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Exploring the Role of Plant Carbonic Anhydrase-like Enzymes in the Synthesis of Neuroactive Alkaloids

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The paper titled "Role of Plant Carbonic Anhydrase-Like Enzymes in Neuroactive Alkaloid Biosynthesis" published in the journal Nature on November 8, 2023, by authors Ryan S. Nett, Yaereen Dho, Chun Tsai, among others, from the Department of Chemical Engineering at Stanford University, the Howard Hughes Medical Institute, and the Department of Molecular and Cellular Biology at Harvard University, etc. This study focuses on a class of plant carbonic anhydrase-like enzymes (CALs) that play a key role in the biosynthesis of neuroactive alkaloids in gymnosperms. Through in-depth analysis of the biosynthetic pathways in ferns, the research team revealed how these CAL proteins participate in the generation of neuroactive gymnosperm alkaloids, such as huperzine A, through a series of complex enzyme-catalyzed reactions. This work not only expands our understanding of plant-specific metabolic pathways but also provides a new perspective for the future discovery and development of plant alkaloid-based medications.

1 Experimental Data Analysis

The CAL proteins identified in the study participate in the formation of polycyclic structures by catalyzing a series of Mannich-type condensation reactions. The experimental data show that this process is crucial for the generation of precursors to alkaloids like huperzine A. Furthermore, through transcriptomic and co-expression analyses, the research team successfully identified genes highly correlated with the expression patterns of these CAL proteins in ferns, providing important clues for further understanding the functions and regulatory mechanisms of CAL proteins.

Figure 1 presents the study of unknown steps in lycopodium alkaloid biosynthesis in lycophyte plants. Figure 1a describes a series of unknown chemical transformations that convert early precursors into a diversified lycopodium alkaloid scaffold. The red and blue structures represent visual representations of the main structural categories of lycopodium alkaloids, not actual compounds found in nature. The box displays representative lycopodium alkaloids, including the cholinesterase inhibitor HupA. Figure 1b illustrates the identification process of new biosynthetic enzyme candidates guided by transcriptomic information, where c.p.m. stands for counts per million. This demonstrates the interdisciplinary approach adopted in studying the alkaloid synthesis mechanisms in lycophyte plants, incorporating both chemical and bioinformatics methods.

Figure 2 illustrates the stepwise discovery of enzymes contributing to compound skeleton formation in the early biosynthetic pathway. The extracted ion chromatograms (EIC) for the assumed intermediates' corresponding m/z values were revealed by transiently co-expressing candidate biosynthetic genes from P. tetrastichus in N. benthamiana (indicated by blue boxes). Generally, compounds appear in the form of [M+H]+ ions. For compound 9, the originating fragment m/z 164.1434 ([M-C5H9N+H]+) served as the primary detection ion, thus used as the diagnostic marker for this compound. All compounds were detected using LC-MS with an HILIC column, except for the enantiomer of 6, which was observed using a C18 column. Note that the y-axis for each set of chromatograms is on different scales, but the scale within an EIC chart is constant. The black arrows indicate the

substrate consumption observed after the addition of CAL-1 and CAL-2. The properties of compounds 4-6 were confirmed by comparison with synthesized or commercially available standards. The structure of compound 8 was proposed based on MS2 and UV analysis (Supplementary Data Figure 2). The structure of compound 9 was proposed based on MS2, UV analysis, NMR of partially purified 9, and MS2 and complete NMR of the oxidation by-product.

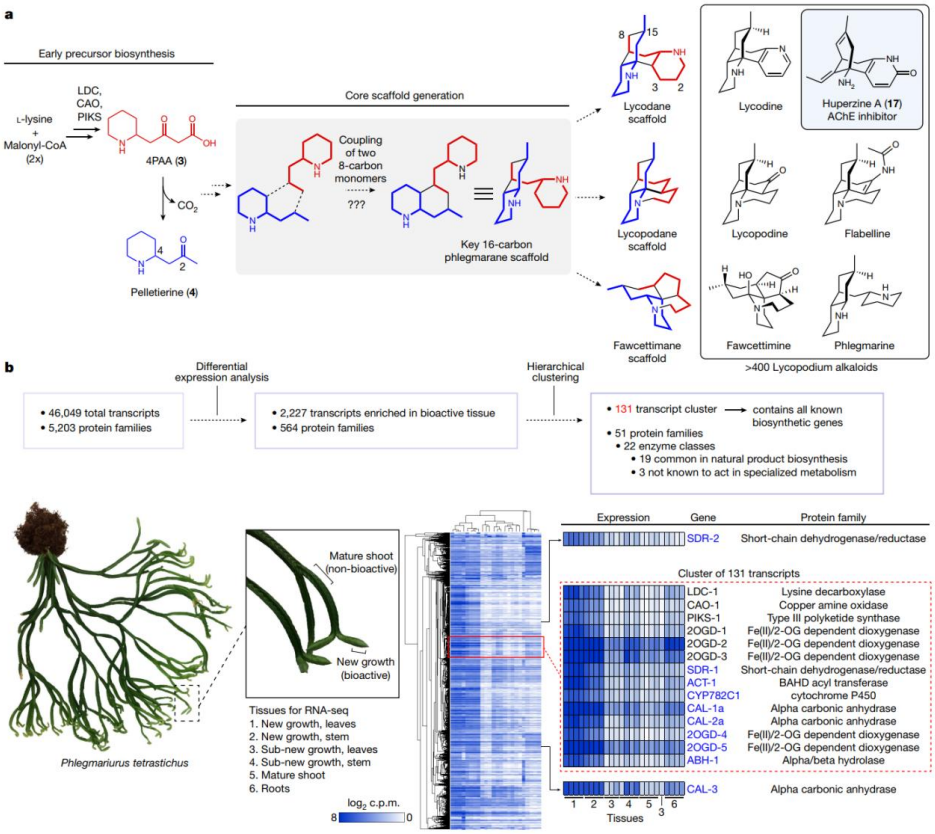

Figure 1 Assessing unknown steps in Lycopodium alkaloid biosynthesis

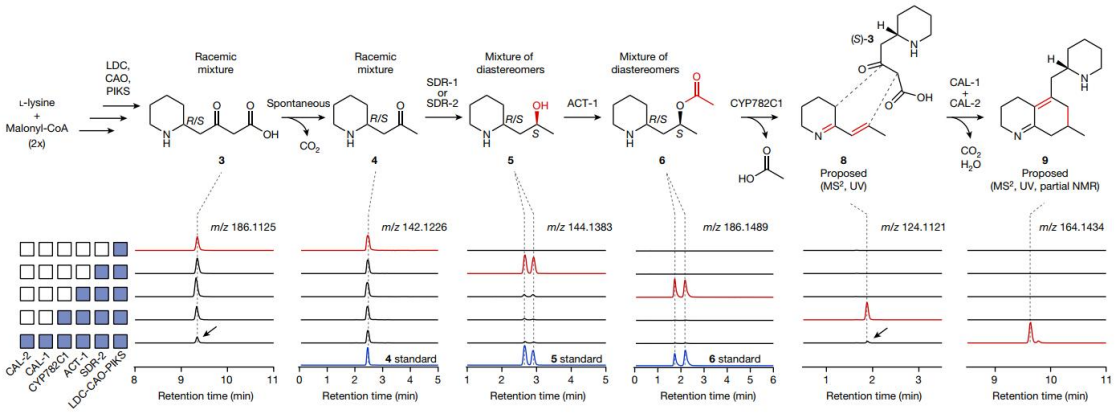

Figure 2 Stepwise discovery of early biosynthetic enzymes contributing to scaffold formation

Figure 3 demonstrates the roles and studies of newly functionalized CAL enzymes in the biosynthesis of lycopodium alkaloids. Figure 3a proposes the mechanism for the synthesis of compound 9 catalyzed by PtCAL-1a/PtCAL-2a. Figure 3b shows a representative Western Blot of 6xHis-tagged CALs expressed alone or co-expressed with untagged gene constructs in N. benthamiana. As an intracellular protein control, a 6xHis-tagged LcLDC construct is included. The experiment was conducted more than three times, with similar results observed. Figure 3c displays the EICs for 9 (m/z 164.1434) produced by extracellular PtCAL-1a and PtCAL-2a (either alone or co-expressed) with different substrate combinations. Figure 3d shows the dynamics of compound 9 production

by extracellular PtCAL-1a/PtCAL-2a compared to the GFP extracellular control over time. Each condition was tested in three replicates. Figure 3e assesses which enantiomer of 3 is used as a substrate in the formation of 9. Since the enantiomers of 3 cannot be directly observed, the chirality is inferred by measuring the enantiomer of 4 formed through the spontaneous decarboxylation of 3. The enantiomers were analyzed as N-acetylated derivatives, with the average ratio shown above each bar graph. Each condition was tested in three replicates. Figure 3f proposes the condensation reaction of 1 and 2 catalyzed by PtCAL-3, producing (S)-3. Figure 3g shows the co-expression of PtCAL-3 with the rest of the pathway required for the production of 9. Three leaves were infiltrated under each condition. Figure 3h displays the EICs for 3 (m/z 186.1125) produced by extracellular PtCAL-3 with different substrates. Figure 3i compares the formation of 3 by extracellular PtCAL-3 with 1 and 2 as substrates over time against the GFP extracellular control. Each condition was tested in three replicates. Figure 3j evaluates the enantiospecific formation of PtCAL-3 products by analyzing the enantiomers of 4 (after N-acetylation) through chiral LC-MS. Figure 3k presents the ratio of (S)-4 to (R)-4 over time in the extracellular PtCAL-3 reaction.

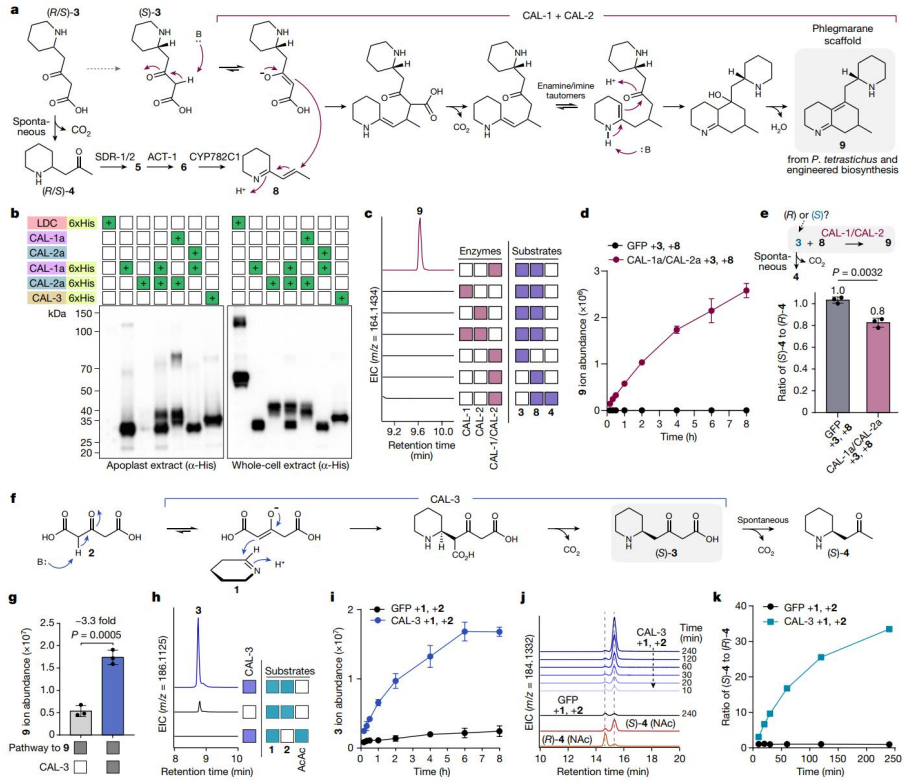

Figure 3 Neofunctionalized CAL enzymes in Lycopodium alkaloid biosynthesis

Figure 4 highlights the crucial role of CAL enzymes in the early biosynthesis of lycopodium alkaloids. Figure 4a presents the biosynthetic proposal for the early chemical transformations of lycopodium alkaloids, noting that the transmembrane transport of intermediates is speculative. Figure 4b is based on an evolutionary tree of CAH family proteins across multiple biological kingdoms (aligned using MUSCLE, with tree construction via the neighbor-joining method), where the bootstrap values (from 100 replicates) indicate support. The key active site residues of each aligned protein are also displayed, with numbering corresponding to human carbonic anhydrase 2 (HsCA2, UniProt ID: P00918). Changes in canonical/conserved sequences are highlighted with colored boxes. Stars mark proteins that have been validated to possess typical CAH activity. A more extensive alignment/evolutionary tree can be found in Supplementary Figure 2. This reflects the evolutionary and functional diversification of CAL enzymes in the biosynthesis of lycopodium alkaloids and their evolutionary journey across different forms of life.

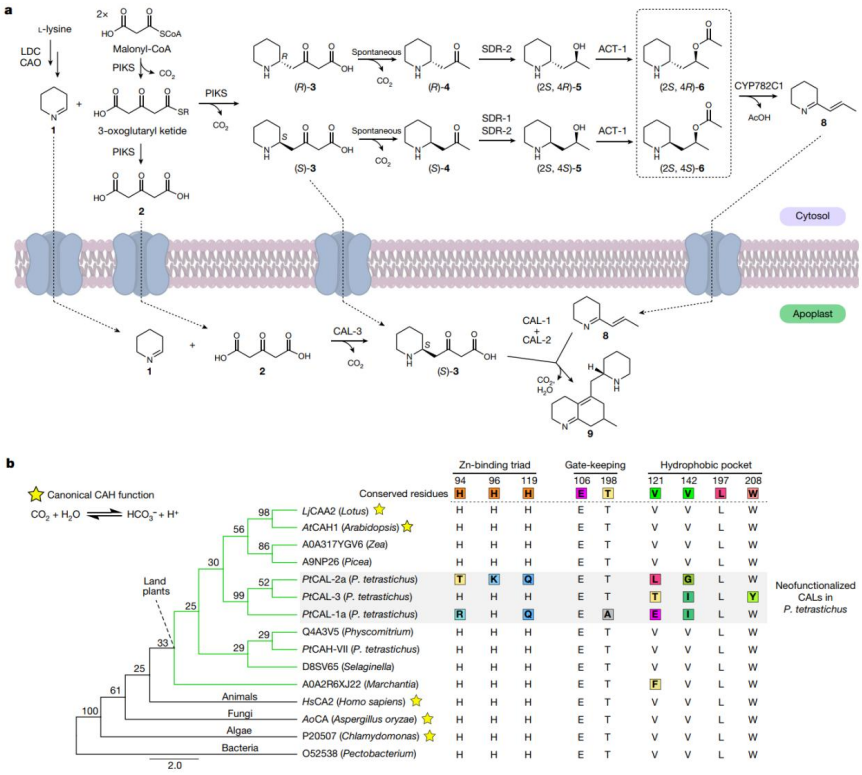

Figure 4 A prominent role for CAL enzymes in early Lycopodium alkaloid biosynthesis

Figure 5 depicts the metabolic network for the generation of the optimized acetylcholinesterase (AChE) inhibitor HupA (compound 17). Enzymes that are newly emerged or previously described enzymes with new reactions are marked in purple. All lycopodium alkaloids with generic names have been validated through real standard substances. The figure also displays the IC50 values of lycopodium alkaloids inhibiting AChE. References for these values can be found in the methods section. The stereochemistry of the methyl in compound 9 is based on the typical stereochemistry observed in isolated lycopodium alkaloids.

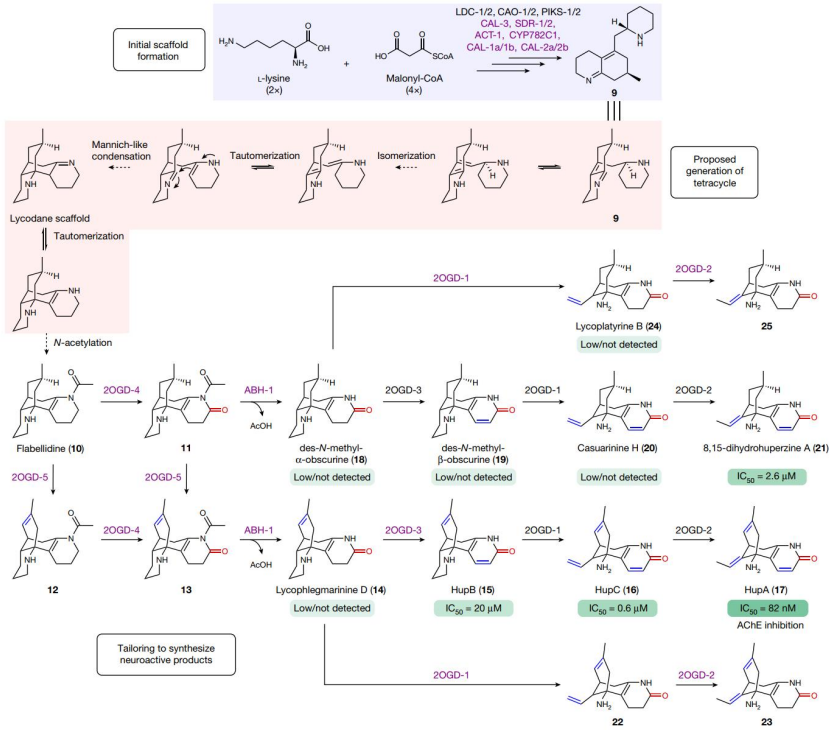

Figure 5 A metabolic network for the generation of an optimized AChE inhibitor, HupA (17)

2 Analysis of Research Findings

The results of this study not only unveil a new role of CAL proteins in the biosynthesis of gymnosperm alkaloids but also demonstrate how plants utilize such enzymes to construct complex bioactive molecules through comparative genomics and functional genomics approaches. The research also suggests that the evolution of these CAL proteins may be associated with an enhancement in plant adaptability to the environment, indicating how plants expand their chemical diversity through gene duplication and functional diversification during evolution.

3 Evaluation of the Research

This research offers a new perspective on the role of CAL proteins in plant-specific metabolic pathways by employing a comprehensive approach that includes biochemistry, molecular biology, transcriptomics, and metabolomics. These findings are not only of significant scientific importance to the field of plant biology but also provide valuable resources for the discovery and development of plant alkaloid-based medications.

4 Conclusions

By delving into the role of plant carbonic anhydrase-like enzymes in the biosynthesis of neuroactive gymnosperm alkaloids, this study not only broadens our understanding of plant metabolic diversity but also offers new strategies for future plant genetic engineering and drug development.

5 Access the Full Text

Nett, R.S., Dho, Y., Tsai, C. et al. Plant carbonic anhydrase-like enzymes in neuroactive alkaloid biosynthesis. Nature 624, 182–191 (2023). https://doi.org/10.1038/s41586-023-06716-y.

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