The Second Site Modifier, Sympathy for the ligule, Encodes a Homolog of Arabidopsis ENHANCED DISEASE RESISTANCE4 and Rescues the *Liguleless narrow* Maize Mutant

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	1st Decision:	December 27, 2108 *revision requested*
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REPORT: (The report shows the major requests for revision and author responses. Minor comments for revision and miscellaneous correspondence are not included. The original format may not be reflected in this compilation, but the reviewer comments and author responses are not edited, except to correct minor typographical or spelling errors that could be a source of ambiguity.)

TPC2018-RA-00840 1st Editorial decision – *revision requested* December 27, 2108

The editorial board agrees that the work you describe is substantive, falls within the scope of the journal, and may become acceptable for publication pending revision, and potential re-review.

We ask you to pay attention to the following points in preparing your revision.

As you can see from the reviewer's comments, all raised significant concerns regarding interpretation of your data. Particularly, they state that the conclusion that the mutant phenotype of Lgn-R derives exclusively from the constitutive induction of PTI, and that the development of ligule and auricle in Lgn-R is abolished as a result of diversion of metabolic resources from normal development to defense is not adequately supported by the data. We suggest softening this claim, and providing alternative models. In addition, the fact that Sol and EDR4 gene share only 33% identity across 29% of the protein suggests that Sol does something different from AtEDR4, thus more caution is warranted in interpreting the significance of this similarity. Lastly, the conclusions made from analyses of the phosphoproteomic data need to be supported by statistical methods, or dropped where the results are not clearly reproduced between repeats.

---------- Reviewer comments:

[Reviewer comments shown below along with author responses]

TPC2018-RA-00840R1 1st Revision received March 29, 2019

Reviewer comments and author responses:

Reviewer #1:

This manuscript deals with a semidominant maize mutant Lgn-R and its suppressor, sol. The Lgn-R mutant, which has a gain-of-function defect in a membrane-associated, grass-specific kinase, was identified in the B73 background where it has a severe dwarf/stunted phenotype with short and narrow leaves that lack both the ligule and auricle. However, when Lgn-R is crossed with Mo17, as well as many other inbreds, its phenotypic expression is suppressed and the mutant phenotype reverts to almost that of a normal plant. Another peculiarity of the mutant phenotype is that it is impacted significantly by temperature, with the warmer temperature enhancing the phenotype and the cooler temperature repressing the mutant phenotype. By crossing Lgn-R::B73 with the IBM RILs and evaluating the resulting testcross progenies, the locus responsible for modifying the expressivity of the mutant was mapped and named sol. In this manuscript the authors describe the successful isolation of sol. It turns out to be an encoder of a homolog of the Arabidopsis EDR4 gene, a negative regulator of immunity. Like EDR4, the transcriptional induction of Sol was shown to be induced by a couple of pathogen-associated conserved molecules with induction kinetics very similar to that of EDR4.
These findings begged the question of what was responsible for the Lgn-R phenotype and how Sol was able to rescue it. Analyses involving RNA-seq and phosphoproteomics revealed that genes and proteins that are normally associated with the induction of the first arm of immunity (PTI) were upregulated in the Lgn-R mutant, and that the Sol allele from Mo17 was somehow able to prevent the induction of these defense response genes/proteins. A key signature of PTI is the activation of MAP kinase cascades, and that is what the authors found when they used a targeted proteomics approach to look for differential phosphorylation at key conserved phosphosites of the map kinase signaling pathway components involved in PTI. The fact that the mutant phenotype is enhanced by high temperature is consistent with this scenario.

Point 1. These data were interpreted to suggest that the mutant phenotype of Lgn-R derives exclusively from the constitutive induction of PTI. Authors further suggest that development of ligule and auricle in Lgn-R is perhaps abolished as a result of diversion of metabolic resources from normal development to defense. While there seems little doubt that the PTI immune response is revved up in Lgn-R, I am not convinced of the cause and effect connection that the authors suggest between autoimmunity and loss of ligule and auricle in Lgn-R. Are the authors aware of any other plant mutant where induction of PTI leads to disruption of a specific developmental program for an organ(s)?

> RESPONSE: We have high-lighted the fact that Lgn-R is unusual in this regard. No other immunity maize mutants have a change in the ligule. In fact, I have been talking to other cereal immunity researchers and they don't see this in their material either.

Point 2. If they are, they should cite that report to support their argument. To me it seems that a direct link between autoimmunity and loss of ligule/auricle development would conflict with what the authors showed in an earlier paper that Lgn-R and other liguleless mutants function in the same pathway. Given the feasibility that a kinase defect can have multiple consequences, it remains possible that some other independent network unique to the development of ligule/auricle is also altered in Lgn-R, along with the PTI-associated map-kinase cascade. It remains unclear what Sol might do to suppress the Lgn-R phenotype; however the findings made in this work provide an exciting opportunity to look further into it.

> RESPONSE: Thanks for these comments. We did not intend to imply that the entire mutant phenotype is simply due to constitutive induction of PTI. We have modified the text and model figure accordingly.

Point 3: What happens to the Lgn-R mutant phenotype if the Sol-Mo17 allele is in hetero condition vs. homo? Also, can Sol-Mo17 suppress the Lgn-R homo phenotype?

> RESPONSE: We grew three self-pollinated Lgn-R/+ NIL families in the greenhouse to find the answer. They are not flowering yet, but by measuring leaf width, we don't see a difference in Sol-M/Sol-M compared to Sol-M/Sol-B in Lgn-R heterozygotes and have added this to the text. It may be that the heights at maturity will be different, but flowering is another couple weeks away. We will adjust the text and figure if we see that difference at the next stage of revision.
>
> We also examined Lgn-R homozygotes segregating for Sol-M. One copy of Sol-M does not change the phenotype of

these very small, nearly dead plants. However, 2 copies of Sol-M provide some statistical increase in size. Both these points are new and in Figure 1I.

Point 4. It would have been appropriate to show that the Lgn-R plants are in fact resistant to pathogens. Given that only the PTI arm of immunity is induced in this mutant, is Lgn-R resistant to both biotrophic and necrotrophic pathogens, as would be expected?

RESPONSE: This is an experiment we have wanted to do. We have now sent seed to Peter Balint-Kurdi who has offered to grow the plants in North Carolina and test them with his pathogens. We don't expect to see Lgn-R B73 survive due to the heat, but it will be interesting to learn if Lgn-R NIL is better than B73 controls. We also attempted small scale infection assays of seeds and seedlings with Fusarium verticillioides. However, as our lab is not equipped for and unfamiliar with these techniques, we struggled to produce consistent and statistically meaningful results.

Point 5. The inbreds CML228 and CML247 are unable to rescue Lgn-R even though the coding region of their sol alleles is the same as Sol-Mo17. As discussed by the authors, one possibility is that their sol alleles have defects in their promoters. I was wondering if this possibility was addressed or not.

RESPONSE: We have not sequenced the promoters, but did carry out Q-RT-PCR to see if Sol is induced in the Lgn-R background, similar to the induction in the Lgn-R B73 background. We used Ms71 and Nc350 as positive and negative controls and found that Sol in CML228 is also induced in the Lgn-R background. These results were added to the text and figures. The results for CML247 were not as clear.

Point 6. Given the possibility of other Sol loci in the maize genome, the authors should consider rewriting sol as sol1. Perhaps the same goes for Lgn. I could not figure out what the significance of R is in the Lgn-R genic designation.

RESPONSE: R stands for reference allele and we have now indicated that. We prefer not to add numbers to Sol and Lgn at this time.

Point 7. The authors mention that the Sol-M missense mutant (G489E) has no phenotype in the absence of Lgn-R. But I wonder if they tried to induce and compare the magnitude of its PTI with that of its progenitor Mo17.

RESPONSE: Good point, but we did not carry out that experiment.

Reviewer #2:

This is a good paper, with a lot of good data. It is well written and generally very clear. My only major concern is that the data may have been selectively over-interpreted in some cases.
Point 1. My main concern is that the authors go all in on the "autoimmune" theory. While I understand the rationale, I don't think one can be very confident about this. The evidence for an autoimmune response is the identity of Sol, the transcriptional and phosphorylation response associated with Lgn-R. I accept this is suggestive but it is notoriously difficult to determine anything by looking at the general transcriptional response and I suspect there may be some post-hoc story making occurring. The basis of the whole thing is the identity of Sol as EDR4 but the characterized Arabidopsis gene shares only 33% identity across 29% of the protein...which does not seem like enough to infer shared function with any confidence.... It is conceivable that Sol does something different to AtEDR4. Therefore to infer the function of Lgn from the presumed function of Sol seems dicey, especially as there are aspects of the Lgn-R phenotype that have not been observed in any known autoimmune response. So I accept that it is reasonable theory but I also suggest that it is reasonably likely to be incorrect.

RESPONSE: Thank you for this point. We have addressed this concern through out the manuscript and in a new model. Additionally, a direct alignment of Sol and EDR4 using a specialized two sequence alignment function of the BLASTp suite revealed a query coverage (Sol as query) of 42% and a percent identity of 61.36%, both of which are greater than previously reported.

Point 2. Given the authors strong advocacy of this theory, it is a little odd that no disease assays have been performed. Can they comment on this?

RESPONSE: We have spoken with various colleagues and they agree that in a limited time frame, it would be hard to find the pathogen and conditions to get a response. We have set up a collaboration with Peter Balint-Kurdi to test these ideas in the field next summer.

Point 3. L141 I am not convinced that Lgn-R NIL plants were less affected by heat that Lgn-R B73-I don't think you can make that claim based on the data presented here. I understand that some T-test results are presented but it looks like you are comparing a difference of 2.7 with one of 2.3 with errors of around 0.6 for each number?

RESPONSE: We carried out additional experiments with growth chambers that did not cycle in temperature. The results were very clear in this comparison and are now included in figure 2.

Point 4. L366. This discussion of what two copies of SolM might do in a B73 background is odd given that it would be easy to examine this directly. Why did the authors not produce Sol-M / Sol-M homozygotes in a B73 background. It would seem that one might be able self the NIL since ear development was rescued. I suspect I may be missing something?

RESPONSE: We have now made these analyses – thanks for the prompt.

Point 5. L377- again I think that the inferences made between the two systems Arabidopis and maize are not justified and based on too little evidence. To invoke RLKs here is not justified.

RESPONSE: We have softened this language.

Reviewer #3:

The manuscript by Anderson et al addresses the basis for the abnormal growth phenotypes observed in the Lgn-R mutant in B73 but suppressed in Mo17, and in particular relation to the interesting observation that the phenotypes are exacerbated under high temperature field conditions. I believe that the authors have provided solid evidence that the genetic basis is associated with a gene related to EDR4 from Arabidopsis (called SOL in this work). The difficulty is that I do not believe they have provided any logical explanation for why or how this occurs. There are a number of descriptive characterizations of various aspects SOL-B vs SOL-M, but none of them actually address how these genes/proteins manifest differences in phenotypic outcomes. Is it differences in expression levels or the activity of the proteins themselves? I realize that an enormous amount of work has already gone into this story just on the genetics alone, and because of that I would like to be sympathetic in support of where to draw the line.

Point 1. However, the main model for the Lgn-R mutant phenotypes provided based on the phosphoproteomic data arguing for autoimmune activation is not supported by a rigorous analysis of the data provided in Supp Data Set 2. Most of the proteins highlighted in the model were only found in a single experiment and/or cannot be argued to be statistically different between WT and mutants. The results shown within the main text in Table 2 for phosphopeptides present in WT but not in the mutant demonstrate the type of data that would be needed to draw conclusions, but the data used to build the model in Fig 6D are nowhere near this level of confidence. As such, the paper provides the identification of a genetic suppressor; but most of the other experiments either provide inconclusive results (detailed below where the results do not appear to support the conclusions) or information that does not inform on why the Lgn-R mutation causes its severe effects in B73 or why the Mo17 SOL suppresses these effects.

RESPONSE: We have removed the model and replaced it with a simpler one that highlights the likelihood that LGN also functions separately in leaf development.

Point 2. Figure 2: The field date indicate substantial differences between genotypes and support the SOL-M modifier contributing to the differences; but the results from the controlled chamber to address temperature alone are not as convincing. The statistical analysis within Figure 2 appears to be insufficient to draw meaningful conclusions about the contributions of high temperature alone between genotypes. Shown are significant differences between Lgn-R in B73 and WT, but comparisons are not indicated between the mutant in B73 and the NIL (or the NIL and WT). These are necessary to state whether the Mo17 SOL modifier reverses the temperature effects as stated in the figure title (it does not appear that it does at least under chamber conditions).

RESPONSE: We now include additional data from growth chamber experiments in which the temperature does not cycle. The data are statistical and presented in figure 2.

Point 3. Supp Figure 3: I am not sure that the results from the water withholding experiment can be interpreted cleanly. The B73 mutants have such a reduced leaf surface area that it wouldn't be surprising that there is less evapotranspirational loss (an added complication is whether the stomatal patterning defect also alters stomatal conductance). The figure title states "Controlled environment experiments clarify the GxE interaction." Neither within the figure nor in the text is it stated what is actually clarified. Please explain. Moreover, without knowing the RWC from the actual field conditions, I'm not sure how this controlled experiment in a growth chamber (at what appears to be lower temperatures) contributes to an understanding of the possible effects of different field irrigation strategies, which at least in the text was indicated as the rationale for performing these experiments.

RESPONSE: We have taken out the water withholding experiments.

Point 4. Supp Figure 3E and 3F: The 3E graph (and text in the results section) indicate that under low temperatures, the B73 mutants "were no longer significantly different from their non-mutant siblings", but the image in 3F clearly shows that the width of leaf 6 in the mutant is about 1/4 of the non-mutant. Please clarify.

RESPONSE: You are right – we have changed the text, and the new growth chamber experiments clearly highlight this difference.

Point 5. Supp Figure 7: It is not clear to me how the authors are concluding that the protein localizes to the plasma membrane rather than the cytosol. In a highly vacuolated cell, the cytosol would be that small area in the proximity of the PM. In addition, an immunoblot is required for this figure to demonstrate that the protein is intact. If YFP is processed/cleaved from the mature protein of SOL-B, YFP is small enough to migrate to the nucleus, which could explain apparent differences. Moreover, I can't see what the point of this experiment is. It isn't really informative and isn't discussed again.

RESPONSE: The localization supports the idea that there is a difference between the two alleles. It is one difference of many and seems worth presenting. Additionally, we have modified our language from “PM” to “cell periphery”.

Point 6. The phosphoprotoemic data in Supp Data Set 2 (mislabeled within the file as Supp Data Set 1) does not support the model provided in Figure 6. For instance, MPK6 is phosphorylated in all samples and arguably more highly in WT in the Data Set, but it is shown if Fig 6D to be only activated in the mutant. A larger issue is that for most of the other proteins shown in Fig 6D, they would not hold up to statistical analysis as most of them are only detect in one out of three biological replicates. While this would not necessarily be surprising as phosphoproteomic analyses of whole tissue lysates would be quite challenging to obtain consistent coverage of all proteins, it means that model provided is not supported by the data.

RESPONSE: We have taken out the model.

TPC2018-RA-00840R1 2nd Editorial decision – *revision requested* April 25, 2019

Thank you for submitting your best work to The Plant Cell. The editorial board agrees that the work you describe is substantive, falls within the scope of the journal, and may become acceptable for publication pending revision, and potential re-review.

As you can see below, both reviewers have made additional suggestions to further improve this manuscript, such as checking interaction of the Lgn mutant with additional pathogens, discussing the whether both PTI and ETI may contribute to the unique phenotype of Lgn, and to clarify the subcellular localization of both Sol-M and Sol-B using maize protoplasts. We encourage you to consider these suggestions, but leave it to your discretion as to which can be reasonably addressed.

TPC2018-RA-00840R2 2nd Revision received May 14, 2019

Reviewer comments and author responses:

Reviewer #1:

This is my second review of this manuscript, which has been resubmitted to Plant Cell. The previous review is attached below. After having gown through the revised manuscript carefully and also looking at the tracked changes, I am satisfied that the authors have done a decent job in addressing many of the concerns raised in the previous reviews.

Point 1. Given that an autoimmune response seems to be revved up in Lgn, I do however wish that the authors looked at the interaction of the Lgn mutant with a couple of pathogens and compared it that of its non-mutant siblings in the B73 background. Although I do not deem it necessary, I feel it would have strengthened the paper significantly. Another minor point concerns the dramatic upregulation (including one with more than 50-fold) of a few NLRs in the RNA-seq data. It is well established that overexpression of NLRs can also trigger an autoimmune response constitutively, and in some cases such overexpression leads to severe stunting of the affected plant. Given this, could it be possible that it is the combination of both PTI and ETI that causes Lgn to have the unique phenotype that it has?

RESPONSE: We appreciate the interest of the reviewer in seeing how Lgn-R interacts with pathogens, but it would take at least a summer field season and still might not give us conclusive data. We don't know what pathogen to try and under what environmental conditions. We are working with Peter Balint-Kurdi to test our plants in his field this summer, but I am worried about how the heat will impact them as well.

It is possible that a combination of PTI and ETI are involved with Lgn-R, but the temperature suggests it is not ETI. We softened the language a little in the discussion. Also, we have carried out ROS staining and it is not as dramatic as with the *narrow odd dwarf* mutant (Rosa et al, 2017) that appears to involve ETI.

Reviewer #4:

Anderson et al. performed substantial genetic experiments to identify the second site modifier, Sol, which encodes the homolog of Arabidopsis EDR4 and can rescue Lgn-R meidated phenotype. The sequence variation and gene expression of Sol are largely correlated with the background effect. Integrated transcriptomic and phosphoproteomic analyses suggest that Lgn-R plants constitutively activate an immune signaling cascade that induces temperature-sensitive responses in addition to defects in leaf development. A model was further proposed. There are some questions need to be further addressed.
Point 1. L150-153: It is better to have more details about the fine-mapping process of Sol. From "Results" and "Materials and Methods", it is hard to learn how Sol was placed between two markers, and what materials or populations were used. Supplemental Table 1 only showed the primers used in this study.

RESPONSE: Sentences were added to the methods to clarify the crosses and analysis for mapping.

Point 2. L227-228: The Sol interval contains four genes, and it is not accurate to say "Sol is induced" or "Sol transcripts are increased" . It is that the expression of GRMZM2g075262 from different lines was detected.

RESPONSE: I would say that the NIL interval contains SOL, but at some point in the paper we determine that GRMZM2g075262 is SOL. At this point, we can say that SOL transcripts are increased.

Point 3. L232-233: Except for allelic difference in protein sequence and in regulation of gene expression, other loci in CML228 and CML247 might also affect the failure to rescue Lgn-R phenotype. In addition, according to the different subcellular localization of SOL-M and SOL-B, it will be interesting to investigate whether the localization of SOL-CML228 and SOL-CML247 also correlated with the phenotype.

RESPONSE: All these are good points. We do know that the F1 of CML228 or CML247 with B73 rescued Lgn-R, so it is possible that we lost the rescuing modifier in the B73 backcrosses.

We have initiated steps to obtain an antibody for SOL to determine the localization of SOL in the different inbreds. These experiments will be interesting, but we don't think they are necessary for the purpose of this paper.

Point 4. L271-275: A significant reduction of Sol expression was detected in Lgn-R B73 tissues treated with PAMP. The reduction is due to the fact that Sol is already up in Lgn-R, so adding the PAMP doesn't increase it at this time point. Probably a good experiment to do would be carrying out a time course with the Lgn-R material and PAMP. What about the Sol expression in Lgn-R Mo17 tissues treated with PAMP?

RESPONSE: We tried the PAMP experiments with Lgn-R in Mo17 and found them inconclusive even for our control. Mo17 is very different from B73 in many ways and we have probably scratched the surface.

Point 5. According to the model (Figure 6D), the subcellular localization of SOL is very important for its function. SOL-M and SOL-B both localized to the cell periphery, but only SOL-B had nuclear localization in N. benthamiana. It is hard to know which compartments SOL localized based on Figure 5A. I suggest that the authors co-express SOL with different subcellular markers, eg. plasma membrane (PM) markers and make sure where SOL localized. And the subcellular localization experiment is better to be verified in maize protoplast to avoid the heterologous misexpression.

RESPONSE: We appreciate these suggestions, but in our mind, the SOL localization is not critical for the model. The model provides testable hypotheses. We hope to obtain a SOL antibody to look at localization by multiple methods and in different genotypes in the future.

TPC2018-RA-00840R2 3rd Editorial decision – *acceptance pending*	May 19, 2019

We are pleased to inform you that your paper entitled "The second site modifier, Sympathy for the ligule, encodes a homolog of Arabidopsis ENHANCED DISEASE RESISTANCE4 and rescues the liguleless narrow maize mutant" has been accepted for publication in The Plant Cell, pending a final minor editorial review by journal staff. At this stage, your manuscript will be evaluated by a Science Editor with respect to scientific content presentation, compliance with journal policies, and presentation for a broad readership.

Final acceptance from Science Editor	June 13, 2019