

Sarcandra glabra genome unveils functional conservation and divergence of O-methyltransferases catalyzing isofraxidin biosynthesis in angiosperms

Corresponding Author: Dr Wei Li

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)

This study presents a high-quality, chromosome-level genome assembly of the medicinal plant *Sarcandra glabra* (Chloranthaceae), representing a valuable genomic resource. By integrating comparative genomics, transcriptome co-expression analysis, phylogenetics, molecular docking, site-directed mutagenesis, and in vitro enzyme assays, the authors demonstrate that the identified enzyme exhibits dual functionality, efficiently catalyzing the conversion of scopoletin to isofraxidin as well as esculetin to scopoletin. Furthermore, the study identifies Leu127 and Leu307 as key residues governing catalytic activity and regioselectivity (7-OH vs. 8-OH), respectively. Notably, comparative analyses reveal functional conservation of this OMT across angiosperms, alongside a distinct evolutionary shift in regioselectivity between early-diverging Chloranthaceae and eudicots. These findings offer important insights into isofraxidin biosynthesis. However, several limitations should be addressed to strengthen the reliability and generalizability of the conclusions.

Major issues:

1. The genome characteristics related to long gene length and the high proportion of dispersedly duplicated genes are discussed across two sections. However, these aspects do not represent central findings and are not directly linked to the main theme of the study. Therefore, it is recommended to streamline the related content and incorporate more general genome analyses, such as additional genome quality assessments (e.g., LAI and Merqury analyses) and whole-genome duplication (WGD) analyses. These could be consolidated under a section titled "Genome Evolution Analyses" or similar. Additionally, although the gene length section is not central, one issue requires clarification: the reported exon length in *S. glabra* is approximately 2.5 times that of other Chloranthaceae species. The authors should verify whether this reflects a true biological characteristic or a statistical artifact, for instance, by analyzing collinear genes among the compared species.
2. One of the main objectives of this study is to identify all candidate OMT genes using the high-quality genome assembly. However, the number of OMT genes appears to correlate with gene annotation quality, as *C. sessilifolius*, which has more predicted genes, also contains more OMTs. To assess the completeness of OMT annotation in *S. glabra*, the authors could perform homologous annotation using tools such as GeMoMa with predicted OMTs from other species (e.g., *C. sessilifolius*, *Liriodendron tulipifera*, *Cinnamomum micranthum*, and *Arabidopsis thaliana*), and also perform the BLAST searches against transcripts that assembled directly by RNA-Seq reads.
3. The manuscript posits the necessity of the catalytic triad (H/E/D) for OMT activity based on sequence alignment and previous studies on flavonoid OMTs, with the natural D266N mutation in SgOMT7 cited as corroborating evidence. However, as the authors note by referencing prior work, the essentiality of this triad has been established primarily in the context of flavonoid OMTs instead of simple coumarins OMT. Furthermore, the rationale for specifically identifying H260, E320, and D261 in SgOMT3 as the catalytic triad, as opposed to other conserved residues like H225, D242, and E325, is not sufficiently elaborated. To unequivocally establish the functional necessity of the proposed triad (H260/E320/D261) in SgOMT3, direct experimental validation, such as site-directed mutagenesis of these residues followed by enzymatic activity assays would enhance the mechanistic interpretation of the findings.

Minor issues:

1. Lines 50–52: Are there references supporting this statement, or is it speculative? Please provide citations or clarify.
2. Lines 97–98: The phrase "The enrichment of isofraxidin in roots of isofraxidin" appears to be an editorial error; please correct.

3. Line 129: The coefficient of determination (R^2) reported in Fig. 2f does not match the value stated in the text; please verify and correct.
4. Lines 136-139: The described result is inconsistent with Supplementary Table 4; please verify the numerical values and revise the description accordingly.
5. Lines 159-160: What was the rationale for selecting 51 OMT genes from *Chrysanthemum indicum* as the query?
6. Lines 171-172: The conclusion appears premature in the absence of gene family contraction/expansion analysis. Consider deleting this sentence or providing supporting evidence.
7. Fig. 1a: The resolution of the pathway diagram and text labels should be improved for clarity.
8. Fig. 3d: Please include a label for the substrate, fraxetin, to ensure completeness.
9. Supplementary Figure 12: The asterisks and short line segments in the sequence alignment are difficult to distinguish; consider improving the visual representation.

Reviewer #2

(Remarks to the Author)

This study presents a chromosome-level genome assembly of *Sarcandra glabra*, a basal angiosperm species in the Chloranthaceae family. The authors report an unusually large genome (~4.23 Gb) with exceptionally long intergenic and intronic regions, likely due to transposable element (TE) expansion. They identify and functionally characterize O-methyltransferases (OMTs), particularly SgOMT3, as key enzymes in the biosynthesis of the bioactive coumarin isofraxidin. Through in vitro assays, structural modeling, and mutagenesis, they show that SgOMT3 catalyzes the methylation of fraxetin to produce both isofraxidin and fraxidin, with regioselectivity influenced by specific amino acid residues. Comparative analysis across angiosperms suggests functional conservation but divergent regioselectivity of OMT orthologs.

Major Concerns

While the genome assembly is high-quality and the OMT characterization is thorough, the study lacks reverse genetics validation (e.g., VIGS, RNAi, or CRISPR knockout) to establish a causal link between SgOMT3/5 and isofraxidin/fraxidin biosynthesis in planta. Without this, the physiological relevance of these enzymes remains correlative. Additionally, the evolutionary significance of the expanded genome is underexplored—no discussion of how this relates to early angiosperm adaptation or TE dynamics.

Minor Comments

- 1 Metabolite Quantification: Reported as $\mu\text{g/g}$ fresh weight (FW), which is sensitive to growth conditions. Consider dry weight (DW) for consistency and reproducibility.
- 2 Figure 1: Add atomic numbering for fraxetin (C7, C8 hydroxyls) to clarify regioselectivity.
- 3 Figure 2: The circus plot lacks a color scale for GC/TE density tracks—add a legend.
- 4 Introduction: Expand to emphasize *S. glabra*'s phylogenetic position as a basal angiosperm and its value for understanding early genome evolution and specialized metabolism.

Reviewer #3

(Remarks to the Author)

A high-quality chromosome-level genome of *S. glabra* was constructed in this study, and the distinct enzymatic activities of O-methyltransferases catalyzing isofraxidin and fraxidin biosynthesis were predicted and analyzed.

1. The experimental evidence is insufficient to conclusively demonstrate the enzymatic activities and regioselectivities of OMTs, as the majority of the conclusions were drawn from software predictions.
2. In the Methods section, several experimental procedures were either streamlined or omitted. (1) qRT-PCR was not conducted to confirm the RNA-sequencing results. (2) The recombinant OMT protein was obtained using a synthesized OMT gene sequence instead of PCR amplification, and the actual presence and transcripts of OMT genes were not verified experimentally. (3) The explanation of site-directed mutagenesis lacks the specificity of an experimental procedure.
3. L127 has been identified as a crucial amino acid in SgOMT3; what about the significance of this amino acid in the other eight orthologous proteins?
4. The writing is not standardized or ambiguous.

The results indicated that SgOMT3 was capable of producing isofraxidin. In the abstract: Notably, SgOMT3 orthologs from eudicot species preferentially produced isofraxidin rather than fraxidin (Lines 26-27). The functions of these orthologs are inconsistent. Therefore, the original description is inappropriate.

L2: Title: catalyzing?

L58-59: SbPFOMT2/5 and then SbFOMT3/5/6?

L97: 'of isofraxidin' was repeated twice.

L177: $R > 0.8$ or $R^2 > 0.8$?

L301-304: Ese08G000662.t1, Ese14G001482.t1 lost near 20 amino acid residues at the N-terminal, which may result in the loss of its catalytic activity. The enzymes of Ese08G000662.t1 (Araliaceae) and chr3g00557471 (Asteraceae) showed relatively strong catalyzing activities. Does Ese08G000662.t1 actually possess catalytic activity?

L372: Our discovery that SgOMT3 converts esculetin to scopoletin reveals a previously unrecognized role for such enzymes in angiosperms.

L395: Previous study revealed that substituting the Leu residue in SbFOMT5 with the polar amino acid.....

Fig3/4/5/6: The figure legends were too simple. Why the same substances of isofraxidin and fraxidin have different retention times in LC-MS/MS? ~3 min in Fig 3, ~8 min in Fig 5b, 6d, ~6 min in Fig6c. Figure 6e lacks an annotation for the color bar

chart. Additionally, the substances corresponding to peaks 1/2/3/4 in the LC-MS/MS analysis were not identified.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have satisfactorily responded to all my concerns, and I have no additional issues to raise.

Reviewer #2

(Remarks to the Author)

While the genome assembly is high-quality and the OMT characterization is thorough, the study lacks reverse genetics validation to establish a causal link between SgOMT3/5 and isofraxidin/fraxidin biosynthesis in planta. Without this, the physiological relevance of these enzymes remains correlative.

Version 2:

Reviewer comments:

Reviewer #2

(Remarks to the Author)

My comments were properly addressed. The draft could be accepted.

Open Access This Peer Review File is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made.

In cases where reviewers are anonymous, credit should be given to 'Anonymous Referee' and the source.

The images or other third party material in this Peer Review File are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.

To view a copy of this license, visit <https://creativecommons.org/licenses/by/4.0/>

We sincerely appreciate the valuable comments and the time and effort of the reviewers. We have carefully considered all suggestions and revised the manuscript accordingly. A detailed point-to-point response to each comment is provided in the response letter.

We incorporated additional genome analyses, including quality assessments and WGD analyses, and re-evaluated the copy number of OMT genes using the method recommended by the reviewer. We experimentally validated the functional necessity of the proposed triad (H260/E320/D261) in SgOMT3 and confirmed the importance of the critical L127 residue in orthologous proteins through *in vitro* assays. We also strengthened the discussion on how genome expansion is linked to TE dynamics and early angiosperm adaptation. We provided more detailed descriptions of the experimental procedures and corrected the inconsistency in retention time for metabolite measurements, as well as other writing errors.

We hope that the response letter and revised manuscript address the reviewers' concerns. Detailed responses to each comment are provided below.

Reviewer #1

Comments to the Author:

*This study presents a high-quality, chromosome-level genome assembly of the medicinal plant *Sarcandra glabra* (Chloranthaceae), representing a valuable genomic resource. By integrating comparative genomics, transcriptome co-expression analysis, phylogenetics, molecular docking, site-directed mutagenesis, and in vitro enzyme assays, the authors demonstrate that the identified enzyme exhibits dual functionality, efficiently catalyzing the conversion of scopoletin to isofraxidin as well as esculetin to scopoletin. Furthermore, the study identifies Leu127 and Leu307 as key residues governing catalytic activity and regioselectivity (7-OH vs. 8-OH), respectively. Notably, comparative analyses reveal functional conservation of this OMT across angiosperms, alongside a distinct evolutionary shift in regioselectivity between early-diverging Chloranthaceae and eudicots. These findings offer important insights into isofraxidin biosynthesis. However, several limitations should be addressed to strengthen the reliability and generalizability of the conclusions.*

Response: We sincerely appreciate the valuable comments and suggestions you have provided. To address these limitations, we have incorporated more comprehensive genome analyses, verified the exon length of *S. glabra*, re-evaluated the copy number of OMT genes, and experimentally validated

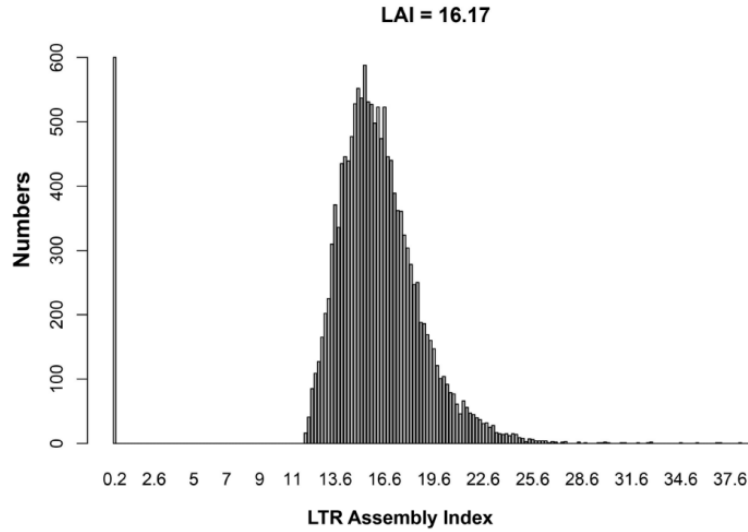
the functional necessity of the catalytic triad residues (H260/E320/D261) in SgOMT3, along with other revisions as suggested. We believe these new results address the reviewer's concerns and strengthen the reliability of our conclusions. Detailed responses to each comment are provided below.

Concern 1 (Major): 1. *The genome characteristics related to long gene length and the high proportion of dispersedly duplicated genes are discussed across two sections. However, these aspects do not represent central findings and are not directly linked to the main theme of the study. Therefore, it is recommended to streamline the related content and incorporate more general genome analyses, such as additional genome quality assessments (e.g., LAI and Merqury analyses) and whole-genome duplication (WGD) analyses. These could be consolidated under a section titled “Genome Evolution Analyses” or similar. Additionally, although the gene length section is not central, one issue requires clarification: the reported exon length in S. glabra is approximately 2.5 times that of other Chloranthaceae species. The authors should verify whether this reflects a true biological characteristic or a statistical artifact, for instance, by analyzing collinear genes among the compared species.*

Response: Thank you for your comments. As you recommended, we have streamlined the content related to gene length and the proportion of dispersedly duplicated genes. In addition, we have incorporated more general genome analyses, including genome quality assessments (e.g., LAI and Merqury analyses) and whole-genome duplication (WGD) analyses under a section titled “Genome Evolution Analyses” (lines 118–163, Fig. 2b; Supplementary Figs. 3, 12; Supplementary Table 1).

The Long Terminal Repeat (LTR) Assembly Index (LAI) score of 16.17 indicates that the continuity of the *S. glabra* genome has reached reference quality (lines 104–106, Supplementary Fig. 3). Meanwhile, Merqury analyse shows that the 15 chromosomes of *S. glabra* have an average base quality value of 48.56, indicating that the chromosomal sequences are highly accurate (lines 106–109, Supplementary Table 1).

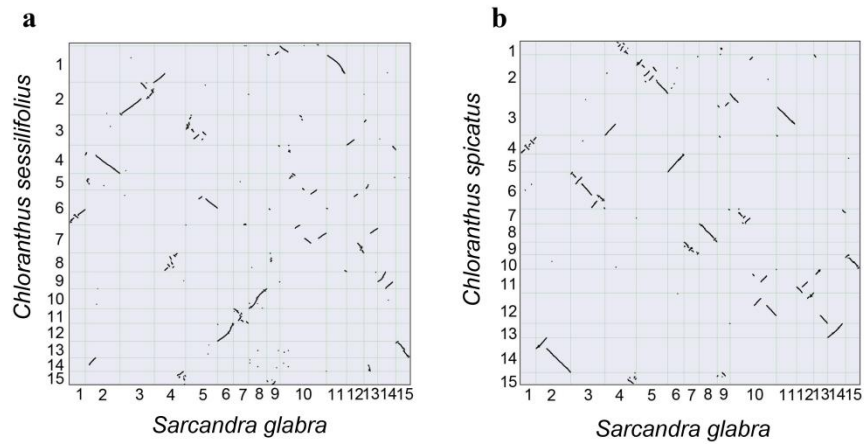
Additionally, we conducted WGD analyses (lines 148–163). The intergenomic syntenic analyses revealed that the depth ratios between *S. glabra* and *Amborella trichopoda*, *Vitis vinifera*, *C. sessilifolius*, and *C. spicatus* were found to 2:1, 2:3, 2:2, and 2:2, indicating that one shared ancient WGD event occurred among *S. glabra*, *C. sessilifolius*, and *C. spicatus* (lines 148–151, Fig. 2b; Supplementary Fig. 11). We identified a clear synonymous substitution rate (*Ks*) peak in *S. glabra*, similar to those in *C. spicatus* and *C. sessilifolius*, suggesting a shared ancient WGD event in Chloranthaceae (lines 151–156, Supplementary Fig. 12). The *Ks* distributions also indicated that *S. glabra* diverged from *C. spicatus* prior to the divergence between *C. sessilifolius* and *C. spicatus*, and revealed an early divergence between magnoliids and Chloranthaceae (Supplementary Fig. 12).



Supplementary Figure 3. The Long Terminal Repeat (LTR) Assembly Index (LAI) distribution in the *S. glabra* genome assembly.

Supplementary Table 1. Consensus quality value (Qv) score of *S. glabra* genome assembly.

Chr	Start	End	Qv	Error rate
Chr01	60,751	191,806,174	48.21	1.51e-05
Chr02	131,207	441,107,858	48.49	1.42e-05
Chr03	120,128	380,427,366	48.23	1.50e-05
Chr04	120,955	327,226,395	47.54	1.76e-05
Chr05	125,041	446,298,543	48.75	1.33e-05
Chr06	62,625	236,511,222	48.99	1.26e-05
Chr07	57,589	220,421,755	49.05	1.24e-05
Chr08	85,193	292,607,625	48.58	1.39e-05
Chr09	57,979	195,791,653	48.51	1.41e-05
Chr10	149,299	493,161,736	48.41	1.44e-05
Chr11	81,469	272,527,644	48.47	1.42e-05
Chr12	64,108	208,629,373	48.35	1.46e-05
Chr13	52,889	202,068,766	49.04	1.25e-05
Chr14	74,887	269,477,952	48.78	1.32e-05
Chr15	63,678	237,374,415	48.94	1.28e-05



Supplementary Figure 11. Chromosome co-linearity among *S. glabra*, *C. sessilifolius*, and *C. spicatus*.

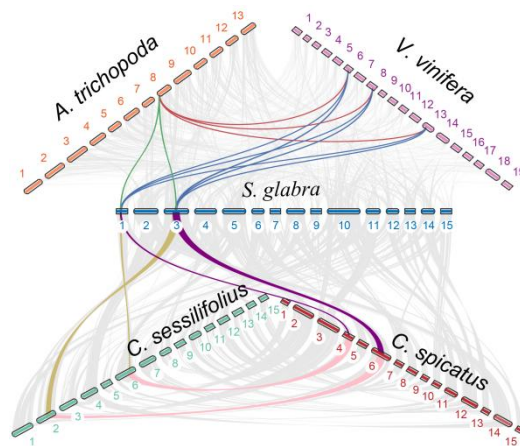
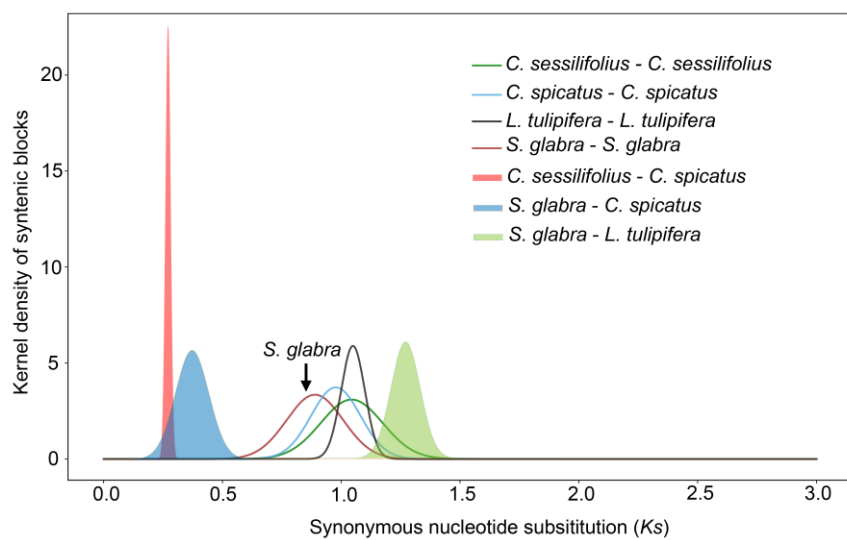


Fig.2b. Intergenomic syntenic analyses between *S. glabra* and *A. trichopoda*, *V. vinifera*, *C. sessilifolius*, and *C. spicatus*.



Supplementary Figure 12. The *Ks* distributions of intragenomic syntenic blocks within *S. glabra* and other three sequenced species.

Thank you for your comment on examination of the exon length in Chloranthaceae species. We carefully re-examined the statistics of average exon length in *S. glabra* and calculated the average exon lengths based on collinear genes among the Chloranthaceae species. We found that the average exon length for *C. sessilifolius* and *C. spicatus* was calculated based solely on CDS regions (Guo et al., 2021; Ma et al., 2021), whereas that for *S. glabra* in our study included both UTR and CDS regions, potentially introducing bias in direct cross-species comparisons. Using whole-genome collinear genes, we standardized the metric to “average exon length within CDS regions” to address this issue. This yielded a corrected value of **0.29 kb** for *S. glabra*, 1.45-fold and 1.2-fold greater than those of *C. sessilifolius* and *C. spicatus*, respectively (**lines 863–864, Table 1**).

Concern 2 (Major): One of the main objectives of this study is to identify all candidate OMT genes using the high-quality genome assembly. However, the number of OMT genes appears to correlate with gene annotation quality, as C. sessilifolius, which has more predicted genes, also contains more OMTs. To assess the completeness of OMT annotation in S. glabra, the authors could perform homologous annotation using tools such as GeMoMa with predicted OMTs from other species (e.g., C. sessilifolius, Liriodendron tulipifera, Cinnamomum micranthum, and Arabidopsis thaliana), and also perform the BLAST searches against transcripts that assembled directly by RNA-Seq reads.

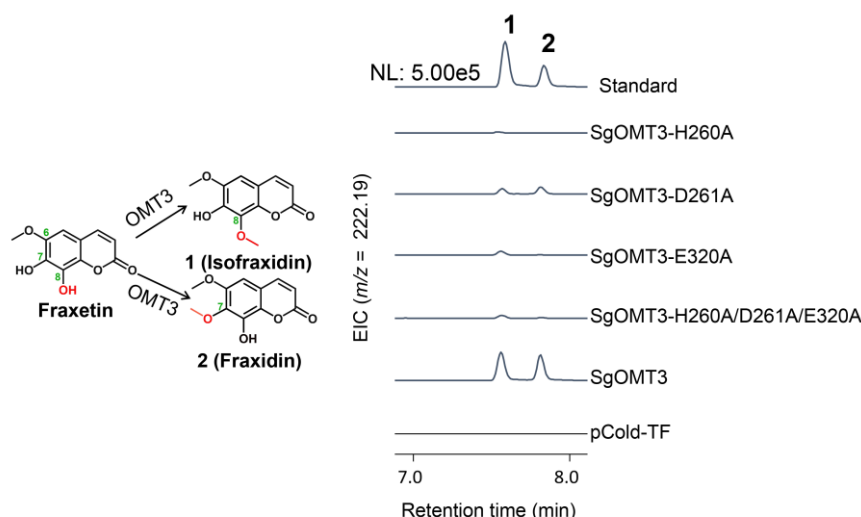
Response: We thank the reviewer for the comment on the OMT annotations. To assess the completeness of OMT annotation in *S. glabra*, we performed homolog-based annotation using GeMoMa, integrating predicted OMTs from several species as query references owing to their high-quality genome assemblies, including *Chrysanthemum indicum* (Deng et al., 2024), *Arabidopsis thaliana*, *Oryza sativa*, and *Glycine max*, along with RNA-Seq reads and hmmsearch database (PF00891.hmm and PF01596.hmm). After filtering out incomplete gene sequences, the numbers of predicted OMTs in *S. glabra*, *C. sessilifolius*, and *C. spicatus* were as follows: *Chrysanthemum indicum*-based annotation (18, 16, and 26), *Arabidopsis thaliana*-based annotation (18, 13, and 24), *Oryza sativa*-based annotation (19, 14, and 25), *Glycine max*-based annotation (20, 14, and 26), RNA-Seq reads-based annotation (22, 15, and 26), and hmmsearch-based annotation (19, 14, and 21). Overall, the number of OMT genes was consistently lower in the three surveyed Chloranthaceae genomes (**lines 166–177, Fig. 3a; Supplementary Fig. 13**).



Supplementary Figure 13. The number of OMT genes predicted from four species, along with RNA-Seq reads and hmmsearch database (PF00891.hmm and PF01596.hmm).

Concern 3 (Major): The manuscript posits the necessity of the catalytic triad (H/E/D) for OMT activity based on sequence alignment and previous studies on flavonoid OMTs, with the natural D266N mutation in SgOMT7 cited as corroborating evidence. However, as the authors note by referencing prior work, the essentiality of this triad has been established primarily in the context of flavonoid OMTs instead of simple coumarins OMT. Furthermore, the rationale for specifically identifying H260, E320, and D261 in SgOMT3 as the catalytic triad, as opposed to other conserved residues like H225, D242, and E325, is not sufficiently elaborated. To unequivocally establish the functional necessity of the proposed triad (H260/E320/D261) in SgOMT3, direct experimental validation, such as site-directed mutagenesis of these residues followed by enzymatic activity assays would enhance the mechanistic interpretation of the findings.

Response: Thank you for your comment. We agree that it is necessary to perform experimental validation on the functional necessity of the proposed triad (H260/E320/D261) in SgOMT3. We performed site-directed mutagenesis on the residues H260, E320, and D261 in SgOMT3, individually substituting them with alanine to generate the mutants H260A, E320A, and D261A, as well as a triple mutant (H260A/E320A/D261A). The results showed that the production of isofraxidin and fraxidin was significantly decreased in all mutants compared with SgOMT3, indicating the functional importance of the proposed catalytic triad (H260/E320/D261) in SgOMT3 (**Supplementary Fig. 18**). We also added the relative content in **lines 252–254**.



Supplementary Figure 18. Enzymatic activity assays of the site-directed mutants of SgOMT3. The site-directed mutagenesis was performed on the residues H260, E320, and D261 in SgOMT3, individually substituting them with alanine to generate the mutants H260A, E320A, and D261A. In addition, a triple mutant (H260A/E320A/D261A) was constructed by simultaneously introducing all three mutations.

Other comments and questions

1. Lines 50–52: Are there references supporting this statement, or is it speculative? Please provide citations or clarify.

Response: Thank you. We have added the reference and revised the sentence accordingly (**lines 50–51**).

2. Lines 97-98: The phrase “The enrichment of isofraxidin in roots of isofraxidin” appears to be an editorial error; please correct.

Response: Thank you. We have corrected it (**lines 98–99**).

3. Line 129: The coefficient of determination (R^2) reported in Fig. 2f does not match the value stated in the text; please verify and correct.

Response: Thank you for bringing this to our attention. The coefficient of determination (R^2) is 0.53. We have accordingly updated the value in the main text (**line 127, Supplementary Fig. 6e**). We apologize for any inconvenience caused by this discrepancy.

4. Lines 136-139: The described result is inconsistent with Supplementary Table 4; please verify the numerical values and revise the description accordingly.

Response: Thank you for bringing this to our attention. We have verified the corresponding values in Supplementary Table 5 and revised the relevant description in the manuscript accordingly (**lines 134–137, Supplementary Table 5**). We apologize for any inconvenience caused by this discrepancy.

5. Lines 159-160: What was the rationale for selecting 51 OMT genes from *Chrysanthemum indicum* as the query?

Response: Thank you for the comment. In previous and recent studies on the OMT gene family, identification was performed using HMM databases (PF00891.hmm and PF01596.hmm) (Cui et al., 2022b; Luo et al., 2026; Wang et al., 2026). However, as this approach resulted in a relatively limited number of identified OMTs in three Chloranthaceae species, we have now incorporated a homologous protein alignment strategy to enhance the reliability of the results. In the study of *Chrysanthemum indicum*, the genome was successfully assembled at the chromosome level, and the predicted OMT data were considered reliable (Deng et al., 2024). Therefore, 51 OMT genes from *C. indicum* were selected as query sequences. As you suggested, we also used *Arabidopsis thaliana*, *Oryza sativa*, and *Glycine max*, together with RNA-Seq reads and the HMMsearch databases (PF00891.hmm and PF01596.hmm) as queries in the revised manuscript, and obtained a similar number of OMT genes.

6. Lines 171-172: The conclusion appears premature in the absence of gene family contraction/expansion analysis. Consider deleting this sentence or providing supporting evidence.

Response: Thank you for your valuable advice. We agree that the conclusion appears premature in the absence of gene family contraction/expansion analysis. We have therefore removed the sentence (**line 180**).

7. Fig. 1a: The resolution of the pathway diagram and text labels should be improved for clarity.

Response: Thanks for your good advice. We have improved the resolution of the pathway diagram and enhanced the clarity of the text labels (**Fig. 1a**).

8. Fig. 3d: Please include a label for the substrate, fraxetin, to ensure completeness.

Response: Thanks for your valuable suggestion. We have added the substrate of fraxetin (**Fig. 3c, d**).

9. Supplementary Figure 12: The asterisks and short line segments in the sequence alignment are

difficult to distinguish; consider improving the visual representation.

Response: Thanks for your good advice, we have improved the visual representation of **Supplementary Fig. 17**.

Reviewer #2

Comments to the Author:

*This study presents a chromosome-level genome assembly of *Sarcandra glabra*, a basal angiosperm species in the Chloranthaceae family. The authors report an unusually large genome (~4.23 Gb) with exceptionally long intergenic and intronic regions, likely due to transposable element (TE) expansion. They identify and functionally characterize O-methyltransferases (OMTs), particularly SgOMT3, as key enzymes in the biosynthesis of the bioactive coumarin isofraxidin. Through in vitro assays, structural modeling, and mutagenesis, they show that SgOMT3 catalyzes the methylation of fraxetin to produce both isofraxidin and fraxidin, with regioselectivity influenced by specific amino acid residues. Comparative analysis across angiosperms suggests functional conservation but divergent regioselectivity of OMT orthologs.*

Response: We sincerely appreciate your thoughtful and constructive feedback. We are truly grateful for the time and effort you dedicated to reviewing our work and for providing such insightful guidance.

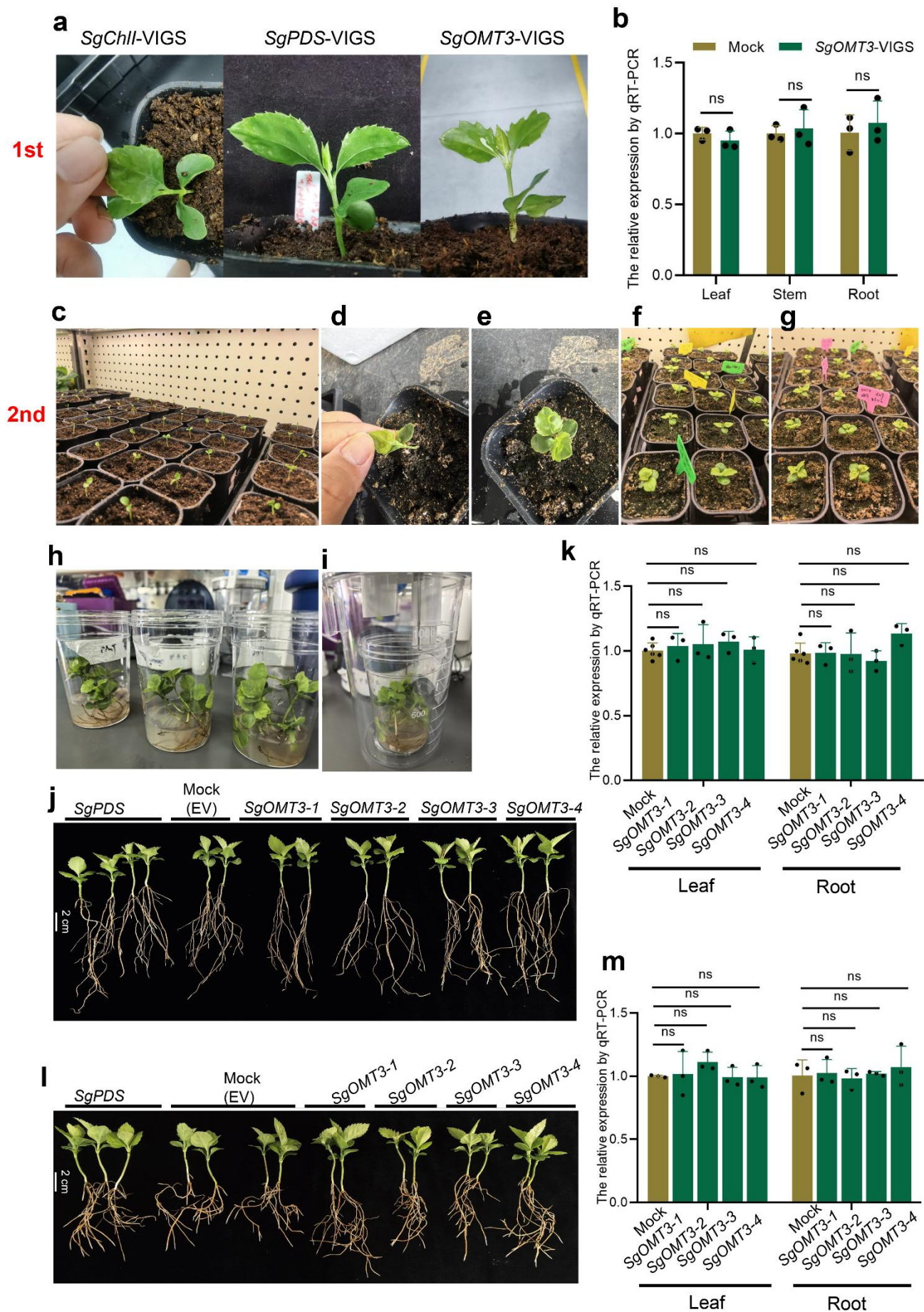
Concern 1 (Major): *While the genome assembly is high-quality and the OMT characterization is thorough, the study lacks reverse genetics validation (e.g., VIGS, RNAi, or CRISPR knockout) to establish a causal link between SgOMT3/5 and isofraxidin/fraxidin biosynthesis in planta. Without this, the physiological relevance of these enzymes remains correlative.*

Response: Thank you for your valuable suggestion. Although VIGS, RNAi, or CRISPR knockout has not previously been reported for *S. glabra*, we performed VIGS as recommended. Four 300-bp fragments of SgOMT3, together with one 300-bp fragment of the SgPDS gene, were cloned into the vector pTRV2 (**Response letter Table 1**). The corresponding recombinant plasmids were then introduced into GV3101 competent cells. Subsequently, GV3101 harboring pTRV1 was mixed at a 1:1 ratio with GV3101 cultures carrying the recombinant plasmids. The resulting inoculum mixtures were infiltrated into *S. glabra* leaves and roots. Meanwhile, we optimized the VIGS experimental procedures, including selection of cotyledon-emergence-stage seedlings, extension of the post-infiltration incubation period, and the use of vacuum infiltration (**Please refer to the detailed VIGS methods provided at the end of this response letter**). However, no visible bleaching phenotype was observed in the infiltrated *S. glabra* leaves, and the expression analysis revealed that the expression levels of

SgOMT3 were not significantly reduced in the *SgOMT3*-VIGS plantlets compared with the control (**Response letter Fig. 1**). To exclude the possibility that this result was due to inefficiency of the VIGS system itself, we applied the same infiltration protocol to tobacco leaves. In contrast to the controls, the tobacco leaves exhibited a clear bleaching phenotype, confirming that the recombinant plasmid pTRV2-*SgPDS* is functional under these experimental conditions (**Response letter Fig. 2**).

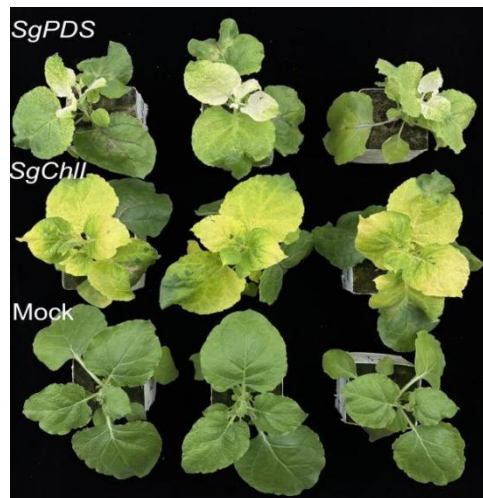
We have carefully evaluated the potential causes for the failure of the VIGS experiment in *S. glabra*. As an early-diverging angiosperm, *S. glabra* appears to exhibit low susceptibility to VIGS-mediated gene silencing, in contrast to most eudicot species (Li et al., 2025; Reynolds et al., 2026) and monocot plants (Holzberg et al., 2002; Liu et al., 2016; He et al., 2023), in which VIGS has been widely and successfully applied. Phylogenetic analyses indicate that Chloranthales and magnoliids are sister groups to eudicots. To date, however, there have been no reports of successful VIGS applications in early-diverging angiosperms, including species from Chloranthales and magnoliids (Dommes et al., 2019; Hu et al., 2025).

Based on the above results, we consider that the VIGS method is not a suitable approach for validating functional genes in *S. glabra*. Isofraxidin is highly enriched in the roots of *S. glabra*, and the expression level of *SgOMT3* shows the highest Pearson's correlation coefficient (r) with isofraxidin content. Moreover, only *SgOMT3* from *S. glabra* and its orthologs across six other species catalyzed isofraxidin formation in *in vitro* enzymatic assays. Taken together, these findings indicate that *SgOMT3* is most likely the key enzyme responsible for isofraxidin biosynthesis in *S. glabra*.



Response letter Figure 1. Phenotypes of *SgPDS*, *SgChlI*, and *SgOMT3* in transient VIGS of *S. glabra* plantlets.

a 1st VIGS experiment: GV3101 harboring pTRV1 was individually mixed, at a 1:1 ratio, with GV3101 cultures carrying pTRV2-*SgPDS*, pTRV2-*SgChlI*, pTRV2-*SgOMT3-1*, pTRV2-*SgOMT3-2*, pTRV2-*SgOMT3-3*, and pTRV2-*SgOMT3-4* or pTRV2. The resulting inoculum mixtures were infiltrated into *S. glabra* leaves. **b** The relative expression levels were compared between empty vector (Mock) and *SgOMT3*-VIGS plantlets. **c**, **d**, and **e** 2nd VIGS experiment: Seedlings at the cotyledon-emergence stage were used for VIGS assays. **f** and **g** The additional round of VIGS inoculation was performed when the first pair of true leaves emerged. **h** and **i** Seedlings were subjected to vacuum infiltration in a bacterial suspension, and their root tissues were partially removed with scissors to create small wounds. **j** Phenotypes of *SgPDS*-VIGS, *SgOMT3*-VIGS, and empty vector (EV, mock) plantlets. **k** The relative expression levels were compared between empty vector (Mock) and *SgOMT3*-VIGS plantlets. **l** Phenotypes of *SgPDS*-VIGS, *SgOMT3*-VIGS, and empty vector (EV, mock) plantlets treated by vacuum infiltration. **m** The relative expression levels were compared between empty vector (Mock) and *SgOMT3*-VIGS plantlets treated by vacuum infiltration.



Response letter Figure 2. Phenotypes of *SgPDS*, *SgChlI*, and empty vector (Mock) in transient VIGS of *Nicotiana tabacum* leaves.

Concern 2 (Major): Additionally, the evolutionary significance of the expanded genome is underexplored—no discussion of how this relates to early angiosperm adaptation or TE dynamics.

Response: Thanks for your valuable advice. We agree that it is necessary to explore the relationship between expanded genome and TE dynamics. Thus, we have added corresponding results (**lines 134–137**) and discussion to the revised manuscript (**lines 381–387**): TEs are major drivers of plant genome expansion, evolution, and adaption (Oliver and Greene, 2012; Oliver et al., 2013; Simonin and Roddy, 2018; Pellicer and Leitch, 2020; Bhadra et al., 2023; Yang et al., 2026). In the *S. glabra* genome, the substantial expansion of *Athila* and *Tekay* elements of the Ty3/Gypsy LTR retrotransposons, which are mainly distributed in intergenic regions and introns, likely contributed to genome expansion

(**Supplementary Fig. 7–9**). Similarly, substantial expansion of *Athila* and *Tekay* elements has also been reported in Liliaceae species, such as *Lilium regale*, *L. davidii*, and *L. sargentiae*, resulting in their gigantic genomes and extended intergenic and intronic regions (Xu et al., 2024; Liang et al., 2025; Sun et al., 2025).

Other comments and questions

1 Metabolite Quantification: Reported as $\mu\text{g/g}$ fresh weight (FW), which is sensitive to growth conditions. Consider dry weight (DW) for consistency and reproducibility.

Response: Thanks for your good advice. We have quantified the product contents using dry weight (DW) tissue samples and revised the relevant descriptions (**lines 90–99, Fig. 1c; Supplementary Fig. 1**). The product concentrations increase by one order of magnitude when the dry weight of tissues is used. However, the accumulation pattern of the products remains the same as when the fresh weight of tissues is used. Accordingly, the selection of candidate SgOMT genes for *in vitro* assays remains unchanged.

2 Figure 1: Add atomic numbering for fraxetin (C7, C8 hydroxyls) to clarify regioselectivity.

Response: Thank you for the helpful suggestion. We have added atomic numbering for fraxetin (**Fig. 1a**).

3 Figure 2: The circus plot lacks a color scale for GC/TE density tracks—add a legend.

Response: Thanks for your good advice. We have added legends into the circus plot (**Fig. 2a, lines 885–892**).

*4 Introduction: Expand to emphasize *S. glabra*'s phylogenetic position as a basal angiosperm and its value for understanding early genome evolution and specialized metabolism.*

Response: Thank you for your suggestion. We have expanded the Introduction to emphasize *S. glabra*'s phylogenetic position (**lines 74–76**).

Reviewer #3

Comments to the Author:

A high-quality chromosome-level genome of S. glabra was constructed in this study, and the distinct enzymatic activities of O-methyltransferases catalyzing isofraxidin and fraxidin biosynthesis were predicted and analyzed.

Response: We sincerely thank you for your insightful and constructive feedback. We greatly appreciate the time and expertise you invested in reviewing our work.

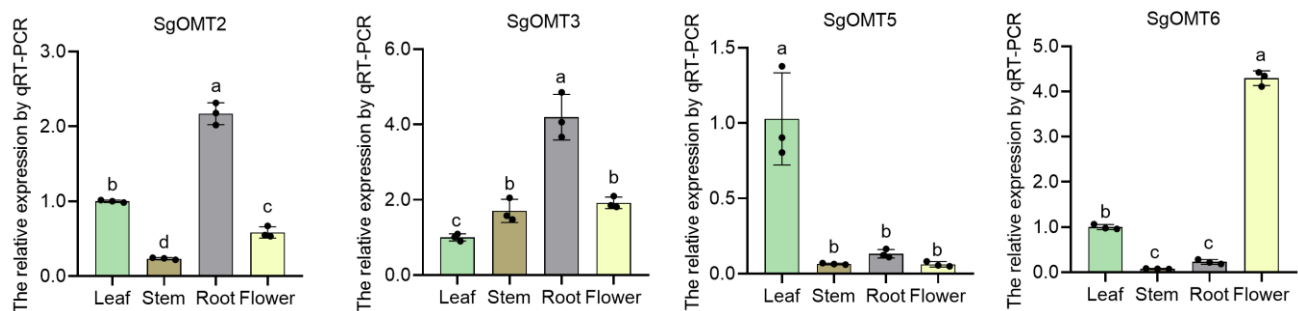
Concern 1 (Major): The experimental evidence is insufficient to conclusively demonstrate the enzymatic activities and regioselectivities of OMTs, as the majority of the conclusions were drawn from software predictions.

Response: Thank you for the comment. To experimentally validate the enzymatic activities of OMTs, we cloned *SgOMT* genes and their orthologs from *Eleutherococcus senticosus* (Araliaceae), *Artemisia annua* (Asteraceae), *Arabidopsis thaliana* (Cruciferae), and *Morus notabilis* (Moraceae), as well as from *S. glabra*, *C. sessilifolius*, and *C. spicatus* (Chloranthaceae). These genes were heterologously expressed and functionally characterized through *in vitro* enzymatic assays (**Figs. 3–6; Supplementary Figs. 16–26**). To investigate regioselectivity, extensive site-directed mutagenesis was performed on *SgOMT* genes and their orthologs, targeting residues L127 (**Fig. 5; Supplementary Fig. 19**) and L307 (**Fig. 6; Supplementary Figs. 25, 26**), to identify the key residues responsible for the specificity of isofraxidin and fraxidin production. All experiments were repeated three times, and relevant statistical analyses were performed to make the results more reliable. Our *in vitro* enzymatic assays and site-directed mutagenesis experiments revealed that Leu127 in *SgOMT3* and Val292 in *SgOMT5* are key residues involved in isofraxidin and fraxidin biosynthesis. Moreover, a Leu-to-Ile substitution at position 307 in *SgOMT3* altered its regioselectivity, shifting the conversion predominantly toward isofraxidin. Our Computational analyses, including Chai-1 modeling and RMSF assessments also support these experimental findings. Collectively, these complementary experimental and computational approaches provide a comprehensive understanding of the observed differences in enzymatic activities. We hope that these experimental results have addressed the reviewers' concerns.

Concern 2 (Major): In the Methods section, several experimental procedures were either streamlined or omitted. (1) qRT-PCR was not conducted to confirm the RNA-sequencing results. (2) The recombinant OMT protein was obtained using a synthesized OMT gene sequence instead of PCR amplification, and the actual presence and transcripts of OMT genes were not verified experimentally. (3) The explanation of site-directed mutagenesis lacks the specificity of an experimental procedure.

Response: Thanks for your good advice. We apologize for the omitted information in the original manuscript, which has been added in the revised version.

(1) We performed qRT-PCR analysis of *SgOMT2*, *SgOMT3*, *SgOMT5*, and *SgOMT6* to validate the RNA-sequencing results. The results showed a consistent expression pattern with the RNA-sequencing data for these *SgOMT* genes (lines 200–204, Supplementary Fig. 15; Supplementary Table 6).



Supplementary Figure 15. The relative expression of four *SgOMT* genes was analyzed using qRT-PCR.

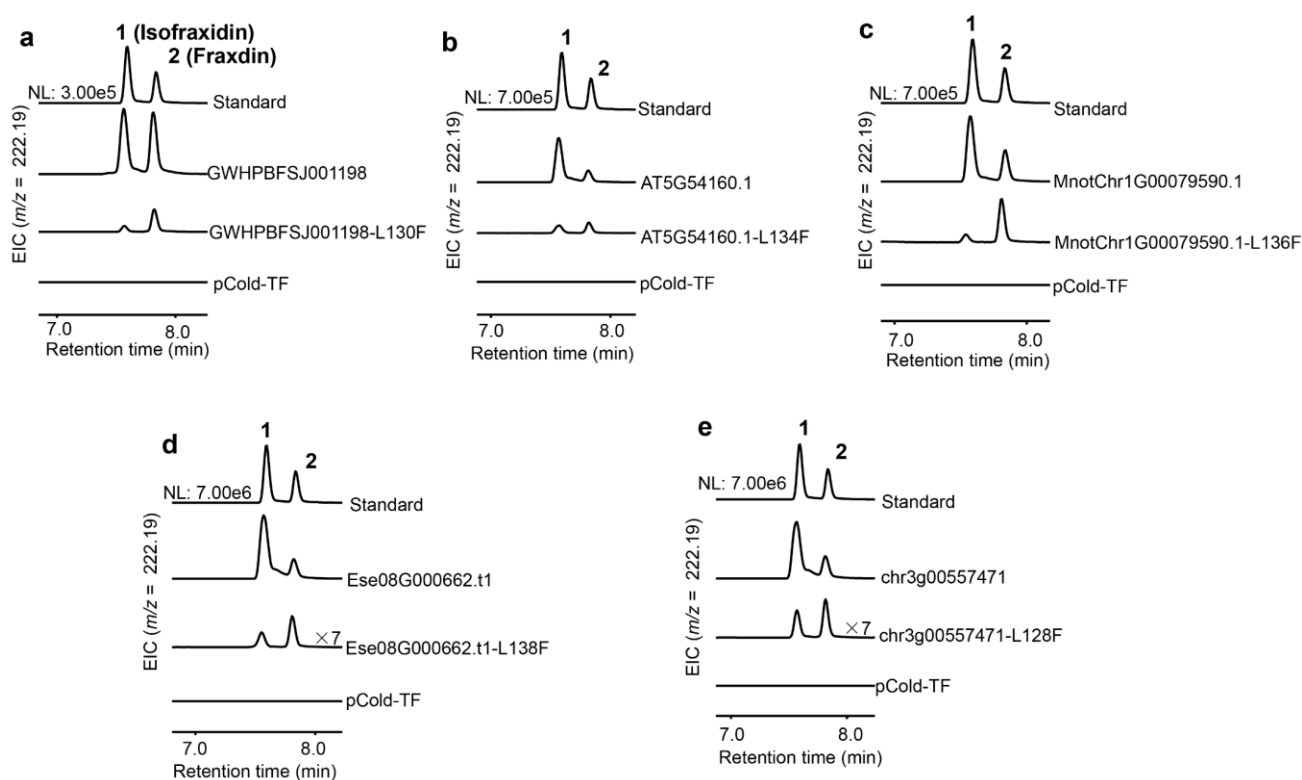
(2) In the original manuscript, the method for gene cloning was described as “the coding sequences of selected OMT genes from *S. glabra*, *C. spicatus*, *E. senticosus*, *A. annua*, *A. thaliana*, and *M. notabilis* were synthesized using SnapGene 6.0.2 software” (lines 600–603). In fact, it was **the primers**, rather than the genes, that were synthesized, while all genes were cloned from plant tissues. Therefore, this description has been rephrased as “**the primers** corresponding to the coding sequences (CDS) of selected OMT genes from *S. glabra*, *C. spicatus*, *E. senticosus*, *A. annua*, *A. thaliana*, and *M. notabilis* were designed using Snapgene 6.0.2 software” in the revised manuscript (lines 600–603). The detailed methods for gene cloning have also been added in the revised version (lines 594–605). We apologize for the inaccurate description and hope that this revision has addressed the reviewers’ concerns.

(3) Overlap PCR was employed to perform site-directed mutagenesis of the target gene. Details of the specific experimental procedures have been added in the revised manuscript (lines 654–661).

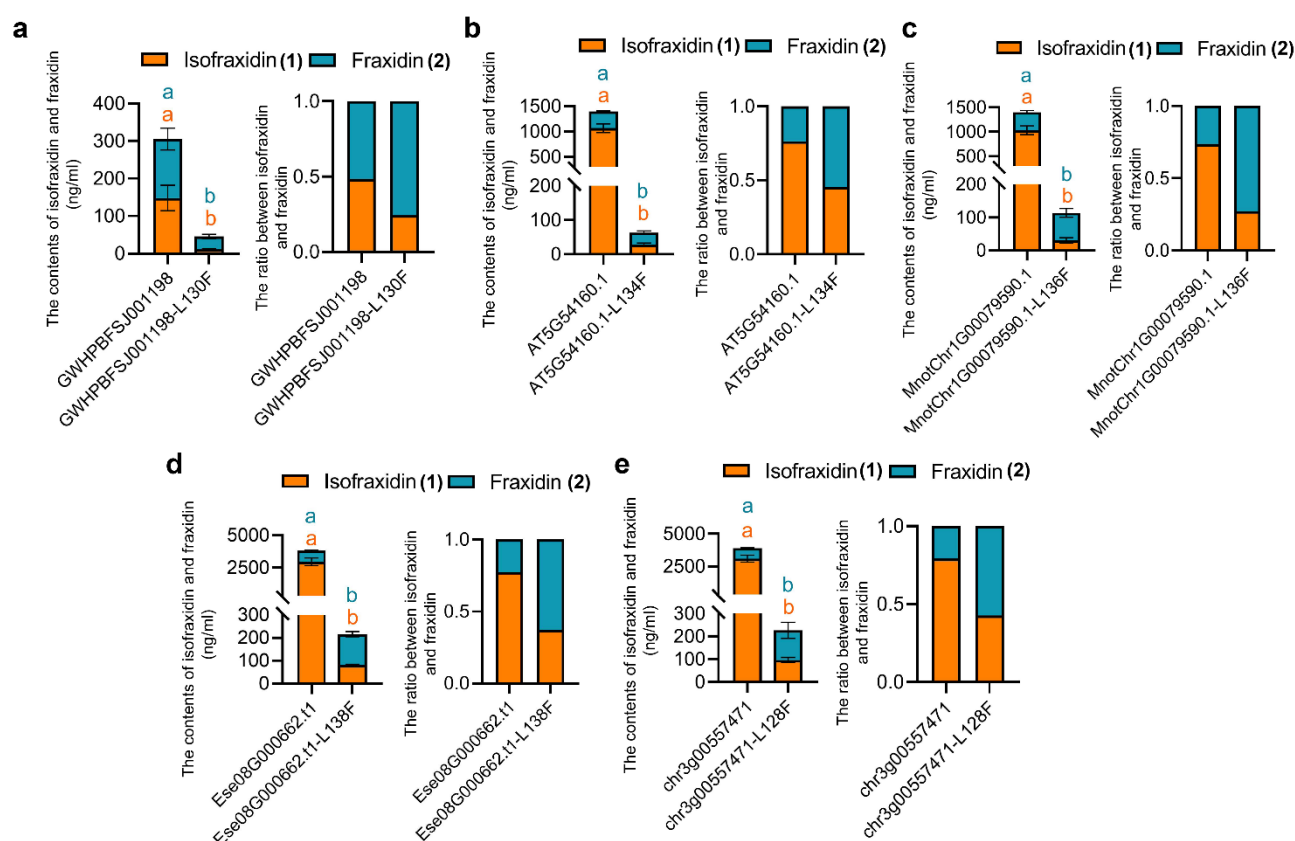
Concern 3 (Major): L127 has been identified as a crucial amino acid in SgOMT3; what about the significance of this amino acid in the other eight orthologous proteins?

Response: Thank you for your valuable suggestion. We selected five additional orthologous proteins: GWHPBFSJ001198 (*C. spicatus*), AT5G54160.1 (*A. thaliana*), MnotChr1G00079590.1 (*M. notabilis*), Ese08G000662.t1 (*E. senticosus*), and chr3g00557471 (*A. annua*) for site-directed mutagenesis. The test was not conducted for LwOMT1 and LwOMT11 from *Lophophora williamsii*, as these enzymes are responsible for hallucinogen mescaline production, rather than producing isofraxidin and fraxidin. We referred to their amino acid sequences solely to assist in identifying key catalytic residues. In addition, Ese14G001482.t1 (*E. senticosus*) was not selected for further assays because it has lost its catalytic activity (**Fig. 6c; Supplementary Fig. 23**).

Substitution of this Leucine (Leu) corresponding to L127 in SgOMT3 with phenylalanine (Phe) in GWHPBFSJ001198, AT5G54160.1, MnotChr1G00079590.1, Ese08G000662.t1, and chr3g00557471 significantly decreased isofraxidin production as well as ratio of isofraxidin to fraxidin, in their mutants, consisted with the result for L127F mutagenesis of SgOMT3 (**Response letter Figs. 3, 4**).



Response letter Figure 3. Enzymatic activity assays of five SgOMT3 orthologous proteins with their corresponding site-specific mutants by LC-MS/MS.



Response letter Figure 4. The contents and ratios of isofraxidin and fraxidin in five SgOMT3 orthologous proteins with their corresponding site-specific mutants.

Other comments and questions

The writing is not standardized or ambiguous.

Response: We apologize for the non-standard and ambiguous writing in the original manuscript. We have revised them in the updated version accordingly.

1. The results indicated that SgOMT3 was capable of producing isofraxidin. In the abstract: Notably, SgOMT3 orthologs from eudicot species preferentially produced isofraxidin rather than fraxidin (Lines 26-27). The functions of these orthologs are inconsistent. Therefore, the original description is inappropriate.

Response: Thank you for your valuable suggestion. In our study, the *in vitro* enzymatic assay showed that SgOMT3 from *S. glabra* produced comparable amounts of isofraxidin and fraxidin, rather than exclusively isofraxidin (lines 23–24). This enzymatic characteristic was observed for both the SgOMT3 gene from *S. glabra* and the GWHPBFSJ001198 gene from *C. spicatus*, but not for their eudicot orthologs (Fig. 6c). In contrast, SgOMT3 orthologs from eudicot species, including *E.*

senticosus, *A. annua*, *A. thaliana*, and *M. notabilis*, predominantly produced higher amounts of isofraxidin than fraxidin (lines 307–325, Fig. 6c; Supplementary Fig. 23). We apologize for the inaccurate description in the abstract, and the sentence has been rephrased as “Notably, SgOMT3 orthologs from eudicot species preferentially produced more isofraxidin than fraxidin” (Lines 26-27). We hope this revision addresses the reviewer’s concern.

2. L2: Title: *catelyzing*?

Response: We apologize for the typographical error and have corrected it to the appropriate term, “catalyzing” (line 2).

3. L58-59: *SbPFOMT2/5 and then SbFOMT3/5/6?*

Response: We are sorry for the confusion. PFOMT refers to Phenylpropanoid and Flavonoid OMTs (Type II OMTs), whereas FOMT refers to Flavonoid OMTs (Type I OMTs), and these are two distinct enzymes with different names (Cui et al., 2022a).

4. L97: *‘of isofraxidin’ was repeated twice.*

Response: We apologize for this error and have removed the duplicate (lines 98–99).

5. L177: *R>0.8 or R²>0.8?*

Response: We apologize for the confusion. The correct notation is “*r*”, which generally refers to the Pearson correlation coefficient and is written in italics or lowercase. We have corrected it in the revised manuscript (line 185, Fig. 3b).

6. L301-304: *Ese08G000662.t1, Ese14G001482.t1 lost near 20 amino acid residues at the N- terminal, which may result in the loss of its catalytic activity. The enzymes of Ese08G000662.t1 (Araliaceae) and chr3g00557471 (Asteraceae) showed relatively strong catalyzing activities. Does Ese08G000662.t1 actually possess catalytic activity?*

Response: We are sorry for missing the phrase “compared to” in the original sentence. In fact, the text in L301-304 should read: “**Compared to** Ese08G000662.t1, Ese14G001482.t1 lost near 20 amino acid residues at the N- terminal, which may result in the loss of its catalytic activity”. The word “Compared to” was missing in the original sentence. This means that only Ese14G001482.t1 lost near 20 amino acid residue and its catalytic activity, whereas Ese08G000662.t1 showed relatively strong catalytic activities. We apologize for the confusion caused by the writing error. The sentence has now been

rephrased for clarification in the revised manuscript (lines 313–315, Fig. 6c; Supplementary Figs. 23, 25, 26).

7. L372: Our discovery that SgOMT3 converts esculetin to scopoletin reveals a previously unrecognized role for such enzymes in angiosperms.

Response: After a literature survey, we confirm that this activity has been reported in *Plagiochasma appendiculatum* (Xu et al., 2016). Therefore, the text has been rephrased to provide a correct description as follows: “Our findings confirm that SgOMT3 indeed possesses enzymatic activity for converting esculetin into scopoletin, which is consistent with its ortholog in *Plagiochasma appendiculatum*” (lines 392–394).

8. L395: Previous study revealed that that substituting the Leu residue in SbFOMT5 with the polar amino acid……

Response: We apologize for this error, and the repeated “that” has been deleted (lines 415–418).

9. Fig3/4/5/6: The figure legends were too simple. Why the same substances of isofraxidin and fraxidin have different retention times in LC-MS/MS? ~3 min in Fig 3, ~8 min in Fig 5b, 6d, ~6 min in Fig6c.

Response: We apologize for the inconvenience. We have added more detailed descriptions in **Figs. 3/4/5/6 (lines 903–983)**. Regarding the inconsistency in retention time, different chromatographic methods were used for some of the measurements, resulting in variations in the retention time values. To address this, we have now standardized the LC–MS/MS mobile phase program so that all samples exhibit peak elution at approximately the same retention time (**Figs. 3–6; Supplementary Figs. 16, 18–20, 25**). In addition, we have revised the Methods section accordingly (**lines 487–490**).

10. Figure 6e lacks an annotation for the color bar chart. Additionally, the substances corresponding to peaks 1/2/3/4 in the LC-MS/MS analysis were not identified.

Response: Thank you for your suggestions. We have added the annotation for the color bar chart in **Fig. 6e**, as well as the substance names corresponding to the peaks in the LC–MS/MS analysis in all figures (**Figs. 3–6**). Please refer to the figures in the revised manuscript for further details.

Supplementary Methods for Reviewer 2, Concern 1:

The detailed procedures for the VIGS experiment in *S. glabra* are described below:

1st VIGS experiment (from January to July 2024):

Four 300 bp fragments of *SgOMT3* gene (*SgOMT3-1*, *SgOMT3-2*, *SgOMT3-3*, and *SgOMT3-4*) were designed using the SGN VIGS Tool (<https://vigs.solgenomics.net/>). The corresponding recombinant plasmids (pTRV2-*SgOMT3-1*, pTRV2-*SgOMT3-2*, pTRV2-*SgOMT3-3*, and pTRV2-*SgOMT3-4*) were then constructed and introduced into GV3101 competent cells (**Response letter Table 1**). Next, GV3101 harboring pTRV1 (OD₆₀₀ = 0.6) was individually mixed, at a 1:1 ratio, with GV3101 cultures carrying pTRV2-*SgPDS*, pTRV2-*SgChlI*, pTRV2-*SgOMT3-1*, pTRV2-*SgOMT3-2*, pTRV2-*SgOMT3-3*, and pTRV2-*SgOMT3-4* or pTRV2 (EV) (each at OD₆₀₀ = 0.6).

The resulting inoculum mixtures were infiltrated into *S. glabra* leaves. After 20 days, no visible bleaching or chlorosis phenotype was observed in the infiltrated *S. glabra* leaves, suggesting that the recombinant plasmids pTRV2-*SgPDS* and pTRV2-*SgChlI* were not functional in *S. glabra* (**Response letter Fig. 1a**). Meanwhile, the expression level of *SgOMT3* was analyzed in *SgOMT3*-VIGS plantlets. The results showed that the expression of *SgOMT3* did not significantly decrease in the *SgOMT3*-VIGS plantlets compared with the control (Mock) (**Response letter Fig. 1b**).

2nd VIGS experiment (from November 2025 to April 2026):

We hypothesized the reasons for the failure of the first VIGS experiment in *S. glabra* and made improvements before conducting the experiment again in the second trial. (1) The previously selected *S. glabra* seedlings were too large for effective VIGS. In subsequent experiments, seedlings at the cotyledon-emergence stage will be used for VIGS assays. Furthermore, an additional round of VIGS inoculation will be performed when the first pair of true leaves emerges (**Response letter Fig. 1c–e**). (2) The post-infiltration incubation period should be extended from 20 days to 40 days to observe leaf bleaching or chlorosis in *S. glabra* leaves (**Response letter Fig. 1f, g**). (3) The inoculation procedure should be optimized by subjecting seedlings to vacuum infiltration in a bacterial suspension, followed by a 30-day incubation to observe leaf bleaching or chlorosis. Prior to infiltration, the root tips of the seedlings were partially removed with scissors to create small wounds, thereby facilitating bacterial infection during the vacuum process (**Response letter Fig. 1h, i**).

Unfortunately, no visible bleaching phenotype was observed in either the infiltrated *S. glabra* leaves or the vacuum-infiltrated *S. glabra* plantlets (**Response letter Fig. 1j, l**). Furthermore, the expression analysis revealed that the expression levels of *SgOMT3* were not significantly reduced in the *SgOMT3*-VIGS plantlets compared with the control (**Response letter Fig. 1k, m**). The second

VIGS experiment in *S. glabra* also failed, indicating that the VIGS method is not a suitable approach for validating functional genes in *S. glabra*.

The detailed procedures for the VIGS experiment in *Nicotiana tabacum* are described below:

The recombinant plasmids pTRV2-*SgPDS* (causing leaf bleaching) and pTRV2-*SgChlI* (inducing leaf chlorosis) were constructed as positive controls to facilitate phenotype observation. The plasmids pTRV1, pTRV2-*SgPDS*, and pTRV2-*SgChlI* were then transformed into *Agrobacterium tumefaciens* strain GV3101 competent cells.

Next, GV3101 harboring pTRV1 (OD₆₀₀ = 0.6) was separately mixed with GV3101 cultures carrying pTRV2-*SgPDS* (OD₆₀₀ = 0.6), pTRV2-*SgChlI* (OD₆₀₀ = 0.6), or pTRV2 (Empty vector, OD₆₀₀ = 0.6) at a 1:1 ratio. The resulting inoculum mixtures were infiltrated into *Nicotiana tabacum* leaves. Phenotypic changes, including leaf bleaching or chlorosis, were examined one week after infiltration.

Compared to the controls, the tobacco leaves exhibited clear bleaching and chlorosis phenotype, confirming that the recombinant plasmids pTRV2-*SgPDS* and pTRV2-*SgChlI* were functional (Response letter Fig. 2).

Response letter Table 1. Primers used for VIGS in the present study.

Primer	Sequence (5' to 3')	Usage
pTRV2- <i>SgPDS</i> -F	gtgagtaaggttacgaattcCTTAGATGGCAACCCGCCT	Gene Clone
pTRV2- <i>SgPDS</i> -R	cgtgagctcgggtaccggatccCTGTCAAACCATATATGGACATTAATCA	
pTRV2- <i>SgChlI</i> -F	gtgagtaaggttacgaattcTTGTATGTTGACGAAGTTAATCTTTTGG	
pTRV2- <i>SgChlI</i> -R	cgtgagctcgggtaccggatccTGCCTTGTAAGAACCTCGAAACT	
pTRV2- <i>SgOMT3-1</i> -F	gtgagtaaggttacgaattcCGTCTTAGAGATTATAGGCAAAGCTG	
pTRV2- <i>SgOMT3-1</i> -R	cgtgagctcgggtaccggatccAAGTGGTACCAGCTCTCCATGAG	
pTRV2- <i>SgOMT3-2</i> -F	gtgagtaaggttacgaattcCACCACACACGATGATGGAAAA	
pTRV2- <i>SgOMT3-2</i> -R	cgtgagctcgggtaccggatccGTCTCAAGAATCTTCTTCATGGTTAAGG	
pTRV2- <i>SgOMT3-3</i> -F	gtgagtaaggttacgaattcCGCATCTCAGCTGCCCCAC	
pTRV2- <i>SgOMT3-3</i> -R	cgtgagctcgggtaccggatccGCTGTCAATCCATATGCCTTGTT	
pTRV2- <i>SgOMT3-4</i> -F	gtgagtaaggttacgaattcAACGGATCCAAGGTTCAATAAGAT	
pTRV2- <i>SgOMT3-4</i> -R	cgtgagctcgggtaccggatccATCCACTTCATGAAAATGGCATC	
qPCR- <i>SgOMT3</i> -F	TTTCGATCTCCCTCACGTCATC	qPCR
qPCR- <i>SgOMT3</i> -R	AAAATGGCATCTCCGCTTGG	
<i>SgCAC</i> -F	TCCGACAAATTGGAGGTTGC	
<i>SgCAC</i> -R	TGCTGCTGACAACAATCACG	PCR
pTRV2-F	CAGGCGGTTCTTGTGTGTCA	
pTRV2-R	AATGTCTTCGGGACATGCCC	Validation

References

- Bhadra, S., Leitch, I. J., and Onstein, R. E.** (2023). From genome size to trait evolution during angiosperm radiation. *Trends in Genetics* **39**:728–735.
- Cui, M.-Y., Lu, A.-R., Li, J.-X., Liu, J., Fang, Y.-M., Pei, T.-L., Zhong, X., Wei, Y.-K., Kong, Y., Qiu, W.-Q., et al.** (2022a). Two types of *O*-methyltransferase are involved in biosynthesis of anticancer methoxylated 4'-deoxyflavones in *Scutellaria baicalensis* Georgi. *Plant Biotechnol. J.* **20**:129–142.
- Cui, X., Meng, F., Pan, X., Qiu, X., Zhang, S., Li, C., and Lu, S.** (2022b). Chromosome-level genome assembly of *Aristolochia contorta* provides insights into the biosynthesis of benzyloquinoline alkaloids and aristolochic acids. *Hortic. Res.* **9**:uhac005.
- Deng, Y., Yang, P., Zhang, Q., Wu, Q., Feng, L., Shi, W., Peng, Q., Ding, L., Tan, X., Zhan, R., et al.** (2024). Genomic insights into the evolution of flavonoid biosynthesis and *O*-methyltransferase and glucosyltransferase in *Chrysanthemum indicum*. *Cell Rep.* **43**:113725.
- Dommes, A. B., Gross, T., Herbert, D. B., Kivivirta, K. I., and Becker, A.** (2019). Virus-induced gene silencing: empowering genetics in non-model organisms. *J. Exp. Bot.* **70**:757–770.
- Guo, X., Fang, D., Sahu, S. K., Yang, S., Guang, X., Folk, R., Smith, S. A., Chanderbali, A. S., Chen, S., Liu, M., et al.** (2021). Chloranthus genome provides insights into the early diversification of angiosperms. *Nat. Commun.* **12**:6930.
- He, G., Zhao, X., Xu, Y., Wang, Y., Zhang, Z., Xiao, L., Hudson, M., Hu, R., and Li, S.** (2023). An efficient virus-induced gene silencing (VIGS) system for gene functional studies in *Miscanthus*. *GCB Bioenergy* **15**:805–820.
- Holzberg, S., Brosio, P., Gross, C., and Pogue, G. P.** (2002). Barley stripe mosaic virus-induced gene silencing in a monocot plant. *The Plant Journal* **30**:315–327.
- Hu, Y., Wang, J., Liu, L., Yi, X., Wang, X., Wang, J., Hao, Y., Qin, L., Li, K., Feng, Y., et al.** (2025). Evolutionary history of magnoliid genomes and benzyloquinoline alkaloid biosynthesis. *Nat. Commun.* **16**:4039.
- Li, Y., Li, R., Shang, J., Zhao, K., Sui, Y., Liu, Z., Yan, H., Bao, M., Liang, M., Zhao, Q., et al.** (2025). A de novo-originated gene drives rose scent diversification. *Cell* **188**:6121–6137.e24.
- Liang, Y., Gao, Q., Li, F., Du, Y., Wu, J., Pan, W., Wang, S., Zhang, X., Zhang, M., Song, X., et al.** (2025). The giant genome of lily provides insights into the hybridization of cultivated lilies. *Nat. Commun.* **16**:45.
- Liu, N., Xie, K., Jia, Q., Zhao, J., Chen, T., Li, H., Wei, X., Diao, X., Hong, Y., and Liu, Y.** (2016). Foxtail mosaic virus-induced gene silencing in monocot plants. *Plant Physiol.* **171**:1801–1807.
- Luo, Y., Wang, R., Zhang, Y., Wang, Y., Liu, J., and Wang, Y.** (2026). A chromosome-level genome assembly and multi-omics analyses provide insights into the biosynthesis of piperlongumine in *Piper sarmentosum*. *Commun. Biol.* **9**:170.
- Ma, J., Sun, P., Wang, D., Wang, Z., Yang, J., Li, Y., Mu, W., Xu, R., Wu, Y., Dong, C., et al.** (2021). The *Chloranthus sessilifolius* genome provides insight into early diversification of angiosperms. *Nat. Commun.* **12**:6929.
- Oliver, K. R., and Greene, W. K.** (2012). Transposable elements and viruses as factors in adaptation and evolution: an expansion and strengthening of the TE-Thrust hypothesis. *Ecol. Evol.* **2**:2912–2933.
- Oliver, K. R., McComb, J. A., and Greene, W. K.** (2013). Transposable elements: powerful contributors to angiosperm evolution and diversity. *Genome Biol. Evol.* **5**:1886–1901.

Pellicer, J., and Leitch, I. J. (2020). The Plant DNA C-values database (release 7.1): an updated online repository of plant genome size data for comparative studies. *New Phytologist* **226**:301–305.

Reynolds, E. E., Trauger, M., Li, F.-S., Huang, J., Moss, T., Christ, B., Xu, M., Knoch, E., and Weng, J.-K. (2026). Elucidation of gene clusters underlying withanolide biosynthesis in ashwagandha through yeast metabolic engineering. *Nat. Plants* **12**:432–446.

Simonin, K. A., and Roddy, A. B. (2018). Genome downsizing, physiological novelty, and the global dominance of flowering plants. *PLoS Biol.* **16**:e2003706-.

Sun, J., Wang, X., Wang, K., Meng, D., Mu, Y., Zhang, L., Wang, J., Yao, G., and Guo, L. (2025). Genomic and epigenomic insight into giga-chromosome architecture and adaptive evolution of royal lily (*Lilium regale*). *Nat. Commun.* **16**:5617.

Wang, H., Ou, X., Zhang, J., Yang, C., Zhang, Z., Kanwal, F., Zou, X., Yang, J., and Wang, S. (2026). Pan-genome analysis reveals the evolutionary dynamics and functional divergence of the *O*-methyltransferase gene family in tomato. *J. Integr. Plant Biol.* **n/a**.

Xu, R.-X., Gao, S., Zhao, Y., Lou, H.-X., and Cheng, A.-X. (2016). Functional characterization of a Mg^{2+} -dependent *O*-methyltransferase with coumarin as preferred substrate from the liverwort *Plagiochasma appendiculatum*. *Plant Physiology and Biochemistry* **106**:269–277.

Xu, S., Chen, R., Zhang, X., Wu, Y., Yang, L., Sun, Z., Zhu, Z., Song, A., Wu, Z., Li, T., et al. (2024). The evolutionary tale of lilies: Giant genomes derived from transposon insertions and polyploidization. *The Innovation* **5**:100726.

Yang, L.-L., Li, Y., Niu, X.-M., and Guo, Y.-L. (2026). The enigma of transposable element abundance: lessons from *Arabidopsis*. *Trends in Genetics* Advance Access published 2026, doi:<https://doi.org/10.1016/j.tig.2025.12.007>.

We sincerely appreciate the valuable comments and the time and effort of both you and the reviewers. We have carefully considered all suggestions and revised the manuscript accordingly. A detailed point-to-point response to each comment is provided in the response letter.

To establish a causal link between genes and metabolites *in planta*, we conducted *Agrobacterium rhizogenes*-mediated hairy root transformation for RNAi-mediated gene silencing.

We hope that the response letter and revised manuscript address the reviewers' concerns. Detailed responses to each comment are provided below.

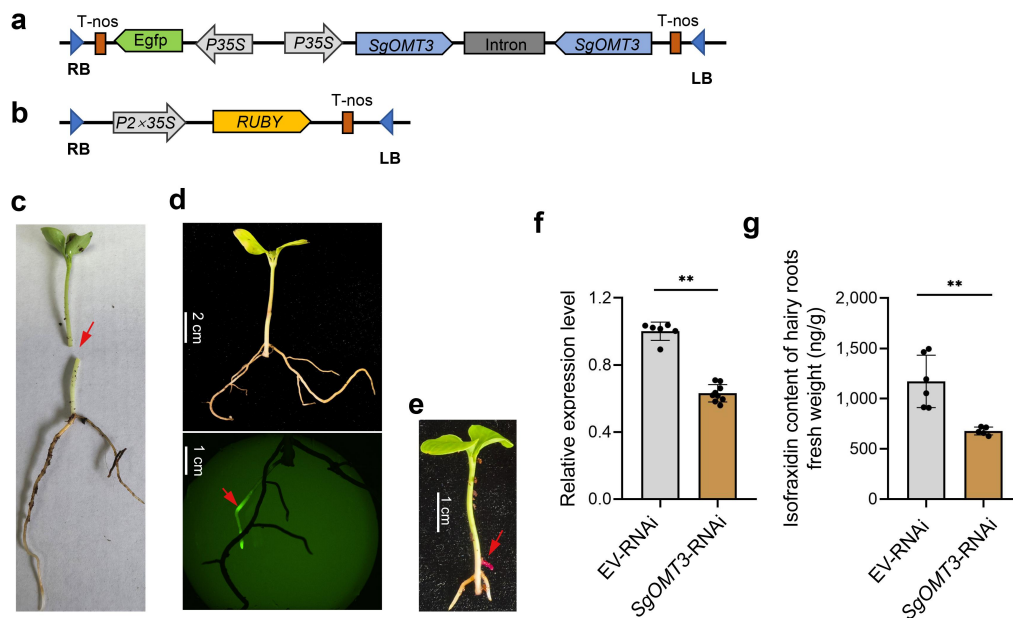
Reviewer #2

Concern 1 (Major): *While the genome assembly is high-quality and the OMT characterization is thorough, the study lacks reverse genetics validation to establish a causal link between SgOMT3/5 and isofraxidin/fraxidin biosynthesis in planta. Without this, the physiological relevance of these enzymes remains correlative.*

Response: Thank you for your valuable suggestion. As recommended in the first-round revision, we performed VIGS via *Agrobacterium tumefaciens* GV3101 infiltration in both leaves and roots. However, no positive results were obtained, indicating that this method is not suitable for *S. glabra*.

To complement reverse genetic validation and establish a causal relationship between SgOMT3/5 and isofraxidin/fraxidin biosynthesis *in planta*, we employed *Agrobacterium rhizogenes*-mediated hairy root transformation for RNAi-based gene silencing. Positive signals were detected using GFP fluorescence and *RUBY* reporter as controls, confirming the successful establishment of the hairy root transformation system in *S. glabra* (**Supplementary Figure 15**).

Our major results show that *SgOMT3* is the primary target gene, as it exhibited the highest Pearson's correlation coefficient with isofraxidin content and the greatest catalytic efficiency toward its biosynthesis. In contrast, *SgOMT5* showed very low expression levels and no detectable activity in isofraxidin production. Therefore, *SgOMT3* was selected for RNAi-mediated gene silencing. RNAi-mediated silencing of *SgOMT3* in the hairy roots of *S. glabra* led to a significant reduction in both expression levels and isofraxidin accumulation, thereby demonstrating that *SgOMT3* plays a critical role in isofraxidin biosynthesis *in planta* (**lines 189–192, Supplementary Table 15, and Response letter Table 1**). In addition, we have added the methods for plasmid construction and adventitious root genetic transformation system in *S. glabra* (**lines 665–687**).



Supplementary Figure 15. The expression levels of *SgOMT3* and the content of isofraxidin in *SgOMT3*-RNAi plantlets. **a** and **b** Schematic representation of the *SgOMT3*-RNAi constructs and the pCAMBIA2300 plasmid carrying the *RUBY* reporter gene. **c** Seedlings of *S. glabra* at the cotyledon-emergence stage were excised at the hypocotyl region (1.5-2 cm above the root base) and used as explants. **d** An accessible adventitious root transformation protocol for *S. glabra* seedlings established after four months of growth. Samples were illuminated under fluorescent light. Red arrow indicates GFP fluorescence in transformed adventitious roots. **e** Phenotypic characterization of *S. glabra* adventitious roots one month following K599-*RUBY* infection. **f** Expression analysis of *SgOMT3* in *SgOMT3*-RNAi (n = 9) and Empty vector (EV)-RNAi (n = 6) adventitious roots. (Student's t tests: **p* < 0.05, ***p* < 0.01). **g** The isofraxidin content in *SgOMT3*-RNAi (n = 5) and EV-RNAi (n = 6) adventitious roots. (Student's t tests: **p* < 0.05, ***p* < 0.01).

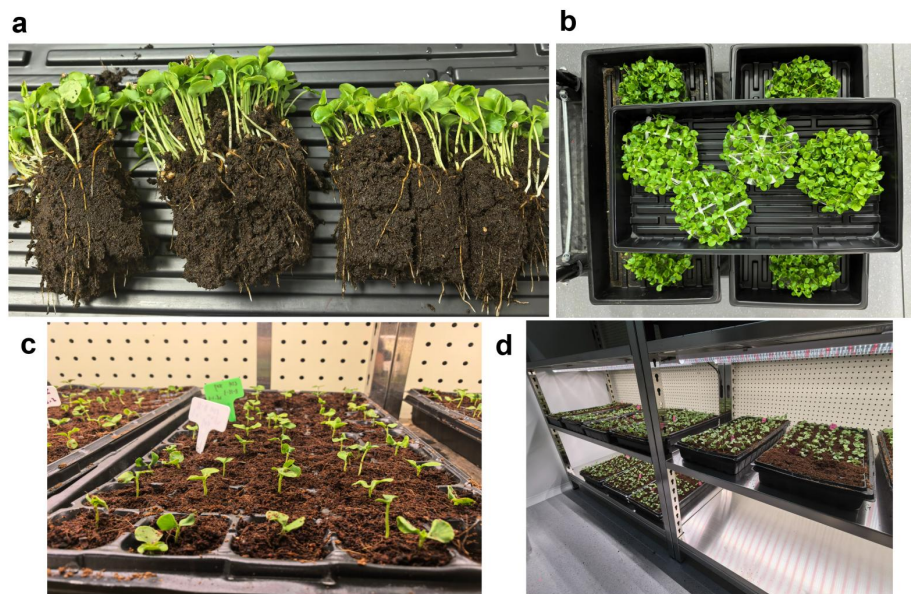
Supplementary Methods for Reviewer 2, Concern 1:

Plasmid construction and adventitious root genetic transformation system in *S. glabra*.

A specific 300-bp fragment of *SgOMT3*, along with its reverse-complement sequence, was cloned into the pWMB110 vector for RNAi interference (RNAi). In addition, a reporter cassette comprising the CaMV 35S promoter (P35S), *eGFP*, and the nopaline synthase terminator (T-Nos) was inserted into the pWMB110 vector to monitor transgene expression in transformed tissues. Meanwhile, we constructed the overexpression vector pCAMBIA2300, harboring the 35S promoter, the *RUBY* reporter gene, and the T-Nos terminator, to verify that *Agrobacterium rhizogenes*-mediated hairy root transformation is effective under soil-grown conditions. The resulting constructs were transformed into competent cells of *A. rhizogenes* strain K599 and plated on TY solid medium supplemented with kanamycin and streptomycin. Then, 200 μ L of the culture was spread onto the

same medium and incubated at 28°C for 15 h. Bacterial cells were subsequently resuspended in MES buffer (10 mM MES, 10 mM MgCl₂, and 200 µM acetosyringone), adjusted to an OD₆₀₀ of 0.6, and incubated at 28°C for 2 h.

Seeds of *S. glabra* were germinated in peat soil, with hypocotyl elongation and cotyledon expansion occurring 60 days post-germination. Seedlings were excised at the hypocotyl region (1.5–2 cm above the root base) and used as explants. The wounded hypocotyls were immersed in the bacterial suspension for 15 h, then immediately transplanted to a moist coco coir substrate and incubated in darkness for 3 days. Co-cultivation was conducted at 25°C under a 16-h photoperiod and 60% relative humidity (**Response letter Figure 1**). One to four months after infection, adventitious roots emerged from infection sites, and transgenic roots were identified using a portable fluorescence observation instrument (LUYOR-3415RG Fluorescent Protein Lamp, Shanghai, China) and a fluorescent stereomicroscope (SMZ25, Nikon, Japan). The transgenic roots were subsequently selected for further characterization.



Response letter Figure 1. *Agrobacterium rhizogenes*-mediated hairy root transformation under soil-grown conditions using an RNAi approach.

Response letter Table 1. Primers used for RNAi in the present study.

Primer	Sequence (5' to 3')	Usage
qPCR- <i>SgOMT3</i> -F	TTTCGATCTCCCTCACGTCATC	qPCR
qPCR- <i>SgOMT3</i> -R	AAAATGGCATCTCCGCTTGG	
<i>SgCAC</i> -F	TCCGACAAATTGGAGGTTGC	
<i>SgCAC</i> -F	TGCTGCTGACAACAATCACG	
RNAi- <i>SgOMT3</i>	CCAAGGTTCAATAAGATCTTCAATAATGGCATGTCAAATC	RNAi plasmid construction
	ACTCCACCTTAACCATGAAGAAGATTCTTGAGACATACAA	
	AGGATTTGAAGGCCTTTCAAGCGTTGTCGATGTTGGCGGG	
	GGTACAGGAGCCACCATTAACATGATCATTCTAAGTATC	
	CTCATATTAAGGGTATTAATTTTCGATCTCCCTCACGTCATC	
	GACGATGCGCCCTCGTATTCTGGTGTGGAGCATGTTGGTG	
	GAGACATGTTTGTAGCGTTCCAAGCGGAGATGCCATTTT	
	CATGAAGTGGATAATTCAT	

Reviewer #1 (Remarks to the Author):

The authors have satisfactorily responded to all my concerns, and I have no additional issues to raise.

Response: We thank the reviewer for confirming that all concerns have been satisfactorily addressed and for the constructive feedback that helped improve our manuscript.

Reviewer #2 (Remarks to the Author):

My comments were properly addressed. The draft could be accepted.

Response: We sincerely thank the reviewer for their positive assessment and recommendation for acceptance.