nature portfolio

Peer Review File

Evolutionary hijacking of fatty acid biosynthesis drives insecticidal acylsugar diversity in tomato

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In their manuscript titled “Evolutionary hijacking of fatty acid biosynthesis drives insecticidal acylsugar diversity in tomato,” Wang et al describe their investigation into differential acylsugar profiles between domesticated tomato and its wild relative. Specifically, the authors use an introgression-based approach to narrow in on two closely related acyl-CoA synthetases for further study. Using a combination of protein biochemistry, stable isotope labeling, reverse genetics, subcellular localization, and expression analysis, the authors conclude that the wild tomato uses a novel strategy in which one acyl-CoA synthetase produces a branched-chain acyl-CoA which is then acted on by canonical fatty acid synthase machinery to form the acyl moieties incorporated into the acylsugars, and they identify localization and expression patterns as likely underlying the reduced ability of domesticated tomato to produce these same acylsugars despite retention of both acyl-CoA synthetases. The authors present this as an example of neofunctionalization of a single enzyme without altering catalytic function leading to metabolic innovation.

Overall, the manuscript is well-written and supports most conclusions made, including providing an example of functional divergence deriving from non-catalytic differences. This divergence is made even more interesting by the fact that the enzyme in question controls a branch of secondary metabolism (acylsugar synthesis) that apparently leverages the catalytic machinery of a core primary metabolic pathway (fatty acid elongation). However, as detailed in my specific comments below, my enthusiasm is tempered by shortcomings in the reverse genetic analyses and generally poor support for the neofunctionalization claim.

Specific Comments:

1) It is incorrectly stated in the text that some analyzed lines do not produce the iC11/iC13 acylsugars (lines 182-183: “transgenic plants harboring proSpBACS2::SpBACS2 did not produce these branched-chain acylsugars;” lines 295-296: “not normally detectable in M82”). Although the difference between these lines and the IL 2-5 line is stark, the small peaks corresponding to these compounds are visible in the sample chromatograms, and indeed the compounds are quantified in the relevant bar graphs. Thus M82 maintains iC11/iC13 acylsugar biosynthetic potential, though it is dramatically reduced, and the text should be modified to reflect this.

2) It is generally accepted in the field that reverse genetic analyses must be performed on multiple (at least two but preferably three) independent lines in order to rule out pleiotropic and positional effects from transgene insertion. However, only single lines per construct were used for expression of BACS isoforms using various promoters in the M82 background (see Figs 2b-c, 4e-f, and associated text).

3) Caution should be taken in interpreting the negative result associated with the expression of SpBACS2 under control of proSpBACS2 in the M82 background. The mere presence of the gene with a relevant promoter in the genome does not guarantee transcriptional function, as other factors such as insertion location, epigenetics, and silencing all contribute, which is readily apparent by the variable expression generally achieved between different independent lines in any genetic modification strategy. This concern is exacerbated by the use of only a single transgenic line as noted in point 2.

4) Lines 184-185 state “These results demonstrate that SpBACS1 is the gene responsible for the branched-chain acylsugar phenotype in IL2-5.” While the results do show (disregarding concerns about independent transgenic lines) that SpBACS1

driven by its native promoter is sufficient to promote the branched-chain acylsugar phenotype, but they do not show that SpBACS1 is necessary for the phenotype. This conclusion. Impeding SpBACS1 functon(knockout or knockdown) in a relevant background is necessary. I note that the authors have already performed VIGS of both BACS isoforms in the S. pennellii background (Fig 5 and associated text), and thus quantifying the iC11 and iC13 acylsugars in those samples could more fully support the conclusion of the necessity of SpBACS1 for production of these compounds.

5) Lines 245-247: “These results support the hypothesis that iC11 and iC13 are formed through fatty acid elongation of iC5 using two-carbon units.” The design of acetate labeling is insufficient to support this claim, as the label from acetate would be incorporated into the final product regardless of whether fatty acid elongation happens downstream of iC5 conjugation by BACS1 or if a parallel biosynthetic strategy is operating in which iC11 and iC13 moieties are synthesized via an independent fatty acid-derived process. I recommend simultaneous feeding with labeled acetate and labeled iC5, as this will lead to the hypothesized pathway structure being readily evident by the appearance of M+3 ions.

6) Expression analysis of the genes (lines 267-278) refers vaguely to trichome transcriptomic datasets. Information about the nature of these transcriptional datasets and their analysis is missing from the manuscript and should be included in the methods.

7) The promoter swap experiment, in which SlBACS1 was expressed under the control of the SpBACS1 promoter, presents compelling evidence (disregarding concerns about independent transgenic lines) for the conclusion of altered expression profile being responsible for differential function of the two BACS1 isoforms. However, no comparable experiment is performed to support the conclusion that altered subcellular localization of BACS2 is similarly solely responsible for the BACS1 vs BACS2 functional divergence. Have the authors considered expression of BACS2 with the SpBACS1 transit peptide?

8) The evolutionary trajectory analysis (lines 359-371), and the derived conclusions regarding metabolic innovation, is very limited. The focus on only two species does not allow for confident reconstitution of the evolutionary trajectory. Furthermore, loss of metabolic function, especially in defense, frequently accompanies domestication as cultivation practices relieve the selective pressure to retain those functions. The authors stated this very succinctly in writing about “robust defensive traits that have been inadvertently selected against during breeding for yield and quality” (lines 414-415). Therefore the divergence between SpBACS1 and SlBACS1 is most likely a loss of an unnecessary functon(akin to a fully deactivating mutation in at a catalytic residue) present in an ancestor rather than a “metabolic innovation” or a “neofunctionalization” as stated in the manuscript. Parts of the manuscript describing such evolutionary trajectory or implying gain of function should be revised to avoid overstating implications of the findings.

9) It would be helpful to provide direct access to the sequences for BACS1 and BACS2 from both S. lycopersicum and S. pennellii, either by providing accession numbers or by providing the sequences themselves in supplementary files.

Reviewer #2

(Remarks to the Author)
Wang et al present an intriguing evolutionary model in which SpBACS1 links amino acid catabolism to plastidial fatty acid elongation, enabling the formation of long branched chain acylsugars in wild tomatoes. The findings regarding divergence in cis regulation and subcellular targeting provide a compelling narrative for how metabolic traits can shift during domestication without changes in catalytic function. This represents an appealing contribution to understanding the evolution of specialized metabolites. However, several major issues prevent the work from being evaluated as reliable in its current form.

1. One of my major concerns is related to figure and data integrity. In Figure S1B, the IL2 to IL5 chromatograms show irregular marks between roughly 790 and 800 m/z that resemble cut and pasted image fragments. Additionally, the axes are inconsistent across subpanels. In some cases the X axis touches the chromatogram trace while in others it does not, and the position of the Y axis varies or appears to start below the X axis. Some panels also lack a clear intersection point for the axes. The authors should provide the raw mass spectrometry files and regenerate the figure directly from source data. Verification with an image integrity tool would also be appropriate.

2. The interpretation of the fatty acid feeding experiments is difficult to assess with the data provided. Exogenous application of long chain fatty acids is often inefficient due to poor membrane permeability, which may explain the lack of iC11 or iC13 signal in Figure S2. In contrast, the nC11 and nC13 traces appear dominated by noise, and the difference from the iC substrates is not fully clear because of peak tailing and smoothing. Application of nC11 and nC13 to M82 leaves as controls is missing. As an alternative strategy, the authors may consider supplying substrates to crude trichome protein extracts, which would bypass membrane uptake limitations and offer a cleaner assessment of enzymatic activity. Although this is an in vitro approach, it can still provide functional evidence.

For Figure 1B, the data are more convincing, but the result would be stronger if authors spiked standards into the biological samples and compared retention times with unspiked samples. This would help rule out matrix related shifts. Conclusions about branched chain versus straight chain species could also be supported by presenting key fragment ions and parent ion differences.

3. Although the results suggest that BACS1 preferentially produces iC5, it is not yet clear that iC11 and iC13 arise from direct elongation of iC5. In the 1-13C iC5 labeling experiment, the expected isotopologues are not shown. For S3:21 (5,5,11)

and S3:23 (5,5,13), one would expect a strong M+3 signal because all three acyl chains should incorporate one 13C each. For S3:22 (5,5,12), the maximum expected shift is M+2. None of these patterns are presented.

The explanation for the low M+1 signal of S3:22 in lines 228 to 231 is also not fully supported. Authors should provide a full isotopologue distribution for each detected species, correct for natural abundance, and add negative controls showing that nC12 fragments from unlabeled samples display no M+1 enrichment. As an alternate theory, if two iC5 units originate from an unlabeled pool, then S3:22 should resemble the control, which is not the case in Fig 3C. Authors should discuss the significant M+2 signal observed for the iC11 fragment of S3:21, which does not fit the simple elongation scenario that would primarily generate M+1.

4. With 13C2 sodium acetate, the expected isotopologues should reflect the full chain assembly from labeled precursors. If iC11 and iC13 are direct elongation products of iC5, then M+6 for iC11 (3 rounds of elongation with 13C2 acetate) and M+8 for iC13 (4 rounds of elongation with 13C2 acetate) are expected rather than only M+2. The absence of these isotopologues weakens the proposed pathway.
In both labeling experiments, controls using M82 and the proSpBACS2::SpBACS2 line are missing. Without labeling patterns from these backgrounds, it is difficult to exclude alternative explanations for the observed M+2 in Figure 3D and M+1 in Figure 3F. Controls are essential to confirm that these signals arise from elongation of iC5 rather than unrelated pathways.

Reviewer #3

(Remarks to the Author)
Wang and co-authors describe the isolation and characterization of a novel plastid-localized acyl-CoA synthetase SpBACS1 that is important for the elongation of the short branched-chain fatty acids iC4 and iC5 to the medium branched-chain fatty acids iC10 and iC11/ iC13, respectively in wild tomato, S. pennellii. These fatty acids are then used to decorate sugars to generate acylsugars that provide protection against insect pests.
The domesticated tomato, S. lycopersicum (which does not produce these particular acyl sugars) possesses a functional ortholog of the enzyme, but it is not expressed in the types of trichomes that produce these acylsugars. The BACS2 paralog in both species also possesses the required activity, but these enzymes localize to the mitochondria, and not the chloroplast where fatty acid elongation can occur. What is particularly interesting about the study therefore, is the observation that changing the localization of expression of the enzyme resulted in the synthesis of a new set of compounds.

Experimentally, the authors provide compelling evidence for this role of SpBACS1 in the elongation process of branch-chain fatty acids. This includes genetic evidence based on finemapping of a RIL, biochemical experiments including enzyme assays and isotope-labeling of elongation products, and work expressing the two BACS1 genes under the control of different promoters.

The experimental strategy is generally well conceived, though as discussed below, a focus on SpBACS1 in the context of native S. pennellii metabolism, rather than production of iC11/iC13 in IL2-5, would have described the function of the enzyme in its natural context. Otherwise, the individual experiments, resulting data and interpretation are solid. The manuscript is well written. I have only a few minor comments:

1. My biggest critique is that much of the chemical analysis, biochemistry and discussion revolves around the production of iC11 and iC13 from iC5 in RIL IL2-5. This focus is somewhat artificial given that IL2-5 is a genotype generated by breeding, and not by evolution. Instead, SpBACS1 underwent evolution in the background of S. pennellii, presumably with selection to produce iC10 from iC4. I think it would have been preferable for the focus of the paper to be on the production of the iC10 in native S. pennellii, but I recognise this might not be easily achieved. I suggest the final results describing the production of iC10 in native S. pennellii could be better emphasized.

2. Along these lines, it is interesting is that SpBACS1 has much higher activity with iC5 (substrate in IL2-5) compared to iC4 (presumably the natural substrate?). Can the authors comment?

3. Fig 3E. The authors mention the appearance of M+2 peaks corresponding to elongation using 13C-labeled acetate. As multiple C2 units will need to be incorporated to elongate from iC5 to iC11 or iC13, were M+4, M+6, etc peaks observed, consistent with the incorporation of multiple 13C-labeled acetate moieties?

4. If I am interpreting the sugar nomenclature correctly, it looks like S. pennellii does contain some iC5 – i.e. it is present in the acylsugars S3:19(4,5,10) and G3:19(4,5,10). Can the authors discuss why this iC5 is not extended to make iC11 or iC13 in S. pennellii? Are there different pools of iC5 – one for incorporation into acyl sugars and one for elongation?

5. Line 329: consider changing to “differing from IL2-5.”

Reviewer #4

(Remarks to the Author)
Wang et al. present a highly interesting study on metabolic innovation in plants, demonstrating how existing primary metabolic pathways can be repurposed through neo-gene expression, neo-subcellular localization, while retaining their

original catalytic functions. This work provides valuable insights into the remarkable metabolic plasticity of plants and offers a compelling example of how plants may rapidly evolve novel biochemical capabilities—such as the production of insecticidal metabolites—during evolution. The manuscript is well-structured, clearly written, and presents its key findings in a logical and engaging manner. I particularly appreciate the hypothesis-driven approach and the elegant presentation of data. The figures are visually appealing and effectively support the conclusions. The biochemical and genetic assays are robust and provide high-quality evidence for the authors' claims.
I have only a few minor suggestions and questions:
The authors should clarify how the chemical structures of the acyl sugars were determined. Were authentic standards used for identification? This information appears to be missing from the main text and figures, and would strengthen the validity of the structural assignments.
While the Discussion is informative and well-written, it would benefit from a deeper exploration of the mechanism by which long-branched-chain fatty acids bypass the conventional fatty acid synthesis (FAS) machinery. Could co-evolved acyl-ACP thioesterases be responsible for premature termination of the plastidial FAS cycle? Addressing this would enhance the mechanistic understanding of how these unusual fatty acid chains are produced.
It would be interesting to know whether these long-branched-chain acyl sugars exhibit enhanced insecticidal activity compared to their straight-chain counterparts? A discussion or comparison of bioactivity could further highlight the ecological and evolutionary significance of this metabolic innovation.
Some figure legends are insufficient. For example, in Fig. S2, the authors should specify how the acyl sugar structures were defined. In Fig. S5, annotations of gene names and appropriate citations would improve clarity and help readers interpret the data more effectively.
In line 81, medium- and long-branched chain fatty acids?
The section from lines 137–145, which describes the detection of new peaks via feeding percussors, is less convincing due to the limited data (only one biological replicate) and small peak intensities. In contrast, the subsequent paragraph of MS-based analyses is more robust. It might be more effective to restructure this part by presenting it as additional evidence, while emphasizing the stronger MS-based results with standards firstly.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Regarding responses to my prior comments (numbering refers to the numbering of my original comments):
1) Changes to the text have fully addressed my concern.
2) Inclusion of additional data on a second independent transgenic line for reverse genetic analysis fully addressed my concern, which I note was critical for this work to meet the minimum standards of the field.
3) Changes to the text have fully addressed my concern.
4) While the authors were unable to complete all suggested experiments, they did make necessary changes to the language to ensure conclusions are adequately supported by the available data. This concern has been addressed.
5) The authors competed the suggested experiment and obtained results that more directly support their original conclusion. This concern has been fully addressed.
6) The authors added information that fully address my concern.
7) While the authors declined to complete the suggested experiment, I acknowledge the difficulty of my suggestion. The authors did make changes to the wording to ensure they are not over-stating conclusions in the absence of the new data, and therefore in the manuscript's present form, while the requested data would provide interesting information, it is not strictly necessary to support the core conclusions. Therefore I am satisfied with how the authors addressed this concern.
8) The modifications made to the text fully address this concern.
9) I thank the authors for fully accommodating this request.

Reviewer #2

(Remarks to the Author)
The authors have adequately addressed all comments raised during the review process, and the manuscript is now significantly improved.

Reviewer #3

(Remarks to the Author)
The manuscript has been strengthened by incorporating results from additional experiments. What was originally an interesting and compelling study that combined biochemical, genetic and cell biology data, is now even more persuasive. I don't have any further comments or concerns.

Reviewer #4

(Remarks to the Author)

The authors have adequately addressed my concerns, and I have no further comments.

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Response to Reviews

We thank the reviewers for their valuable comments, which have made it possible for us to significantly improve this manuscript. All comments have been addressed with corresponding revisions or explanations.

Reviewer #1 (Remarks to the Author):

In their manuscript titled "Evolutionary hijacking of fatty acid biosynthesis drives insecticidal acylsugar diversity in tomato," Wang et al describe their investigation into differential acylsugar profiles between domesticated tomato and its wild relative. Specifically, the authors use an introgression-based approach to narrow in on two closely related acyl-CoA synthetases for further study. Using a combination of protein biochemistry, stable isotope labeling, reverse genetics, subcellular localization, and expression analysis, the authors conclude that the wild tomato uses a novel strategy in which one acyl-CoA synthetase produces a branched-chain acyl-CoA which is then acted on by canonical fatty acid synthase machinery to form the acyl moieties incorporated into the acylsugars, and they identify localization and expression patterns as likely underlying the reduced ability of domesticated tomato to produce these same acylsugars despite retention of both acyl-CoA synthetases. The authors present this as an example of neofunctionalization of a single enzyme without altering catalytic function leading to metabolic innovation.

Overall, the manuscript is well-written and supports most conclusions made, including providing an example of functional divergence deriving from non-catalytic differences. This divergence is made even more interesting by the fact that the enzyme in question controls a branch of secondary metabolism (acylsugar synthesis) that apparently leverages the catalytic machinery of a core primary metabolic pathway (fatty acid elongation).

Response: We thank the reviewer for recognizing the value of our study. In response to the constructive suggestions, we have conducted additional experiments and made corresponding revisions, which have substantially strengthened the quality of the manuscript.

However, as detailed in my specific comments below, my enthusiasm is tempered by shortcomings in the reverse genetic analyses and generally poor support for the neofunctionalization claim.

Specific Comments:

1. It is incorrectly stated in the text that some analyzed lines do not produce the iC11/iC13 acylsugars (lines 182-183: "transgenic plants harboring proSpBACS2::SpBACS2 did not produce these branched-chain acylsugars;" lines 295-296: "not normally detectable in M82"). Although the difference between these lines and the IL 2-5 line is stark, the small peaks corresponding to these compounds are visible in the sample chromatograms, and indeed the compounds are quantified in the relevant bar graphs. Thus M82 maintains iC11/iC13 acylsugar biosynthetic potential, though it is dramatically reduced, and the text should be modified to reflect this.

1.1 Response: We deeply appreciate the reviewer's careful examination of our chromatograms and quantitative data. Strictly speaking, very minor peaks with the

exact same m/z and retention times can occasionally be observed and integrated in the EIC mode for M82 and the *proSpBACS2*::*SpBACS2* transgenic lines, which accounts for the small, non-zero values presented in our bar graphs. The reason we originally described them as "not detectable" or "did not produce" is that these signals are extremely weak, often bordering on the instrument's limit of detection with very poor signal-to-noise ratios, making definitive MS/MS fragmentation confirmation highly challenging in these specific background lines.

However, we fully agree with the reviewer that using absolute terms like "did not produce" is scientifically inaccurate. We have modified the text throughout the manuscript to accurately reflect that these compounds are present at "trace, near-background levels" or are "almost undetectable," rather than being completely absent.

2. It is generally accepted in the field that reverse genetic analyses must be performed on multiple (at least two but preferably three) independent lines in order to rule out pleiotropic and positional effects from transgene insertion. However, only single lines per construct were used for expression of BACS isoforms using various promoters in the M82 background (see Figs 2b-c, 4e-f, and associated text).

1.2 Response: We thank the reviewer for raising this important point. We fully agree that transgenic complementation and promoter-swap experiments should be supported by multiple independent transgenic lines to minimize the possibility that the observed phenotypes result from positional effects or other insertion-related artifacts.

To address this concern, we added additional independent transgenic lines and reanalyzed the relevant experiments. For the experiments shown in Fig. 2B–C, we added one additional independent line for each construct. The revised analysis now includes two independent *proSpBACS1::SpBACS1* lines and two independent *proSpBACS2::SpBACS2* lines in the M82 background. Both independent *proSpBACS1::SpBACS1* lines accumulated iC11/iC13-containing acylsugars, whereas both independent *proSpBACS2::SpBACS2* lines showed only trace or near-undetectable levels comparable to M82. These results confirm that the contrasting effects of *SpBACS1* and *SpBACS2* are reproducible across independent transformation events.

For the experiments shown in Fig. 4E–F, we expanded the analysis to three independent *proSpBACS1::SlBACS1* transgenic lines. All three independent lines consistently accumulated iC11/iC13-containing acylsugars at levels clearly elevated above M82, confirming that expression of the cultivated tomato *SlBACS1* coding sequence under the *SpBACS1* promoter is sufficient to restore long-branched-chain acylsugar accumulation.

We have updated Fig. 2B–C, Fig. 4E–F, and the associated Results text accordingly. These additional independent lines strengthen the genetic evidence supporting our conclusions that *SpBACS1*, but not *SpBACS2*, drives robust long-branched-chain acylsugar production, and that the functional difference between *SpBACS1* and *SlBACS1* is primarily attributable to promoter-mediated expression differences rather than loss of enzymatic activity in SlBACS1.

3. Caution should be taken in interpreting the negative result associated with the expression of SpBACS2 under control of proSpBACS2 in the M82 background. The mere presence of the gene with a relevant promoter in the genome does not guarantee

transcriptional function, as other factors such as insertion location, epigenetics, and silencing all contribute, which is readily apparent by the variable expression generally achieved between different independent lines in any genetic modification strategy. This concern is exacerbated by the use of only a single transgenic line as noted in point 2.

1.3 Response: We thank the reviewer for this thoughtful comment. We agree that a negative result from a transgenic experiment should be interpreted cautiously, because the presence of a transgene and its native promoter in the genome does not necessarily guarantee robust transcriptional activity. As the reviewer notes, insertion position, epigenetic effects, and transgene silencing can all influence expression and may vary among independent transformation events.

To address this concern, we added an additional independent *proSpBACS2::SpBACS2* transgenic line in the M82 background. The revised Fig. 2B–C now includes two independent *proSpBACS2::SpBACS2* lines. Both lines showed only trace or near-undetectable levels of iC11/iC13-containing acylsugars, in contrast to the independent *proSpBACS1::SpBACS1* lines, which consistently accumulated these compounds. This additional independent line reduces the likelihood that the lack of the long-branched-chain acylsugar phenotype in the *proSpBACS2::SpBACS2* plants was caused by unfavorable insertion events or other unexpected factors.

At the same time, we agree that this result alone should not be overinterpreted as definitive evidence for the absence of any biological function of *SpBACS2*. Indeed, silencing *SpBACS2* in *S. pennellii* did not measurably alter the acylsugar profile under our experimental conditions, and our current data do not establish the native biological role of *SpBACS2*. and We have therefore revised the manuscript to soften the interpretation of the *proSpBACS2::SpBACS2* negative result. We now present it as one piece of evidence, alongside the subcellular localization data and the *S. pennellii* VIGS results, indicating that *SpBACS2* does not contribute to long-branched-chain acylsugar accumulation in the contexts tested.

We also revised the relevant Results and Discussion text to describe the *proSpBACS2::SpBACS2* data more cautiously and to avoid implying that *SpBACS2* lacks biological function altogether. Our conclusion is now limited to the specific pathway examined here: *SpBACS2* did not promote detectable long-branched-chain acylsugar accumulation in cultivated M82 and did not affect the measured acylsugar profile when silenced in *S. pennellii*.

4) Lines 184-185 state “These results demonstrate that SpBACS1 is the gene responsible for the branched-chain acylsugar phenotype in IL2-5.” While the results do show (disregarding concerns about independent transgenic lines) that SpBACS1 driven by its native promoter is sufficient to promote the branched-chain acylsugar phenotype, but they do not show that SpBACS1 is necessary for the phenotype. This conclusion. Impeding SpBACS1 functon(knockout or knockdown) in a relevant background is necessary. I note that the authors have already performed VIGS of both BACS isoforms in the S. pennellii background (Fig 5 and associated text), and thus quantifying the iC11 and iC13 acylsugars in those samples could more fully support the conclusion of the necessity of SpBACS1 for production of these compounds.

1.4 Response: We agree that the original statement was a little strong. The transgenic complementation experiment in M82 demonstrates that *SpBACS1* driven by its native promoter is sufficient to promote accumulation of long-branched-chain acylsugars in

the M82 background, but by itself it does not demonstrate that SpBACS1 is necessary for this phenotype. We have therefore revised the wording in the manuscript to avoid overstatement.

To address the reviewer's concern, we made use of the native *S. pennellii* background, where SpBACS1 contributes to endogenous long-branched-chain acylsugar production. Although stable CRISPR/Cas9 knockout lines would provide an ideal genetic test, transformation and regeneration of wild tomato *S. pennellii* remain technically challenging, inefficient, and substantially more time-consuming than in cultivated tomato. We therefore used VIGS as a feasible and direct approach to reduce *SpBACS1* expression in *S. pennellii* and analyzed the acylsugar profiles of the resulting plants. In the revised study, we re-performed VIGS assays in *S. pennellii* to generate fresh *SpBACS1*- and *SpBACS2*-silenced plants for the newly added insect bioassays, and we also used these independently generated VIGS materials to re-evaluate the effects of *SpBACS1* and *SpBACS2* silencing on acylsugar accumulation.

In the revised manuscript, we show that VIGS effectively reduced *SpBACS1* transcript levels, as confirmed by RT-qPCR (Fig. 5D). Importantly, LC–MS analysis revealed that silencing *SpBACS1* significantly decreased the abundance of long-branched-chain acylsugars in *S. pennellii*, particularly the iC10-containing acylsugars S3:18(4,4,10), S3:19(4,5,10), G3:18(4,4,10), and G3:19(4,5,10) (Fig. 5E). In contrast, silencing *SpBACS2* did not significantly affect these acylsugars. These results support the conclusion that *SpBACS1* is required for the robust accumulation of long-branched-chain acylsugars in native *S. pennellii*.

We also appreciate the reviewer's specific suggestion to examine iC11- and iC13-containing acylsugars in the VIGS samples. However, in *S. pennellii*, iC11/iC13-containing acylsugars are present at much lower levels than iC10-containing acylsugars. Previous work has shown that *S. pennellii* acylsugars are enriched in iC4-derived acyl chains, whereas cultivated tomato backgrounds contain relatively higher levels of iC5-derived acyl chains. In our proposed biosynthetic model, these short branched-chain fatty acids are activated by SpBACS1 and then serve as primers for subsequent two-carbon elongation by the fatty acid biosynthetic machinery. Thus, in native *S. pennellii*, iC4-derived primers are expected to yield iC10-containing acyl groups after three rounds of elongation, whereas in the IL2-5/M82 background, iC5-derived primers give rise to iC11- and iC13-containing acyl groups. Therefore, in the native *S. pennellii* background, iC10-containing acylsugars are the predominant products of SpBACS1-dependent long-branched-chain acylsugar biosynthesis.

Accordingly, we have revised the relevant statement from "These results demonstrate that *SpBACS1* is the gene responsible for the branched-chain acylsugar phenotype in IL2-5" to a more precise and softer conclusion "These results demonstrate that SpBACS1 is sufficient to promote the accumulation of iC11- and iC13-containing long-branched-chain acylsugars in the M82 background" (Line 197-198).

5. Lines 245-247: "These results support the hypothesis that iC11 and iC13 are formed through fatty acid elongation of iC5 using two-carbon units." The design of acetate labeling is insufficient to support this claim, as the label from acetate would be incorporated into the final product regardless of whether fatty acid elongation happens downstream of iC5 conjugation by BACS1 or if a parallel biosynthetic strategy is

operating in which iC11 and iC13 moieties are synthesized via an independent fatty acid-derived process. I recommend simultaneous feeding with labeled acetate and labeled iC5, as this will lead to the hypothesized pathway structure being readily evident by the appearance of M+3 ions.

1.5 Response: We thank the reviewer for this insightful and constructive suggestion. We agree that acetate labeling alone cannot fully distinguish between elongation of an iC5-derived primer and an alternative parallel fatty acid-derived biosynthetic route, because acetate-derived two-carbon units could be incorporated into fatty acyl chains regardless of upstream precursors.

To address this point, we performed a new dual-isotope feeding experiment as suggested by the reviewer. Specifically, we simultaneously fed IL2-5 leaves with [1-^{13}C]-iC5 and [1,2-$^{13}C_2$]-sodium acetate, followed by LC–MS/MS analysis of the relevant acylsugar fragments. Under this dual-labeling condition, our proposed model predicts that the elongated iC11/iC13 acyl chains should contain both the one-carbon label from iC5 and the two-carbon label from acetate, producing diagnostic $[M+3]^-$ acyl-chain fragment ions.

Consistent with this prediction, we detected not only $[M+1]^-$ signals corresponding to incorporation of labeled iC5 and $[M+2]^-$ signals corresponding to incorporation of labeled acetate-derived two-carbon units, but also clear $[M+3]^-$ signals in the long-branched-chain acyl fragments. In particular, MS/MS analysis revealed a distinct $[M+3]^-$ iC11 fragment ion, demonstrating simultaneous incorporation of carbon from both labeled iC5 and labeled acetate into the same elongated branched acyl chain. These new data are now presented in Fig. 3G.

This dual-labeling result provides much stronger evidence for our proposed pathway architecture: iC5 is first activated by SpBACS1 and then elongated by fatty acid biosynthetic machinery through the addition of acetate-derived two-carbon units to form long-branched-chain acyl moieties. We have revised the Results section (Line 284-293) accordingly to make clear that the acetate-only experiment supports incorporation of two-carbon units, whereas the new dual-labeling experiment provides more direct evidence that iC5 and acetate-derived carbon are incorporated into the same elongated branched acyl chain.

6. Expression analysis of the genes (lines 267-278) refers vaguely to trichome transcriptomic datasets. Information about the nature of these transcriptional datasets and their analysis is missing from the manuscript and should be included in the methods.

1.6 Response: We thank the reviewer for pointing this out. To address this concern, we have revised the relevant Results text to specify the source of the trichome transcriptomic datasets used for expression analysis. We have also added the appropriate citations where these datasets are first introduced.

7. The promoter swap experiment, in which SlBACS1 was expressed under the control of the SpBACS1 promoter, presents compelling evidence (disregarding concerns about independent transgenic lines) for the conclusion of altered expression profile being responsible for differential function of the two BACS1 isoforms. However, no comparable experiment is performed to support the conclusion that altered subcellular

localization of BACS2 is similarly solely responsible for the BACS1 vs BACS2 functional divergence. Have the authors considered expression of BACS2 with the SpBACS1 transit peptide?

1.7 Response: We thank the reviewer for this thoughtful suggestion. We agree that expressing SpBACS2 with the SpBACS1 chloroplast transit peptide would be a valuable experiment to directly test whether altered subcellular localization alone is sufficient to explain the functional divergence between SpBACS1 and SpBACS2.

We considered this experiment carefully. However, generating and validating stable transgenic tomato plants expressing a transit-peptide-swapped SpBACS2 construct would require a substantial amount of additional time, beyond the practical revision timeframe. Importantly, we agree with the reviewer that, without such an experiment, we should not conclude that altered subcellular localization is the sole cause of the functional divergence between SpBACS1 and SpBACS2. We have therefore revised the relevant text to make this interpretation more cautious.

In addition, our biochemical data indicate that SpBACS2 is not identical to SpBACS1 in catalytic performance. Although SpBACS2 can activate short branched-chain fatty acids, its catalytic efficiency toward iC5 is lower than that of both SpBACS1 and SlBACS1. Specifically, the calculated kcat/Km for iC5 is approximately 0.096 for SpBACS2 (Fig. 5B), compared with approximately 0.34 for SpBACS1 (Fig. 3B) and 0.61 for SlBACS1 (Fig. 4B). SpBACS2 also shows the highest Km among these enzymes, suggesting reduced substrate affinity. These differences indicate that catalytic divergence may also contribute to the distinct behavior of SpBACS2.

Accordingly, we have softened our conclusion in the revised manuscript. We now state that the varied localization of SpBACS2 is likely an important factor limiting its participation in plastidial fatty acid elongation and long-branched-chain acylsugar biosynthesis. The revised text (Line 372-377, Line 388-395) acknowledges that both subcellular relocalization and differences in catalytic properties may have both contributed to the functional divergence between SpBACS1 and SpBACS2.

8. The evolutionary trajectory analysis (lines 359-371), and the derived conclusions regarding metabolic innovation, is very limited. The focus on only two species does not allow for confident reconstitution of the evolutionary trajectory. Furthermore, loss of metabolic function, especially in defense, frequently accompanies domestication as cultivation practices relieve the selective pressure to retain those functions. The authors stated this very succinctly in writing about “robust defensive traits that have been inadvertently selected against during breeding for yield and quality” (lines 414-415). Therefore the divergence between SpBACS1 and SlBACS1 is most likely a loss of an unnecessary function (akin to a fully deactivating mutation in at a catalytic residue) present in an ancestor rather than a “metabolic innovation” or a “neofunctionalization” as stated in the manuscript. Parts of the manuscript describing such evolutionary trajectory or implying gain of function should be revised to avoid overstating implications of the findings.

1.8 Response: We thank the reviewer for this thoughtful comment. We agree that the evolutionary history of the BACS-mediated pathway is complex and that a comparison between *S. pennellii* and *S. lycopersicum* alone cannot fully resolve the ancestral state or reconstruct the complete evolutionary trajectory across the *Solanum* lineage. Our intention was not to claim a definitive lineage-by-lineage reconstruction, but rather to

highlight the genetic and biochemical mechanisms associated with divergence in long-branched-chain acylsugar production between wild and cultivated tomato.

We appreciate the reviewer's point that the reduced production of these metabolites in cultivated tomato may reflect loss or attenuation of a defensive metabolic trait during domestication. This is a plausible interpretation and is consistent with the broader view that domestication can reduce specialized metabolic defenses when selection pressure for such traits is relaxed. At the same time, our data show that the cultivated tomato ortholog SlBACS1 retains catalytic activity, and that the absence of long-branched-chain acylsugars in cultivated tomato is largely associated with altered tissue-specific expression rather than loss of enzyme function. Thus, rather than being analogous to a fully deactivating mutation in a catalytic residue, the divergence observed here appears to involve changes in regulatory deployment and subcellular pathway context.

In response, we have revised the manuscript to avoid overinterpreting the evolutionary directionality. Specifically, we have softened or removed statements implying definitive neofunctionalization, metabolic innovation, or a fully reconstructed evolutionary trajectory. We now describe our findings more cautiously as evidence that changes in gene expression and subcellular localization are associated with divergence in long-branched-chain acylsugar production between wild and cultivated tomato.

We have also revised the Discussion (Line 552-555) to emphasize that the SpBACS1-mediated pathway represents a mechanistic example of how existing primary metabolic modules can be connected to generate specialized metabolic diversity, while avoiding a strong claim about when or in which lineage this capacity first arose. The revised manuscript now frames the work as identifying the genetic and biochemical basis of a defensive metabolic trait enriched in wild tomato and reduced in cultivated tomato, rather than as definitively proving a specific evolutionary gain-of-function event.

9. It would be helpful to provide direct access to the sequences for BACS1 and BACS2 from both S. lycopersicum and S. pennellii, either by providing accession numbers or by providing the sequences themselves in supplementary files.

1.9 Response: In the revised manuscript, we have now provided the corresponding accession numbers for these genes (Line 656-658). In addition, we have included the BACS1 and BACS2 sequences from both species in Supplementary Table S4. These additions will allow readers to directly access and compare the sequences used in our phylogenetic, biochemical, and functional analyses.

Reviewer #2 (Remarks to the Author):

Wang et al present an intriguing evolutionary model in which SpBACS1 links amino acid catabolism to plastidial fatty acid elongation, enabling the formation of long branched chain acylsugars in wild tomatoes. The findings regarding divergence in cis regulation and subcellular targeting provide a compelling narrative for how metabolic traits can shift during domestication without changes in catalytic function. This represents an appealing contribution to understanding the evolution of specialized metabolites.

Response: We thank the reviewer for recognizing the value of our study. In response to

the constructive suggestions, we have conducted additional experiments and made corresponding revisions, which have substantially strengthened the quality of the manuscript.

However, several major issues prevent the work from being evaluated as reliable in its current form.

1. One of my major concerns is related to figure and data integrity. In Figure S1B, the IL2 to IL5 chromatograms show irregular marks between roughly 790 and 800 m/z that resemble cut and pasted image fragments. Additionally, the axes are inconsistent across subpanels. In some cases the X axis touches the chromatogram trace while in others it does not, and the position of the Y axis varies or appears to start below the X axis. Some panels also lack a clear intersection point for the axes. The authors should provide the raw mass spectrometry files and regenerate the figure directly from source data. Verification with an image integrity tool would also be appropriate.

2.1 Response: We thank the reviewer for raising this important concern. We fully appreciate that image and data integrity are essential for the reliable evaluation of MS-based evidence, and we have taken this comment very seriously.

We would like to state clearly that no manipulation or alteration of the underlying mass spectrometry data was performed. After carefully re-examining the original files and the assembled figure, we found that the irregular marks noted by the reviewer in the original Fig. S1B were caused by unintentional vector-graphic artifacts introduced during figure assembly in Adobe Illustrator. Specifically, several orphaned vector paths remained in the figure after chromatogram traces were edited and arranged for presentation. These artifacts affected only the visual appearance of the assembled figure.

The inconsistent axis intersections, slight differences in axis position, and variable spacing between chromatogram traces and axes resulted from manual formatting during figure preparation. These did not affect the chromatographic data, peak detection, quantification, statistical analysis, or any conclusion of the study.

To address the reviewer's concern transparently and comprehensively, we have taken the following steps in the revised submission. First, we regenerated Fig. S1B directly from the source chromatogram data and removed the unintended vector artifacts. Second, we standardized the formatting of all chromatogram panels, including axis position, axis intersection, trace placement, and stroke weight, to ensure consistent presentation across subpanels. Third, we carefully re-examined all MS-related figures in the manuscript and corrected any similar formatting inconsistencies where present.

In addition, to allow independent verification of the underlying data, we have deposited all raw MS data used in this study in the public repository Figshare (https://figshare.com/s/d8ece53f660c98464934). The deposited dataset includes the raw files corresponding to the LC-MS, LC-MS/MS, GC-MS, and stable isotope-labeling analyses reported in the manuscript. The repository information has been added to the Data availability section of the revised manuscript.

Furthermore, as requested, we have included screenshots of the original spectra for the relevant MS-based panels in the Source Data files submitted with the revised manuscript. These original instrument-derived screenshots allow direct comparison between the raw chromatographic/spectral evidence and the processed figure panels.

2. The interpretation of the fatty acid feeding experiments is difficult to assess with the data provided. Exogenous application of long chain fatty acids is often inefficient due to poor membrane permeability, which may explain the lack of iC11 or iC13 signal in Figure S2. In contrast, the nC11 and nC13 traces appear dominated by noise, and the difference from the iC substrates is not fully clear because of peak tailing and smoothing. Application of nC11 and nC13 to M82 leaves as controls is missing. As an alternative strategy, the authors may consider supplying substrates to crude trichome protein extracts, which would bypass membrane uptake limitations and offer a cleaner assessment of enzymatic activity. Although this is an in vitro approach, it can still provide functional evidence.

For Figure 1B, the data are more convincing, but the result would be stronger if authors spiked standards into the biological samples and compared retention times with unspiked samples. This would help rule out matrix related shifts. Conclusions about branched chain versus straight chain species could also be supported by presenting key fragment ions and parent ion differences.

2.2 Response: We thank the reviewer for this careful and constructive comment. We agree that the fatty acid feeding experiment alone is not sufficient to definitively establish the structures of the C11 and C13 acyl chains, particularly because exogenous application of long-chain fatty acids can be affected by differences in uptake, membrane permeability, and cellular transport. We also agree that the most direct and convincing evidence for distinguishing branched-chain versus straight-chain C11/C13 acyl groups comes from our GC–MS analysis of derivatized acyl chains released from the native acylsugars (Fig.1B).

In response to the reviewer's suggestion, we have repeated and strengthened the GC–MS experiment shown in Fig. 1B. In the revised analysis, we included additional samples in which authentic iC11 and iC13 standards were spiked directly into the biological IL2-5 acylsugar-derived samples. These spiked samples were analyzed together with the unspiked biological samples and the corresponding straight-chain and branched-chain standards. The spiked IL2-5 samples showed a single co-eluting peak at the same retention time as the iC11 or iC13 standard, rather than the appearance of separate peaks or shifted retention times. This result rules out matrix-related retention-time shifts and further supports the conclusion that the C11 and C13 acyl chains in IL2-5 acylsugars correspond to terminally branched iC11 and iC13 species. The revised data are now presented in Fig. 1B.

We also considered the reviewer's suggestion to use diagnostic fragment ions to support the structural assignment. However, under our GC–MS conditions, the mass spectra of the straight-chain and terminally branched C11/C13 ethyl ester standards were highly similar and did not provide sufficiently distinctive fragment ions for confident discrimination. Therefore, we consider retention-time matching with authentic standards, together with the new standard-spiking experiment in the biological matrix, to be the most reliable basis for assigning the acyl-chain structures.

Regarding the fatty acid feeding experiment, we agree that differences in uptake efficiency and cellular accessibility could influence the results. In particular, the failure or weak incorporation of exogenously applied iC11/iC13 may reflect inefficient delivery rather than the absence of biochemical competence. Therefore, we have revised the manuscript to avoid relying on the feeding experiment as the primary

evidence for C11/C13 structural assignment. In the revised Results section, we now present the GC–MS analysis of acyl-chain ethyl esters, including the new spiking experiment, as the principal evidence that the IL2-5 C11 and C13 acyl chains are terminally branched. The fatty acid feeding experiment has been repositioned as supporting evidence.

We also carefully considered the reviewer's suggestion to perform substrate-feeding assays using crude trichome protein extracts. While this would in principle bypass limitations associated with fatty acid uptake, it is technically challenging in this system. Obtaining trichome protein extracts with minimal contamination from leaf tissues requires physical separation of glandular trichomes from leaves, which is typically performed under liquid-nitrogen freezing conditions. Such conditions may compromise enzyme activity. In addition, the very small proportion of trichome tissue relative to whole leaves makes it difficult to obtain sufficient active protein for reliable biochemical assays. For these reasons, we did not pursue this experiment in the current revision.

Overall, we have revised the manuscript to clarify the hierarchy of evidence (Line 145-151): the structural conclusion that IL2-5 produces iC11- and iC13-containing acylsugars is now based primarily on GC–MS retention-time comparison with authentic standards and biological-matrix spiking, while the fatty acid feeding experiment is treated only as supplementary supporting evidence.

3. Although the results suggest that BACS1 preferentially produces iC5, it is not yet clear that iC11 and iC13 arise from direct elongation of iC5. In the 1-13C iC5 labeling experiment, the expected isotopologues are not shown. For S3:21 (5,5,11) and S3:23 (5,5,13), one would expect a strong M+3 signal because all three acyl chains should incorporate one 13C each. For S3:22 (5,5,12), the maximum expected shift is M+2. None of these patterns are presented.

2.3a Response: We thank the reviewer for this insightful comment. We agree that the original presentation of the isotope-labeling data did not sufficiently show the expected isotopologue patterns, particularly for acylsugars containing multiple iC5-derived acyl chains. We have therefore substantially revised and expanded this part of the analysis.

In the revised manuscript, in addition to the $[M+1]^-$ signals, we now present the $[M+3]^-$ isotopologue changes for S3:21(5,5,11) and S3:23(5,5,13) following [1-^{13}C]-iC5 feeding. As predicted, because these acylsugars contain two direct iC5 chains and one elongated iC11 or iC13 chain derived from iC5, all three acyl chains can incorporate one ^{13}C atom. Consistent with this expectation, we observed a clear increase in the $[M+3]^-/M^-$ for both S3:21(5,5,11) and S3:23(5,5,13). These new data are now included in Fig. 3C.

We have also replaced and expanded the MS/MS fragmentation data in Fig. 3D. In the previous version, we mainly showed the labeled iC11/iC13 fragments. In the revised figure, we now present both the labeled iC5 fragments and the labeled iC11 or iC13 fragments from the same acylsugars (Fig. S5B). These MS/MS data show that, after [1-^{13}C]-iC5 feeding, both the directly incorporated iC5 chains and the elongated long-branched chains become labeled. This pattern is consistent with the interpretation that iC5 contributes not only to the short iC5 acyl chains but also serves as the precursor for the elongated iC11/iC13 moieties.

For S3:22(5,5,12), we have added MS/MS fragmentation data in Fig. S5B. As the reviewer predicted, this acylsugar is expected to incorporate label into the two iC5 chains, but not into the nC12 chain. Consistent with this model, the labeled iC5 fragment signal increased markedly, whereas the C12 fragment did not show evidence of labeling. This result further supports the conclusion that the iC5-derived label is incorporated specifically into iC5-derived acyl chains rather than broadly into all fatty acyl moieties.

In addition, to more directly test whether iC5 is elongated by acetate-derived two-carbon units, we performed a new dual-isotope feeding experiment. IL2-5 leaves were simultaneously supplied with [1-^{13}C]-iC5 and [1,2-$^{13}C_2$]-sodium acetate, followed by LC–MS/MS analysis of acyl-chain fragments. Under our proposed pathway, the elongated iC11/iC13 chain should contain both the one-carbon label from iC5 and the two-carbon label from acetate, resulting in diagnostic $[M+3]^-$ fragment ions. Consistent with this prediction, we detected $[M+1]^-$ signals from iC5 labeling, $[M+2]^-$ signals from acetate labeling, and importantly, a clear $[M+3]^-$ iC11 fragment signal reflecting simultaneous incorporation of both labeled substrates into the same long-branched-chain acyl moiety. These new data are presented in Fig. 3G.

Together, these additional isotopologue and MS/MS fragmentation analyses provide stronger evidence that iC11 and iC13 arise through elongation of an iC5-derived precursor using two-carbon units, rather than through an independent parallel fatty acid-derived pathway.

The explanation for the low M+1 signal of S3:22 in lines 228 to 231 is also not fully supported. Authors should provide a full isotopologue distribution for each detected species, correct for natural abundance, and add negative controls showing that nC12 fragments from unlabeled samples display no M+1 enrichment. As an alternate theory, if two iC5 units originate from an unlabeled pool, then S3:22 should resemble the control, which is not the case in Fig 3C. Authors should discuss the significant M+2 signal observed for the iC11 fragment of S3:21, which does not fit the simple elongation scenario that would primarily generate M+1.

2.3b Response: We thank the reviewer for this careful and constructive comment. We agree that the original explanation of the isotope-labeling results, particularly for S3:22(5,5,12), was not sufficiently clear and that a more complete presentation of the isotopologue patterns is needed.

In our original interpretation, S3:22(5,5,12) was used as a comparison because it contains two iC5 chains and one straight-chain C12 chain. After feeding [1-^{13}C]-iC5, the two iC5 chains are expected to incorporate the label, whereas the nC12 chain is not expected to be labeled through this precursor. Therefore, compared with the unlabeled control, S3:22(5,5,12) should show increased labeled isotopologues, including $[M+1]^-$ and $[M+2]^-$ species, depending on whether one or both iC5 chains incorporate the isotope. In contrast, S3:21(5,5,11) and S3:23(5,5,13) contain two iC5 chains plus one elongated iC11 or iC13 chain that, according to our model, is also derived from iC5. Thus, these acylsugars are expected to have a greater probability of isotope incorporation than S3:22(5,5,12).

To strengthen this interpretation, we have added MS/MS fragmentation data for labeled S3:22(5,5,12). These data show a clear labeled iC5 fragment, whereas the C12 fragment does not show detectable ^{13}C labeling. This provides direct fragment-level evidence that

the isotope from [1-^{13}C]-iC5 is incorporated into the iC5 chains, but not into the nC12 chain. These new data are now presented in Fig. S5B.

Following the reviewer's suggestion, we also expanded the isotopologue analysis. In Fig. S5A, S5C, and S5E of the revised manuscript, we now provide the relative distributions of M^-, $[M+1]^-$, $[M+2]^-$, $[M+3]^-$, and $[M+4]^-$ for the four long-branched-chain acylsugars under three labeling conditions: [1-^{13}C]-iC5 feeding, [1,2-$^{13}C_2$]-sodium acetate feeding, and combined [1-^{13}C]-iC5 plus [1,2-$^{13}C_2$]-sodium acetate feeding. The corresponding unlabeled iC5 or sodium acetate treatments were used as controls. Compared with the natural-abundance isotopologue distributions in the controls, the labeled treatments showed clear enrichment of higher isotopologues, including $[M+2]^-$, $[M+3]^-$, and $[M+4]^-$ species, consistent with incorporation of labeled carbon into these acylsugars.

We agree that full molecular isotopologue distributions alone provide supportive rather than definitive evidence, because they can be influenced by natural isotope abundance and by labeling of multiple acyl chains within the same molecule. Therefore, we have placed greater emphasis on the more direct MS/MS fragment evidence. In particular, the revised figures now show that labeled iC11 and iC13 fragments are significantly enriched after [1-^{13}C]-iC5 feeding, and the new dual-labeling experiment further detects diagnostic $[M+3]^-$ long-branched-chain fragments after simultaneous feeding with labeled iC5 and labeled acetate. These fragment-level data directly support incorporation of iC5-derived carbon into the elongated iC11/iC13 acyl chains.

Regarding the reviewer's point about the observed $[M+2]^-$ signal for the iC11 fragment of S3:21(5,5,11), we believe this signal is most likely influenced by natural isotope abundance superimposed on the experimentally introduced ^{13}C label. In other words, once an iC11 fragment contains one introduced ^{13}C atom from [1-^{13}C]-iC5, the presence of an additional naturally occurring heavy isotope in the same fragment can increase the apparent $[M+2]^-$ signal.

Overall, we have revised this section to more clearly show three levels of evidence: acyl-chain-specific MS/MS fragmentation, and dual-substrate isotope labeling, and molecular isotopologue distributions. Together, these expanded analyses provide stronger support for our model that iC11 and iC13 acyl chains are generated by elongation of an iC5-derived precursor using two-carbon elongation units.

4. With 13C2 sodium acetate, the expected isotopologues should reflect the full chain assembly from labeled precursors. If iC11 and iC13 are direct elongation products of iC5, then M+6 for iC11 (3 rounds of elongation with 13C2 acetate) and M+8 for iC13 (4 rounds of elongation with 13C2 acetate) are expected rather than only M+2. The absence of these isotopologues weakens the proposed pathway.

2.4a Response: We thank the reviewer for this technically insightful comment. We agree that, under our proposed model, elongation of an iC5-derived primer by multiple rounds of two-carbon addition should, in principle, generate isotopologues higher than $[M+2]^-$ after [1,2-$^{13}C_2$]-sodium acetate feeding. Specifically, complete incorporation of labeled acetate units through three elongation rounds would generate an $[M+6]^-$ iC11 chain, whereas four labeled elongation rounds would generate an $[M+8]^-$ iC13 chain.

To address this concern, we repeated the [1,2-$^{13}C_2$]-sodium acetate labeling experiment with a slightly extended labeling period and performed a more detailed isotopologue analysis. Using Q-TOF MS, we were able to detect $[M+4]^-$ signals for S3:21(5,5,11) and S3:23(5,5,13), indicating incorporation of two labeled two-carbon unit into these acylsugars. The new data of increased $[M+4]^-/M^-$ are now included in Fig. 3E.

Because the $[M+4]^-$ signals were relatively low in abundance, Q-TOF MS/MS did not provide sufficient sensitivity to reliably obtain the corresponding acyl-chain fragment ions. We therefore further analyzed the labeled samples using a high-sensitivity TIMS-TOF mass spectrometer. With this approach, we successfully fragmented the $[M+4]^-$ acylsugar ions and detected an $[M+4]^-$ labeled C11 acyl-chain fragment. These new MS/MS data are now presented in Fig. S5D. This fragment-level evidence demonstrates that multiple acetate-derived two-carbon units are incorporated into the long-branched acyl chain itself, providing stronger support for the model that iC11 and iC13 are generated through repeated two-carbon elongation.

We did not detect $[M+6]^-$ or $[M+8]^-$ signals under our experimental conditions. We believe this is most likely due to the low probability of consecutive incorporation of three or four fully labeled acetate units into the same elongating acyl chain in vivo, together with the limited abundance of these specific long-branched-chain acylsugars. As the number of required labeled elongation cycles increases, the expected abundance of the fully labeled $[M+6]^-$ or $[M+8]^-$ species decreases substantially, making these isotopologues difficult to detect even with highly sensitive MS instrumentation.

We have revised the Results section to acknowledge this limitation explicitly (Line 271-283). Rather than implying that the acetate-labeling experiment alone proves complete sequential elongation, we now state that the detection of $[M+4]^-$ molecular isotopologues and, importantly, an $[M+4]^-$ labeled C11 fragment provides evidence for incorporation of multiple acetate-derived two-carbon units into the long-branched-chain acyl moiety. Together with the [1-^{13}C]-iC5 labeling and the new dual-labeling experiment, these data provide strong support for our proposed model that iC11/iC13 acyl chains arise through repeated two-carbon elongation of an iC5-derived precursor.

In both labeling experiments, controls using M82 and the proSpBACS2::SpBACS2 line are missing. Without labeling patterns from these backgrounds, it is difficult to exclude alternative explanations for the observed M+2 in Figure 3D and M+1 in Figure 3F. Controls are essential to confirm that these signals arise from elongation of iC5 rather than unrelated pathways.

2.4b Response: We agree that appropriate genetic-background controls are needed for interpreting the isotope-labeling experiments and for excluding the possibility that the observed labeled signals arise from unrelated pathways.

As the reviewer notes, both M82 and the *proSpBACS2::SpBACS2* line accumulate iC11/iC13-containing acylsugars only at trace or near-undetectable levels under our analytical conditions. To address the reviewer's concern, we performed an additional feeding experiment using M82, with IL2-5 included as a positive control. M82 leaves were fed either unlabeled iC5 or [1-^{13}C]-iC5, and the corresponding acylsugar profiles were analyzed.

These new data show that feeding unlabeled iC5 did not induce detectable accumulation of the iC11/iC13-containing acylsugars in M82. Similarly, after [1-^{13}C]-iC5 feeding, M82 still did not show detectable $[M+1]^-$ signals for the iC11/iC13-containing

acylsugars. In contrast, under the same labeling conditions, IL2-5 showed clear enrichment of $[M+1]^-$ signals for the four iC11/iC13-containing acylsugars. These results, included in Fig. S5G in the reviesed manuscript, indicate that exogenous iC5 labeling alone is not sufficient to generate detectable long-branched-chain acylsugars in M82, supporting that the that the labeled iC11/iC13 signals observed in IL2-5 depend on the elongation of iC5 rather than a broadly operating unrelated pathway.

Interestingly, in M82 fed with [1-^{13}C]-iC5, we did observe increased $[M+1]^-$ signal for S3:22(5,5,12). MS/MS analysis of this labeled S3:22(5,5,12) showed that the label was incorporated into the C5 fragment, whereas the C12 fragment was not labeled (Fig. S5F). This result is consistent with our expectation that exogenous labeled iC5 can contribute to iC5 acyl chains in acylsugars, but does not by itself lead to detectable production of iC11/iC13 long-branched-chain acylsugars in M82.

In addition to this new M82 control experiment, the new dual-labeling experiment in IL2-5 by simultaneously feeding [1-^{13}C]-iC5 and [1,2-$^{13}C_2$]-sodium acetate (Fig. 3G) also provide more direct evidence that the long-branched chains arise through elongation of an iC5-derived precursor.

Together, the absence of labeled iC11/iC13 acylsugars in M82 after [1-^{13}C]-iC5 feeding, the specific labeling of iC5 but not C12 fragments in M82 S3:22(5,5,12), and the detection of dual-labeled iC11 fragments in IL2-5 provide stronger support that the iC11/iC13 labeling observed in IL2-5 arises from elongation of iC5 rather than an unrelated fatty acid pathway.

Reviewer #3 (Remarks to the Author):

Wang and co-authors describe the isolation and characterization of a novel plastid-localized acyl-CoA synthetase SpBACS1 that is important for the elongation of the short branched-chain fatty acids iC4 and iC5 to the medium branched-chain fatty acids iC10 and iC11/ iC13, respectively in wild tomato, S. pennellii. These fatty acids are then used to decorate sugars to generate acylsugars that provide protection against insect pests.

The domesticated tomato, S. lycopersicum (which does not produce these particular acyl sugars) possesses a functional ortholog of the enzyme, but it is not expressed in the types of trichomes that produce these acylsugars. The BACS2 paralog in both species also possesses the required activity, but these enzymes localize to the mitochondria, and not the chloroplast where fatty acid elongation can occur. What is particularly interesting about the study therefore, is the observation that changing the localization of expression of the enzyme resulted in the synthesis of a new set of compounds.

Experimentally, the authors provide compelling evidence for this role of SpBACS1 in the elongation process of branch-chain fatty acids. This includes genetic evidence based on finemapping of a RIL, biochemical experiments including enzyme assays and isotope-labeling of elongation products, and work expressing the two BACS1 genes under the control of different promoters.

The experimental strategy is generally well conceived, though as discussed below, a focus on SpBACS1 in the context of native S. pennellii metabolism, rather than production of iC11/iC13 in IL2-5, would have described the function of the enzyme in its natural context. Otherwise, the individual experiments, resulting data and

interpretation are solid. The manuscript is well written.

Response: We thank the reviewer for recognizing the value of our study. In response to the constructive suggestions, we have conducted additional experiments and made corresponding revisions, which have substantially strengthened the quality of the manuscript.

I have only a few minor comments:

1. My biggest critique is that much of the chemical analysis, biochemistry and discussion revolves around the production of iC11 and iC13 from iC5 in RIL IL2-5. This focus is somewhat artificial given that IL2-5 is a genotype generated by breeding, and not by evolution. Instead, SpBACS1 underwent evolution in the background of S. pennellii, presumably with selection to produce iC10 from iC4. I think it would have been preferable for the focus of the paper to be on the production of the iC10 in native S. pennellii, but I recognise this might not be easily achieved. I suggest the final results describing the production of iC10 in native S. pennellii could be better emphasized.

3.1 Response: We thank the reviewer for this thoughtful comment. We agree that the native biological context of *SpBACS1* is *S. pennellii*, where the major long-branched-chain acylsugars contain iC10 rather than iC11/iC13. The previous version of the manuscript placed relatively strong emphasis on the IL2-5-derived iC11/iC13 phenotype, and that the role of *SpBACS1* in native *S. pennellii* should be more clearly highlighted.

To address this point, we have revised the manuscript to give greater emphasis to the function of *SpBACS1* in its native *S. pennellii* background. Ideally, this would be tested using stable CRISPR/Cas9 knockout lines in *S. pennellii*. However, stable transformation and regeneration of this wild tomato species remain technically challenging and inefficient, and generating validated knockout lines would require a much longer timeframe. Therefore, to directly assess *SpBACS1* function in native *S. pennellii*, we have repeated the VIGS experiments to knock down *SpBACS1* and *SpBACS2*, and used the VIGS materials to perform insect bioassays.

In the revised manuscript, we show that *SpBACS1* silencing in *S. pennellii* significantly reduced the abundance of iC10-containing acylsugars, whereas *SpBACS2* silencing did not have a detectable effect on these metabolites. The successful knockdown of the target genes was confirmed by RT-qPCR, and the corresponding metabolic changes are shown in Fig. 5D–E. These results support the conclusion that *SpBACS1* is required for the robust accumulation of long-branched-chain acylsugars in its native *S. pennellii* background. We have revised the Results section to emphasize this point more prominently.

We also performed new insect bioassays using the *S. pennellii* VIGS materials to evaluate the ecological relevance of SpBACS1-mediated long-branched-chain acylsugars. These data are now presented in Fig. S10. Although *SpBACS1* silencing reduced iC10-containing acylsugars, we did not observe a statistically significant change in resistance to the insects tested. We have added this result (Line 396-411) and discussed (Line 556-567) its interpretation. In particular, we note that plant chemical defense is often mediated by the combined effects of multiple specialized metabolites. Thus, reduction of one acylsugar subclass may not be sufficient to produce a strong resistance phenotype when other abundant acylsugars and defensive compounds remain

present. This functional redundancy may contribute to the robustness of the defensive metabolite system.

At the same time, we believe that the IL2-5/M82 background remains an important part of the study. First, the discovery of SpBACS1 was enabled by the allelic difference between IL2-5 and M82, making this system essential for identifying the responsible gene. Second, the comparison between *S. pennellii* and the cultivated tomato background reveals an important principle relevant to crop improvement: the phenotypic output of an introduced wild allele can depend strongly on the metabolic precursor pool of the recipient background. In native *S. pennellii*, where iC4-derived precursors predominate, SpBACS1 mainly promotes iC10-containing acylsugars. In the IL2-5/M82 background, where iC5-derived precursors are more prominent, the same enzyme promotes iC11/iC13-containing acylsugars. We have emphasized this point in Discussion to present how SpBACS1 activity can generate different long-branched-chain acylsugar outputs depending on precursor availability in different genetic background.

Overall, we have rebalanced the manuscript to better distinguish between the native role of SpBACS1 in *S. pennellii* and the mechanistic insights gained from IL2-5. The revised version now more clearly emphasizes that iC10 production in *S. pennellii* represents the native context of SpBACS1 function, while the IL2-5/M82 experiments reveal how this wild-tomato enzyme behaves when introduced into a cultivated-tomato metabolic background.

2. Along these lines, it is interesting is that SpBACS1 has much higher activity with iC5 (substrate in IL2-5) compared to iC4 (presumably the natural substrate?). Can the authors comment?

3.2 Response: We thank the reviewer for highlighting this interesting point. We agree that it is notable that SpBACS1, which originates from *S. pennellii*, where iC4-derived acylsugars are more abundant, shows strong activity toward iC5 in vitro.

We do not view this as contradictory. Many metabolic enzymes, especially those involved in specialized metabolism, show a degree of substrate promiscuity. Such latent or "silent" biochemical capacity may not be fully manifested in the native metabolic context if the relevant substrate is present only at low abundance, but it can become apparent in vitro or when the enzyme is expressed in a different metabolic background. In our case, SpBACS1 is capable of activating both iC4 and iC5. Although it shows relatively strong activity toward iC5 in vitro, the metabolic output in planta is also determined by substrate availability.

This interpretation is consistent with our genetic-background comparison. In native *S. pennellii,* where the iC4 precursor pool is relatively abundant, SpBACS1 primarily contributes to the production of iC10-containing acylsugars. By contrast, when SpBACS1 is introduced into the M82/IL2-5 background, where iC5-derived acylsugar precursors are more prominent, the same enzyme promotes production of iC11- and iC13-containing acylsugars. Thus, the distinct products observed in different genetic backgrounds likely reflect the interaction between SpBACS1 substrate promiscuity and the available branched-chain fatty acid precursor pools.

We have added this point to the Discussion (Line 530-537). The text emphasizes that SpBACS1 has the biochemical capacity to activate related short branched-chain substrates, but the dominant acylsugar products formed in vivo depend on the relative

availability of iC4 versus iC5 in a given tomato background. This provides an example of how a single enzyme can generate different specialized metabolic outputs when placed into different precursor environments.

3. Fig 3E. The authors mention the appearance of M+2 peaks corresponding to elongation using 13C-labeled acetate. As multiple C2 units will need to be incorporated to elongate from iC5 to iC11 or iC13, were M+4, M+6, etc peaks observed, consistent with the incorporation of multiple 13C-labeled acetate moieties?

3.3 Response: We thank the reviewer for raising this important point. Reviewer 2 also raised this question, where we responded in "2.4a Response". We agree that, if iC11 and iC13 are generated by elongation of an iC5-derived precursor, incorporation of more than one acetate-derived C2 unit should be detectable in principle. Therefore, isotopologues higher than $[M+2]^-$ would provide stronger evidence for repeated two-carbon elongation.

To address this, we repeated the [1,2-$^{13}C_2$]-sodium acetate labeling experiment with a slightly extended labeling period and performed a more detailed isotopologue analysis. Using Q-TOF MS, we detected clear $[M+4]^-$ signals for S3:21(5,5,11) and S3:23(5,5,13), consistent with incorporation of two labeled acetate-derived C2 units. These data are now included in Fig. 3E.

Because the abundance of the $[M+4]^-$ labeled molecular ions was relatively low, Q-TOF MS/MS did not provide sufficient sensitivity to obtain reliable acyl-chain fragment ions. We therefore further analyzed the labeled samples using a high-sensitivity TIMS-TOF mass spectrometer. With this approach, we successfully fragmented the $[M+4]^-$ labeled acylsugar ions and observed an $[M+4]^-$ labeled C11 acyl-chain fragment. These new MS/MS data are presented in Fig. S5C. This result provides fragment-level evidence that multiple acetate-derived two-carbon units are incorporated into the long-branched acyl chain itself.

We did not reliably detect $[M+6]^-$ or $[M+8]^-$ isotopologues under our experimental conditions. We believe this is most likely due to the low probability of consecutive incorporation of three or four fully labeled acetate units into the same elongating acyl chain in vivo, together with the limited abundance of these specific long-branched-chain acylsugars. As the number of required labeled elongation cycles increases, the expected abundance of the fully labeled $[M+6]^-$ or $[M+8]^-$ species decreases substantially, making these isotopologues difficult to detect even with highly sensitive MS instrumentation.

We have revised the Results section to acknowledge this limitation explicitly (Line 271-283). Rather than implying that the acetate-labeling experiment alone proves complete sequential elongation, we now state that the detection of $[M+4]^-$ molecular isotopologues and, importantly, an $[M+4]^-$ labeled C11 fragment provides evidence for incorporation of multiple acetate-derived two-carbon units into the long-branched-chain acyl moiety. Together with the [1-^{13}C]-iC5 labeling and the new dual-labeling experiment, these data provide strong support for our proposed model that iC11/iC13 acyl chains arise through repeated two-carbon elongation of an iC5-derived precursor.

4. If I am interpreting the sugar nomenclature correctly, it looks like S. pennellii does contain some iC5 – i.e. it is present in the acylsugars S3:19(4,5,10) and G3:19(4,5,10). Can the authors discuss why this iC5 is not extended to make iC11 or iC13 in S. pennellii? Are there different pools of iC5 – one for incorporation into acyl sugars and one for elongation?

3.4 Response: We thank the reviewer for raising this important point. The reviewer is correct that the acylsugar nomenclature S3:19(4,5,10) and G3:19(4,5,10) indicates the presence of a C5 acyl chain in these *S. pennellii* acylsugars. However, based on previously published structural analyses of *S. pennellii* acylsugars using NMR (DOI: 10.3390/metabo10100401), the C5 chain in these compounds is assigned as anteiso-C5 (aiC5) rather than iso-C5 (iC5) (See the below attached table from that publication). These two branched C5 fatty acids have different branching patterns.

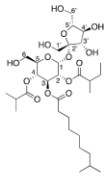
This distinction is important for interpreting our results. In our enzyme activity assays, SpBACS1 showed strong activity toward iC5 but only very weak activity toward aiC5 (Fig. 3A). Therefore, even though C5-containing acylsugars are present in *S. pennellii*, the relevant C5 substrate may not be efficiently activated by SpBACS1 if it is predominantly aiC5 rather than iC5. This substrate-structure difference may help explain why the major long-branched-chain acylsugars in *S. pennellii* are iC10-containing compounds derived from iC4, rather than iC11/iC13-containing compounds derived from iC5.

We have revised the Discussion to clarify this point (Line 538-547). We now distinguish between iC5 and aiC5 and note that the C5 moieties previously reported in *S. pennellii* acylsugars are aiC5, which is a poor substrate for SpBACS1 in our biochemical assays. This provides a plausible explanation for why these C5-containing acylsugars do not necessarily imply efficient iC5-dependent elongation in *S. pennellii*.

At the same time, we agree that we cannot completely exclude the possibility that *S. pennellii* contains a small iC5 pool. If low amounts of iC5 are present and elongated to iC11 or iC13, the resulting acylsugars may be below the detection limit of our current analytical platform and present at much lower abundance than the dominant iC10-containing acylsugars. We have therefore revised the manuscript to avoid stating that iC11/iC13-containing acylsugars are entirely absent from *S. pennellii*. Instead, we now state more cautiously that iC10-containing acylsugars are the predominant detectable long-branched-chain products in *S. pennellii* under our analytical conditions**.**

Overall, this revision clarifies that the dominant product profile in *S. pennellii* likely reflects both substrate availability and substrate specificity: abundant iC4 can be efficiently activated and elongated by SpBACS1 to produce iC10, whereas the detected C5 acyl chains in major *S. pennellii* acylsugars are likely aiC5, which is a poor SpBACS1 substrate and therefore less likely to be elongated into long-terminal branched products(aiC11/aiC13).

Table S9 NMR chemical shifts for S3:19(4,5,10)-1 purified from *S. pennellii* LA0716.



S3:19(4,5,10)-1

Purified from *S. pennellii* LA0716

Chemical Formula: $C_{31}H_{54}O_{14}$

HRMS: (ESI) *m/z* calculated for $C_{31}H_{54}O_{14}$ ($[M+NH_4]^+$): 668.3852

Experimental *m/z*: 668.3856

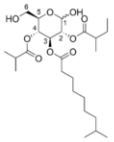
InChI Key: WWMDOPWHTJPJMW-KSMFYLDCSA-N

NMR (500 MHz, $CDCl_3$)

Sample mass: 2 mg

Carbon # (group)	^{1}H (ppm)	^{13}C (ppm) (from HSQC and HMBC)
1 (CH)	5.78 (d, J = 4.0 Hz, 1H)	88.80
2 (CH)	4.85 (dd, *J* = 10.4, 4.0 Hz, 1H)	70.84
- 1 (CO)	-	176.77
- 2 (CH)	2.40 (sextet, *J* = 6.8 Hz, 1H)	40.61
- 3 (CH_3)	1.12 (m, 3H)	16.08
- 4 (CH_2)	1.42, 1.60 (m, 2H)	26.78
- 5 (CH_3)	0.86 (t, *J* = 7.3 Hz, 3H)	11.42
3 (CH)	5.56 (t, J = 10.0 Hz, 1H)	69.00
- 1 (CO)	-	173.04
- 2 (CH_2)	2.20 (t, J = 7.5 Hz, 2H)	34.16
- 3 (CH_2)	1.51(m, 2H)	24.68
- 4,5,6 (CH_2)	1.24 (m)	29.34
- 7 (CH_2)	1.13 (m)	38.82
- 8 (CH)	1.48 (m)	28.08
- 9,10 (CH_3)	0.85 (m)	22.71
4 (CH)	4.90 (t, *J* = 10.0 Hz, 1H)	68.49
- 1 (CO)	-	176.16
- 2 (CH)	2.53 (hept, *J* = 7.0 Hz, 1H)	34.03
- 3,4 (CH_3)	1.11 (d, *J* = 7.0 Hz, 6H)	18.85
5 (CH)	4.20 (m, 1H)	71.85
6 (CH_2)	3.60, 3.60 (m, 2H)	61.55
1' (CH_2)	3.61 (m, 1H), 3.51 (d, *J* = 11.9 Hz, 2H)	64.60
2' (C)	-	104.52
3' (CH)	4.24 (m, 1H)	78.07
4' (CH)	4.30 (t, *J* = 8.5 Hz, 1H)	72.96
5' (CH)	3.74 (m, 1H)	81.35
6' (CH_2)	3.88 (d, *J* = 13.1 Hz, 1H), 3.74 (m, 1H)	60.06

Table S14 NMR chemical shifts for G3:19(4,5,10)-1 purified from *S. pennellii* LA0716.



G3:19(4,5,10)-1

Purified from *S. pennellii* LA0716

Chemical Formula: $C_{28}H_{42}O_9$

HRMS: (ESI) *m/z* calculated for $C_{28}H_{42}O_9$ ($[M+NH_4]^+$): 506.3324

Experimental *m/z*: 506.3328

InChI Key: UCMUJVLLMOVWPV-VSLCRTCVSA-N
InChI Key (α): UCMUJVLLMOVWPV-ONCJQFAMSA-N
InChI Key (β): UCMUJVLLMOVWPV-MBFSEYTRSA-N

NMR (500 MHz, $CDCl_3$)

Sample mass: 2 mg

Carbon # (group)	^{1}H (ppm)		^{13}C (ppm) (from HSQC and HMBC)	
	α	β	α	β
1 (CH)	5.51 (d, *J* = 3.6 Hz)	4.74 (d, *J* = 8.1 Hz)	90.29	95.86
2 (CH)	4.88 (m)	4.87 (m)	71.17	73.35
- 1 (CO)	-	-	176.22	176.22
- 2 (CH)	2.41 (sextet, *J* = 6.9 Hz)	2.41 (sextet, *J* = 6.9 Hz)	40.92	40.92
- 3 (CH_3)	1.13 (m, 3H)	1.13 (m, 3H)	16.35	16.35
- 4 (CH_2)	1.45, 1.65 (m, 2H)	1.45, 1.65 (m, 2H)	26.49	26.49
- 5 (CH_3)	0.88 (t, *J* = 7.3 Hz, 3H)	0.88 (t, *J* = 7.3 Hz, 3H)	11.50	11.50
3 (CH)	5.69 (t, J = 9.9 Hz)	5.41 (t, J = 9.6 Hz)	68.69	71.09
- 1 (CO)	-	-	172.71	172.71
- 2 (CH_2)	2.23 (t, *J* = 7.4 Hz)	2.23 (t, *J* = 7.4 Hz)	34.05	34.05
- 3 (CH_2)	1.54(m)	1.54(m)	24.76	24.76
- 4,5,6 (CH_2)	1.25(m)	1.25(m)	29.27	29.27
- 7 (CH_2)	1.24 (m)	1.24 (m)	27.22	27.22
- 8 (CH)	1.50 (m)	1.50 (m)	27.88	27.88
- 9,10 (CH_3)	0.88 (d, *J* = 7.0 Hz, 6H)	0.88 (d, *J* = 7.0 Hz, 6H)	22.48	22.48
4 (CH)	5.04 (m)	5.04(m)	68.55	68.55
- 1 (CO)	-	-	176.73	176.73
- 2 (CH)	2.54 (hept, J = 7.0 Hz)	2.54 (hept, J = 7.0 Hz)	33.98	33.98
- 3,4 (CH_3)	1.13 (m)	1.13 (m)	18.31	18.31
5 (CH)	4.07 (ddd, *J* = 10.2, 4.0, 2.2 Hz)	3.58 (m)	69.49	74.58
6 (CH_2)	3.58, 3.69 (m)	3.58, 3.69 (m)	61.10	61.10

5. Line 329: consider changing to "differing from IL2-5."

3.5 Response: We thank the reviewer for this suggestion. We have revised the wording as suggested, changing "differs from IL2-5" to "differing from IL2-5."

Reviewer #4 (Remarks to the Author):

Wang et al. present a highly interesting study on metabolic innovation in plants, demonstrating how existing primary metabolic pathways can be repurposed through neo-gene expression, neo-subcellular localization, while retaining their original catalytic functions. This work provides valuable insights into the remarkable metabolic plasticity of plants and offers a compelling example of how plants may rapidly evolve novel biochemical capabilities—such as the production of insecticidal metabolites—during evolution. The manuscript is well-structured, clearly written, and presents its key findings in a logical and engaging manner. I particularly appreciate the hypothesis-driven approach and the elegant presentation of data. The figures are visually appealing and effectively support the conclusions. The biochemical and genetic assays are robust and provide high-quality evidence for the authors' claims.

Response: We thank the reviewer for recognizing the value of our study. In response to the constructive suggestions, we have conducted additional experiments and made corresponding revisions, which have substantially strengthened the quality of the manuscript.

I have only a few minor suggestions and questions:

1. The authors should clarify how the chemical structures of the acyl sugars were determined. Were authentic standards used for identification? This information appears to be missing from the main text and figures, and would strengthen the validity of the

structural assignments.

4.1 Response: We thank the reviewer for this important comment. We agree that the basis for the acylsugar structural assignments should be described more clearly in the main text and figures.

In the revised manuscript, we have added additional text in the Material and Methods (Line 619-628) to explain how the acylsugar structures were assigned and how the acylsugar nomenclature is used. Many of the major tomato acylsugars discussed in this study have been structurally characterized in previous work by NMR, including representative cultivated tomato acylsugars such as S3:22(5,5,12) and S4:24(2,5,5,12), as well as *S. pennellii* acylsugars such as S3:19(4,5,10) and G3:19(4,5,10). For these previously characterized compounds, both the acyl chain lengths and branching patterns have been established. We have now added the relevant citations and explanatory text to make this clearer for readers.

For the newly characterized C11- and C13-containing acylsugars in IL2-5, we assigned the overall acylsugar composition using high-resolution LC–MS and MS/MS fragmentation. These analyses allowed us to infer the sugar core, total acyl chain number, and acyl chain composition, including the presence of C11 or C13 acyl moieties (Fig. S1F). However, LC–MS/MS alone cannot unambiguously distinguish straight-chain from terminally branched C11/C13 acyl chains.

To determine the branching pattern of the C11 and C13 acyl chains, we therefore performed acyl-chain release and derivatization followed by GC–MS comparison with authentic fatty acid ethyl ester standards. In the revised manuscript, we further strengthened this analysis by including standard-spiking experiments, in which authentic iC11 and iC13 standards were added directly to the biological IL2-5-derived samples. The spiked samples showed single co-eluting peaks at the same retention times as the corresponding iC11 or iC13 standards, supporting the assignment of these acyl chains as terminally branched iC11 and iC13 rather than straight-chain nC11 or nC13 species. These data are now presented more clearly in Fig. 1B.

2. While the Discussion is informative and well-written, it would benefit from a deeper exploration of the mechanism by which long-branched-chain fatty acids bypass the conventional fatty acid synthesis (FAS) machinery. Could co-evolved acyl-ACP thioesterases be responsible for premature termination of the plastidial FAS cycle? Addressing this would enhance the mechanistic understanding of how these unusual fatty acid chains are produced.

4.2 Response: We thank the reviewer for this insightful suggestion. We agree that the mechanism by which these unusual long-branched-chain acyl moieties are released from, or diverted away from, the canonical plastidial fatty acid synthesis pathway is an important unresolved question.

Our data support a model in which SpBACS1 generates short branched-chain acyl-CoA substrates, such as iC4-CoA or iC5-CoA, that can enter the plastidial fatty acid elongation machinery. However, production of discrete medium-length branched acyl chains such as iC10, iC11, and iC13 likely also requires mechanisms that terminate elongation at specific chain lengths and release the products for subsequent incorporation into acylsugars.

We agree with the reviewer that acyl-ACP thioesterases are attractive candidates for this role. In canonical plastidial fatty acid biosynthesis, chain-length specificity is strongly influenced by enzymes that determine when elongating acyl-ACP intermediates are released. Specialized acyl-ACP thioesterases could, in principle, hydrolyze branched medium-chain acyl-ACP intermediates at defined chain lengths, thereby allowing unusual iC10, iC11, or iC13 fatty acids to accumulate rather than continuing through the conventional fatty acid elongation program.

Furthermore, in canonical de novo fatty acid synthesis, KAS III catalyzes the initiating condensation between an acyl-CoA primer and malonyl-ACP, whereas subsequent elongation steps are carried out by other KAS enzymes using acyl-ACP substrates. Because our model proposes that branched-chain acyl-CoA substrates such as iC4-CoA or iC5-CoA are used as non-canonical primers, an important open question is whether tomato trichomes contain a KAS III or KAS III-like activity capable of accepting these branched acyl-CoA substrates. To our knowledge, such an enzyme has not yet been identified in tomato acylsugar biosynthesis.

Accordingly, we have modified the Discussion (Line 484-499) to emphasize that SpBACS1 is likely the enzyme that enables entry of branched amino-acid-derived carbon into plastidial fatty acid metabolism, but that it may not be the only specialized component required for production of these unusual acyl chains. The full pathway may involve coordinated or co-evolved activities at both the primer-entry step, potentially involving KAS III-like substrate specificity, and the termination/release step, potentially involving specialized acyl-ACP thioesterases.

These revisions improve the mechanistic framing of the pathway and clarify that SpBACS1 provides the metabolic bridge into fatty acid elongation, while additional fatty acid synthesis and release enzymes are likely required to define the final chain-length distribution of long-branched-chain acylsugar precursors.

3. It would be interesting to know whether these long-branched-chain acyl sugars exhibit enhanced insecticidal activity compared to their straight-chain counterparts? A discussion or comparison of bioactivity could further highlight the ecological and evolutionary significance of this metabolic innovation.

4.3 Response: We thank the reviewer for this thoughtful suggestion. To address the biological relevance of SpBACS1-mediated long-branched-chain acylsugars, we performed new insect bioassays using both disruption-of-function-like and gain-of-function materials. First, we used VIGS to silence *SpBACS1* in native *S. pennellii*. RT-qPCR confirmed reduced *SpBACS1* expression (Fig. 5D), and LC–MS analysis showed that silencing *SpBACS1* significantly reduced the abundance of long-branched-chain iC10-containing acylsugars (Fig. 5E). Second, we used *proSpBACS1::SpBACS1* transgenic M82 plants, which accumulate newly detectable iC11- and iC13-containing acylsugars. We then performed western flower thrips preference assays and cotton bollworm resistance assays using these materials. These new data are now presented in Fig. S10.

In these assays, reduction of long-branched-chain acylsugars in *S. pennellii* or increased accumulation of iC11/iC13-containing acylsugars in M82 did not result in a statistically significant change in insect resistance under the conditions tested. These results suggest that SpBACS1-mediated long-branched-chain acylsugars alone do not act as a dominant determinant of resistance in these assays.

We agree that a direct comparison between purified long-branched-chain and straight-chain acylsugars would be an ideal way to evaluate relative bioactivity. However, we currently do not have suitable plant materials that differ only in this specific structural feature, such as lines producing exclusively long-branched-chain acylsugars versus lines producing only matched straight-chain counterparts. Because acylsugar profiles differ in multiple chain lengths, branching patterns, sugar cores, and abundance, a clean biological comparison between these two compound classes is not yet feasible in planta.

We have revised the Discussion (Line 556-567) to propose that acylsugar-mediated defense is likely determined by the combined action of multiple acylsugar species rather than by any single acyl chain class alone. Thus, even if individual long-branched-chain acylsugars differ in bioactivity from straight-chain analogs, the effect of adding or removing one subclass may be buffered by the broader acylsugar mixture. This chemical redundancy may contribute to the robustness of plant defense.

4. Some figure legends are insufficient. For example, in Fig. S2, the authors should specify how the acyl sugar structures were defined. In Fig. S5, annotations of gene names and appropriate citations would improve clarity and help readers interpret the data more effectively.

4.4 Response: We thank the reviewer for this helpful suggestion. In the revised manuscript, we have expanded the Results text to explain how the acylsugar structures and nomenclature were defined. We now clarify that the acylsugar annotations are based on high-resolution LC–MS/MS analysis, acyl-chain fragmentation patterns, and, where applicable, previously reported NMR-based structural assignments from tomato acylsugar studies. We have also added the relevant citations to support these assignments.

For Fig. S2, we have revised the legend to more clearly state how the indicated acylsugar structures were assigned and what evidence supports the distinction between endogenous C11/C13-containing acylsugars and products associated with exogenous fatty acid feeding.

For the previous Fig. S5, now revised as Fig. S6, we have included appropriate citations so that readers can better understand the identities and roles of the genes shown in the figure. These changes should improve the clarity and interpretability of the supplementary figures.

5. In line 81, medium- and long-branched chain fatty acids?

4.5 Response: We thank the reviewer for pointing this out. We have revised the wording in line 82 from **"**medium- and long-chain fatty acids**"** to "medium- and long-branched-chain fatty acids" for greater accuracy.

6. The section from lines 137–145, which describes the detection of new peaks via feeding percussors, is less convincing due to the limited data (only one biological replicate) and small peak intensities. In contrast, the subsequent paragraph of MS-based analyses is more robust. It might be more effective to restructure this part by presenting it as additional evidence, while emphasizing the stronger MS-based results with standards firstly.

4.6 Response: We thank the reviewer for this helpful suggestion. We agree that the fatty acid feeding experiment is less definitive than the GC–MS-based structural analysis, particularly because the feeding data were based on limited biological replication and relatively small peak intensities.

In the revised manuscript, we have reorganized this section to make the hierarchy of evidence clearer (Line 140-163). We now present the GC–MS analysis of acyl chains released from IL2-5 acylsugars as the primary evidence for the structural assignment. In this experiment, acyl chains from IL2-5 acylsugars were derivatized and compared directly with authentic straight-chain and terminally branched fatty acid standards. These data, now strengthened by standard-spiking analysis in Fig. 1B, provide the key evidence that the C11 and C13 acyl chains in IL2-5 are terminally branched rather than straight-chain species.

We have moved the fatty acid feeding result to a supporting role. In the revised Results section, this experiment is described as additional evidence that initially suggested the endogenous C11/C13-containing acylsugars differed from straight-chain nC11/nC13-containing products, but we no longer rely on it as the principal basis for structural assignment.

In summary, this restructuring emphasizes the stronger standard-based GC–MS evidence first and presents the feeding experiment more cautiously as supplementary support.