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Recruitment of Sugar Transport and Scent Volatile Genes for Prey Attraction in the Nectar Spoon of *Heliamphora tatei*

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ABSTRACT

Prey attraction is an integral component of the carnivorous syndrome, yet its molecular adaptations have remained largely unexplored. Our study utilized tissue-specific transcriptomic data from the South American marsh pitcher plant, *Heliamphora tatei*, to explore the molecular and developmental basis of prey attraction. Carnivorous plants often present specialized structures associated with prey attraction and in *Heliamphora*, that function is carried out by the nectar spoon, a colorful extension of the top of the pitcher that is densely covered in nectaries. Through comparisons of gene expression in the nectar spoon with the rest of the pitcher, we identified a suite of differentially expressed genes that likely contribute to prey attraction, including enzymes involved in volatile synthesis and sugar transporters. We found that one lineage of sugar transporters, the 14a clade of *SWEET*s (Sugars Will Eventually Be Exported Transporters), is highly upregulated in the nectar spoon and has evolved more rapidly in Sarraceniaceae, consistent with specialization for nectar transport as part of prey attraction. Among the genes related to volatile production, we found several enzymes best known for their role in floral scent. These results suggest that, similar to prey digestion, ancient genes are repurposed for novel functions during the transition to carnivory and may facilitate the repeated convergent origins of carnivory across angiosperms.

1 | Introduction

Carnivorous plants have evolved to thrive in nutrient-poor environments by capturing and digesting animal prey to obtain additional nitrogen and phosphorus (Ellison and Adamec 2018; Givnish et al. 2018). Among carnivorous plants, pitcher plants present an exceptional case of phenotypic convergence across unrelated lineages (Fleischmann et al. 2018). Pitcher plants have independently evolved in at least six different lineages across five plant families, including *Nepenthes* (Nepenthaceae, Caryophyllales), Sarraceniaceae (Ericales), and *Cephalotus follicularis* (Cephalotaceae, Oxalidales) (Fleischmann et al. 2018). These lineages, known as true pitcher plants, present remarkably similar trap morphologies despite their independent origins (Thorogood et al. 2018). Derived from modified leaves, these specialized pitfall traps not only capture and digest prey but also employ various signals such as scent, color, and pattern to attract insect visitors (Cross et al. 2018; Clarke et al. 2018; Naczi 2018; Bonhomme et al. 2011). This suite of adaptations (prey attraction, capture, and digestion) is referred to as the carnivorous syndrome (Ellison and Adamec 2018; Juniper et al. 1989; Sinnott-Armstrong et al. 2022).

Recent genomic and transcriptomic studies have highlighted numerous convergent molecular and developmental adaptations related to prey digestion in pitcher plants (de Vries and de

Summary

- Tissue-specific transcriptomics reveal genes underlying prey attraction in *Heliamphora tatei*.
- *SWEET14a* transporters are upregulated and rapidly evolving, suggesting specialization for sugar transport for nectar production.
- Ancient genes are repurposed for novel functions, enabling convergent evolution of carnivory.

Vries 2020; Freund et al. 2022; Matušíková et al. 2018; Renner et al. 2018; Rottloff et al. 2016). For example, in *Cephalotus follicularis*, *Sarracenia purpurea*, and *Nepenthes* species, several digestive enzymes, including chitinases, proteases, and β−1,3-glucanases, are expressed and secreted into the carnivorous pitchers to facilitate prey digestion (Renner and Specht 2012; Fukushima 2017; Yilamujiang et al. 2016; Wan Zakaria et al. 2018; Buch et al. 2015). These enzymes exhibit molecular specialization for prey digestion, characterized by convergent amino acid substitutions, which improve enzymatic activities within the unique physiological environment of pitcher fluids (Freund et al. 2022; Fukushima 2017; Senevirathna et al. 2019). Additionally, mutations in amino acid coding sequences likely facilitate the extracellular secretion of these digestive enzymes into the pitcher fluids (Renner and Specht 2012), and gene expression, as well as enzyme secretion, is induced upon prey capture (Buch et al. 2015; Luciano and Newell 2017; Saganová et al. 2018). Interestingly, the genes encoding these digestive enzymes are not unique to pitcher plants but ubiquitous in all flowering plants, where they play roles in plant defense against pathogens and herbivory (de Vries and de Vries 2020; Freund et al. 2022; Matušíková et al. 2018; Renner et al. 2018). This suggests that the digestive enzymes secreted by pitcher plants were co-opted or repurposed from ancestral gene copies associated with pathogenesis (de Vries and de Vries 2020; Freund et al. 2022; Matušíková et al. 2018; Renner et al. 2018). While much is known about the molecular adaptation as well as convergence associated with prey digestion, the mechanisms underlying prey attraction remain largely unexplored (Mithöfer 2022).

Nectar production plays a major role in luring insects to pitcher plants, enticing them to explore the pitcher long enough to fall into the trap (Płachno 2007; Dávila-Lara et al. 2020). Accordingly, the extrafloral nectaries (EFNs) that produce the nectar are typically concentrated around the opening (the peristome) and the lid (e.g., the nectar spoon of *Heliamphora tatei*, Figure 1A), although they can also be found along highly pigmented nectar guides on the outer pitcher surface (Płachno 2007). In addition to their importance in prey attraction (Di Giusto et al. 2008), EFNs are widespread in non-carnivorous plants, where they function in plant defense by attracting invertebrate predators to reduce herbivory (Weber and Keeler 2012; Marazzi et al. 2013). Beyond the nectar secreted by EFNs, pitchers also produce volatile organic compounds (VOCs) to signal to prey and amino acids as an additional source of nutrition (Dávila-Lara et al. 2020; Dress et al. 1997; Plachno et al. 2007; Wells et al. 2011). The production of nectar, scent, and color in pitcher plants suggests potential evolutionary links among mechanisms used by carnivorous plants for prey attraction, non-carnivorous plants for defense, and flowers for pollination.

The mechanisms underlying prey attraction in carnivorous plants have only begun to be explored, but as with prey digestion, molecular convergence appears to be an emerging theme. For instance, the EFN-rich rim of the Venus flytrap (*Dionaea muscipula*) and the EFN-covered pitchers of *Cephalotis*

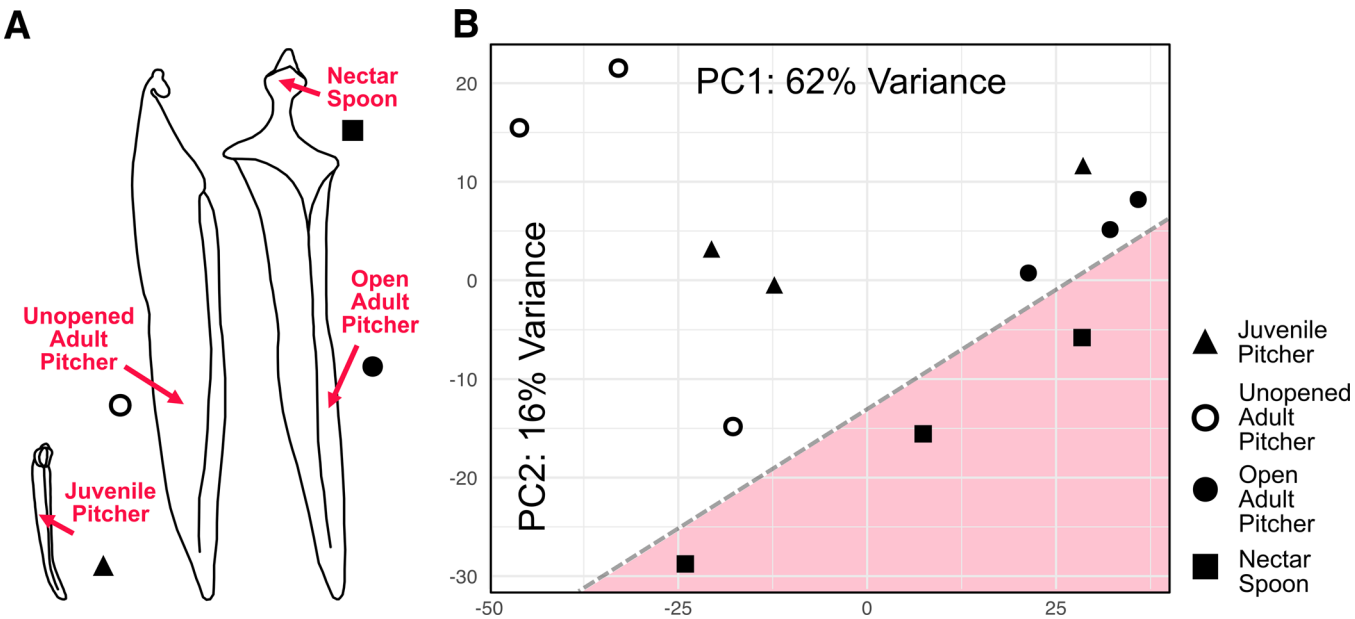

FIGURE 1 | A. Drawing of the open juvenile, unopened (developing) adult, and open adult pitchers of *H. tatei* sampled in this study. For pitcher tissue samples, a 2 mm × 4 mm section was collected from the pitcher body at the location indicated by the red arrow (see detailed tissue sampling information in Appendix J). For the nectar spoon, the nectar chamber (concentrated with EFNs) was sampled from the open adult pitcher, as indicated by the red arrow. B. PCA plot of the expression profiles of *H. tatei* pitcher tissues. The pink background was added to visually distinguish the expression profiles of nectar spoon and pitcher tissues along the PC1 and PC2 axes.

follicularis and *Nepenthes* × *ventrata* exhibit upregulation several classes of sugar-related genes, including sucrose synthase, sucrose phosphate synthase, and the *SWEET*s (Sugars Will Eventually Be Exported Transporters) (Fukushima 2017; Filyushin et al. 2018; Shchennikova et al. 2021; Palfalvi et al. 2020). These genes are part of a widely conserved network responsible for sugar synthesis and transport in other organs (e.g., floral nectaries) and other taxa (Julius et al. 2017; Hu et al. 2020; Chatt et al. 2021; Kaur et al. 2021), consistent with the notion that carnivory commonly involves existing toolkits (Fukushima 2017). Moreover, as VOCs represent another major component of prey attractants emitted by carnivorous plants, it is likely that genes involved in the biosynthesis of various VOCs were also convergently recruited for carnivory. Indeed, genes associated with terpenoid synthesis are significantly expanded in the Venus flytrap and its relatives (Droseraceae), and while the genes have not been identified, terpenoid VOCs are important for prey attraction in pitcher plants as well (Di Giusto et al. 2010). Furthermore, many pitcher plants secrete toxic compounds in their EFNs, which help immobilize prey and facilitate capture (Lathika et al. 2025; Mody et al. 1976).

To investigate the mechanisms involved prey attraction in carnivorous plants and potential convergence at the molecular and developmental levels, we generated tissue-specific transcriptomic data from the South American marsh pitcher plant, *H. tatei* (Figure 1). The genus *Heliamphora* (Sarraceniaceae), represents one of the most species-rich lineages of pitcher plants where the molecular adaptations associated with carnivory need further elucidation. This clade is endemic to the Guiana Highlands of Venezuela and comprises 24 described species and several yet to be described taxa (>70% of the entire family Sarraceniaceae) (Golos et al. 2024; McPherson 2011). *H. tatei* is a member of the western clade of *Heliamphora* (Liu and Smith 2021) and presents similar pitcher morphology (Liu and Smith 2023) and ecology (McPherson 2011) as other members of the genus, with an erect pitcher and a well-developed reddish nectar spoon (Figure 1A). Its carnivorous functions, such as prey digestion and attraction, have been previously characterized both in the lab and in the field (Jaffé et al. 1992; Jaffé et al. 1995). These studies have established the ability of *H. tatei* to secrete proteolytic enzymes and digest a range of insect prey, from ants to flies (Jaffé et al. 1992). Gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) analyses of the *H. tatei* secretions from its nectar spoons revealed that, in addition to sugars, various VOCs, such as sarracenin (a monoterpene), xylene, alkanes, and phenylacetaldehyde, are secreted as prey attractants (Jaffé et al. 1995).

Through gene expression profiling, phylogenetic comparisons, and molecular evolution analyses, we test several predictions regarding the mechanisms of prey attraction in *Heliamphora*. First, we compare gene expression between the EFN-rich nectar spoon and multiple pitcher stages to identify significantly differentially expressed genes in the spoon (Figure 1). We predict that these will include genes associated with rewards and attractants, such as sugars and VOCs. Next, we examine patterns of co-expression for these differentially expressed genes to determine if they are coregulated and identify possible regulators. As the results from these two sets of analyses indicated a central role for *SWEET* sugar transporters, we documented their diversity in *Heliamphora* and tested for changes in selection associated with carnivory within one clade of *SWEET*s (14a) showing elevated expression in the nectar spoon. Our findings suggest that *SWEET14a* may be evolving more rapidly in *Heliamphora* and its sister genus *Sarracenia*, consistent with the notion that this gene copy has undergone functional specialization. We discuss these findings in the context of the growing body of literature on prey attraction in carnivorous plants, suggesting that molecular convergence may extend beyond prey digestion to other aspects of the carnivorous syndrome.

2 | Materials and Methods

2.1 | Transcriptome Assembly and Annotation

The search for candidate genes for prey attraction was carried out in transcriptomes of *Heliamphora tatei*, grown under controlled environmental conditions (photoperiod 15 h per day; temperature range 12°C–24; relative humidity >80%) inside growth chambers equipped with artificial lights and temperature/humidity regulating units at the University of Colorado Boulder before RNA extraction. All *Heliamphora* species, including *H. tatei* produce two pitcher forms, the small juvenile pitchers that are still capable of prey and nutrient absorption but lack a nectar spoon, and the larger adult pitchers, which at maturity possess a nectar spoon. We sampled three biological replicates for four developmental stages/tissues (Figure 1A, Appendix J): (1) the pitcher body of open juvenile pitchers, (2) the pitcher body of unopened adult pitchers, (3) the pitcher body of open adult pitchers, and (4) the nectar spoon of open adult pitchers. In juvenile pitchers, smaller extrafloral nectaries are found sparsely on the inner pitcher wall, and in adult pitchers, extrafloral nectaries are found on both the interior and exterior (Płachno 2007; Plachno et al. 2007; McPherson 2011). However, EFNs are most abundant in the nectar spoon, where they also appear enlarged relative to those on the body (Płachno 2007; Plachno et al. 2007; McPherson 2011).

Fresh tissue samples were flash frozen with liquid nitrogen and shipped to GENEWIZ, USA for RNA extraction and sequencing. RNA extraction, at GENEWIZ, was performed using Plant RNA Isolation Aid (Thermo Fisher Scientific) and the RNeasy Plant Mini Kit (Qiagen, USA). The quantity and integrity of the sample RNA were analyzed by TapeStation (Agilent Technologies). RNASeq libraries were sequenced using Illumina HiSeq (GENWIZ) to generate 150 bp paired-end reads. The quality of raw reads was first assessed using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc), and adapter contamination was removed using Trimmomatic v0.38 (Bolger et al. 2014). All post-processed paired-end reads were pooled together and assembled *de novo* with Trinity v2.8.5 (Haas et al. 2013) under default parameters. The completeness of the transcriptome assembly was then assessed by Benchmarking Universal Single-Copy Orthologs (BUSCO) v2.0 using the "Eudicotyledons odb10" data set (Mosè Manni et al. 2021). Coding regions within the assembled transcripts were identified using the TransDecoder program (https://github.com/TransDecoder/TransDecoder). The complete cds were then extracted from the output of TransDecoder. To annotate the coding sequences,

BLASTx was performed against the complete protein coding sequences of *Solanum lycopersicum* and *Arabidopsis thaliana* from Swiss-Prot and TrEMBL (Bairoch 2000). In downstream weighted gene co-expression network analysis (WGCNA), candidate genes and genes of interests were further BLASTed against the nucleotide collection (nt) database via Blast2GO (Conesa et al. 2005) to improve annotation quality and perform Gene Ontology (GO) enrichment analysis.

2.2 | Differential Expression (DE) Analysis between Nectar Spoon and Pitcher Tissues

To quantify the expression of protein-coding genes, each sample read was mapped to the filtered transcriptome using Salmon v0.13.1 (Patro et al. 2017). Salmon transcript quantification files were then imported into the R environment v4.3.2 using the tximport package v1.30.0 (Soneson et al. 2015). DE analysis was conducted using the DESeq. 2 package v1.42.0 (Love et al. 2014), which performs read normalization by default. Using the "vst" function implemented in DESeq. 2, principal components analysis (PCA) (Li et al. 2024) was performed for all samples to explore the relationships between nectar spoon and pitcher tissues. To identify candidate genes differentially expressed in the nectar spoon, we performed three gene expression comparisons: between the nectar spoon ($n = 3$) and the juvenile pitcher ($n = 3$), between the nectar spoon and the unopened adult pitcher ($n = 3$), and between the nectar spoon and the open adult pitcher ($n = 3$). For each comparison, significantly differentially expressed transcripts were identified with a false discovery rate adjusted p-value ≤ 0.05 and a fold change ≥ 8 (Appendix A).

2.3 | WGCNA of Gene Module Associated With Nectar Reward Biosynthesis and Secretion

Normalized counts of the significantly differentially expressed transcripts ($n = 2330$) were then used as input for WGCNA to identify modules of co-expressed genes (Langfelder and Horvath 2008), as we predict that regulators of candidate genes associated with nectar secretion in the nectar spoon would potentially regulate other prey attractant biosynthesis genes. WGCNA computes pairwise Pearson's correlation coefficients, which are then converted to an adjacency matrix with the raw values raised to a soft-thresholding power (β) to approximate a scale-free network (Langfelder and Horvath 2008). For our data, we selected a β of 12, which corresponds to an R^2 value of 0.57 with the scale-free model and a mean connectivity of 134 (Appendix B).

We also used WGCNA to calculate the correlation of each module eigengene with each tissue type (i.e., nectar spoon, juvenile pitcher, unopened adult pitcher, and open adult pitcher). Next, we conducted Gene Ontology (GO) enrichment analysis to determine if the genes within highly correlated modules share similar biological functions. Short alternatively spliced isoforms and transcripts with no BLAST hit were identified and removed before GO analysis. Finally, we explored connectivity within modules correlated with the nectar spoon. We identified highly connected hub genes based on their cumulative weighted correlations (CWC, or the sum of all weighted correlations). To simplify network visualization and subsequent analysis, we used an arbitrary cutoff to filter out gene connections (weighted correlations) with values below 0.64. This allowed us to extract 27 genes with strongest weighted correlations from the most highly associated module (Figure 2) to visualize their connections as a network in Cytoscape (Shannon et al. 2003).

2.4 | Phylogenetic Analysis of *SWEET* Genes

Differential expression (DE) analysis and WGCNA indicated that a copy of the *SWEET* transporters is one of the highly upregulated hub genes in the nectar spoon. *SWEET* genes are often present in multiple copies due to their biological roles (see discussion), and thus, we searched the transcriptomic data to extract *SWEET* genes from *Heliamphora* and estimate the phylogenetic placement of the upregulated copy.

Potential *H. tatei SWEET* genes were identified through BLAST searches using known *SWEET* coding sequences from relatives *Camellia sinensis* (tea plant) and *Actinidia eriantha* (kiwi). Identified transcripts were then aligned and clustered into groups of alternatively spliced isoforms. In each cluster, isoform with the highest expression level (typically also the longest isoforms) was selected for phylogenetic analysis. These selected transcripts were searched against the nucleotide collection (nt) database via BLAST and assigned to *SWEET* clade/gene membership based on the most similar matches.

We also examined *SWEET* genes in the sister genus of *Heliamphora*, *Sarracenia*, to isolate molecular changes that might coincide with the shift to carnivory in their common ancestor. For the identification of potential *SWEET* genes in *Sarracenia purpurea*, raw Illumina short reads data from pitcher primordia, unopened pitchers (fed vs. unfed with prey), and opened pitchers were downloaded (Appendix C) and first mapped to *Heliamphora SWEETs* using the Geneious Mapper in Geneious v11.1.5 under default settings. These mapped reads were pooled and *de novo* assembled using the Geneious Assembler in Geneious v11.1.5. Raw sample reads were mapped to these newly assembled *S. purpurea SWEETs* (Appendix D) to verify and quantify the expression of the longest isoform used in subsequent phylogenetic analysis.

The amino acid coding sequences of *H. tatei and S. purpurea SWEET* transporters were aligned and annotated with sequences of *SWEET* transporters identified in other angiosperms (e.g., *Arabidopsis*, *Nicotiana*, *Vitis*, *Jasminum*, *Actinidia*, *Camellia*, *Capsicum*, etc.) using Geneious Alignment in Geneious v11.1.5 (Appendix E).

Phylogenetic inference of *SWEET* transporters was conducted using FastTree v2.1.11 (JTT with optimized Gamma20 likelihood) (Price and Arkin 2010) based on the amino acid sequence alignment and RAxML v8.2.10 (GTRGAMMA with 1000 bootstrap replicates) (Stamatakis 2014) based on the nucleotide sequence alignment.

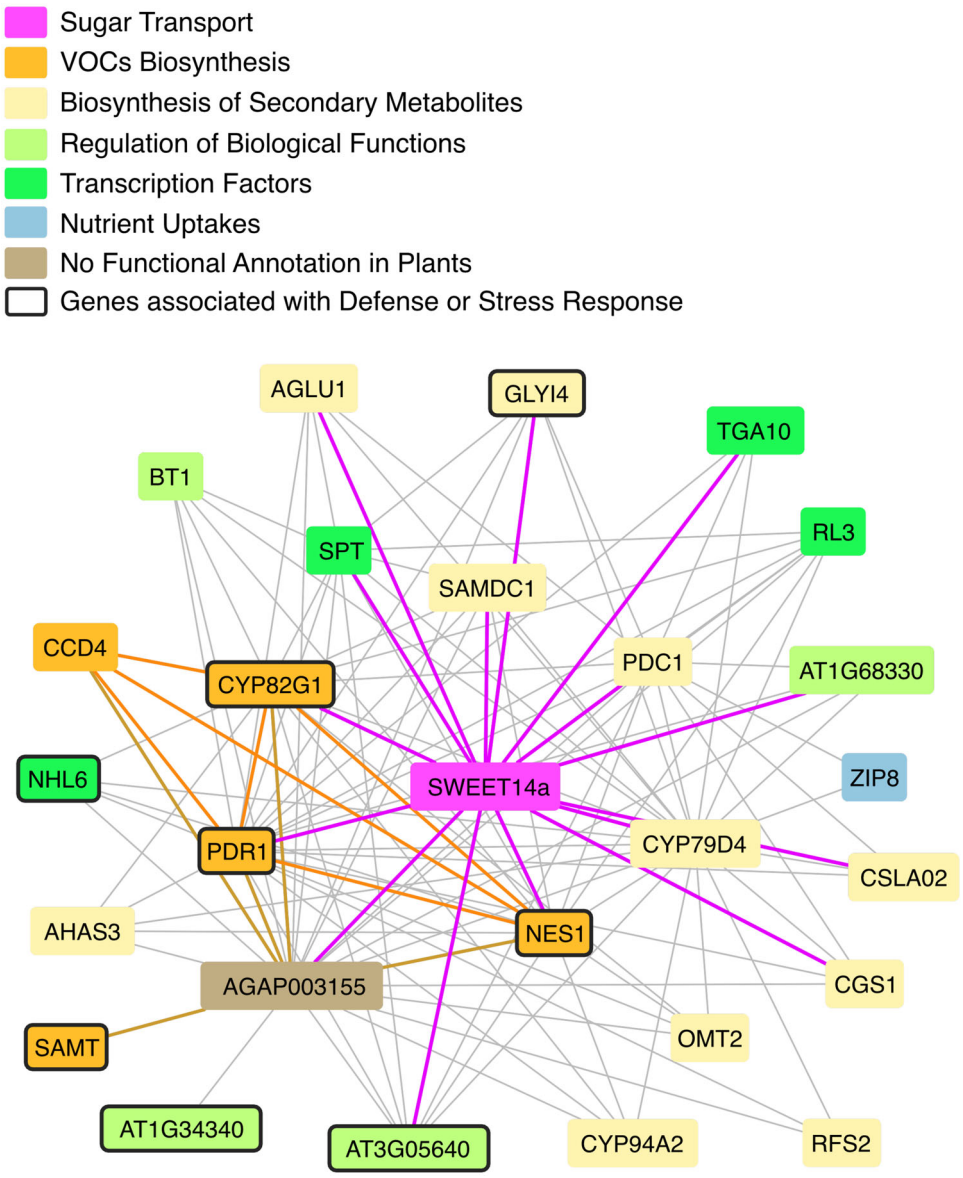

FIGURE 2 | Top hub genes within the NS2 module containing sugar and volatile-related genes. The network shows the 27 genes with edge weights above 0.64 (arbitrary cut-off, see text); the full list of 170 genes in NS2 and their edge weights is given in Supplement F. Genes are colored based on their biological functions as indicated in the legend (see Appendix H for detailed annotation information). Genes outlined in black are associated with plant defense and stress responses. To highlight co-expression patterns relating to sugar and volatile synthesis, edges connected to SWEET14a are shown with bold pink lines; edges connecting genes related to VOC biosynthesis are in bold orange; and edges connecting AGAP003155 (a putative esterase) to VOC genes are in bold tan. Although AGAP003155 has not yet been functionally characterized in plants, its connection to all of the VOC genes, as well as the presence of esters among nectar secretions, suggests it may also be involved with VOC production.

2.5 | Molecular Evolution of SWEET 14 Sucrose Transporters

We estimated rates of nonsynonymous (dN) and synonymous substitutions (dS) within one clade of *SWEET* genes (*SWEET14*, see below) to test the hypothesis that the copy upregulated in the nectar spoon experienced a shift in selective regime after the emergence of the pitcher trap in Sarraceniaceae. We used the *SWEET14* gene tree topology containing sequences from *H. tatei*, *S. purpurea*, *Actinidia eriantha*, *Rhododendron vialii*, *Solanum lycopersicum* (tomato), *Alnus glutinosa*, and *Oryza sativa* (rice). *SWEET14* is present in two copies (*a*, *b*) in the Ericales (*H. tatei*, *S. purpurea*, *A. eriantha*, *R. vialii*) and single copy in the remaining taxa, suggesting a duplication event at or before the origin of the Ericales. We estimated branch lengths on the *SWEET14* gene tree as numbers of nucleotide substitutions per codon using maximum likelihood (ML) methods in the CODEML program of PAML v4 (Yang 2007). We used a series of branch models (Figure 4B) to test the hypothesis that the upregulated copy of *SWEET14* (14a) has experienced altered selective pressures in Sarraceniaceae compared to related homologs.

First, we fit the one-ratio model (M0) as the null model, which assumes all branches have only one ω (dN/dS, the ratio of nonsynonymous vs. synonymous mutations). We then

compared M0 to the M1 model that included two estimated ω (one for the 14a and 14b clades and one for outgroup lineages), M2 model that included two estimated ω (one for the 14a clade and one for the other lineages), M3 model that included two estimated ω (one for the 14b clade and one for the other lineages). As M1 was the best fit (indicating different rates before the duplication and for post-duplication clades), we tested an additional nested M4, allowing for separate rates in the two *SWEET* paralogs for post-duplication clades (a separate ω for 14a, 14b, and outgroups), and M5, nested within M4 and allowing for one additional estimation of ω in *H. tatei* and *S. purpurea* 14a. Finally, we fit the free-ratio model, which estimates ω separately for each branch in the phylogeny, to obtain the phylogram of *SWEET14*.

3 | Results

3.1 | De Novo Assembly of H. tatei Transcriptome

A total of 398,159,702 raw reads were generated from the 12 *H. tatei* tissue samples, with each sample yielding approximately 30–40 million reads. The raw data were deposited in the National Center for Biotechnology Information (NCBI) Short Read Archive database under the accession number PRJNA1098758. After the removal of adaptor sequences and low-quality reads (Phred score < 30), a total of 393,859,427 reads were retained and used as input for the Trinity assembler. The Trinity assembly produced a total of 888,618 assembled transcripts, with a median size of 406 bp and a mean size of 797 bp. BUSCO analysis revealed the presence of 94.2% complete BUSCO genes in the assembly, with 2.0% of all BUSCO genes present in fragmented form and 3.8% missing in this *de novo* transcriptome assembly. From the raw assembler output, a total of 90,194 transcripts (median size = 747 bp, mean size = 1009 bp), representing potential isoforms of 37,299 protein-coding genes, were extracted for differentially expressed gene analysis (Supplement A).

To assess the relationship between *H. tatei* and other closely related carnivorous species in Sarraceniaceae, we performed a reciprocal BLAST analysis between the *de novo* assembled *H. tatei* transcriptome (37,299 predicted protein-coding genes) and the *S. purpurea* transcriptome (18,122 protein-coding genes (Fukushima 2017)). The lower gene count in *S. purpurea* is likely due to differences in sequencing depth and the limited range of tissue types sampled. Our analysis identified 13,744 reciprocal BLAST hits, indicating that approximately 75.8% of *S. purpurea* genes have homologous counterparts in *H. tatei*—a level of similarity comparable to the 78.7% of orthologous genes shared between *S. purpurea* and *S. psittacina* (Srivastava et al. 2011). The substantial overlap in transcriptome content between these sister lineages of American pitcher plants may reflect conservation of functional genomic variation associated with carnivory.

3.2 | Expression Profiles of Nectar Spoons and Pitcher Tissues

The PCA plot (Figure 1B) of *H. tatei* transcriptome gene expression profile across four tissue conditions showed that the replicates were most tightly clustered for the adult pitchers, with the remaining conditions showing some spread among the replicates. This variation was likely due to developmental differences among the replicates (e.g., slightly younger or older juvenile pitchers), as all samples were collected from individuals living in the same environment at the same time. Nevertheless, the nectar spoon replicates were segregated from the juvenile pitchers as well as the unopened and open adult pitchers, suggesting distinct expression profiles between tissues responsible for different ecological functions (pitcher body for photosynthesis and prey digestion vs. nectar spoon for prey attraction).

A total of 351, 392, and 654 transcripts (Supplement B) were found to be differentially expressed in the nectar spoon when compared to open juvenile, unopened adult, and open adult pitchers, respectively (Appendix A). We also identified 486, 923, and 436 transcripts (Supplement B) that were differentially expressed in open juvenile, unopened adult, and open adult pitchers, among these comparisons, respectively (Appendix A). In total, these account for 2330 unique transcripts that are significantly differentially expressed among the three comparisons between the nectar spoon and pitcher tissues.

3.3 | WGCNA Reveals Gene Module Associated With Nectar Rewards and Volatile Production

To identify the various co-expressed gene modules in nectar spoon tissue, WGCNA was performed on these 2330 transcripts, resulting in 21 modules (Appendix F). Of these, four modules showed significant Pearson's correlation with the nectar spoon — NS1 (correlation coefficient = 0.98, $p < 0.0001$), NS2 (0.75, $p = 0.006$), NS3 (0.69, $p = 0.01$), and NS4 (0.62, $p = 0.03$)—containing 77, 214, 43, and 36 transcripts, respectively (see blast results in Supplement C). Of the 77 transcripts in NS1, 61 were uniquely annotated, 11 were found to be isoforms, and 5 did not receive any BLAST results. In NS2, 170 out of 214 transcripts were uniquely annotated (of these, 103 have annotated functions in *A. thaliana*), while 41 were isoforms and 3 received no BLAST hits. For NS3 and NS4, 42 and 39 uniquely annotated genes were retained after filtering, respectively.

GO enrichment analyses revealed that "transcription regulation" is the most significantly enriched biological process across all modules (Appendix G). In NS1, additional enriched processes include "DNA replication," "signal transduction," and "electron transport chain." For NS2, the key enriched processes are "transmembrane transport," "carbohydrate metabolic process," and "protein ubiquitination." NS3 is characterized by significant enrichment in "carbohydrate metabolic process," "DNA damage response," and "DNA repair." Lastly, NS4 shows enrichment in "proton transmembrane transport," "DNA metabolic process," and "photosynthesis."

Among these four modules, NS2 was further investigated in relation to prey attraction because it contains genes related to both nectar reward and volatiles. All genes in NS2 were significantly upregulated in the nectar spoon in at least one comparison to pitcher tissues, and none were upregulated in any pitcher tissues when compared to the nectar spoon. Although gene modules NS1, NS3, and NS4 also showed strong

correlations with nectar spoon tissue (Appendix F & G), their predicted biological processes (Supplement C) suggested they may be involved in the growth and development of nectar spoon tissue.

3.4 | Hub Genes Analysis Reveal Co-Expressed Genes Associated With Prey Attraction

The CWC was calculated for each gene of the NS2 module, allowing us to rank all 170 genes in the network based on total weighted interactions. To simplify network visualization and facilitate subsequent analysis, we focused on the top 27 hub genes (CWC $\geq$ 0.64; Appendix H). This cut-off was applied solely for network visualization to improve interpretability (Figure 2); the full set of 170 genes in NS2 with their CWC values is given in Appendix H and the unfiltered network edges with their associated weighted correlation value are given in Supplement F.

Among these co-expressed hub genes, two-thirds are involved in the metabolism, biosynthesis, and transport of various forms of primary and secondary metabolites, while the rest are genes associated with regulatory roles (with exception of ZIP8). Several of the metabolite-related genes are tied to sugar production (AGLU1, alpha-glucosidase; RFS2, galactinol-sucrose galactosyltransferase 2), with the *SWEET14a* sucrose transporter emerging as the top hub gene, showing the highest connectivity (CWC) in the module (when considering the total number of direct interactions per gene, *SWEET14a* ranked fifth in connectivity within the network). Eight of the 27 hub genes are associated with plant defense and stress responses, including AT1G34340 (Sánchez et al. 2004; Ascencio-Ibáñez 2008), AT3G05640 (Babu et al. 2008; Xin et al. 2007), CYP82G1 (Rajarammohan et al. 2018; Lee et al. 2010), NHL6 (Bao et al. 2016), NES1 (Aharoni et al. 2003), GLYI4 (Proietti et al. 2019, 2018), PDR1 (Stukkens et al. 2005; Jasiński et al. 2001), SAMT (Zou et al. 2021; Wang et al. 2019). The last of these (SAMT) is required for the production of the volatile methyl salicylate, which is commonly produced in fruits and flowers (Zou et al. 2021; Wang et al. 2019; Chu et al. 2024; Ross et al. 1999). Other NS2 genes likely involved in VOC biosynthesis and secretion include CYP82G1 (CYP82 family cytochrome P450 monooxygenase), PDR1 (pleiotropic drug resistance-type ATP-binding cassette transporter), NES1 [(3S,6E)-nerolidol synthase 1], and CCD4 (carotenoid cleavage dioxygenase 4) (Appendix H). The first three (CYP82G1, PDR1, NES1) are all part of the biosynthetic pathway of terpenes or terpenoids, which are generally secreted to attract pollinators or defend against herbivores (Lee et al. 2010; Stukkens et al. 2005; Aharoni et al. 2004; Zheng et al. 2021). In addition to these sugar and VOC related genes, several hub genes are involved in the biosynthesis and metabolism of other organic compounds, including flavonoids (OMT2, O-methyltransferase 2) and amino acids (CGS1, cystathionine gamma-synthase 1; AHAS3, acetolactate synthase).

3.5 | Identification, Expression and Phylogeny of *H. tatei* and *S. purpurea SWEETs*

Our estimated amino acid phylogeny of *SWEETs* showed strong support for four major clades, I, II, III, and IV (Figure 3), in accordance with previous studies (Filyushin et al. 2018; Eom et al. 2015; Wang et al. 2018; Ji et al. 2022; Zhu et al. 2023; Zhanga 2023). Given variation in the numbering schemes for these copies across taxa, we labeled the Sarraceniaceae *SWEETs* following the annotations from the reference quality wild kiwifruit (*A. eriantha*) genome (NCBI RefSeq: GCF_019202715.1 (Tang et al. 2019)), as it also belongs to the Ericales. We identified a total of 8 *SWEET* homologs in the *H. tatei* transcriptome (*SWEET 1*, *2*, *3*, *4*, *14a*, *14b*, *16*, and *17*). All but *SWEET 17* were also recovered from the transcriptome of *S. purpurea*. Following the species relationships, the *H. tatei* and *S. purpurea SWEET* copies form sister pairs within clades containing sequences from other Ericales (e.g., *Camellia*, *Actinidia*, *Rhododendron*, Figure 3). Finally, we found a likely Ericales-specific duplication of *SWEET14*, where each taxon retains two paralogous copies (Figures 3, 4A). When we included the 17 *SWEET* copies from *A. thaliana* in the phylogeny, we again recover the four major clades with strong support (see Supplement D). We noted, however, that in both trees (Figure 3, Supplement D), annotations do not always correspond to clades, especially within Clade III. For example, the copies annotated as *SWEET14* from Ericales, Brassicales, and Poales to not form a monophyletic group, but are intermingled with other clade III copies. Thus, any assignment of names to copies should be considered tentative pending a more comprehensive survey across angiosperms.

We examined the expression of the eight *SWEET* genes in the *H. tatei* transcriptome and found varying degrees of differential expression. For example, *SWEET1* showed similar expression across all tissues while *SWEET4* is active in all tissues except for the nectar spoon. *SWEET14a*, the hub gene that emerged from the co-expression analyses, was the only copy upregulated in the nectar spoon (Figure 3), consistent with the idea that it has a specialized function in prey attraction. Like their *H. tatei* orthologs, *S. purpurea SWEETs* were also differentially expressed across developmental stages (Appendix C). However, the lid wasn't separately sequenced in *S. purpurea* (Fukushima 2017), preventing us from testing for similar patterns of DE. The amino acid alignments, nucleotide alignments, and phylogeny inferred from aligned nucleotide sequences can be found in Supplement D; DE comparison results and the expression data (normalized read counts and TPM) of *H. tatei SWEETs* are available in Supplement E.

3.6 | Molecular Evolution of SWEET 14

In the first round of model comparisons (M1–M4 vs. M0), M1 and M4 emerged as the best-fitting models ($p < 0.001$), indicating changes in selection pressures for *SWEET 14a* and *14b* following the ancestral duplication of *SWEET14* (Figure 4, Appendix I). Even though a direct comparison between the nested models M4 and M1 revealed no significant improvement in fit ($p = 0.861$), further comparisons of M5 against M0, M1, and M4 showed M5 model significantly improved model fit. The parameter estimates from M5 suggest an increased rate of nonsynonymous mutations in *SWEET 14a* ($\omega 1$) of *H. tatei* and *S. purpurea*. The value for dn/ds for this lineage is 0.826, which is almost 3 times higher than the nonsynonymous mutations rate ($\omega 2 = 0.277$) of their non-carnivorous relatives.

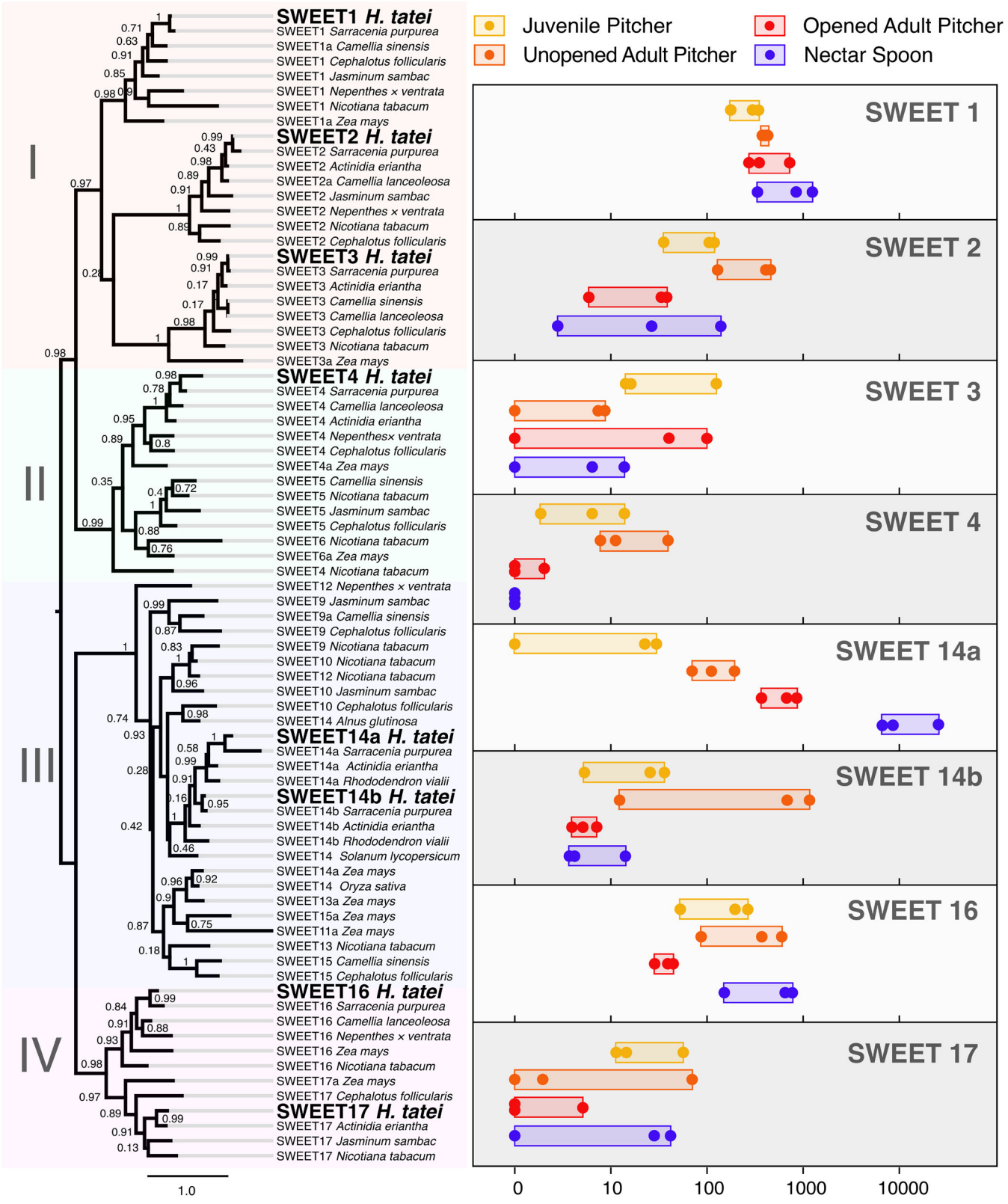

FIGURE 3 | Phylogeny of Heliamphora SWEET genes and their expression across tissues and developmental stages. The phylogeny, rooted using the midpoint method, was estimated based on amino acid sequences, with branch lengths representing amino acid substitutions per site. The expression profile displays normalized read counts across different tissues and developmental stages. Major clades of *SWEET* homologs are indicated in the background for reference. A more inclusive phylogeny incorporating *Arabidopsis thaliana* and additional model species, along with a *SWEET* gene phylogeny estimated from nucleotide sequences, is provided in Supplement D.

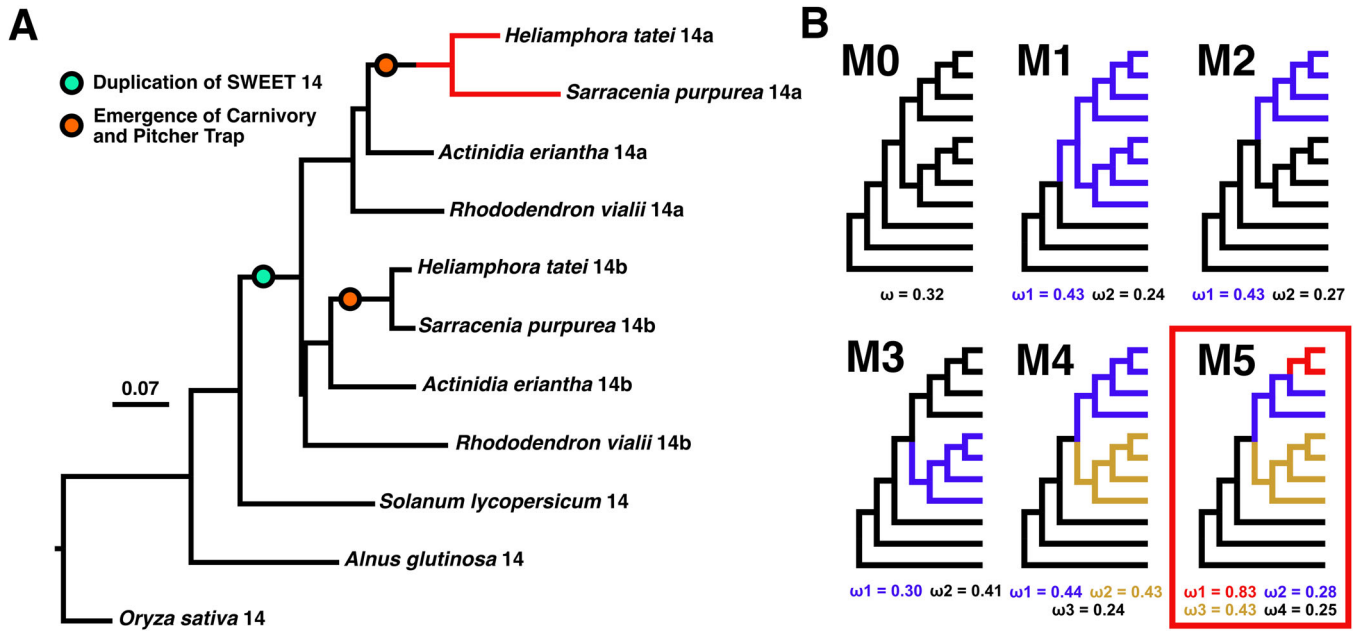

FIGURE 4 | Molecular evolution of *SWEET14* Sucrose Transporters. A. Phylogeny of *SWEET14* with branch length inferred using the CODEML program (PAML) with the free-ratio model, which allows for a separate ω (dN/dS ratio) for each branch. Branch lengths represent non-synonymous substitution rates. The open circle highlighted in cyan marks the ancestral duplication event of *SWEET14*, giving rise to the *14a* and *14b* paralogs. Open circles highlighted in orange indicate the time points in each *SWEET14* paralog gene tree when carnivory and pitcher trap morphology first emerged in the ancestral populations of Sarraceniaceae. The branch highlighted in red represents a possible evolutionary scenario in which *SWEET14a* was co-opted for prey attraction following the emergence of carnivory in Sarraceniaceae. This scenario is supported by the best-fit model's (M5) estimation of accelerated non-synonymous substitution rates in branches leading to pitcher plant species. B. Base model (M0) and nested branch models (M1–M5) estimated with CODEML, with parameter (ω) estimations reported below. The best-fit model (M5) is highlighted in red.

4 | Discussion

Our DE analysis revealed a multitude of genes differentially expressed across tissues and developmental stages of *H. tatei*. Through WGCNA of these DE genes, we found many were co-expressed and clustered into several gene modules that could be associated with various biological functions and processes. Among these gene modules, we identified genes in the NS2 module likely associated with the biosynthesis, transport, and secretion of various prey attractants, like sugars and VOCs. Furthermore, molecular evolution analyses of the *SWEET14a* sucrose transporter, the top hub gene of NS2, suggested that it has accumulated increased non-synonymous mutations in *Heliamphora* and *Sarracenia* after the emergence of the pitcher trap in Sarraceniaceae, consistent with functional evolution and specialization.

4.1 | Modularity and Evolution of Prey Attraction at the Genetic Level

In carnivorous plants, ecological functions such as photosynthesis, carnivory, and pollination are highly modular and are often performed through distinct specialized tissues (Givnish et al. 2018; Płachno and Muravnik 2018). In the convergently evolved pitcher plants, this modularity is manifested in the distinct functional zones (pitcher body – digestion, pitcher hood/nectar spoon – prey attraction) found on their carnivorous pitchers (Thorogood et al. 2018). Our transcriptomic analyses of *H. tatei* further reveal that distinct carnivorous functions, such as prey attraction, are modular not only at morphological (Liu and Smith 2023) and ecological (Plachno et al. 2007) levels but also at the genetic level, as demonstrated by co-expressed gene networks.

In the co-expressed gene network associated with prey attraction (i.e., the NS2 module), most of the top hub genes are involved in the transport and biosynthesis of various organic compounds, such as sugars and VOCs. In particular, we observed co-expression of volatile genes related to the production of terpenes and terpenoids (*CYP82G1*, *PDR1*, *NES1*), apocarotenoids (*CCD4*), and methylsalicylate (*SAMT*). These expression patterns align well with the terpene compounds known to be emitted from *Heliamphora* pitchers. By far the most abundant volatile is the monoterpene sarracenin, with other components including cinerone (another terpene), xylene, phenol, phenylacetaldehyde, esters, and various alkanes (Jaffé et al. 1995). While no methylsalicylate (MeS) was detected in the two *Heliamphora* pitcher samples analyzed in (Jaffé et al. 1995), this compound and its derivatives were detected in *Sarracenia* and *Darlingtonia californica* pitchers in the extensive metabolomic surveys of Jurgens et al. (2009) (Jürgens et al. 2009) and Hotti et al. (2017) (Hotti et al. 2017). who found over 50 unique volatiles across 8 species. Given the patterns of differential expression we observe and sister-group relationship of *Heliamphora* and *Sarracenia*, we predict that more extensive metabolomic studies of *Heliamphora* pitchers would reveal that they produce a similarly diverse volatile mixture as *Sarracenia* (Dupont et al. 2023), including methylsalicylate and possibly also apocarotenoids. Such a complex profile may allow *Heliamphora* to lure a range of insects, as the peppermint-like

MeS, for example, attracts lepidopterans (Plepys et al. 2002) while the terpenes attract ants and flies (Jaffé et al. 1995).

The remaining hub genes in NS2 include several transcription factors and proteins that regulate various biological processes. The transcription factor *SPATULA*, a basic helix-loop-helix (bHLH) transcription factor, primarily controls growth at the margins of organs (e.g., the edges of carpels) (Wu et al. 2018; Liang and Hu 2024; Heisler et al. 2001), and thus could be involved in the expansion of the mouth of the pitcher to form the nectar spoon in *Heliamphora*. The *RAD*-like genes, a group of MYB transcription factors including RL3, are involved in floral symmetry (Baxter et al. 2007) and may also contribute to the development of the spoon's shape. The basic leucine-zipper transcription factor TGA10, linked to both volatile and sugar gene in our analysis (Figure 2), is known to regulate anther development as well as defense responses mediated by reactive oxygen species in model systems (Schiermeyer et al. 2003; Thurow et al. 2005; Murmu et al. 2010). While none of these precise transcription factors have previously been implicated in controlling volatile production, the families of transcription factors to which they belong (e.g., *bHLH* and *MYB* genes) are well known to regulate terpenoid volatiles across plant species (e.g., Yang et al. 2020; Zhang et al. 2023). Sugar transport via the *SWEET* genes is similarly influenced by a diverse array of transcription factors in other taxa (Hua et al. 2022), although posttranscriptional regulation is also common (Han et al. 2023). While *Heliamphora* is far from a model system, in vitro experiments (e.g., yeast one-hybrid assays), targeted sequencing (e.g., ChIP-Seq, CUT&RUN) or even gene silencing (e.g., Kellenberger et al. 2019) could be used to validate the functional interactions suggested by our study and identify additional players in the nectar spoon gene regulatory network.

4.2 | Convergent Recruitment of *SWEET* Genes for Carnivorous Functions

The function and evolution of *SWEET* family transporters have been extensively studied in model systems (Chen et al. 2011), as well as in many economically important species, such as *Oryza sativa* (Eom et al. 2015), *Solanum lycopersicum* (Zhang et al. 2021), and *Camellia sinensis* (Wang et al. 2018). *SWEET* sugar transporters function as bidirectional uniporters, enabling the movement of sugars across cell membranes through concentration gradients (Ji et al. 2022). These transporters are crucial to a variety of biochemical processes, including phloem loading of sugar for long-distance transport, nectar secretion, pollen nutrition, seed filling, fruit development, and responses to biotic and abiotic stress (Ji et al. 2022). *SWEET* genes are divided into four subfamilies (designated Clades I to IV) based on sequence similarity, with proteins within the same subfamilies sharing similarities in structure, function, and cellular localization. For example, the Clade I (*SWEET 1–3*) and II (*SWEET 4–8*) subfamilies specifically transport hexoses (Eom et al. 2015). The Clade III subfamily (*SWEET* 9–15) is dedicated to the transport of sucrose (Eom et al. 2015). Meanwhile, the SWEET proteins of the Clade IV subfamily (*SWEET 16* and *17*) are located on the vacuolar membrane and generally transport fructose (Eom et al. 2015).

Our DE and WGCNA analyses suggested that *H. tatei SWEET14a*, sucrose transporter belonging to clade III of the *SWEETs*, likely plays a crucial role in nectar secretion as a prey attractant. While most other copies of *SWEET* genes are expressed in the nectar spoon at similar levels to juvenile and mature pitcher tissue, *SWEET14a* is significantly upregulated in the nectar spoon (Figure 3). Our phylogenetic analyses suggested that *SWEET 14a* and its paralog *14b* arose from an ancestral gene duplication event before the emergence of carnivory in Ericales, allowing for specialization of the descendant duplicate copies. Moreover, our molecular evolution analyses (Figure 4) show that *SWEET14* paralogs evolved at nearly three times higher rate after the emergence of carnivory in Sarraceniaceae. If *SWEET14a* was indeed co-opted for sucrose transport at the base of the Sarraceniaceae, we predict that the third genus in the family (*Darlingtonia*) would share the elevated dN/dS ratio observed in *Sarracenia* and *Heliamphora*. Additionally, we would expect *SWEET14a* to be upregulated in the homologous attractive organs of *Darlingtonia* (the showy nectar-producing forked tongue) and *Sarracenia* (the lid).

Given that sugar, and often sucrose, is the dominant reward in EFNs of carnivorous plants (Płachno 2007; Plachno et al. 2007; Lathika et al. 2025; Joel 1988), the *SWEET* clade III transporters may be involved in prey attraction in other distantly related pitcher plant lineages. Indeed, although no other transcriptomic studies have specifically sampled the EFNs of pitcher plants, diverse *SWEET* transporters have been identified in the transcriptomes of several species, including all four gene subfamilies. These *SWEET* genes also show patterns of differential expression, consistent with functional specialization (Fukushima 2017; Filyushin et al. 2018). For example, *SWEET12a* (another clade III *SWEET* like *SWEET14*) upregulated in late-stage pitcher development in *Nepenthes* and similar patterns of *SWEET* differential expression are seen in *Cephalotus (*Fukushima 2017). Interestingly, genomic and transcriptomic analyses of the EFNs in the Venus flytrap also point to the recruitment of *SWEET* transporters despite the drastically different trap morphology and prey capture strategy from pitcher plants (Palfalvi et al. 2020). These patterns suggest that the specialization of *SWEET* genes for prey attraction may be a common feature of *SWEET* gene family evolution in pitcher plants and other carnivorous plants, with additional developmental work targeting EFNs representing a clear next step.

4.3 | Is the Nectar Spoon Also a Scent Spoon?

Although the nectar reward is often thought of as the primary attractant offered to prey, our study underscores the key role of volatile compounds in luring insects to carnivorous plants. In particular, we found that sugar synthesis related genes (including *SWEET14a*) are tightly co-expressed in the nectar spoon with a large suite of volatile genes that align with known classes of insect-attracting specialized metabolites (Jürgens et al. 2009; Hotti et al. 2017). Indeed, volatile genes in *H. tatei* are among the most highly DE genes in the nectar spoon (Appendix H, Supplement B), accounting for 5 of the top 27 most highly connected genes in the NS2 module (Figure 2). This pattern parallels the co-expression of similar genes involved in sugar transport, terpenoid biosynthesis, and other VOC production in the EFNs of the Venus flytrap (Palfalvi et al. 2020). Among the convergently evolved carnivorous plant EFNs, certain VOCs (e.g., the monoterpenoid 6-methyl-5-hepten-2-one)

are shared across independently evolved carnivorous lineages, although many others exhibit lineage-specific patterns (Di Giusto et al. 2010; Jürgens et al. 2009; Hotti et al. 2017). These lineage-specific VOCs fall into four major chemical classes—alcohols, esters, terpenoids, and benzenoids—all of which have been detected in various forms across all carnivorous species studied to date (Di Giusto et al. 2010; Jürgens et al. 2009; Hotti et al. 2017). These findings emphasize that carnivorous plants EFNs are not merely nectar-producing structures but also function as scent-producing organs, with both nectar rewards and volatile emissions playing an essential role in attracting prey.

5 | Conclusions

The function of carnivorous pitchers relies on a combination of attractive traits to lure prey, morphological structures to trap them, and physiological adaptations for digestion. Moreover, the features involved in attraction are themselves a complex assemblage, including the production of scent, nectar and amino acids. Our work suggests that the development of prey attraction traits may be coordinated by shared regulators, although whether this genetic architecture predates the origin of carnivory or evolved as part of the emergence of the syndrome remains to be explored. Moreover, increased expression of sugar transporters, already present in the genome for multiple physiological functions, along with a large swath of scent volatile genes, appears to be fundamental to the nectar spoon's role in prey attraction. One sugar transporter, *SWEET14a*, is not only specialized for the nectar spoon, but also evolves more quickly in Sarraceniaceae. This study suggests that, as with prey digestion, many genes ancestrally involved in defense and stress response are repurposed for new functions to develop prey attraction traits during the evolution of carnivory. Our work opens the door for investigating the degree of molecular convergence in prey attraction in other carnivorous plant lineages, many of which use similar combinations of sugary reward and scent volatiles to lure prey.

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Data Availability Statement

The data that supports the findings of this study are available in the supporting material of this article.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.