non-functional kinase domain based on earlier work from the same lab. I think this needs to be revised in the manuscript since they do not provide any data that shows a way to differentiate activated SUB from inactive SUB.*

We applied the term "*SUB* activity" in a genetic sense. Essentially, we meant activity levels without referring to a particular biochemical parameter. In the revised version of the manuscript we keep this term in only two instances. All other occurrences were removed and more specific terms were added (abundance, transcript levels etc).

Second decision letter

MS ID#: DEVELOP/2021/199425

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

AUTHORS: Ajeet Chaudhary, Xia Chen, Barbara Leśniewska, Rodion Boikine, Jin Gao, Sebastian Wolf, and Kay Schneitz
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

This work opens exciting avenues towards understanding SUB-mediated signaling arising from defective cell walls.

Comments for the author

The authors have addressed all my major concerns, including providing additional evidence and strengthening their statistical analysis. The revised manuscript is clearly presented, the data rigorously analyzed, and findings represent important advances on SUB function during cellulose alterations. The author's arguments on cell fate are also better explained and convincing in the revised version. Kudos!

Reviewer 2

Advance summary and potential significance to field

See first review, the message did not change and everything became more clear and better explained in the revised version.

Comments for the author

The authors have answered all my comments in a convincing way and where applicable have added new data and made changes in the text. This has significantly improved the manuscript.