nature portfolio

Peer Review File

Chromosome-scale pearl millet genomes reveal CLAMT1b as key determinant of strigolactone pattern and Striga susceptibility

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
In this manuscript the authors describe the generation of two high quality genomes for pearl millet. They use a combination of comparative genomics an RNA-seq analysis to identify two copies of the gene family known to catalyze the conversion of inactive precursors into biologically active strigolactones across many species which are present in a striga susceptible pearl millet line and absence in the striga resistant line. They validated that one of these two gene copies is competent to produce biologically active strigalactones when expressed in another species (tobacco) and that the structural variant carrying the two gene copies is segregating across a wide range of pearl millet germplasm.

Overall I found the science presented in this paper which I was qualified to assess to be sound. The logical flow was challenging at times. By line 114 the authors have identified a set of two candidate genes using comparative genomics. Then lines 117-148 divert to a separate RNA-seq effort which identified a larger set of candidate genes. Then on lines 149-154, the authors come back and test the two candidate genes from the comparative genomics efforts. But this could be fixed with structural editing rather than new experiments.

If this were a paper focused on having produced a better pearl millet reference genome I'd expect a bit more discussion of whether one of the two lines sequenced would make a good reference genotype and comparisons to other sequenced grass genomes, do these new more complete genomes tell us things that were missed in the Varshney genomes? But that's not the focus of the paper.

If this were a paper about identifying molecular markers to aid breeding efforts to deal with striga in pearl millet production I'd expect more efforts to verify that a marker designed based on the presence or absence the region containing the gene we know is needed to convert inactive precursors to strigalactones is a good predictor of which lines will and won't be susceptible to striga in the field. The only relevant GWAS study I could find was focused solely on west african germplasm and used really small sample sizes, but there is not a strong and obvious signal from chromosome 2. But the paper is somewhere in between which makes it harder to assess.

I have one major (relevant to the last paragraph above) and some minor comments:

Major:

1. In Supplemental Figure 18, if I understand correctly, lines at the top of the graph should lack the striga susceptibility locus while lines at the bottom of the graph carry CLAMT 1b and are predicted to be susceptible to striga. The relative frequencies of these two groups using reasonable cutoffs should be reported in the main text. However, it seems like the vast majority of pearl millet tested already carries the resistance allele. The result they report that only 2 of 8 independently sequenced pearl millet lines carrying the allele that confers susceptibility is also consistent with that conclusion. This, in turn, creates one of two problems. Either the vast majority of the work is already done and most pearl millet is already resistant to striga, or there are alternative pathways/alleles that can also induce striga susceptibility in pearl millet. In either of these situation, the authors' conclusion about the impact of their paper (lines 210-212) becomes harder to support.

Minor:

2. Lines 103-108: Yes it makes sense a more complete genome should have a higher proportion of transposon sequences. But what is meant by the values being in more detail than those reported in the other papers cited?

3. Lines 126-127: Is the thinking here that there will be feedback inhibition of transcriptional activity of the entire pathway? That's certainly possible but it is also possible only certain key steps in the pathway would experience feedback inhibition and/or the inhibition could occur at the translational or post-translational stage. I think the experiment is a reasonable one and the issues I raised speak more to the potential for false negatives than false positives, I just urge the authors to better capture the uncertainty.

4. Line 339: shouldn't "the R Edger" be either "edgeR" or "the R package edgeR"?

Reviewer #2:
Remarks to the Author:
The study focused on understanding the role of strigolactones in Striga susceptibility in pearl millet. Four SLs were tentatively identified in the susceptible line P10 but were absent in the resistant line Aw. Furthermore, the resistant line Aw was found to lack a specific genome segment containing ClAMT1, which is associated with pennilactone production. The study highlights the importance of the CLAMT1 genes in SL diversity and Striga susceptibility in pearl millet. This paper holds important value for the field, however, it requires thorough revision, particularly regarding numerous errors in figure numbering and description. Additionally, supplementary data should be treated with equal importance Therefore, please carefully edit the figure legends and chromatograms.
1. It would enhance clarity for readers if Figure 1 is organized as it appears in the text. Figure 1a is only referenced later, whereas figures 1b, 1c, etc., have already been discussed.
2. Could you please clarify the meaning of MiZax3 and MiZax5?
3. In Supplementary Figure 3a, is pennilactone referred to as PL1? There seems to be inconsistency in its labeling.
4. 'Additionally, the treatment with zaxinone growth regulators reduced Striga infestation, underscoring the role of SLs in P10 Striga susceptibility'.
when treating with zaxinone growth regulators, were SLs the only factors altered? Could other factors also influence Striga susceptibility? Considering Supplementary Figure 3a and b, where the MZ5 treatment had the lowest strigolactone levels, and the Striga emergence showed no significant difference compared to the control, how can the author conclude that striga susceptibility is solely due to the role of SLs?
5. In the statement '… Aw, lacking the four non-canonical SLs (PL1, PL2, PL3, and PL4), produced an average of 10 tillers, while P10 developed only one tiller under normal growth conditions.', it appears the article isn't structured logically. At this point, the author hasn't yet mentioned or elucidated that PL1-4 are non-canonical SLs. How can this information be used here?
6. 'Our results suggest that the differing SL compositions of Aw and P10 likely account for their contrasting pre-attachment Striga resistance phenotypes.'
Could the author specify what the pre-attachment Striga resistance phenotypes entail? Is it the germination that was investigated?
7. Omni-C should be Omni-CTM.
8. Please ensure consistency in formatting; gene names in the main text and figure including supplementary figure legends should be italicized.
9. What does the MP3 treatment refer to in Line 144?
10. The statement CLAMT1c was upregulated only under low Pi, and CLAMT1b transcripts increased strongly with both treatments (Supplementary figure 11)' doesn't seem to match with Supplementary figure 11. Is this referencing the wrong figure?
11. Could the legend for Figure 3b be clarified? What do these chromatograms represent? Are they total ion chromatograms?
12. In Figure 3c, please explain the reason for the retention time shift. Additionally, compared to Supplementary Figure 1, the retention time for PL1 appears different again. It might be necessary to include a negative control, such as Aw without feeding, and also to display the MeCLA itself.
13. Line 165 should maintain consistency in naming between PL1 and pennilactone.

14. Figure 3d content should be included in the text.
15. In Figure 3a, gene expression of MAX1-1500 is shown, while in the genome map (Figure 2), MAX1-1400 is depicted. Could you explain this discrepancy?
16. 'The absence of this branch in Aw may account for the higher orobanchol and orobanchyl acetate content, as the metabolic flux is not divided, and there is no competition for the CL or CLA precursor.' Alternatively, could it be that in Aw, the genes responsible for producing orobanchol and orobanchyl are more highly expressed compared to P10, as indicated in Figure 3a?
17. If a detailed breakdown of the command workflow (Supplementary Figure 19) is available on our GitHub page, should it be referenced as figure 20?
18. Supplementary Figure 19c is not mentioned in the text.
19. In Figure 14b, why do the retention times of PL2 in P10 and Aw with MeCLA feeding differ? Also, there appear to be two peaks after feeding. Moreover, the relative abundance of the mass fragments seems different. Based on these observations, it's unclear whether they are the same compounds.
20. Could you indicate where Supplementary Figure 12 is referenced in the text, and provide information on where the isoform information originates from?
21. In Supplementary Figure 6, what does MAKR3 refer to?

Reviewer #3:
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Very clear differences in SL content were observed between the cultivars Aw and P10 - with high levels of Orobanchol and Orobanchol acetate and low levels of PL1-4 in Aw compared to P10. Aw lacks the CLAMT1a,b genes and could not produce products of CLAMT, which are MeCLA and PL1-4. Biochemical tests support MeCLA being the precursor for PL1-4. Cultivars that contain CLAMT are all at the high end of stimulating striga germination and indeed, this region is common to many pearl millet varieties.

This manuscript is a beautiful flow from phenotype to gene discovery to biochemical causation. This study will be an important contribution to addressing striga resistance in plants.

The point that mycorrhizal colonization is not impaired in the absence of CLAMT needs to be emphasised such as with its own paragraph.

A similar statement is needed for the tillering. What is known about this part of the pathway with respect to tillering and how might this study indicate future directions? Fig 1 and Supp fig 4 shows decreased tiller number in Aw under with striga. This should be mentioned with some brief speculation as to the cause. Also, Aw has much higher tillering than P10, yet it has higher levels on Orobanchol type SLs. Whilst the point is taken well that tillering is regulated by many factors and these are not near-isogenic lines, it is curious. Also there is some discussion that MeCLA is the precursor of the bioactive SLs for branching in some species (Brewer papers). This would be consistent with the reduced branching of P10 which accumulates in this pathway.

The data are consistent with Aw lacking and P10 having a pathway via CLAMT to MeCLA and PL1. The Figure 3 showing this could also add the information on the phenotype, particularly the striga effect.

Most figures have Aw on left and P10 on right – please modify for supp 19.

[EDITOR]

Dear Professor Al-Babili,

Thank you again for submitting your manuscript "Chromosome-scale pearl millet genomes reveal a CARLACTONOIC ACID METHYL TRANSFERASE as key determinant of strigolactone pattern and Striga susceptibility" to Nature Communications. We have now received reports from 3 reviewers and, after careful consideration, we have decided to invite a major revision of the manuscript.

As you will see from the reports copied below, the reviewers raise important concerns. We find that these concerns limit the strength of the study, and therefore we ask you to address them with additional work. Without substantial revisions, especially the GWAS sampling size problem concerned by Reviewer #1, we will be unlikely to send the paper back to review.

If you feel that you are able to comprehensively address the reviewers' concerns, please provide a point-by-point response to these comments along with your revision. Please show all changes in the manuscript text file with track changes or colour highlighting. If you are unable to address specific reviewer requests or find any points invalid, please explain why in the point-by-point response.

Important: In addition to the above, you must comply with the following editorial requests; we will not be able to proceed with your revised manuscript otherwise. Please also see the Nature Communications formatting instructions, which you may find useful while preparing your revised manuscript.

[Reviewer #1] (Remarks to the Author):

In this manuscript the authors describe the generation of two high quality genomes for pearl millet. They use a combination of comparative genomics an RNA-seq analysis to identify two copies of the gene family known to catalyze the conversion of inactive precursors into biologically active strigolactones across many species which are present in a striga susceptible pearl millet line and absence in the striga resistant line. They validated that one of these two gene copies is competent to produce biologically active strigalactones when expressed in another species (tobacco) and that the structural variant carrying the two gene copies is segregating across a wide range of pearl millet germplasm.

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If this were a paper about identifying molecular markers to aid breeding efforts to deal with striga in pearl millet production I'd expect more efforts to verify that a marker designed based on the presence or absence the region containing the gene we know is needed to convert inactive precursors to strigalactones is a good predictor of which lines will and won't be susceptible to striga in the field. The only relevant GWAS study I could find was focused solely on west african germplasm and used really small sample sizes, but there is not a strong and obvious signal from chromosome 2. But the paper is somewhere in between which makes it harder to assess.

We thank the reviewer for taking the considerable time and effort to review our work and provide their insightful feedback. We agree that our work cannot be categorized as a pure genome paper or as a manuscript that identifies molecular markers. It is actually a combination of both, complemented by the discovery of characterization of an important metabolic pathway that leads to a plant hormone with a crucial role in the infestation by a root parasitic plant impacting global food security. Our paper is, as described by Reviewer 3, "a beautiful flow from phenotype to gene discovery to biochemical causation"**.**

We determined the strigolactone pattern of two lines with a contrasting phenotype in Striga resistance and showed that the difference in the pattern of released strigolactones is a reason for this phenotype. Our primary objective behind sequencing the genomes of the two lines was to find the genetic origin of the different phenotypes.

The cost and expertise required to produce high quality draft genomes have reduced noticeably. But even in the increasingly crowded space of high-quality genomes, some genome assemblies score better

in some respects. In this work, we publish high quality genomes of two novel pearl millet accessions that have important traits for farmers and breeders. The initial HiFi draft assemblies for both accessions produced 4 out of 7 complete chromosomes with the other three chromosomes getting scaffolded with OmniC out of two or three contigs, each. This is an indication of the quality of HMW DNA and library prep works. To address the point raised by the reviewer about considering our paper as a "genome paper", we have compared our assemblies against available pearl millet assemblies (as of March 2024). We included in the comparison the latest Varshney genomes (three genomes, Ramu 2023), ten Yan et al 2023 genomes and one Salson et al 2023 genome. Our results indicate that our assemblies score the highest in a few metrics followed by Vashney's assemblies. For example, the number of gaps in our assemblies (excluding the unanchored scaffold) is three and five for P10 and Aw, respectively. Whereas in Vashney's latest assembly, we counted a total of 303 gaps. In terms of BUSCO scores, our assemblies score higher albeit by a small margin (98.5/98.6% vs 98.4%). See Supplementary Note 1 and Supplementary Table 3.

Having said this, our objective was not to outperform any other assembly out there. In fact, we believe that the genomics community appreciates that pangenomes are indeed more useful as they capture greater variability (https://doi.org/10.1038/s41576-020-0210-7; https://jasbsci.biomedcentral.com/articles/10.1186/s40104-023-00860-1). Therefore, we do not believe that our assemblies should supersede previous assemblies. To the contrary, we strongly encourage the community to embrace the diversity and build better reference pangenomes; to which our assemblies will be of great value.

With this view, there is not necessarily one reference genome per species anymore. The most suitable reference can be selected based on a biological question, or in this case, created *de novo*. Again, we primarily sequenced these genomes to find the genetic origin of their contrasting phenotypes in Striga resistance and strigolactone production. Presumably further research on these topics, which are of high importance in pearl millet, will use these high-quality assemblies as well. Whether the Aw and P10 assemblies will be used as a reference for wider pearl millet research is up to the community.

To better reflect this position, we have changed the wording in line 229-230 from "..can serve as the reference genome for global pearl millet research." to "..can serve as a set of reference genomes for global pearl millet research.".

Public GWAS data would indeed have been a great addition to our paper, but as Reviewer 1 mentioned, and as we reported in line 121-123, there are unfortunately not enough suitable studies done on pearl millet. However, even in the more studied and related species maize and sorghum, there is a puzzling lack of SL related findings from GWAS, even though it is an established knowledge that SLs are crucial for Striga infestation. It is an important open question in the field, but our speculations on the cause of this GWAS blind spot are outside the scope of this paper. Specifically for Rouamba et al., the GWAS study in pearl millet, we suspect the abundant application of fertilizer in both their pot and field experiments would suppress the SL pathway, which is highly induced under low phosphate conditions, thereby leaving SL related loci under the detection limit.

I have one major (relevant to the last paragraph above) and some minor comments:

Major:

1. In Supplemental Figure 18, if I understand correctly, lines at the top of the graph should lack the striga susceptibility locus while lines at the bottom of the graph carry CLAMT 1b and are predicted to be susceptible to striga. The relative frequencies of these two groups using reasonable cutoffs should be reported in the main text. However, it seems like the vast majority of pearl millet tested already carries the resistance allele. The result they report that only 2 of 8 independently sequenced pearl millet lines carrying the allele that confers susceptibility is also consistent with that conclusion. This, in turn, creates one of two problems. Either the vast majority of the work is already done and most pearl millet is already resistant to striga, or there are alternative pathways/alleles that can also induce striga susceptibility in pearl millet. In either of these situation, the authors' conclusion about the impact of their paper (lines 210-212) becomes harder to support.

This is a great comment that speaks to the core of the relevancy of the results presented in the paper.

Indeed, in most of the re-sequenced lines, we do not find the CLAMT fragment, as depicted in supplemental Figure 19. However, this dataset is not necessarily representative for pearl millet lines used in agriculture in areas where Striga is a dominant problem. SOSAT-C88 P10 was specifically used in this study for its agricultural relevance in West Africa, the region with the most severe Striga pressure in the world. This also means that mentioning the frequencies found in this non-representative dataset in the main text may be misleading. We have added it to the figure description instead.

Additionally, the widespread presence of the CLAMT fragment in all regions and all categories of cultivars, combined with the outcrossing nature of pearl millet, means our results will be relevant to any research program looking at Striga susceptibility in pearl millet, every pearl millet breeding program, as well as any seed company working with pearl millet. Regardless of whether a specific line currently contains the CLAMT fragment.

As indicated by the reviewer, there could be additional unknown SLs or yet-to-be-identified compounds produced in other pearl millet lines through other enzymes, which contribute to Striga seed germination. Looking at Pl343841 and Pl521612 in Figure 4 could be interpreted to hint at other Striga germination stimulants, possibly another SL branch. This would make removing the *CLAMTb* allele, and maintaining its absence, an incomplete but necessary part of conferring pre-attachment resistance to Striga.

We agree with the reviewer that the conclusion in the final sentence of the paper was worded too strong, considering the other potential factors in both pre- and post-attachment resistance. Therefore, we rephrased the statement "…confer Striga resistance." to "… reduce Striga susceptibility". We feel this better encompasses the idea that this finding is not by itself enough to stop Striga infestation, but nonetheless an essential factor in reducing it, as shown in Figure 4.

Minor:

2. Lines 103-108: Yes it makes sense a more complete genome should have a higher proportion of

transposon sequences. But what is meant by the values being in more detail than those reported in the other papers cited?

The phrasing "..assigned in more detail" refers to the additional subcategories of transposable elements specified in Supplementary table 2. For clarity, we have rephrased it to "..assigned to more specific subcategories".

3. Lines 126-127: Is the thinking here that there will be feedback inhibition of transcriptional activity of the entire pathway? That's certainly possible but it is also possible only certain key steps in the pathway would experience feedback inhibition and/or the inhibition could occur at the translational or post-translational stage. I think the experiment is a reasonable one and the issues I raised speak more to the potential for false negatives than false positives, I just urge the authors to better capture the uncertainty.

We thank the reviewer for this comment. That is correct; there was certainly a chance of false negatives. However, we did have prior information of the regulation of this pathway from related grass species. Li et al (2023) successfully identified additional members of the SL biosynthesis pathway in maize, based on co-expression, indicating regulation at the transcriptional level. Feedback regulation of SL biosynthesis genes upon application of (synthetic) SLs has also been established, first in Arabidopsis (Mashiguchi et al., 2009), and later leading to the identification of additional SL biosynthesis genes in rice (Haider et al., 2023).

To clarify the reasoning behind this decision, we adjusted the wording from "..with the SL analog MP3, which was expected to modulate the transcript levels of SL biosynthetic genes." to "..with MP3, which is an SL analog, and therefore expected to decrease the transcript levels of SL biosynthetic genes through feedback regulation (Haider et al., 2023).".

4. Line 339: shouldn't "the R Edger" be either "edgeR" or "the R package edgeR"?

Yes; this has been edited to "the R package edgeR"

Reviewer #2 (Remarks to the Author):

The study focused on understanding the role of strigolactones in Striga susceptibility in pearl millet. Four SLs were tentatively identified in the susceptible line P10 but were absent in the resistant line Aw. Furthermore, the resistant line Aw was found to lack a specific genome segment containing ClAMT1, which is associated with pennilactone production. The study highlights the importance of the CLAMT1 genes in SL diversity and Striga susceptibility in pearl millet. This paper holds important value for the field, however, it requires thorough revision, particularly regarding numerous errors in figure numbering and description. Additionally, supplementary data should be treated with equal importance Therefore, please carefully edit the figure legends and chromatograms.

We thank the reviewer for the positive evaluation of our manuscript and the recognition of its value for the field. We have revised the manuscript, including Supplementary data.

1. It would enhance clarity for readers if Figure 1 is organized as it appears in the text. Figure 1a is only referenced later, whereas figures 1b, 1c, etc., have already been discussed.

Response: We thank the reviewer for this notice. Figure 1a is now mentioned first, upon introduction of the Aw and P10 phenotypes.

2. Could you please clarify the meaning of MiZax3 and MiZax5?

Mimic of Zaxinone 3 (MiZax3) and Mimic of Zaxinone 5 (MiZax5) have now been clarified in the text.

3. In Supplementary Figure 3a, is pennilactone referred to as PL1? There seems to be inconsistency in its labeling.

Yes, PL1 is correct. We have revised the PL1 in 5 instances throughout the text and figure descriptions.

4. 'Additionally, the treatment with zaxinone growth regulators reduced Striga infestation, underscoring the role of SLs in P10 Striga susceptibility'.
when treating with zaxinone growth regulators, were SLs the only factors altered? Could other factors also influence Striga susceptibility? Considering Supplementary Figure 3a and b, where the MZ5 treatment had the lowest strigolactone levels, and the Striga emergence showed no significant difference compared to the control, how can the author conclude that striga susceptibility is solely due to the role of SLs?

We thank the reviewer for this notice. We have now adjusted the text to reflect more clearly that the reduction in Striga germination under MiZax3 and Mizax5 treatment did not reach significance, while it did for zaxinone.

Zaxinone and its mimics do indeed have effects beyond regulating SL biosynthesis. Additionally, the effects of the mimics differ from those of natural zaxinone in some respects (Wang et al., 2020). Therefore, we cannot exclude that MiZax3 or MiZax5 affects Striga emergence through other mechanisms, such as improving root growth and altered root architecture (Wang et al., 2020) that would, however, make the plant more accessible for Striga seeds in the soil. Looking at the host phenotype in Supplementary figure 3, it is clear that the Striga in the mock treated pot germinated early (likely because of higher SL exudation), thereby affecting the host from an early age, while the emergence in the MiZax5 treated pot has not affected the host much and is therefore likely a late germination through additional root spreading. This would not alter the conclusion as it relates to SLs.

Ultimately, the most relevant test remains that involving zaxinone itself. Both because its effects are better understood, and because it represents the natural metabolite. And Striga emergence is significantly reduced under zaxinone treatment. Therefore, we believe that the conclusion remains valid, despite the unfortunate statistical near-miss of the MiZax treatments.

5. In the statement '… Aw, lacking the four non-canonical SLs (PL1, PL2, PL3, and PL4), produced an average of 10 tillers, while P10 developed only one tiller under normal growth conditions.', it appears the article isn't structured logically. At this point, the author hasn't yet mentioned or elucidated that PL1-4 are non-canonical SLs. How can this information be used here?

We thank the reviewer for this observation. We have moved this section to a much later part of the manuscript, grouping it with further discussion of tillering.

6. 'Our results suggest that the differing SL compositions of Aw and P10 likely account for their contrasting pre-attachment Striga resistance phenotypes.'
Could the author specify what the pre-attachment Striga resistance phenotypes entail? Is it the germination that was investigated?

Yes, the Striga seed germination is the main result, which is how 'pre-attachment resistance' is used in literature (Dayou et al., 2021). If the SLs exclusively produced by P10 are sufficient to induce Striga seed germination, and we only see weak germination stimulation from Aw total root exudate, it follows that these additional SLs make that difference.

Pre-attachment resistance could also be related to the growth of Striga towards the host root, which is also based on sensing SLs, or to haustorium development.

7. Omni-C should be Omni-CTM.

Five instances of Omni-C have been changed into Omni-C™.

8. Please ensure consistency in formatting; gene names in the main text and figure including supplementary figure legends should be italicized.

Seven gene names have been italicized in the main figure descriptions, nine in Figure 3 and many in the supplementary descriptions.

9. What does the MP3 treatment refer to in Line 144?

This has been clarified in place (now line 150). It refers to treatment with the SL analog MP3, as described earlier in the paragraph (line 130-132).

10. The statement CLAMT1c was upregulated only under low Pi, and CLAMT1b transcripts increased strongly with both treatments (Supplementary figure 11)' doesn't seem to match with Supplementary figure 11. Is this referencing the wrong figure?

We apologize for this mistake. This figure reference was misplaced; it has been relocated to six words earlier. Now the correct link between expression and the later reference to Figure 3a is clarified.

11. Could the legend for Figure 3b be clarified? What do these chromatograms represent? Are they total ion chromatograms?

We thank the reviewer for this comment. We have revised the legend.

12. In Figure 3c, please explain the reason for the retention time shift. Additionally, compared to Supplementary Figure 1, the retention time for PL1 appears different again. It might be necessary to include a negative control, such as Aw without feeding, and also to display the MeCLA itself.

We thank the reviewer for this notice. Upon feeding with MeCLA, we observed the formation of two isomers, including one (the second overlapping peak) that corresponds to PL1 in P10 and an isomer that eluted slightly earlier. The difference in retention time between Supplementary Figure 1 and Figure 3c was due to the usage of two different LC-MS instruments: IDEX-Orbitrap was used for Supplementary Figure 1 while Figure 3c was produced by Altis-Quadrupole. The Supplementary figure accompanying Figure 3c has been split into two and MeCLA measurement data was added (Supplementary figure 13).

13. Line 165 should maintain consistency in naming between PL1 and pennilactone.

Agreed; pennilactone has been changed to PL1.

14. Figure 3d content should be included in the text.

We have added this in line 174.

15. In Figure 3a, gene expression of MAX1-1500 is shown, while in the genome map (Figure 2), MAX1-1400 is depicted. Could you explain this discrepancy?

Our apologies for this oversight; these refer to the same gene. The naming confusion comes from rice, where *OsMAX1-1400* and *OsMAX1-1500* are both homologs of this one pearl millet gene. This has now been changed to *MAX1-1400* in Figure 3 and its description.

16. 'The absence of this branch in Aw may account for the higher orobanchol and orobanchyl acetate content, as the metabolic flux is not divided, and there is no competition for the CL or CLA precursor.' Alternatively, could it be that in Aw, the genes responsible for producing orobanchol and orobanchyl are more highly expressed compared to P10, as indicated in Figure 3a?

We thank the reviewer for this comment. Indeed, we have to consider this possibility, though the enzymes producing orobanchol and orobanchyl acetate in pearl millet have not yet been identified, and we can only speculate about their expression. To account for this possibility, we have added the following sentence: In addition, enzymes responsible for the biosynthesis of these two SLs may be present in Aw at a higher level, compared to P10.

17. If a detailed breakdown of the command workflow (Supplementary Figure 19) is available on our GitHub page, should it be referenced as figure 20?

Yes, apologies. That should refer to Supplementary Figure 22 in line 523.

18. Supplementary Figure 19c is not mentioned in the text.

Yes, this has been corrected in line 224 (now renumbered as Supplementary Figure 20c).

19. In Figure 14b, why do the retention times of PL2 in P10 and Aw with MeCLA feeding differ? Also, there appear to be two peaks after feeding. Moreover, the relative abundance of the mass fragments seems different. Based on these observations, it's unclear whether they are the same compounds.

We thank the reviewer for this comment. Indeed, we detected two PL2 isomers, one of which (retention time: 10.69) co-eluted with PL2 in P10 (retention time: 10.78). We assume that the formation of two PL2 isomers is a result of the two isomers of PL1 (with overlapping peaks), which again were converted into two PL2 (a likely hydroxyl-PL1) isomers. The presence of the two isomers explains the difference in the relative abundance of MS fragments.

20. Could you indicate where Supplementary Figure 12 is referenced in the text, and provide information on where the isoform information originates from?

Supplementary Figure 12 is now referenced together with supplementary Figure 13, where both the a- and c-variants show no CLAMT enzymatic activity on CLA substrate.

No sufficiently convincing single transcript could be found for CLAMT1c, so we tested a version from the automated annotation as well as one from manual re-annotation. An additional explanation on the provenance of the CLAMT1c isoforms was added to the methods section of the transient expression in tobacco leaf.

21. In Supplementary Figure 6, what does MAKR3 refer to?

One of the finds from a GWAS experiment in sorghum looking for Striga resistance related markers is *Membrane-associated kinase regulator 3* (*MAKR3*; Mallu et al., 2022). This information was cut from the main text earlier to save space, but has now been added to the Figure description.

Reviewer #3 (Remarks to the Author):

Very clear differences in SL content were observed between the cultivars Aw and P10 - with high levels of Orobanchol and Orobanchol acetate and low levels of PL1-4 in Aw compared to P10. Aw lacks the CLAMT1a,b genes and could not produce products of CLAMT, which are MeCLA and PL1-4. Biochemical tests support MeCLA being the precursor for PL1-4. Cultivars that contain CLAMT are all at the high end of stimulating striga germination and indeed, this region is common to many pearl millet varieties.

This manuscript is a beautiful flow from phenotype to gene discovery to biochemical causation**.** This study will be an important contribution to addressing striga resistance in plants.

The point that mycorrhizal colonization is not impaired in the absence of CLAMT needs to be emphasised such as with its own paragraph.

This is indeed an important finding. The discussion of the AM colonization has been promoted to its own paragraph.

A similar statement is needed for the tillering. What is known about this part of the pathway with respect to tillering and how might this study indicate future directions? Fig 1 and Supp fig 4 shows decreased tiller number in Aw under with striga. This should be mentioned with some brief speculation as to the cause. Also, Aw has much higher tillering than P10, yet it has higher levels on Orobanchol type SLs. Whilst the point is taken well that tillering is regulated by many factors and these are not near-isogenic lines, it is curious. Also there is some discussion that MeCLA is the precursor of the bioactive SLs for branching in some species (Brewer papers). This would be consistent with the reduced branching of P10 which accumulates in this pathway.

A new paragraph on tillering has been formed collecting all tillering remarks together and expanding their discussion.

The data are consistent with Aw lacking and P10 having a pathway via CLAMT to MeCLA and PL1. The Figure 3 showing this could also add the information on the phenotype, particularly the striga effect.

That is a good addition; it has been added into Figure 3d, to increase its capacity as a summary of the findings.

Most figures have Aw on left and P10 on right – please modify for supp 19.

Correct; supplementary Figure 19 has been modified to have Aw on the left, and further changes have been made to format it more similar to the other figures.

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
The authors have successfully addressed my concerns regarding methodology, data analysis and conclusions. I think this is a sound paper and represents a great deal of effort and work on the part of the authors.

Some of my concerns about the potential scale of the significance and impact from the first round of review remain in this revision -- specifically regarding the lack of a link between this fragment and data on variation in striga resistance in pearl millet populations in association studies and the potentially largely fixed nature of the haplotype identified -- but 1) these are not concerns it would be straightforward to address in any reasonable length of time and 2) significance and impact are areas where, as a reviewer, I believe I should defer to the editor's judgement.

Congratulations to the authors of a very fine piece of science.

Reviewer #2:
Remarks to the Author:
My concerns were sufficiently addressed.

Reviewer #3:
Remarks to the Author:
Thank you for your modifications. I have no further critique.