

Chemically guided single-cell transcriptomics reveals sulfotransferase-mediated scaffold remodeling in securinine biosynthesis

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Abstract

Alkaloids are a structurally diverse group of nitrogen-containing natural products. Unlike other specialized metabolite classes, alkaloids lack a unified biosynthetic pathway or enzyme family. Their scaffold formation and remodeling often use unexpected intermediates and ubiquitous enzymes that have evolved novel, noncanonical functions, making it challenging to elucidate biosynthetic pathways of alkaloids^{1–3}. To address this, we integrated chemical insights acquired from biomimetic synthesis with single-cell transcriptomics and uncovered key biosynthetic steps of securine alkaloids in *Flueggea suffruticosa*. Feeding experiments using stable isotope-labeled candidate intermediates guided us to identify biosynthetic precursors and the corresponding enzyme classes responsible for each transformation. We found that neosecurinanes, (–)-virosine A and (–)-virosine B, are formed through conjugation between 1-piperidine and menisdaurilide. Subsequently, the [2.2.2]-bicyclic neosecurinanes undergo a sulfotransferase-mediated 1,2-amine shift, yielding [3.2.1]-bicyclic securinanes: allosecurinine and securinine. This transformation revealed an unexpected catalytic role of sulfotransferases, not as conventional tailoring enzymes, but as key mediators of scaffold remodeling. We also found a precursor and biosynthetic gene of menisdaurilide. These findings highlight the power of chemically guided single-cell transcriptomics in unravelling complex biosynthetic pathways.

Introduction

Biosynthetic alkaloid scaffold remodeling is a common feature across alkaloid families and a major driver of their structural diversification. Alkaloid scaffold formation and remodeling are often mediated by enzymes that have acquired specialized catalytic activities from conventional metabolic roles. For example, scaffold formation in *Lycopodium* alkaloids involves neofunctionalized carbonic anhydrases¹. Catharanthine is formed from dehydrosecodine via an α/β hydrolase-mediated [4 + 2]-cycloaddition², and scaffold remodeling in colchicine biosynthesis is catalyzed by specialized cytochrome P450³. In contrast to other specialized metabolites, alkaloids lack common precursors or conserved intermediates, making it difficult to predict the sequence of reactions leading to the core scaffold. This biochemical ambiguity further complicates the identification of biosynthetic genes using conventional approaches based on known enzyme functions.

To identify biosynthetic genes for plant specialized metabolites, tissue-specific RNA sequencing has been widely used. Co-expression analyses using known biosynthetic genes as baits are effective in uncovering biosynthetic pathways^{3–11}. To enhance the resolution of co-expression analysis, researchers have adopted sampling strategies that consider tissue developmental stages^{1,12}, inducible conditions¹³, and fine-scale tissue dissection. While these approaches improve the resolution of biosynthetic gene discovery, a gene expression analysis at the single-cell level would offer an even higher resolution. This advantage stems from both the substantially increased number of samples (individual cells) and the expectation that sequential genes within a biosynthetic pathway are co-expressed in a single cell.

Recently, co-expression analyses based on single-cell RNA sequencing (scRNA-seq) have been applied to identify biosynthetic genes for benzyl acetone¹⁴, vinblastine^{15,16}, hyperforin¹⁷, and taxol^{18,19}.

In parallel, chemical insights into biosynthetic intermediates would aid in the elucidation of corresponding biosynthetic genes. Historically, biomimicry has played a pivotal role in guiding the synthesis of complex natural products^{20–22}. Conversely, access to presumed biosynthetic precursors and insights into their inherent chemical reactivity have significantly advanced the understanding of biosynthetic pathways. Such information enables the prediction of biosynthetic transformations and candidate gene functions. In addition, isotopically labeled intermediates have proven critical for validating the involvement of candidate molecules in biosynthetic pathways. Hence, synthetic access to (multiply) isotope-labeled biosynthetic candidate intermediates can expedite the elucidation of biosynthetic pathway. Thus, the integration of chemical knowledge is essential to fully harness the potential of high-resolution transcriptomics, especially when most biosynthetic intermediates remain unidentified.

The biosynthesis of securinega alkaloids (SeAs) offers an ideal case study for applying an integrative, chemically guided single-cell transcriptomics approach. SeAs have captivated the scientific community for over six decades owing to their structural complexity and biological activities^{23–25}. Recently, SeAs have emerged as promising medicinal compounds for the treatment of cancer^{26,27} and neurological diseases^{28–30}. Despite their pharmaceutical significance, the biosynthetic origin of SeAs in plants remains largely unresolved. Beyond their known precursors, L-tyrosine (**1**) and the L-lysine-derived 1-piperidine (**11**), no intermediates bridging these precursors to the SeA scaffold have been identified^{31–35}. Nevertheless, extensive efforts toward the total synthesis of securinane scaffold (**8**, **9**) have proposed various candidate intermediates (**Supplementary Fig. 1**), offering critical insights into potential intermediates and core chemical transformations. Here, we elucidate the biosynthetic pathway of monomeric SeAs in *Flueggea suffruticosa* by integrating chemical synthesis with single-cell transcriptomics. Putative intermediates labeled with stable isotopes were synthesized (**Supplementary Fig. 8**), and their intrinsic biochemical reactivities were evaluated (Fig. 1a). Single-cell transcriptomic analysis identified a specific cell type that exhibits highly enriched expression of the two previously known SeA-associated genes^{36,37} (Fig. 1b). This multidisciplinary approach led to the identification of key biosynthetic intermediates and the discovery of enzymes responsible for their conversion into SeA (Fig. 1c).

Single-cell RNA sequencing proposed the cell cluster responsible for SeA biosynthesis

To address the absence of a reference genome, *de novo* genome assembly and gene annotation of *F. suffruticosa* were performed. Using PacBio HiFi sequencing, over 65 Gbps of reads with an average length of 15.9 kb and a Phred quality score of Q27 were generated from genomic DNA extracted from *F. suffruticosa* leaves. These reads were assembled into a high-quality genome, from which 34,960 protein-

coding genes were predicted. A contig N50 of 30.93 Mbps and a BUSCO gene completeness ratio of 95.8% demonstrated the reliability of the genome assembly and gene annotation (Fig. 2a).

With a reference genome in hand, tissue-specific expression patterns of two known SeA biosynthetic genes, *FsBBE2*³⁶ and *FsPS*³⁷, were examined. *FsBBE2*, which catalyzes the conversion of allosecurinine (**8**) to 2,3-dehydroallosecurinine, showed higher expression in leaves compared to other tissues. In contrast, *FsPS*, an early-stage biosynthetic gene, showed no tissue-specific expression pattern (**Extended Data Fig. 1**). Finally, a D₂O labeling assay showed that the leaf is a tissue of active biosynthesis (**Extended Data Fig. 2**). Therefore, scRNA-seq was performed on *F. suffruticosa* leaves to identify candidate genes in the SeA biosynthetic pathway through co-expression analysis with higher resolution.

For scRNA-seq experiments, protoplasts were isolated from five-week-old *F. suffruticosa* leaves and used to generate 10X Genomics libraries in two biological replicates. Microscopic observation and viability staining confirmed that both replicates yielded high-quality protoplasts suitable for downstream analyses (**Supplementary Fig. 3**). The libraries were sequenced, aligned, and demultiplexed by using the assembled *F. suffruticosa* genome and annotation. Replicate 1 and 2 captured 6,313 and 7,146 cells, with median genes per cell of 1,425 and 1,782, and median reads per cell of 19,482 and 17,590, respectively. Mapping showed 34.9% and 45.6% of reads confidently aligned to the genome, with 32.8% and 42.9% mapped to exonic regions, respectively (**Extended Data Fig. 3a**). The validated cell-by-gene matrices were used for downstream analyses.

The single cells were grouped into 12 distinct cell clusters based on their gene expression levels (Fig. 2b, **Extended Data Fig. 3b**). By comparing known cell-type markers of leaf tissues with cluster-specific marker genes, the clusters were predicted as mesophyll, epidermis, vasculature, and guard cells^{15,38–42}. The marker genes used for cell type annotation and their expression level across clusters were shown in a dot plot (**Extended Data Fig. 4**). Interestingly, two known SeA biosynthetic genes, *FsPS* and *FsBBE2*, showed enriched expression in cluster 7 (Fig. 2c, **Extended Data Fig. 3c**). In addition, genes enriched in cluster 7 were associated with processes such as L-lysine (**10**) biosynthesis via the diaminopimelate pathway (GO:009089), aromatic amino acid family biosynthesis (GO:0009073), and sulfate assimilation (GO:0000103) (Fig. 2c and **Extended Data Fig. 5**). Therefore, we hypothesized that cluster 7 is the cell cluster responsible for SeA production in a *F. suffruticosa* leaf.

Identification and biosynthesis of menisdaurilide, a precursor to neosecurinane alkaloids

L-Tyrosine (**1**) and L-lysine-derived 1-piperidine (**11**) have been identified as biosynthetic intermediates of SeA by radioactive isotope feeding assays^{31–35} (Fig. 1c). However, the specific molecule derived from L-tyrosine (**1**) that forms the scaffold with 1-piperidine (**11**) has remained elusive³³. Inspired by previous biomimetic syntheses of SeAs (Fig. 3a), we postulated menisdaurilide (**5**) as a potential biosynthetic intermediate. In 2008, de March and coworkers reported the synthesis of allosecurinine (**8**) employing

vinyllogous Mannich reaction as a key step⁴³(Fig. 3a). The silyl enol ether derivative of the *O*-TBDPS menisdaurilide (**13**) was allowed to react with iminium ion intermediate **14** via Diels–Alder-like transition state to furnish **15**, which was further converted to allosecurinine (**8**) through a four-step transformation. In 2017, the Gademann group reported that compound **15** can be converted into (–)-viroisine A (**6**) via an intramolecular *aza*-Michael reaction as a key step⁴⁴. Recently, our group reported that lithium enolate **17** from *O*-TBDPS menisdaurilide (**12**) reacts with enone **18** to furnish vinyllogous Michael adduct **19**, which was further transformed into compound **21** with neosecurinane core⁴⁵ (Fig. 3a).

These synthetic precedents allowed us to envision that menisdaurilide (**5**) would react with 1-piperidine (**11**) to generate neosecurinane scaffold (Fig. 3b). We hypothesized that menisdaurilide (**5**) and 1-piperidine (**11**) would react to form four different transition states (Fig. 3b, TS-A to D) and produce (–)-viroisine A (**6**), (–)-viroisine B (**7**), (+)-episecurinol A (**23**), and (+)-securinol A (**24**), respectively. Although previous works employed highly activated derivatives of menisdaurilide (**13**, **17**) and 1-piperidine (**14**, **18**), we exposed a mixture of these biosynthetically relevant fragments in a buffer at physiologically reasonable pH levels. Surprisingly, the formation of (–)-viroisine A (**6**), (–)-viroisine B (**7**), and (+)-securinol A (**24**) was observed when the mixture of menisdaurilide (**5**) and 1-piperidine (**11**) was treated with phosphate buffer in pH 7 or 8 (Fig. 3c). Importantly, the production of these monomeric SeAs was more efficient under more basic pH. Intrigued by these observations, we conducted a feeding experiment of [¹³C₂]-menisdaurilide ([¹³C₂]-**5**) to the leaf lysate of *F. suffruticosa* (pH 8) to further verify its biosynthetic plausibility (Fig. 3d). Consistent with the observed chemical reactivity, [¹³C₂]-menisdaurilide ([¹³C₂]-**5**) was incorporated into (–)-viroisine A (**6**) and (–)-viroisine B (**7**), regardless of the prior thermal denaturation of the leaf lysate, suggesting that this vinyllogous Mannich reaction can occur in the absence of enzymes. Hence, we presumed that menisdaurilide (**5**) is the L-tyrosine (**1**)-derived metabolite that can react with 1-piperidine (**11**) to generate the neosecurinane scaffold. However, this does not, by any means, undermine the possibility of the existence of enzymes that mediate this biosynthetic event.

We further analyzed the distribution of menisdaurilide (**5**), 1-piperidine (**11**), and SeAs across the leaf, stem, and root of five-week-old *F. suffruticosa*. Menisdaurilide (**5**), 1-piperidine (**11**), (–)-viroisine A (**6**), (–)-viroisine B (**7**), allosecurinine (**8**), and securinine (**9**) were detected in all three tissue types (**Extended Data Fig. 6**). Additionally, (+)-securinol (**24**) was also identified in all tissues at levels comparable to those of (–)-viroisine A/B (**6**, **7**), whereas (+)-episecurinol (**23**) was not detected within our detection limit. These observations are consistent with the results of the *in vitro* assay, in which mixing menisdaurilide (**5**) and 1-piperidine (**11**) in an aqueous buffer did not yield detectable levels of (+)-episecurinol (**23**). Furthermore, the existence of menisdaurilide (**5**) in other SeA-producing plants belonging to the *Flueggea* and *Phyllanthus* genera^{46,47}, further supports its role as a common biosynthetic precursor derived from L-tyrosine (**1**). Notably, a wide range of neonorsecurinane alkaloids containing a pyrrolidine A ring have been isolated from *Phyllanthus* genera, suggesting that an analogous reaction employing 1-pyrroline instead of 1-piperidine (**11**) may lead to the formation of the neonorsecurinane scaffold.

Encouraged by the successful coupling of menisdaurilide (**5**) with 1-piperidine (**11**), we focused on identifying the penultimate biosynthetic precursor of menisdaurilide and the enzyme responsible for its formation. Menisdaurilide (**5**) is a bicyclic molecule composed of a cyclohexenol (C ring) and a butenolide (D ring) (Fig. 4a). From a retrobiosynthetic perspective, the C ring of menisdaurilide (**5**) likely originates from the phenol moiety of L-tyrosine (**1**), and the D ring is presumed to be formed via an intramolecular *oxa*-Michael reaction⁴⁸. This hypothesis led us to propose that the allylic alcohol moiety on the C ring is formed by reducing the enone group after the D-ring is generated. Accordingly, we came up with two plausible penultimate precursors of menisdaurilide (**5**): a ketone candidate (**3**) requiring reduction and a diol candidate (**4**) requiring dehydration to be converted into the target molecule, menisdaurilide (**5**) (Fig. 4a).

[¹³C₂]-labeled putative precursors were synthesized (**Supplementary Fig. 10**) and incubated with crude leaf lysates of *F. suffruticosa* in the presence of appropriate cofactors for each reaction. Notably, incubation of the [¹³C₂]-ketone candidate ([¹³C₂]-**3**) in the presence of NAD(P)H resulted in the formation of [¹³C₂]-menisdaurilide ([¹³C₂]-**5**) (Fig. 4b, c). In contrast, no [¹³C₂]-menisdaurilide ([¹³C₂]-**5**) was observed from the [¹³C₂]-diol ([¹³C₂]-**4**) candidate. Thus, we identified the ketone candidate, named premenisdaurilide (**3**), as the biosynthetic precursor of menisdaurilide (**5**) and proposed that a NAD(P)H-dependent ketoreductase is responsible for this biotransformation. It is noteworthy that premenisdaurilide (**3**) is unstable in water, which likely accounts for its absence in plant extracts (**Supplementary Fig. 4**). Markedly, the premenisdaurilide (**3**) has been frequently employed as a penultimate precursor to menisdaurilide in chemical syntheses^{49–51} (**Supplementary Fig. 2**).

With the precursor identified and the enzyme's functional ontology defined, we focused on searching for the candidate ketoreductase on cluster 7, which exhibits enriched expression of genes associated with monomeric SeA biosynthesis (Fig. 2d, **Extended Data Fig. 3d**). Ten ketoreductases that showed enriched expression in cluster 7 were selected as candidate genes (**Supplementary Fig. 5**). Upon transient expression of these candidates in *Nicotiana benthamiana* leaves followed by feeding with premenisdaurilide (**3**), a significant increase in the menisdaurilide (**5**) production was observed only in *N. benthamiana* expressing g07207 (Fig. 4d). We thus annotated g07207 as a *menisdaurilide synthase* (*FsMS*).

Neosecurinanes are converted to securinanes via sulfotransferase-mediated 1,2-amine shift

In 2017, Gademann and colleagues reported a chemical transformation converting the neosecurinane scaffold into the securinane scaffold⁴⁴ (Fig. 5a). This transformation involves chemical activation (mesylation) of the *anti*-periplanar hydroxyl group on the neosecurinane scaffold, which results in the formation of an aziridinium ion intermediate (**27**) via an intramolecular S_N2 reaction, followed by an elimination reaction to yield the securinane scaffold. Based on this observed chemical reactivity, they

proposed that neosecurinane alkaloids are biosynthetic precursors of securinane alkaloids. Shortly afterward, Peixoto and coworkers reported a general 1,2-amine shift from neo(nor)securinane scaffold into (nor)securinane scaffold using Mitsunobu's alcohol-activating conditions⁵².

While these chemical syntheses provided insights regarding the scaffold remodeling, there was no direct evidence that this conversion occurs in SeA-producing tissues of *F. suffruticosa*. Moreover, the mode of hydroxyl group activation in neosecurinane alkaloids had remained unclear, as the leaving group is eliminated from the molecule during the transformation. To solve this enigma, we first chemically converted the hydroxyl group of (–)-viroisine B (**7**) into plausible biological leaving groups: the acetyl (**28**) and the sulfate (**29**) groups (Fig. 5b). *O*-Acetylvirosine B (**28**) was isolated from the reaction mixture in 92% yield, implying that a stronger activating group might be required to facilitate the desired transformation. However, treatment of the hydrochloride salt of (–)-viroisine B (**7**) with SO₃•pyridine, followed by incubation in phosphate buffer (pH 8), resulted in the formation of securinine (**9**) in 34% yield (Fig. 5b). The reaction between the hydrochloride salt of (–)-viroisine B (**7**•HCl) and SO₃•pyridine would furnish the *O*-sulfated intermediate **29** and pyridinium hydrochloride, maintaining an acidic reaction environment. The ammonium moiety of **29** would be deprotonated only upon exposure to pH 8 buffer, enabling a 1,2-amine shift. This observation suggests that the 1,2-amine shift may proceed spontaneously, without enzymatic assistance.

Building on these results, we conducted a feeding experiment using [¹³C₂](–)-viroisine B ([¹³C₂]-**7**) to the crude *F. suffruticosa* seedling lysate supplemented with either ATP or PAPS (3'-phosphoadenosine-5'-phosphosulfate). This experiment compared sulfation and phosphorylation *in planta*, due to the difficulty associated with chemical phosphorylation in mild conditions (Fig. 5c). We observed a significantly higher conversion of [¹³C₂](–)-viroisine B ([¹³C₂]-**7**) into [¹³C₂]-securinine ([¹³C₂]-**9**) with the addition of PAPS to the lysate, compared to ATP-supplemented lysate and control groups. In addition, we supplemented [¹³C₂](–)-viroisine A ([¹³C₂]-**6**) and virosine B ([¹³C₂]-**7**) into *F. suffruticosa* leaf discs and confirmed that allosecurinine (**8**) and securinine (**9**) were specifically derived from (–)-viroisine A (**6**) and (–)-viroisine B (**7**), respectively, in *F. suffruticosa* leaves (Fig. 5d, e). These observations confirmed that neosecurinane alkaloids are biosynthetic precursors of securinane alkaloids, and this biosynthetic transformation is mediated by sulfotransferase.

To identify the sulfotransferase responsible for securinane biosynthesis in *F. suffruticosa*, we selected candidate genes annotated as sulfotransferases and enriched in cluster 7. Three genes were used as promising baits for co-expression analysis: *FsPS*, *FsMS*, and *FsBBE2*. Two sulfotransferases originally annotated as flavonol-4-sulfotransferases strongly correlated with *FsPS* and showed high expression levels in cluster 7 at the single cell level (Fig. 2c, d). Three additional sulfotransferases highly expressed in leaves, g00640, g21405, and g01123, were also included in the candidates. *In vitro* enzyme assay revealed that two cluster 7-specific sulfotransferases produce allosecurinine (**8**) and securinine (**9**) from (–)-viroisine A (**6**) and (–)-viroisine B (**7**), respectively (Fig. 5f, g, i, j, **Supplementary Fig. 6**). The catalytic activity was PAPS-dependent *in vitro* (Fig. 5g, j, **Extended Data Fig. 7a, b**). The *in planta* enzyme assay of

sulfotransferases using *N. benthamiana* was consistent with the *in vitro* enzyme assay (Fig. 5h, k, **Extended Data Fig. 7c, d**). Thus, we named the two genes having a PAPS-dependent securinane biosynthetic activity *FsNSST1* and *FsNSST2* (Neosecurinane sulfotransferase 1 and 2). Other candidate sulfotransferases did not show catalytic activity except g21405, which showed weak activity on (–)-viroside B (**7**).

Discussion

SeAs and their precursor were isolated across leaves, stems, and roots of *F. suffruticosa* (**Extended Data Fig. 6**). Such tissue-wide distribution complicates the discovery of candidate biosynthetic genes via traditional bulk transcriptomics. However, scRNA-seq only requires any active biosynthetic tissues for proposing biosynthetic candidate genes, enabling the identification of distinct cell types within the tissue. Through scRNA-seq analysis of the leaf, we discovered a specialized cell cluster that showed enriched expression of two known SeA biosynthetic genes. Cluster 7 was predicted to correspond to a vasculature-associated cell type based on known marker genes. (Fig. 2c, **Extended Data Fig. 3c**, **Extended Data Fig. 4**). Within these cells, we found that genes involved in producing L-tyrosine (**1**) and L-lysine (**10**), the two starting molecules of SeA biosynthesis, were actively expressed.

Deciphering the transcriptome at the cell type level provides valuable insights into alkaloid biosynthesis in plants (Fig. 6). In qPCR analysis, monomeric SeA biosynthetic genes *FsPS*, *FsMS*, *FsNSST1*, and *FsNSST2* showed no significant differences in expression across tissues (**Extended Data Figs. 1, 8**), suggesting that tissue-specific bulk RNA-seq would provide limited insight for identifying these genes. This limitation was successfully overcome by scRNA-seq analysis. ATP sulfurylase (ATPS) and adenylyl-sulfate kinase (APSK) are enzymes producing PAPS, which is a co-factor of sulfotransferase, from ATP. While two *ATPS*s and two *APSK*s are present in the *F. suffruticosa* genome, only *FsATPS2* and *FsAPSK2* were specifically expressed in cluster 7, suggesting their potential specialized roles in SeA biosynthesis. In addition, further modification of allosecurinine (**8**) may occur in cluster 7 and cluster 4, as the key downstream enzyme *FsBBE2*³⁶, which is the first enzyme to direct the flux from allosecurinine (**8**) to various SeAs with elevated oxidation levels, was also enriched in these clusters. Notably, the enrichment of catalases and peroxidases in this cluster implies that detoxification of H₂O₂ produced during piperidine biosynthesis and sulfur assimilation may be activated (Fig. 6). Interestingly, genes related to jasmonate (JA) biosynthesis and signaling were also enriched in cluster 7 (**Extended Data Fig. 5**), consistent with previous reports that *FsBBE2* was induced by JA³⁶. This suggests that the SeA biosynthesis might be regulated by JA signaling in cluster 7. This single-cell transcriptome of *F. suffruticosa* could facilitate the identification of additional SeA-associated genes, including transporters, downstream biosynthetic enzymes, and regulatory transcription factors involved in enriched primary metabolism or in mechanisms that support recovery from amino acid depletion (Fig. 6).

Menisdaurilide (**5**) has been isolated from multiple plant genera^{46,47,53–58}, and its glucoside (phyllanthurinolactone) acts as the leaf-closing factor of *Phyllanthus urinaria*^{59,60}. Despite its abundance

and ecological significance, its biosynthetic pathway remains completely unknown. In this study, we found that menisdaurilide (**5**) is produced from premenisdaurilide (**3**), and we identified a reductase, *FsMS*, responsible for this conversion. *FsMS* showed a strong co-expression with *FsPS* and *FsBBE2*, further supporting the role of menisdaurilide (**5**) as a precursor of SeA. However, the intermediates linking L-tyrosine (**1**) to premenisdaurilide (**3**) remain unidentified. Following Parry³³ and Spenser's hypothesis³⁵, which proposed 4-hydroxyphenylpyruvic acid (4HPP) (**2**) as a putative precursor from L-tyrosine (**1**), we chemically synthesized [¹³C₂]-4HPP ([¹³C₂]-**2**, **Supplementary Fig. 11**) and fed it to the leaf of *F. suffruticosa*, but we were unable to detect any significant increase of [¹³C₂]-allosecurinine ([¹³C₂]-**8**), securinine ([¹³C₂]-**9**), and menisdaurilide ([¹³C₂]-**5**) compared to the control (**Extended Data Fig. 9**). Furthermore, orthologs of the L-tyrosine aminotransferase, which converts L-tyrosine (**1**) into 4HPP (**2**), did not show meaningful expression correlation with SeA biosynthetic genes in our single-cell transcriptomics (Fig. 2 and **Extended Data Fig. 10**). These results imply the involvement of an alternative biosynthetic scenario that converts L-tyrosine (**1**) to premenisdaurilide (**3**) and, eventually, menisdaurilide (**5**). We propose that the elucidation of the biosynthetic pathway from L-tyrosine (**1**) to premenisdaurilide (**3**) be pursued through an integrated approach combining comparative genomics and chemically guided single-cell transcriptomics.

Sulfotransferase is a ubiquitous enzyme class that transfers sulfate groups to the hydroxyl groups of various substrates, using PAPS as a biological sulfate donor^{61,62}. While numerous sulfotransferases have been discovered across different organisms, plant sulfotransferases are primarily known for enhancing solubility or facilitating the catabolism of metabolites^{62–64}. Although sulfotransferases have been occasionally reported as tailoring enzymes in the biosynthesis of plant specialized metabolites^{65–67}, their involvement in biosynthetic pathways associated with skeletal arrangement remains largely unexplored⁶⁸. Scaffold formation or remodeling via alcohol activation is a well-documented mechanism in complex natural product biosynthesis. However, compared to phosphorylation^{69,70}, acetylation⁶, and malonylation¹¹, which are commonly observed in biosynthetic modifications, the role of sulfation in scaffold remodeling has not been previously described. In this study, we identified two sulfotransferases that catalyze the transformation of the [2.2.2]-bicyclic neosecurinane scaffold into the [3.2.1]-bicyclic securinane scaffold. To the best of our knowledge, this represents the first report of a sulfotransferase involved in alkaloid scaffold remodeling through a 1,2-amine shift of a 1,2-aminoalcohol moiety.

The scaffold remodeling step from neo(nor)securinane to (nor)securinane represents a crucial point of structural diversification in SeA biosynthesis. Although belonging to the same genus, *Flueggea virosa* is rich in norsecurinine and its oligomers, which are absent in *F. suffruticosa*^{71–73}. In addition, *F. virosa* contains virosecurinine and viroallosecurinine, the enantiomers of securinine (**9**) and allosecurinine (**8**), respectively⁷⁴. A wide range of securinane-type alkaloids has been discovered in other genera of the Phyllanthaceae family, *Phyllanthus*⁷⁵, *Margaritaria*⁷⁶, and *Breynia*⁷⁷ species. These observations suggest that the emergence of a sulfotransferase capable of converting neosecurinane to securinane played a pivotal role in driving the chemical diversification of securinane alkaloids. Exploring the evolutionary

trajectory of these sulfotransferases across Phyllanthaceae species and correlating their presence with metabolomic profiles offers promising avenues for future research. Such studies may illuminate the evolutionary adaptation of sulfotransferases in alkaloid biosynthesis and their contribution to the metabolic diversity observed within this plant family.

Conclusion

The biosynthetic pathway of monomeric SeA in *F. suffruticosa* was revealed by combining biomimetic synthesis and scRNA-seq. Guided by a biosynthetic hypothesis, putative biosynthetic intermediates labeled with stable isotopes were synthesized, and their innate biochemical reactivity was examined. Single-cell transcriptomic analysis revealed a specific cell type with highly enriched expression of the only two previously known SeA biosynthetic genes. Based on these findings, premenisdaurilide and menisdaurilide were identified as L-tyrosine-derived SeA intermediates, and the enzyme responsible for menisdaurilide biosynthesis (*FsMS*) was characterized. In addition, two sulfotransferases (*FsNSST1* and *FsNSST2*) were found to perform a noncanonical biosynthetic function, catalyzing a 1,2-amine shift that converts the neosecurinane ([2.2.2]-bicyclic) scaffold into the securinane ([3.2.1]-bicyclic) scaffold. This highlights a specialized function of sulfotransferases, a universally distributed enzyme class across all domains of life, in scaffold remodeling of plant alkaloids.

Methods

Analysis of securinega alkaloids (SeAs), menisdaurilide, and 1-piperideine

Securinanes, neosecurinanes, and their intermediate menisdaurilide (**5**) were analyzed using an HPLC-MS/MS (LCMS-8050, Shimadzu, Japan). Leaf, stem, and root tissue from five-week-old *F. suffruticosa* seedlings were ground in liquid nitrogen, and 100 mg of ground materials were extracted with 1 mL of extraction solution (0.1% formic acid aqueous solution). Extracts were filtered and diluted 100-fold in methanol. Samples were injected into the Acquity UPLC® BEH C18 column (1.7 μ m, 2.1 $\times$ 100 mm; Waters, Milford, MA, USA). The solvents were (A) 20 mM ammonium acetate (aq.) and (B) methanol. The LC-time program was as follows ((B) concentration in %): 0–3 min: 5–20%, 3–15 min: 20–40%, 15–17 min: 40–85%, 17–17.5 min: 85–95%, 17.5–20.5 min: 95%, 20.5–21 min: 5%, 21–24 min: 5%. The flow rate was 0.3 mL/min, the injection volume was 1 μ L, and the column oven was set at 40°C. Retention times, precursor ions, and product ions of target metabolites (including ^{13} C-labeled and unlabeled) were compared with synthesized authentic compounds. Subsequent mass spectrometry was performed in the positive-ion mode via ESI (interface voltage, 3 kV; interface temperature, 300°C; DL temperature, 250°C; hat block temperature, 400°C; nebulizing gas, 3 L/min; drying gas, 10 L/min; heating gas, 10 L/min). Multiple reaction monitoring (MRM) was used to detect allosecurinine (**8**), securinine (**9**), (–)-viroisine A (**6**), (–)-viroisine B (**7**), and menisdaurilide (**5**) are listed in Supporting Information (Supplementary Table 1).

To analyze 1-piperideine (**11**), samples were injected into the XBridge[®] Amide Column (3.5 μ m, 2.1 $\times$ 150 mm) (Waters). The mobile phase and LC-time program were used as the reference⁷⁸. For the relative quantification, the 1-piperideine dimer peak was used for the peak integration.

To assess the chemical stability of premenisdaurilide (**3**) in aqueous solution, 1 mg of premenisdaurilide (**3**) was dissolved in 1 mL of water and diluted at different time points. Diluted samples were immediately injected into the XBridge[®] Amide Column (3.5 μ m, 2.1 $\times$ 150 mm) (Waters). The solvents were (A) 10 mM ammonium hydroxide (aq.) and (B) acetonitrile. The LC-time program was as follows ((B) concentration in %): 0–5 min: 98 – 60%, 5–6 min: 60 – 10%, 6–7 min: 10 – 5%, 7–10 min: 5%, 10–11 min: 5–98%, 11–14min: 98%. The flow rate was 0.3 mL/min. Injection volume and column oven temperature were identical to those above. ESI negative-ion mode was used. MRM transitions and MS parameters are listed in **Supplementary Table 1**.

Plant materials

F. suffruticosa leaves were collected from a natural population at Bulgok-san Mountain, Gyeonggi-do, Republic of Korea (37°21'20.5"N 127°07'40.7"E) for genomic DNA isolation and sequencing. For other biochemical assays, commercially available *F. suffruticosa* seeds were obtained from a local vendor (Cheonnyangssiat, Hwacheon-gun, Republic of Korea) and germinated in the laboratory. The seeds were sterilized using 2% (w/v) sodium dichloroisocyanurate dihydrate (Sigma-Aldrich) and 0.1% (v/v) Tween 20 (Glenthams), and sown in moist soil. The seeds were incubated under a 16 h dim light /8 h dark cycle. After germination, the plants were grown under 16 h light and 8 h dark conditions in a plant growth room. The growth condition was 26 $\pm$ 2°C with 60% relative humidity (RH). *Nicotiana benthamiana* plants were also grown in the plant growth room under the same conditions: 16 h-8 h long day cycle, 26°C, and 60% RH. Three to four-week-old plants were used for *Agrobacterium* infiltration in transient expression experiments.

Genomic DNA and total RNA sequencing of *F. suffruticosa*

Genomic DNA from *F. suffruticosa* leaves ($2n = 26$)⁷⁹ was extracted following the cetyltrimethyl ammonium bromide (CTAB) method by Macrogen (Seoul, Republic of Korea)⁸⁰. To estimate the genome size of *F. suffruticosa*, the extracted DNA was sequenced using the TruSeq Nano DNA (350) library kit and the HiSeqXten platform. Additionally, the genomic DNA was sequenced for genome assembly using the Revio platform from Pacific Biosciences (PacBio, Menlo Park, CA, USA) with the PacBio HiFi Library Kit 3.0. For gene annotation, total RNA was extracted from seven leaves, three stems, and five roots of *F. suffruticosa* using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) in combination with the RNase-free DNase I (Qiagen). Each extracted RNA was prepared using the TruSeq Stranded Total RNA with Ribo-Zero Plant Kit and sequenced on the NovaSeq6000 platform.

Genome assembly and annotation of *F. suffruticosa*

Python (v3.9.10), Anaconda3 (v22.9.0), and R (v4.3.3) were used for the subsequent analysis. Genome assembly and annotation generally followed the pipeline outlined in our previous reports^{81,82}. The *de novo* genome assembly of *F. suffruticosa* was generated using the Hifiasm genome assembler (v0.18.5-r499) with default options⁸³. The quality of the genome assembly was evaluated by using the benchmarking universal single-copy ortholog (BUSCO, v5.2.2)⁸⁴ analysis on the eudicots_odb10 database and assembly-stats (v1.0.1). The primary genome assembly was used for further analysis based on the quality assessment.

The repetitive elements of the genome assembly were annotated by using RepeatModeler (v2.0.3) with NINJA (NINJA-0.95-cluster_only) database and RepeatMasker (v4.1.2)^{85,86}. The specific repeat library for *F. suffruticosa* and the predefined eudicot repeat library were used to annotate repetitive elements with default options. The plant canonical telomeric repeat sequence "TTTAGGG" and its synonymous forms were identified using a "grep" command in the masked repetitive elements^{85,86}.

To annotate protein-coding genes, 15 bulk RNA sequencing reads were aligned to the soft-masked genome assembly with hisat2 (v2.2.1) with *-no-unal -dta -max-introlen 5000* and other default options⁸⁷. The protein-coding genes of the *F. suffruticosa* genome were annotated by using the BRAKER pipeline (v2.1.6 using Genemark-ES/ET/EP v.4.61_lic). The Viridiplantae protein database of OrthoDB (v11)⁸⁸⁻⁹⁷ and the 11 bulk RNA-seq alignment BAM files were used in the BRAKER pipeline to generate hints to train Augustus. The annotation of protein-coding genes was evaluated by using BUSCO (v5.2.2) analysis on the eudicots_odb10 database and assembly-stats (v1.0.1)⁸⁴. The protein-coding genes were functionally annotated by using MMseqs2 (v13.4511) against the UniProt Knowledgebase (release 2023.01) and the NCBI NR database^{98,99}. The Gene Ontology (GO) terms and Enzyme Commission (EC) numbers were assigned through UniProt API based on the functional annotation results.

D₂O labeling assay

For the D₂O labeling assay, leaves from five-week-old *F. suffruticosa* plants were carefully excised and placed in individual 10 mL vials containing either 2 mL of H₂O (control) or 99% D₂O, following the protocol^{100,101}. The vials were incubated for six days in a growth room under a 16 h light/8 h dark photoperiod. After the incubation period, 8 mm (diameter) leaf discs were collected from each leaf using a leaf borer and immediately frozen in liquid nitrogen. The frozen samples were ground using a TissueLyser II (Qiagen) at 27 Hz for 2 min. The ground tissue was extracted with 400 µL of 0.1% formic acid by vortexing for 1 min, followed by sonication at 50% amplitude for 5 min. The extracts were centrifuged at 18,000 × g for 5 min, filtered through a 0.22 µm PTFE filter, and diluted 20-fold in methanol before being subjected to LC-MS analysis.

The LC-MS analysis was conducted using the same LC time program previously optimized for the separation of SeAs, with the mass spectra scanned to identify isotope ratios. The isotope ratios were statistically analyzed to assess the incorporation of D₂O into allosecurinine (**8**) and securinine (**9**).

Protoplast isolation in *F. suffruticosa* leaves

The protoplasts were isolated from leaves of five-week-old *F. suffruticosa* plants. Young leaves (the 10th to 12th from the base of the main stem) were used for each replicate in scRNA-seq. Seven leaves per plant were diagonally incised across the primary vein using a scalpel. The cut leaves were treated under vacuum with 25 mL of enzyme solution containing 1% (v/v) Viscozyme L, 0.5% (v/v) Celluclast, 0.5% (v/v) Pectinex (all from Novozymes, Bagsværd, Denmark), 9% (w/v) mannitol, 5 mM 2-(N-morpholino) ethanesulfonic acid (MES), 1 mM KNO₃, 1 mM MgSO₄, 0.2 mM KH₂PO₄, 10 µM CaCl₂, 0.1 µM KI, and 0.1 µM CuSO₄ (all from Duchefa Biochemie, Haarlem, the Netherlands). The vacuum treatment and subsequent release were performed for 5 and 15 min, respectively. Leaves were then incubated in the dark for 1 hour at 25°C on an orbital shaker (25 rpm). The resulting protoplast solution was filtered through a 40 µm cell strainer (Corning Inc., Corning, NY, USA) to remove debris. The protoplasts were gently collected by centrifugation at 100 × g for 5 min with low brake in pre-chilled 9% (w/v) mannitol solution. The resulting protoplasts were then filtered again using a 40 µm tip strainer (Bel-Art SP Scientificware, Wayne, NJ, USA), right before loading into nanoliter-scale Gel Beads-in-Emulsion (GEMs).

To validate the viability and integrity of enzymatically isolated protoplasts, fluorescein diacetate (FDA; Thermo Fisher Scientific, MA, USA) staining was performed. FDA was dissolved in acetone at a concentration of 5 mg/mL to prepare the stock solution. For the FDA working solution, 4 µL of the stock solution was added to 1 mL of 9% (w/v) mannitol solution. 100 µL FDA working solution was added to 1 mL of the protoplast suspension and gently mixed. The stained protoplasts were immediately observed under a fluorescent microscope (M205FA, Leica microsystems, Wetzlar, Germany) with the optical filter set Leica 10447408 (Excitation, 450–490 nm; emission, 500–550 nm).

Generation and analysis of single-cell transcriptome data from *F. suffruticosa* leaves

To generate single-cell transcriptome data from *F. suffruticosa* leaves, protoplasts were isolated and validated as described above. Two biological replicates of the protoplast suspensions were used for scRNA-seq library preparation. Libraries were prepared by using the 10X Genomics Chromium single-cell microfluidics device and the Chromium single-cell 3' RNA library v4 kit (10X Genomics, Pleasanton, CA, USA) according to the manufacturer's protocol. The prepared libraries were sequenced on a NovaSeq 6000 platform (Illumina, San Diego, CA, USA) at Macrogen to generate paired-end NGS reads. Raw sequencing data were processed using the Cell Ranger pipeline (v8.0.1, 10X Genomics) for demultiplexing, alignment to the *F. suffruticosa* reference genome, and generation of gene expression matrices with mkref and count mode with default options. These matrices were subsequently used for downstream analyses, including cell clustering, differential gene expression, and functional annotation.

The cell-by-gene matrices were further analyzed and visualized using the R package Seurat (v5.1.0)¹⁰². Ambient RNA contamination in the raw matrices was removed using SoupX (v1.6.2) with the roundToInt = TRUE option in the adjustCounts function and other default parameters¹⁰³. The data were then normalized using the SCTransform method¹⁰⁴. Cells with less than 300 or more than 3000 detected

features (nFeature_RNA) were filtered out based on the distribution observed in the violin plots of nFeature_RNA and nCount_RNA.

The pre-processed matrices were subjected to dimensionality reduction, and 20 principal components (PCs) were selected for clustering and UMAP analysis based on the elbow plot of the PCA.

DoubletFinder¹⁰⁵ was applied to identify and remove doublets in a Seurat object using 20 PCs. The optimal pK value was determined using the sweep.stats function and corresponding plots. The estimated doublet formation ratio, neighborhood size, and ratio of artificial doublet were set to 0.08, 0.08, and 0.25, respectively. The identified doublets were subsequently removed from further analysis. PCA, UMAP dimensionality reduction, and clustering were re-performed using only the singlet cells as described above.

Marker genes for each cluster were identified using the FindAllMarkers function with the parameters only.pos = TRUE, min.pct = 0.1, and logfc.threshold = 0.2. To predict the cell type of each cluster, a reference set of cell type marker genes was constructed for mesophyll cells, epidermal cells, vascular cells, bundle sheath cells, and Dev. Cells^{15,38–42}. From the 124 cell type marker genes, 35 experimentally validated genes were primarily used as markers to assign cell types to each cluster. Among the marker genes of each cluster, those that ranked within the top 200, expressed at levels greater than 10, and identified as orthologs by a BLAST search against the proteome of *F. suffruticosa* were selected as cell type indicators¹⁰⁶. The expression patterns of the marker genes used to predict the cell type of each cluster were depicted as a DotPlot function in the Seurat package (**Extended Data Fig. 4**).

To identify specialized biological processes associated with each cluster, Gene Ontology (GO) enrichment analysis was performed on the marker gene sets of each cluster. Marker genes were annotated with GO terms derived from functional annotations as described above. GO annotations were processed to extract biological process terms using the GO.db (v3.18.0) and GOSemSim (v2.28.1) R packages^{107,108}. Enrichment analysis was performed using the enricher function of the clusterProfiler (v4.10.1) package, with a p-value cutoff of 0.1. GO terms were filtered, and descriptions of enriched terms were retrieved from the GOTERM database¹⁰⁹. Dot plots and bar plots were generated to display the most significantly enriched GO terms, labeled with their descriptions.

Co-expression analysis was performed on the processed cell-by-gene matrices. Spearman's correlation coefficient matrix was calculated based on gene expression levels across all cells. *FsBBE2* and *FsPS* (g19768) were used as bait genes for the co-expression analysis^{36,37}. The expression levels and patterns of *FsBBE2*, *FsPS*, and candidate genes involved in SeA biosynthesis were visualized using the VlnPlot and FeaturePlot functions in the Seurat package¹¹⁰.

Identification of nonenzymatic reaction to produce neosecurinane scaffold from menisdaurilide and 1-piperidine

A standard nonenzymatic reaction contained 100 mM 1-piperidine (**11**) and 100 mM menisdaurilide (**5**) dissolved in buffer solutions having various pH (50 mM phosphate buffer (pH 6, 7, and 8)). Reaction mixtures were incubated for 48 hours at 30°C in a 500 rpm mixing block (Bioer, Hangzhou, China). After the incubation, reactants were extracted with the same volume of 0.1% formic acid water and 20-fold diluted with methanol, filtered, and neosecurinanes were measured as described above.

F. *suffruticosa* crude lysate feeding assay

To confirm whether hypothetical metabolites are the biosynthetic intermediates of allosecurinine (**8**) or securinine (**9**), ^{13}C -labeled potential intermediates were added to the crude tissue lysate with various cofactors. Five-week-old *F. suffruticosa* seedlings were harvested from the soil and rinsed with deionized water before sampling. Sampled seedlings were snap-frozen in liquid nitrogen and ground using a mortar and pestle with liquid nitrogen. 50 mg of ground seedlings were transferred into 2 mL microcentrifuge tubes and were redissolved in 1 mL of pre-chilled lysis buffer to obtain crude seedling lysates. To assess the enzymatic transition from premenisdaurilide (**3**) to menisdaurilide (**5**), 50 mM HEPES buffer (pH 7.0) was used as the lysis buffer, while 100 mM Tris-HCl buffer (pH 8.0) was used to validate the enzymatic transition from neosecurinane to the securinane scaffold. Crude leaf lysates were also prepared using the same method. For negative control, lysates boiled at 98°C for 10 min were used. Prepared lysates were aliquoted into 0.2 mL clear PCR tubes, placed on ice, and used for the assay within 30 min.

To evaluate premenisdaurilide reductase activity assay in crude tissue lysate, [$^{13}\text{C}_2$]-premenisdaurilide ([$^{13}\text{C}_2$]-**3**) was added (final concentration 1 mM) to crude leaf lysate and boiled leaf lysate. NADH or NADPH was added (final concentration 500 μM) as a cofactor. Substrate-fed lysates were incubated at 30°C for 20 h with mild agitation. After the incubation, reacted lysates were diluted 100-fold in 50% methanol and filtered for [$^{13}\text{C}_2$]-menisdaurilide quantification using the HPLC-MS/MS system as described above. Lysates prepared from four independent plants were used as biological replicates.

To assess neosecurinane sulfotransferase activity in crude tissue lysate, [$^{13}\text{C}_2$]-(-)-viroisine A ([$^{13}\text{C}_2$]-**6**) and [$^{13}\text{C}_2$]-(-)-viroisine B ([$^{13}\text{C}_2$]-**7**) were supplied to crude lysate from whole seedlings (final concentration 500 μM). PAPS and ATP were tested as cofactors. Substrate-fed lysates were incubated at 30°C for 20 h with mild agitation. Reacted lysates were diluted 10-fold in DW and serially diluted 10-fold in methanol. Diluted samples were filtered and analyzed using the HPLC-MS/MS system.

Expression and purification of proteins for in vitro enzyme assay

Coding sequences of putative neosecurinane sulfotransferases were amplified from cDNA produced from RNA from *F. suffruticosa* leaves. The pET50 plasmids for the expression of candidate sulfotransferases with C-terminal 8 $\times$ His tag were transformed into *Escherichia coli* Rosetta™ (DE3) strain. Transformed cells were cultured in 250 mL of LB broth media containing kanamycin (50 mg/L) and chloramphenicol (30 mg/L) in a shaking incubator (200 RPM, 37°C) until the optical density

measured at 600 nm (OD₆₀₀) reached 0.4–0.6. At the OD₆₀₀, protein induction was started by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. The cells were then incubated at 18°C for 20 h and harvested by centrifugation.

Cell pellets were resuspended in 10 mL of lysis buffer solution (50 mM Tris-Cl (pH 8.0), 300 mM NaCl, 20 mM imidazole, 10 mM phenylmethylsulfonyl fluoride, 1 tablet of protease inhibitor cocktail (cOmplete mini EDTA-Free) (Roche), 10% (v/v) glycerol, 0.05% (v/v) tween-20) and subsequently lysed by sonication on ice using the Q125 sonicator (10 min, 30% amplitude, 4s:2s / on:off) (Qsonica, Newtown, CT, USA). Whole-cell lysates were centrifuged at 16500 × g, 4°C for 40 min, and soluble fractions were loaded onto Ni-NTA agarose resin (Thermo Fisher Scientific). After washing with washing buffer (lysis buffer with 50 mM imidazole), the resin was eluted with elution buffer (lysis buffer with 250 mM imidazole). The purification result was confirmed by SDS-PAGE. The purified proteins were then desalted using the PD-10 column (Cytiva, Marlborough, MA, USA) following the manufacturer's instructions. Protein concentrations of desalted proteins were measured using the Pierce™ BCA assay kits (Thermo Fisher Scientific) and were immediately used for the *in vitro* enzyme assay.

In vitro sulfotransferase activity assay

Purified, desalted proteins (10 µg) were added into 200 µL of the reaction mixture (final concentration of 100 mM Tris-HCl pH 8.0, 10 mM MgCl₂, 2 mM 3'-phosphoadenosine-5'-phosphosulfate (PAPS) (Merck KGaA, Darmstadt, Germany), and 0.5 mM (–)-virosine A (**6**) or (–)-virosine B (**7**)) and incubated for 22 h at 30°C. The reaction mixtures were diluted 100-fold with methanol and filtered using syringe filters (0.22 µm) for metabolite analysis. Produced allosecurinine (**8**) or securinine (**9**) was measured using HPLC-MS/MS as described above.

Functional evaluation of biosynthetic genes in *N. benthamiana*

A pCambia-derived binary vector harboring the CDS of target genes downstream of the 35S promoter was transformed into the *Agrobacterium tumefaciens* AGL1 strain. *Agrobacterium* incubation and infiltration for transient expression were performed as previously reported (Rolland, 2018). After 72 h of infiltration, substrates were fed by injecting 1 mM intermediate solutions (premenisdaurilide (**3**), (–)-virosine A (**6**), and (–)-virosine B (**7**)) into the AGL1-infiltrated leaf. All substrate solutions were prepared and used immediately before use. After 24 h of substrate feeding, the leaf was collected, and products were extracted as described above.

***F. suffruticosa* leaf disc assay**

Leaf discs (8 mm diameter) were excised from five-week-old *F. suffruticosa* plants and submerged in a feeding solution containing 1 mM of ¹³C-labeled compounds ((–)-virosine A (**6**), (–)-virosine B (**7**), and 4HPP (**2**)) or in control buffer (50 mM HEPES, pH 7.0) using 24 well plates. The plates were incubated in a plant growth chamber for 72 h under controlled temperature and light conditions (26°C, 16 h light / 8 h dark). After incubation, leaf discs were harvested and snap-frozen for extraction as described above.

Ground leaf discs were extracted with 1 mL of methanol, filtered, and diluted ten-fold for HPLC-MS/MS analysis.

Statistical analysis

To determine statistical significance, all data in this study were analyzed using GraphPad Prism (v.10.4.1) with one of the following statistical tests: one-way ANOVA followed by Tukey's HSD *post-hoc* test, Student's *t*-test, or Welch's *t*-test.

Declarations

Data availability

The sequencing datasets used during the current study will be released after acceptance.

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Author contributions

SH and S-GK conceived and supervised the project. SC, GK, TK, SK, SH, and S-GK wrote the manuscript. SC, GK, TK, HY, and YH performed biochemical experiments. GK and SK conducted chemical synthesis. SC, GK, TK, HY, and HL performed metabolite analysis. TK, HY, and HK conducted single-cell transcriptomic analysis. All authors reviewed the manuscript.

Competing interests

The authors declare no competing interests.

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Figures

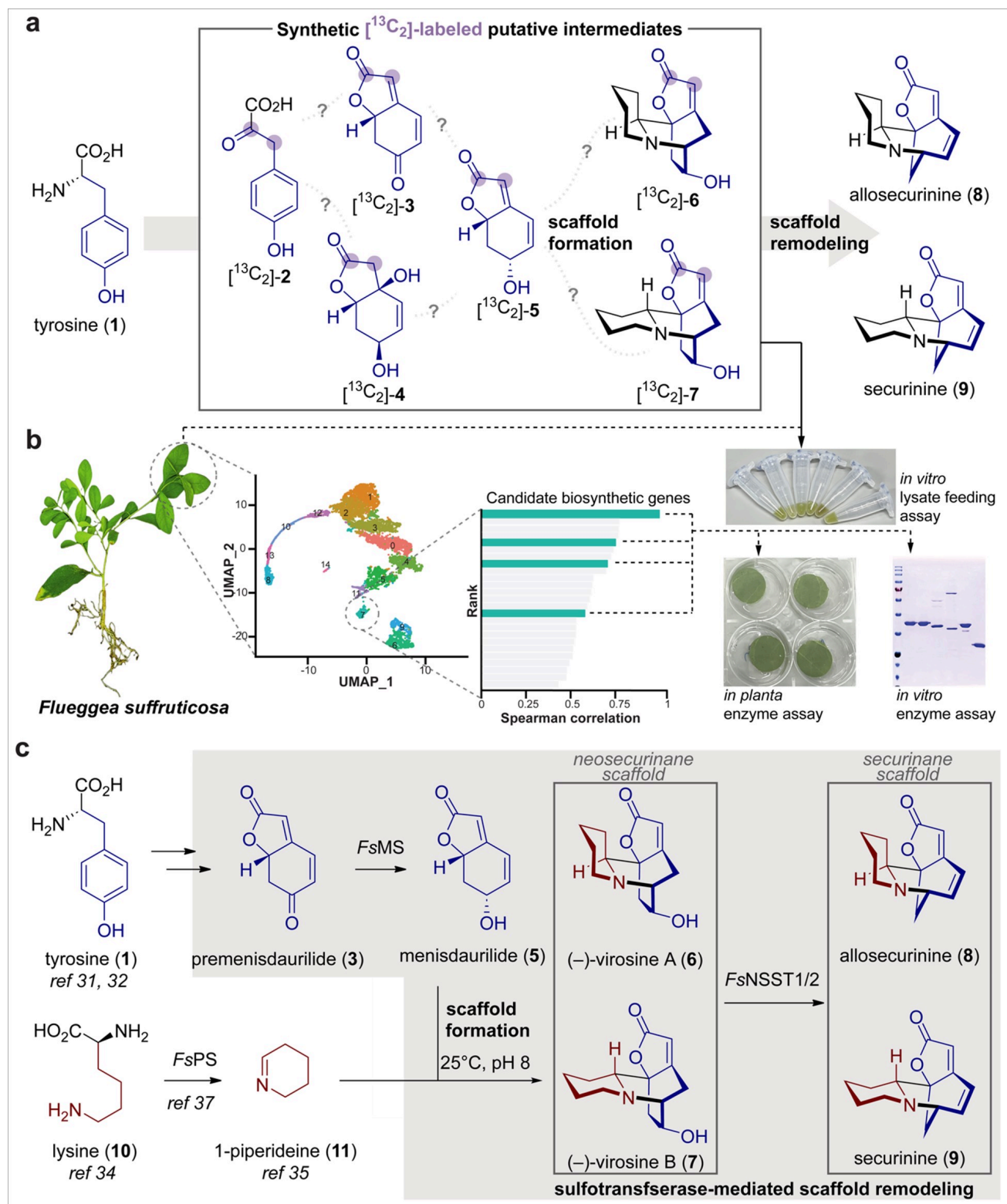


Figure 1

Schematic overview of the chemically guided single-cell transcriptomics strategy for elucidating SeA biosynthesis. **a** To elucidate L-tyrosine-derived biosynthetic intermediates of SeAs, hypothetical intermediates doubly labeled with ^{13}C were synthesized. **b** Single-cell transcriptomics and coexpression analysis were performed on *F. suffruticosa* leaves. Intermediates doubly labeled with ^{13}C were biochemically assessed, and candidate biosynthetic genes were functionally validated both *in vitro* and

in planta. **c** We discover the scaffold formation and remodeling of SeAs in *F. suffruticosa* (Grey box). *FsMS*, menisdaurilide synthase; *FsPS*, piperidine synthase; *FsNSST1/2*, neosecurinane sulfotransferase 1/2.

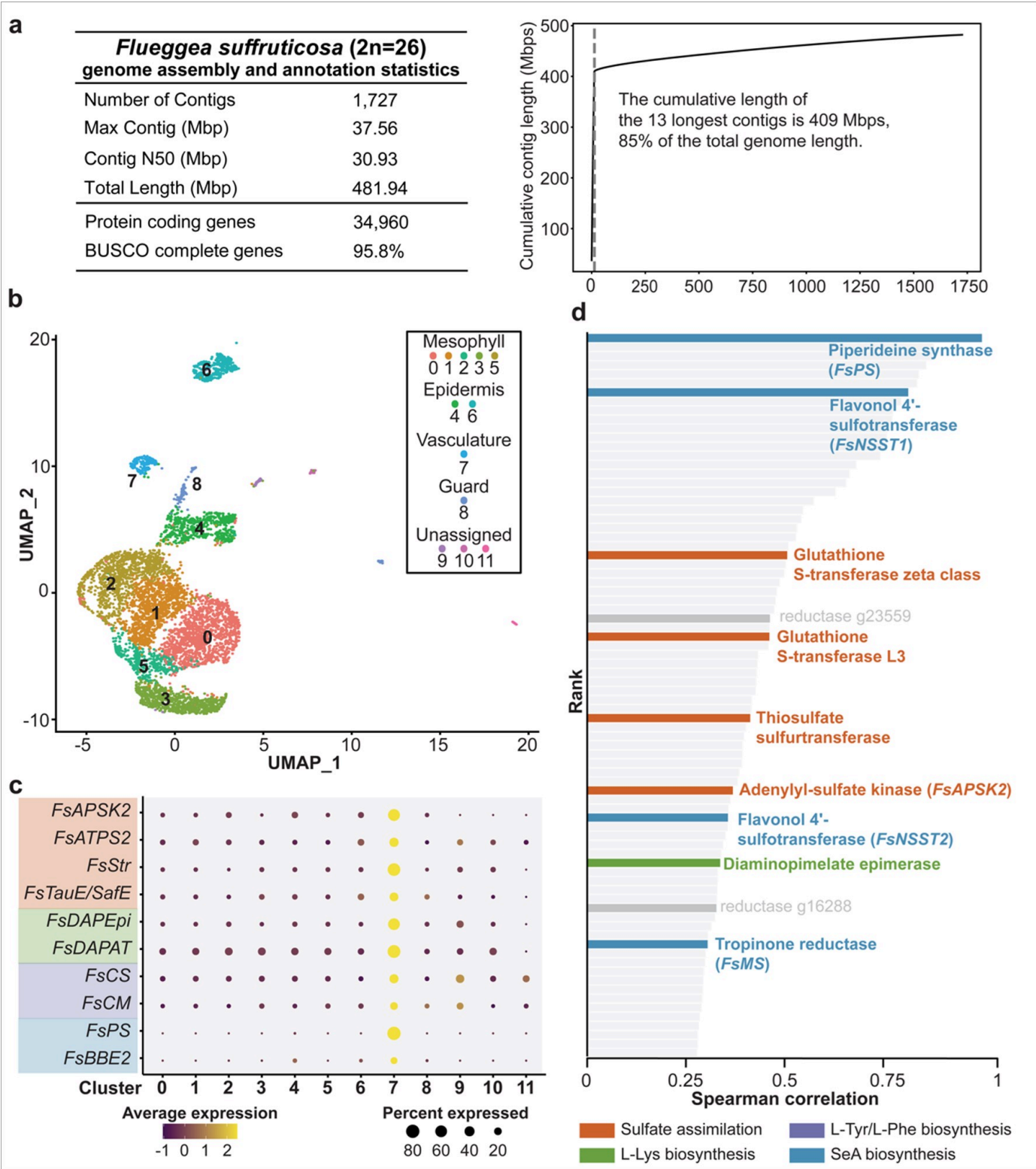


Figure 2

Genome assembly and single-cell transcriptome analysis of *F. suffruticosa*. **a** Statistics of *F. suffruticosa* genome assembly and gene annotation. **b** Uniform manifold approximation and projection (UMAP) plot

of cells after processing showed clusters classified into five major cell types. Cluster numbers were assigned in descending order based on the number of cells in each cluster. **c** Dot plot for genes involved in sulfate assimilation, L-Lys biosynthesis, L-Tyr/L-Phe biosynthesis, and SeA biosynthesis across cell clusters; they were highly enriched in clusters 7. *FsAPSK2*, *APS kinase 2*; *FsATPS2*, *ATP sulfurylase 2*; *FsStr*, *sulfite transferase*; *FsTauE/SafE*, *sulfite transporter*; *FsDAPEpi*, *diaminopimelate epimerase*; *FsDAPAT*, *diaminopimelate aminotransferase*; *FsCS*, *chorismate synthase*; *FsCM*, *chorismate mutase*; *FsPS*, *piperideine synthase*; *FsBBE2*, *berberine bridge-like enzyme 2*. **d** Co-expression analysis using *FsPS* as a bait gene. Genes are ranked based on their Spearman correlation to *FsPS*, up to rank 80. *FsPS*, *piperideine synthase*; *FsNSST1* and *2*, *neosecurinane sulfotransferase 1* and *2*; *FsMS*, *menisdaurilide synthase*. Genes related to the following biosynthetic pathways were color-coded: sulfate assimilation (red), L-Lys biosynthesis (green), L-Tyr/L-Phe biosynthesis (purple), and SeA biosynthesis (blue).

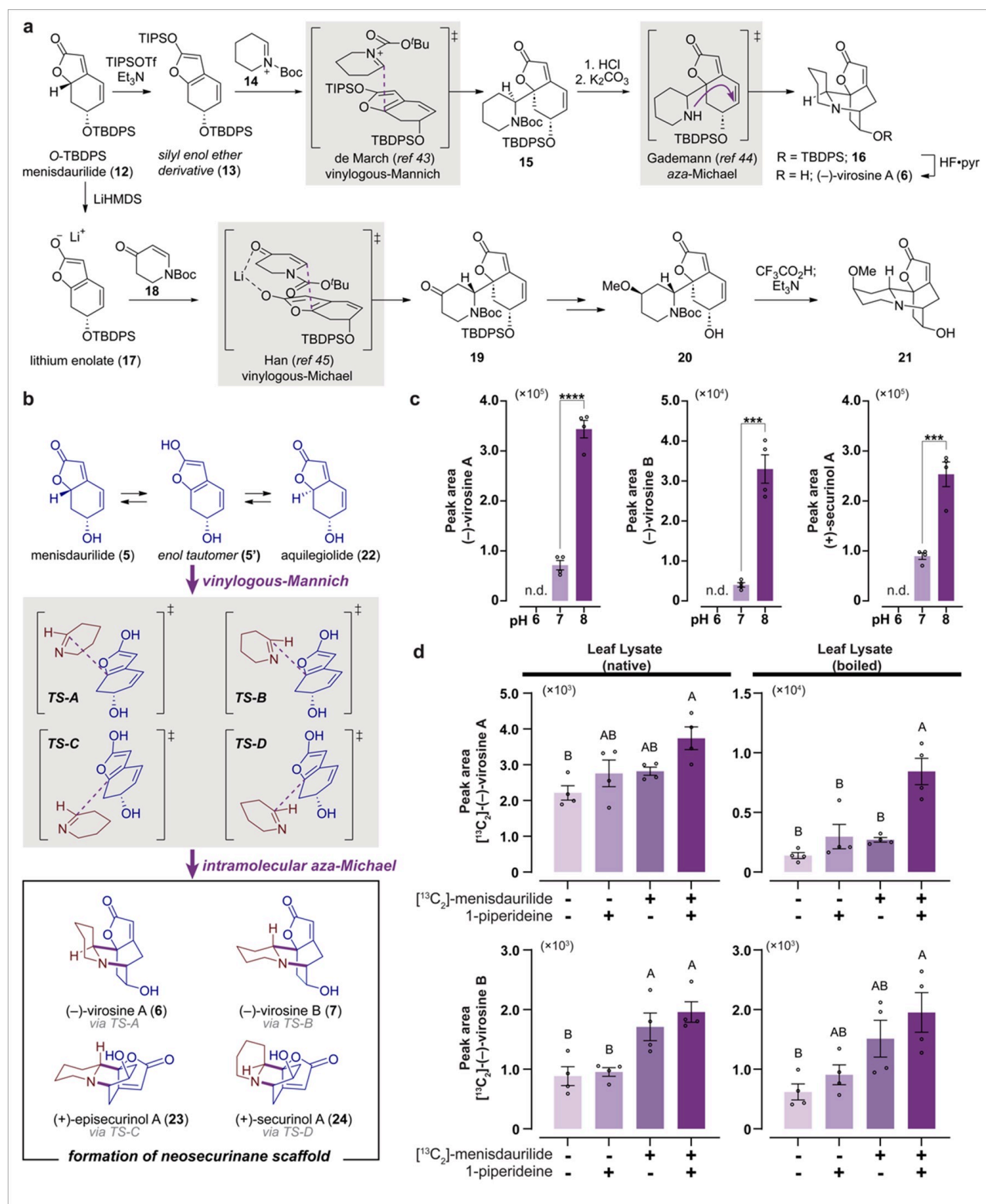


Figure 3

Nonenzymatic formation of neosecurinane scaffold in *F. suffruticosa* leaf lysate. **a** Chemical reactivity of menisdaurilide (**5**) and 1-piperidine (**11**) (TBDPS, *tert*-butyldiphenylsilyl; TIPS, triisopropylsilyl; Boc, *tert*-Butyloxycarbonyl). **b** Possible reaction pathways regarding the formation of neosecurinane scaffold from menisdaurilide (**5**) and 1-piperidine (**11**) (TS, transition state). **c** Reaction between menisdaurilide (**5**) and 1-piperidine (**11**) in aqueous buffer with varying pH levels ($N = 4$, mean $\pm$ SEM, Student's t -test, ***,

$P < 0.001$; ****, $P < 0.0001$). **d** [$^{13}\text{C}_2$]-menisdaurilide (**5**) was supplied with or without 1-piperidine (**11**) to native (left) or boiled (right) leaf lysate (pH 8). Produced [$^{13}\text{C}_2$]-virosine A ([$^{13}\text{C}_2$]-**6**) and [$^{13}\text{C}_2$]-virosine B ([$^{13}\text{C}_2$]-**7**) were measured ($N = 4$, mean $\pm$ SEM. One-way ANOVA, post-hoc Tukey's HSD). Letters indicate statistical differences among treatments.

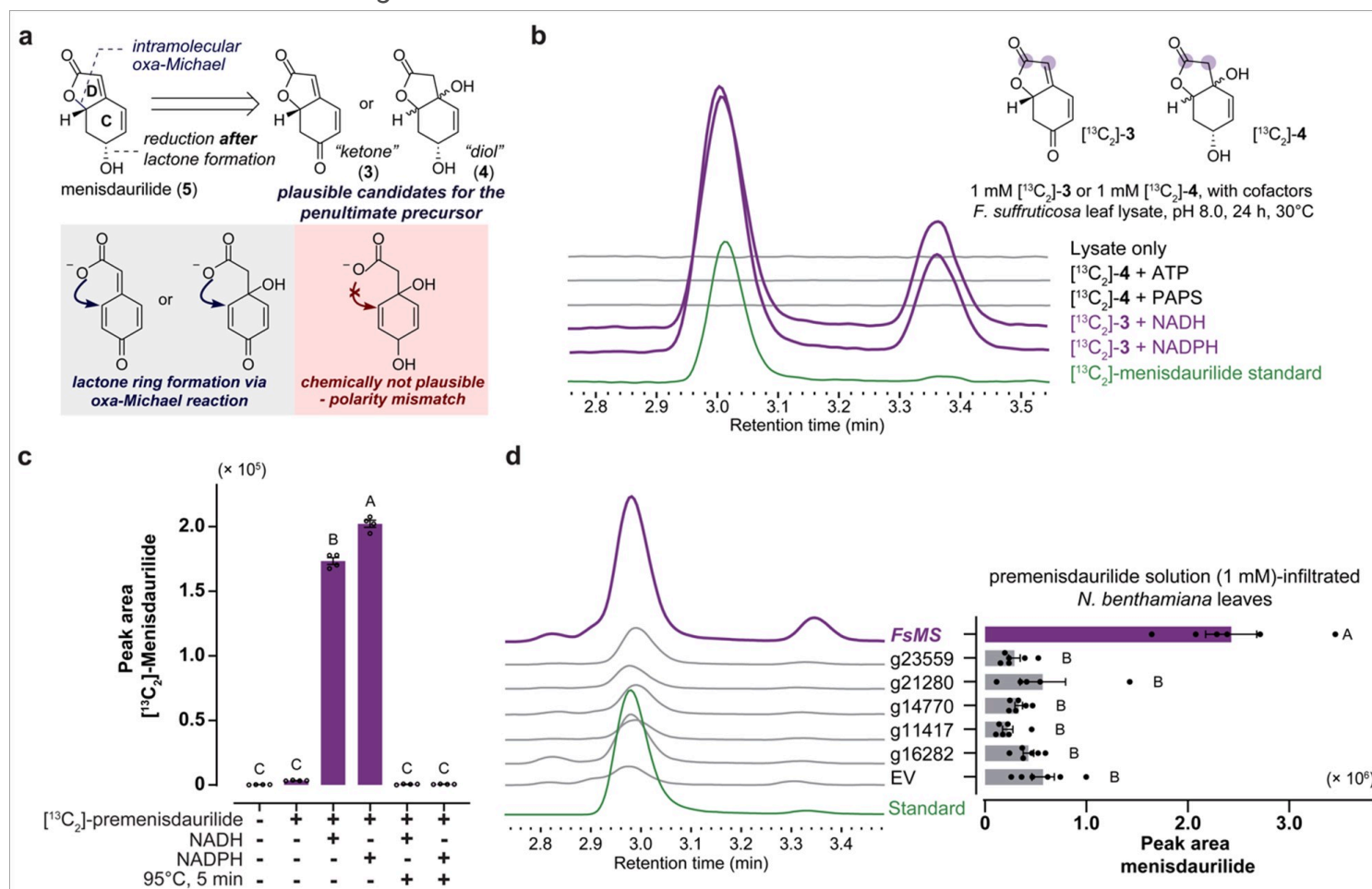


Figure 4

Discovery of premenisdaurilide and its biosynthetic enzyme. **a** Retrobiosynthetic perspective of the menisdaurilide (**5**) biosynthesis. Ketone intermediate (**3**) and diol intermediate (**4**) are shown. **b** Lysate feeding assay with cofactor supplements. [$^{13}\text{C}_2$]-**4** was supplemented with ATP or PAPS, while [$^{13}\text{C}_2$]-**3** was supplemented with reductants. PAPS, 3'-phosphoadenosine-5'-phosphosulfate. **c** [$^{13}\text{C}_2$]-premenisdaurilide ([$^{13}\text{C}_2$]-**3**) was converted to [$^{13}\text{C}_2$]-menisdaurilide ([$^{13}\text{C}_2$]-**5**) in crude lysate reductant dependently. [$^{13}\text{C}_2$]-menisdaurilide ([$^{13}\text{C}_2$]-**5**) peak area was measured ($N = 4$, mean $\pm$ SEM. One-way ANOVA, post-hoc Tukey's HSD). Letters indicate statistical difference among treatments). **d** Functional characterization of menisdaurilide synthase (*FsMS*) with other ketoreductase candidates in *N. benthamiana*. The HPLC-MS/MS chromatograms of menisdaurilide (**5**) are shown, and the peak area was measured 24 hours post-premenisdaurilide (**3**) supplement ($N = 5-6$, mean $\pm$ SEM. One-way ANOVA, post-hoc Tukey's HSD). Letters indicate statistical differences among agroinfiltrated genes. *FsMS*, *Flueggea suffruticosa* menisdaurilide synthase; EV, empty vector.

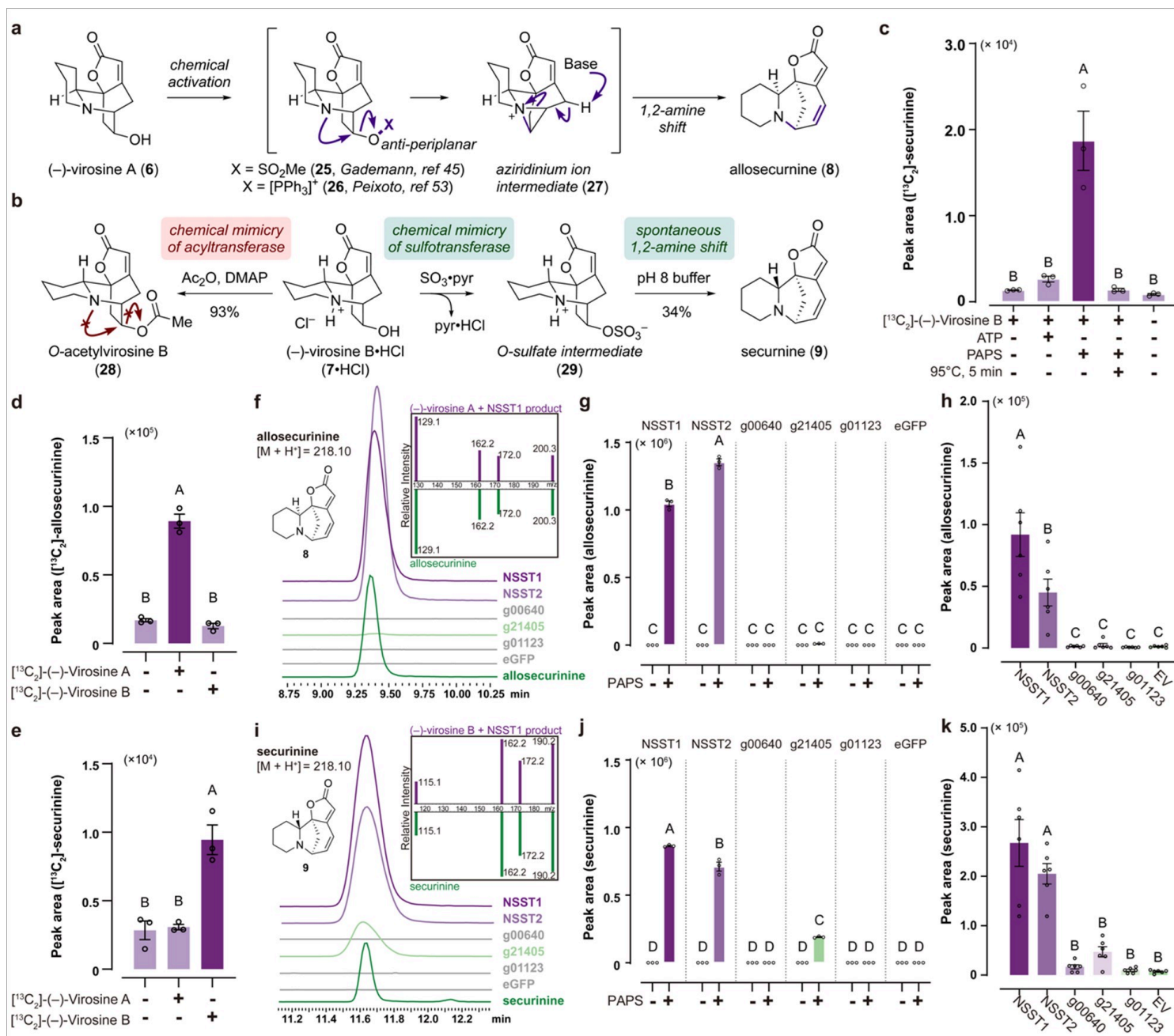


Figure 5

Neosecurinane sulfotransferases (*Fs*NSST1/2) mediate scaffold remodeling to produce the securinane scaffold. **a** Chemical activation of the hydroxyl group on the neosecurinane scaffold triggers its remodeling into the securinane scaffold. **b** Chemical mimicry of acyltransferase and sulfotransferase activity. **c** [¹³C₂]-(-)-viroside B ([¹³C₂]-**7**) was supplemented with either PAPS or ATP in pH 8.0 *F. suffruticosa* seedling lysates (*N* = 3, mean ± SEM. One-way ANOVA, *post-hoc* Tukey's HSD). **d, e** Leaf disc feeding assay of (-)-viroside A (**6**) (**d**) and (-)-viroside B (**7**) (**e**) (*N* = 3, mean ± SEM. One-way ANOVA, *post-hoc* Tukey's HSD). **f-k** Functional validation of *Fs*NSST1/2. HPLC-MS/MS chromatograms of *in vitro* enzyme assay products (**f, i**). Allosecurnine (**8**) or securinine (**9**) was observed only when PAPS was supplemented (**g, j**). Candidate sulfotransferases were transiently expressed in *N. benthamiana*, and

either (-)-virosine A (6) (h) or (-)-virosine B (7) (k) was supplemented. (*N* = 6, mean ± SEM. One-way ANOVA, *post-hoc* Tukey's HSD). Letters indicate statistical differences among treatments.

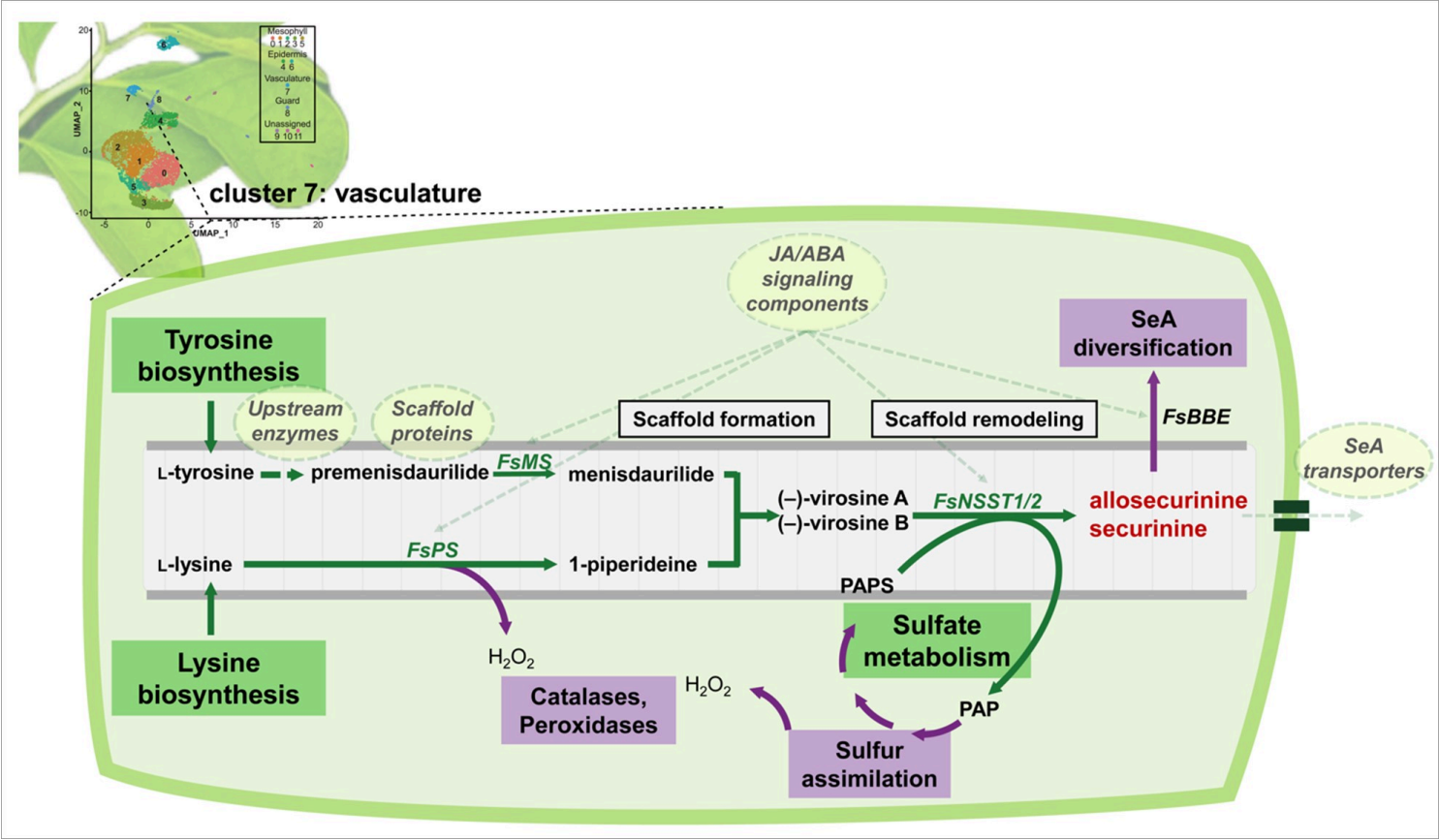


Figure 6

Schematic overview of SeA biosynthesis in *F. suffruticosa* leaves. Single-cell transcriptomic analysis identified cluster 7 as the SeA biosynthetic cell type. This cluster showed enrichment not only of core SeA biosynthetic genes (green boxes and arrows) but also of auxiliary genes (purple boxes and arrows) expected to facilitate SeA biosynthesis. The single-cell transcriptomic data may support the discovery of additional SeA-associated genes, including upstream biosynthetic genes, transporters, and regulators involved in phytohormone-responsive SeA production (circles).

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