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Comparative genomics reveals the diversification of triterpenoid biosynthesis and origin of ocotillol-type triterpenes in *Panax*

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ABSTRACT

Gene duplication is assumed to be the major force driving the evolution of metabolite biosynthesis in plants. Freed from functional burdens, duplicated genes can mutate toward novelties until fixed due to selective fitness. However, the extent to which this mechanism has driven the diversification of metabolite biosynthesis remains to be tested. Here we performed comparative genomics analysis and functional characterization to evaluate the impact of gene duplication on the evolution of triterpenoid biosynthesis using *Panax* species as models. We found that whole-genome duplications (WGDs) occurred independently in Araliaceae and Apiaceae lineages. Comparative genomics revealed the evolutionary trajectories of triterpenoid biosynthesis in plants, which was mainly promoted by WGDs and tandem duplication. Lanosterol synthase (LAS) was likely derived from a tandem duplicate of cycloartenol synthase that predated the emergence of Nymphaeales. Under episodic diversifying selection, the LAS gene duplicates produced by γ whole-genome triplication have given rise to triterpene biosynthesis in core eudicots through neofunctionalization. Moreover, functional characterization revealed that oxidosqualene cyclases (OSCs) responsible for synthesizing dammarane-type triterpenes in *Panax* species were also capable of producing ocotillol-type triterpenes. Genomic and biochemical evidence suggested that *Panax* genes encoding the above OSCs originated from the specialization of one OSC gene duplicate produced from a recent WGD shared by Araliaceae (Pg-β). Our results reveal the crucial role of gene duplication in diversification of triterpenoid biosynthesis in plants and provide insight into the origin of ocotillol-type triterpenes in *Panax* species.

Keywords: gene duplication, *Panax* genomes, whole-genome duplications, triterpenoid biosynthesis, ocotillol-type triterpenes, specialization

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INTRODUCTION

Plants have evolved to synthesize a diverse array of metabolites that play essential roles in various biological processes. The adaptivity derived from these metabolites has driven the evolution of plants and even their interactors. For decades, biologists have been intrigued by the evolutionary mechanism underlying

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the diversification of metabolite biosynthesis in the plant kingdom. Gene duplication is proposed to be the major force driving the evolution of metabolite biosynthesis: relaxed from functional constraints, one duplicate can accumulate mutations. In most cases, such mutations will result in gene loss, but some may be fixed owing to selective advantages conferred by their altered function, whether neofunctionalization, subfunctionalization, or specialization (Ober, 2005). These novelties in function or expression pattern would gradually reshape the biosynthetic pathway for metabolites. In land plants, pervasive whole-genome duplications (WGDs) or polyploidizations serve as the primary sources of gene duplicates. These frequent WGDs are thought to have a key causal role in species diversification, phenotypic evolution, and chemical diversification in both gymnosperm and angiosperm lineages (Landis et al., 2018; Lichman et al., 2020; Stull et al., 2021). The causal linkage between WGDs and diversification of metabolite biosynthesis, although supported on a theoretical basis, remains to be rigorously tested.

Triterpenoids are one of the most diverse metabolites present in plants. Their biosynthesis is catalyzed by enzymes known as oxidosqualene cyclases (OSCs), which can cyclize the precursors 2,3-oxidosqualene and 2,3; 22,23-dioxidosqualene. Two different types of substrate conformation exist during the cyclization process: the chair-boat-chair (CBC) conformation and the chair-chair-chair (CCC) conformation (Thimmappa et al., 2014). Sterols, including cycloartenol and lanosterol, are produced via CBC folding, whereas triterpenes are produced via CCC folding. Based on the catalytic products, plant OSCs can be broadly classified into cycloartenol synthase (CAS), lanosterol synthase (LAS), lupeol synthase (LUS), β-amyrin synthase, and other multifunctional triterpene synthases (bAS and other mTTSs). Sterols function as important membrane components and also as plant hormones that regulate growth and development (Schaller, 2003). The "nonessential" triterpenes are considered to have more specialized functions in plant defense and microbiome interactions (Delis et al., 2011; Khakimov et al., 2015; Miettinen et al., 2018; Huang et al., 2019; Li et al., 2021b; Busta et al., 2021). Genomic screening of the Viridiplantae phylogeny revealed that angiosperms are a hotspot of OSC diversification. Both divergent and convergent evolutionary processes are thought to have influenced the evolution of OSCs, and it is generally accepted that expansion of OSCs has been driven mainly by tandem duplications and that the triterpene synthases of eudicots likely originated from LAS rather than CAS (Pichersky and Lewinsohn, 2011; Xue et al., 2012; Zhou et al., 2016; Cárdenas et al., 2019; Dong et al., 2021). However, the impact of WGDs on the diversification of OSCs and the corresponding evolutionary trajectory remain unresolved.

The genus *Panax* L. (Araliaceae), which contains seven well-defined species and one species complex, is one of the most medicinally important plant genera. The pharmaceutical activities of *Panax* species have been attributed mainly to ginsenosides (glycosylated triterpenoids) (Leung and Wong, 2010; Fan et al., 2020). Biochemical approaches have revealed a wide variety of triterpenoids in *Panax* species, including the dammarane, α/β-amyrin, and ocotillol types (Hou et al., 2021). To date, OSC genes responsible for synthesis of dammarane-type triterpenes have been reported for several *Panax* species (Tansakul et al., 2006; Wang et al., 2014), but the biosynthetic pathway of ocotillol-type triterpenes remains unclear. As one *Panax* species with high medicinal value, *Panax vietnamensis* var. *fuscidiscus* is widely cultivated in Yunnan, China. The high content of ocotillol-type saponins in *P. vietnamensis* var. *fuscidiscus* make it a suitable model for exploring the mechanism of ocotillol-type triterpene biosynthesis (Zhang et al., 2015). *Panax* species have experienced several rounds of WGD in their evolutionary history, but whether extra WGDs have occurred in the common ancestor of all Apiales species after the γ whole-genome triplication (WGT) remains a topic of controversy (Kim et al., 2018a; Li et al., 2021a; Yang et al., 2021a, 2021b; Song et al., 2021). Regardless of disputes about WGD history, genomic and phytochemical evidence indicates that the evolution of triterpenoid biosynthesis in *Panax* species is likely to have been affected by WGDs (Li et al., 2021a). The diversity of triterpenoids and the presence of WGDs in *Panax* species make this genus a suitable model for examining the effects of WGDs on the evolution and diversification of OSCs.

Here we report a high-quality chromosome-level assembly for *P. vietnamensis* var. *fuscidiscus*, together with an improved assembly for *Panax notoginseng*. We found that WGDs have occurred independently in Araliaceae and Apiaceae species rather than being shared by Apiales. Comparative genomics revealed that the diversification of triterpenoid biosynthesis was promoted mainly by WGDs and tandem duplications. Notably, the dammarenediol-II synthases (DDSs) in *Panax* species were functionally characterized as mTTSs. These *Panax* DDS genes originated from the specialization of one OSC gene duplicate produced by the Pg-β WGD. Our findings systematically reveal how gene duplication drives the diversification of triterpenoid biosynthesis in plants and reveal the origin of ocotillol-type triterpenes in *Panax* species.

RESULTS

Panax genome sequencing, assembly, and annotation

PacBio long reads were used to build a *de novo* assembly for *P. vietnamensis* var. *fuscidiscus* (Supplemental Figure 1A). This preliminary assembly was polished with Illumina short reads and scaffolded using Hi-C technology. The final chromosome-level assembly of *P. vietnamensis* var. *fuscidiscus* spans 1.73 Gb, with a scaffold N50 of 144.08 Mb (Supplemental Table 1). The largest 12 scaffolds, which correspond to the karyotype of *P. vietnamensis* var. *fuscidiscus* (2n = 2x = 24), covered 91.04% of the assembly (1.57 Gb) (Supplemental Figures 1B, 2A). The size of the pseudochromosomes is close to the flow cytometry (1.61 Gb) and k-mer-based estimates (1.43 Gb) (Supplemental Figure 3A; Supplemental Tables 2, 3). To evaluate the quality of the *P. vietnamensis* var. *fuscidiscus* genome, 229.47 Gb of the Illumina short reads (132.64×) were mapped to the assembly. The mapping rate of properly paired reads and genome coverage rate were 94.47% and 97.40%, respectively (Supplemental Table 4). We annotated 36 454 protein-coding genes in the *P. vietnamensis* var. *fuscidiscus* genome, with an average gene length of 6166.47 bp (Supplemental Table 5). A total of 33 570 (92.09%) predicted genes could be functionally annotated (Supplemental Table 6). Benchmarking Universal Single-Copy Orthologs (BUSCO) completeness of the assembly and annotated genes were 95.3% and 92.6% (Supplemental

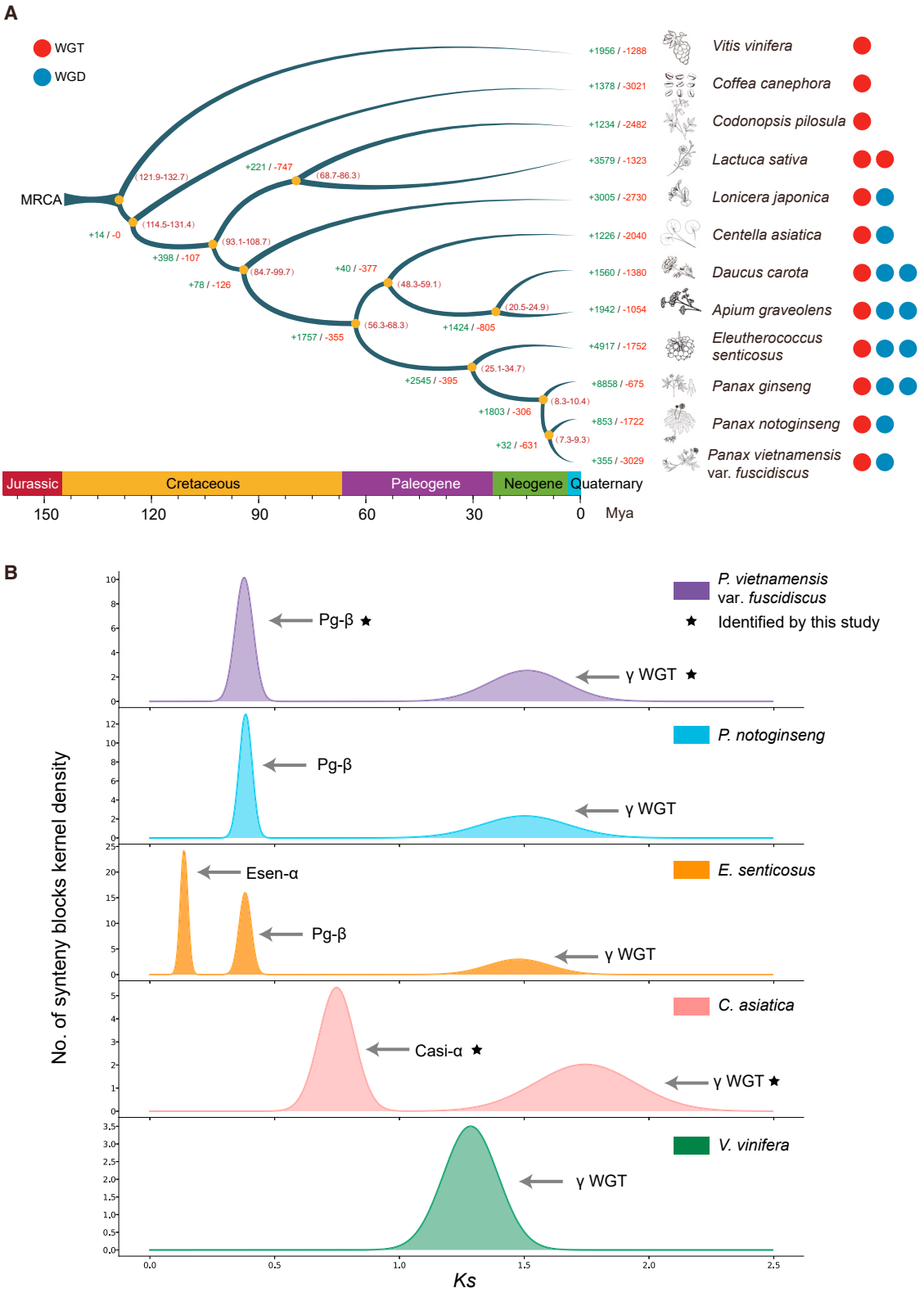

Figure 1. Evolutionary analysis of *P. vietnamensis* var. *fuscidiscus*.

(A) Species tree for 12 eudicots including *P. vietnamensis* var. *fuscidiscus*. Numbers in parentheses indicate estimated divergence times in Mya with 95% confidence intervals. Expansion and contraction of gene families are indicated with plus and minus signs. Whole-genome duplications (WGDs) and whole-genome triplications (WGTs) in each species are shown in blue/red circles. MRCA, most recent common ancestor.

(B) *Ks* distribution of intraspecific collinear blocks. *Ks* peaks of polyploidizations are labeled for each species. Esen, *E. senticosus*; Casi, *C. asiatica*.

Figure 3B; Supplemental Table 7). The reported genome assembly size for *P. vietnamensis* Ha *et* Grushv. (2n = 2x = 24) is 3.00 Gb (Tien et al., 2021), which is 1.73-fold larger than the genome size of *P. vietnamensis* var. *fuscidiscus*. Thus, *P. vietnamensis* var. *fuscidiscus* is likely to be an independent species rather than a variety of *P. vietnamensis* Ha *et* Grushv. If not, *P. vietnamensis* var. *fuscidiscus* is still worth studying for its different genome size compared with *P. vietnamensis* Ha *et* Grushv. and well-developed biosynthetic modules based on *P. vietnamensis* var. *fuscidiscus*.

We also provide an improved chromosome-level assembly of *P. notoginseng* (2n = 2x = 24) created using a previous contig-level assembly (Supplemental Table 8). More sequences (94.29%) were anchored to the 12 pseudochromosomes compared with the previous assembly (86.87%) (Supplemental Figure 2B). We annotated 36 747 protein-coding genes in the updated *P. notoginseng* genome, 92.79% of which were functionally annotated (Supplemental Tables 5 and 9). BUSCO analysis suggested 97.5% and 93.3% completeness of the updated *P. notoginseng* assembly and annotated genes (Supplemental Figure 3B; Supplemental Table 7).

Species-specific LTR expansion produced genome size variation in *Panax*

Repetitive elements constitute 86.79% and 88.18% of the *P. vietnamensis* var. *fuscidiscus* and *P. notoginseng* assemblies. LTR-RTs are the most abundant type of transposable elements (TEs) in both *Panax species*, accounting for 78.94% and 80.66% of the *P. vietnamensis* var. *fuscidiscus* and *P. notoginseng* assemblies. Among the LTR-RTs in *P. vietnamensis* var. *fuscidiscus*, Gypsy elements (54.52% of the genome) are far more abundant than Copia elements (5.67%). A similar phenomenon was observed in the *P. notoginseng* genome, with Gypsy and Copia elements accounting for 55.49% and 4.55% of the genome. DNA transposons are the second most abundant type of TE and constitute 2.90% and 3.13% of the *P. vietnamensis* var. *fuscidiscus* and *P. notoginseng* genomes (Supplemental Tables 10 and 11). Based on intact LTR-RTs (21 251 in *P. vietnamensis* var. *fuscidiscus* and 24 899 in *P. notoginseng*), we estimated the insertion times for LTR-RTs. *P. vietnamensis* var. *fuscidiscus* was found to have experienced a more recent burst of LTRs compared with *P. notoginseng* (Supplemental Figure 4). Clade-level classification of TEs revealed that the numbers of several Gypsy-clade elements (mainly Tekay, Ogre, and Athila) are much higher in *P. notoginseng* than in *P. vietnamensis* var. *fuscidiscus* (Supplemental Tables 12 and 13). These results indicate that *P. notoginseng* experienced a more intense expansion of LTR insertions compared with *P. vietnamensis* var. *fuscidiscus* after their divergence; the difference in genome sizes between the two *Panax* species (~680 Mb) can be attributed mainly to the more intense expansion of LTRs in *P. notoginseng* (LTR size difference, ~609 Mb).

Phylogenomics and evolution of *P. vietnamensis* var. *fuscidiscus*

To study the evolutionary history of *Panax* species, we first performed gene family analysis using 12 eudicots: *Vitis vinifera*, *Coffea canephora*, *Codonopsis pilosula*, *Lactuca sativa*, *Lonicera japonica*, *Centella asiatica*, *Daucus carota*, *Apium graveolens*, *Eleutherococcus senticosus*, *Panax ginseng*, *P. notoginseng*, and *P. vietnamensis* var. *fuscidiscus*. A total of 30 074 ortholog groups, harboring 93.1% of all the studied genes, were identified for the 12 species, and 168 groups are presented as single-copy orthogroups (Supplemental Figure 5A; Supplemental Table 14). Investigation of gene families in *P. vietnamensis* var. *fuscidiscus* and five other Apiales species suggested that *P. vietnamensis* var. *fuscidiscus* and *P. notoginseng* contain 436 and 673 unique gene families, respectively (Supplemental Figure 5B).

Single-copy orthogroups were used to construct maximum likelihood (ML) phylogenetic trees. The species trees inferred by the concatenation method and coalescence-based phylogenetic analysis are identical and well supported (Supplemental Figure 6A and 6B). *P. vietnamensis* var. *fuscidiscus* is placed as a sister lineage to *P. notoginseng* rather than *P. ginseng*, consistent with a *Panax* phylogeny based on chloroplast genomes and ribosomal DNA (Ji et al., 2019). Divergence times were estimated using MCMCTree with time calibrations. The estimated divergence between Araliaceae and Apiaceae occurred ~56.3–68.3 million years ago (Mya). In the *Panax* genus, the speciation of *P. ginseng* occurred first (~8.3–10.4 Mya), followed by the divergence of *P. vietnamensis* var. *fuscidiscus* and *P. notoginseng* (7.3–9.3 Mya) (Figure 1A). We also noted the early divergence of *C. asiatica* in the family Apiaceae, which occurred approximately 48.3–59.1 Mya, validating the basal group position of *C. asiatica* in Apiaceae (Li et al., 2020).

Finally, we estimated the expansion and contraction of gene families during the phylogenetic history of the 12 species using the resolved species tree. In *P. vietnamensis* var. *fuscidiscus*, 355 gene families had undergone expansion, whereas 3029 gene families had undergone contraction ($P < 0.05$) (Figure 1A). Expanded gene families in *P. vietnamensis* var. *fuscidiscus* showed functional enrichment in sesquiterpenoid and triterpenoid biosynthesis ($P < 0.05$) (Supplemental Figure 7A; 7B, Supplemental Tables 15 and 16).

Polyploidization history in Apiales

Polyploidizations in Apiales were systematically characterized to study their impact on the evolution of triterpenoid biosynthesis. The *V. vinifera* genome was used as an outgroup because only one polyploidization event (γ WGT) occurred during its evolution. We inferred WGDs and speciation events by examining the synonymous substitutions per synonymous site (*Ks*) of collinear gene pairs and intra/interspecific syntenic relationships. Two clear peaks were observed in the *Ks* distribution of intraspecific collinear gene pairs for *P. vietnamensis* var. *fuscidiscus*, suggesting an extra round of WGD after the γ WGT (Figure 1B). Interspecific synteny comparison between genomes of *P. vietnamensis* var. *fuscidiscus* and *V. vinifera* revealed that for each genomic region in *V. vinifera*, there are up to six syntenic matches in *P. vietnamensis* var. *fuscidiscus*, validating the extra round of WGD in the latter species (Supplemental Figure 8). In addition to recent peaks attributed to speciation, extra peaks were detected in the *Ks* distribution of collinear gene pairs between *P. vietnamensis* var. *fuscidiscus* and the other two Araliaceae species (*P. notoginseng* and *E. senticosus*) (Supplemental Figure 9). The ancient peaks indicate the shared γ WGT, and the relatively young peaks may

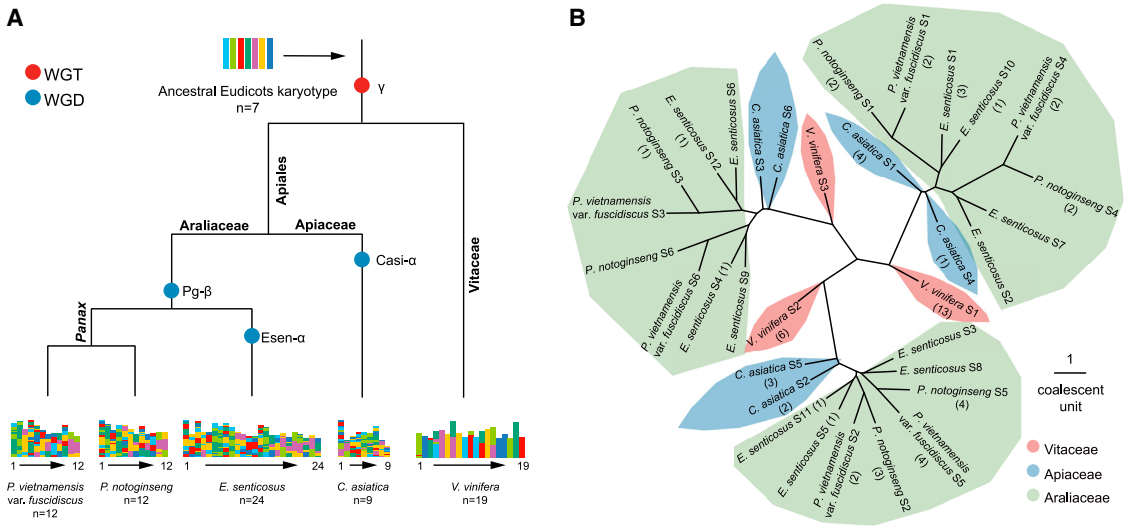

Figure 2. Inference of polyploidization and speciation history in Apiales.

(A) The inferred phylogeny of Apiales species with placement of polyploidizations. Karyotypes were painted in seven colors, corresponding to the seven ancestral eudicot chromosomes.

(B) Synteny-based coalescent species tree showing independent WGDs in Apiaceae and Araliaceae. Genomes were classified into collinear subsets based on polyploidization history (denoted with S). Branch lengths are shown in coalescent units. Because the ASTRAL tree leaves the branch length of terminal branches empty, the lengths of terminal branches were all set to one. Numbers in parentheses represent the number of putative OSC genes.

represent the Pg-β WGD, which is presumed to be shared by Araliaceae species. The *Ks* peak values for the Pg-β WGD and the γ WGT are nearly identical in the three Araliaceae species, suggesting little variation in evolutionary rates among Araliaceae. Synteny comparisons of the updated *P. notoginseng* assembly with that of *E. senticosus* showed exactly 1:2 ratios for the best-matched regions in the largest 12 pseudochromosomes, validating the high quality of the updated *P. notoginseng* assembly compared with an older version (Supplemental Figure 10A and 10B). *C. asiatica* experienced two WGDs according to our analysis (Figure 1B). The *Ks* distribution of interspecific collinear gene pairs between *C. asiatica* and *P. vietnamensis* var. *fuscidiscus* showed two peaks, which correspond to speciation (~0.53) and the shared γ WGT (~1.63) (Supplemental Table 17). The absence of additional peaks suggested that the younger WGDs in Apiaceae and Araliaceae may have occurred independently after their speciation (Figure 2A).

To examine Apiales evolution with greater resolution, we performed synteny-based phylogenetic analysis. Five species (*V. vinifera*, *C. asiatica*, *E. senticosus*, *P. notoginseng*, and *P. vietnamensis* var. *fuscidiscus*) that exhibit a well-preserved ancestral eudicot karyotype (AEK) were selected for the analysis. Using the AEK and the *V. vinifera* genome as references, collinear regions were partitioned into different copies for each species with consideration of WGDs (Supplemental Figures 11–15; Supplemental Table 18). Based on 2255 collinear gene pairs (23 821 genes), ASTRAL produced a phylogenetic tree for the five species with a normalized quartet score of 0.8146. The topology of the synteny-based species tree provides solid evidence that the Pg-β WGD occurred independently in Araliaceae and was shared by Araliaceae species (Figure 2B and Supplemental Figure 16). Interestingly, the collinear subsets for *C. asiatica* in all three lineages produced by the γ WGT did not form sister groups but split successively instead. This suggested that the relatively recent WGD in *C. asiatica* may have been induced by an ancient hybridization.

Evolution of OSCs was mainly promoted by WGDs and tandem duplications

Previous studies have suggested that OSCs for sterol biosynthesis in Eukarya have a bacterial origin and that plant OSCs have likely undergone divergent evolution, with triterpene biosynthesis derived from sterol biosynthesis (Xue et al., 2012; Santana-Molina et al., 2020). Thus CAS likely served as the foundation of OSC evolution. Here, we performed phylogenetic and comparative genomics analyses to clarify the evolution of OSCs in plants. Nine species (*Amborella trichopoda*, *Aristolochia fimbriata*, *V. vinifera*, *C. asiatica*, *E. senticosus*, *P. ginseng*, *P. vietnamensis* var. *fuscidiscus*, *P. notoginseng*, and *Panax quinquefolius*) were included in the analysis, including six Apiales species selected for their diversity in triterpenoid biosynthesis and well-characterized phylogenetic history. We included *A. trichopoda* (ANA-grade) and *A. fimbriata* (Magnoliids) in the analysis for their absence of WGD since the emergence of flowering plants (Amborella Genome Project, 2013; Qin et al., 2021). First, we performed genome-wide identification of OSCs based on conserved protein domains. For *P. quinquefolius*, which lacks a reference assembly, one DDS was used (Supplemental Table 19). In contrast to the abundant OSC genes in eudicots, we identified only one putative OSC in *A. trichopoda* and two putative OSCs in *A. fimbriata*, implying an important role for WGDs in the expansion of OSCs. An ML phylogenetic tree was built for the identified putative OSCs using codon alignments (Figure 4A). On the basis of conserved motifs (Supplemental Figure 17) and phylogenetic relationships with functionally characterized OSCs, the OSCs were classified into putative functional groups (CAS, LAS, LUS, and bAS and other mTTSs)

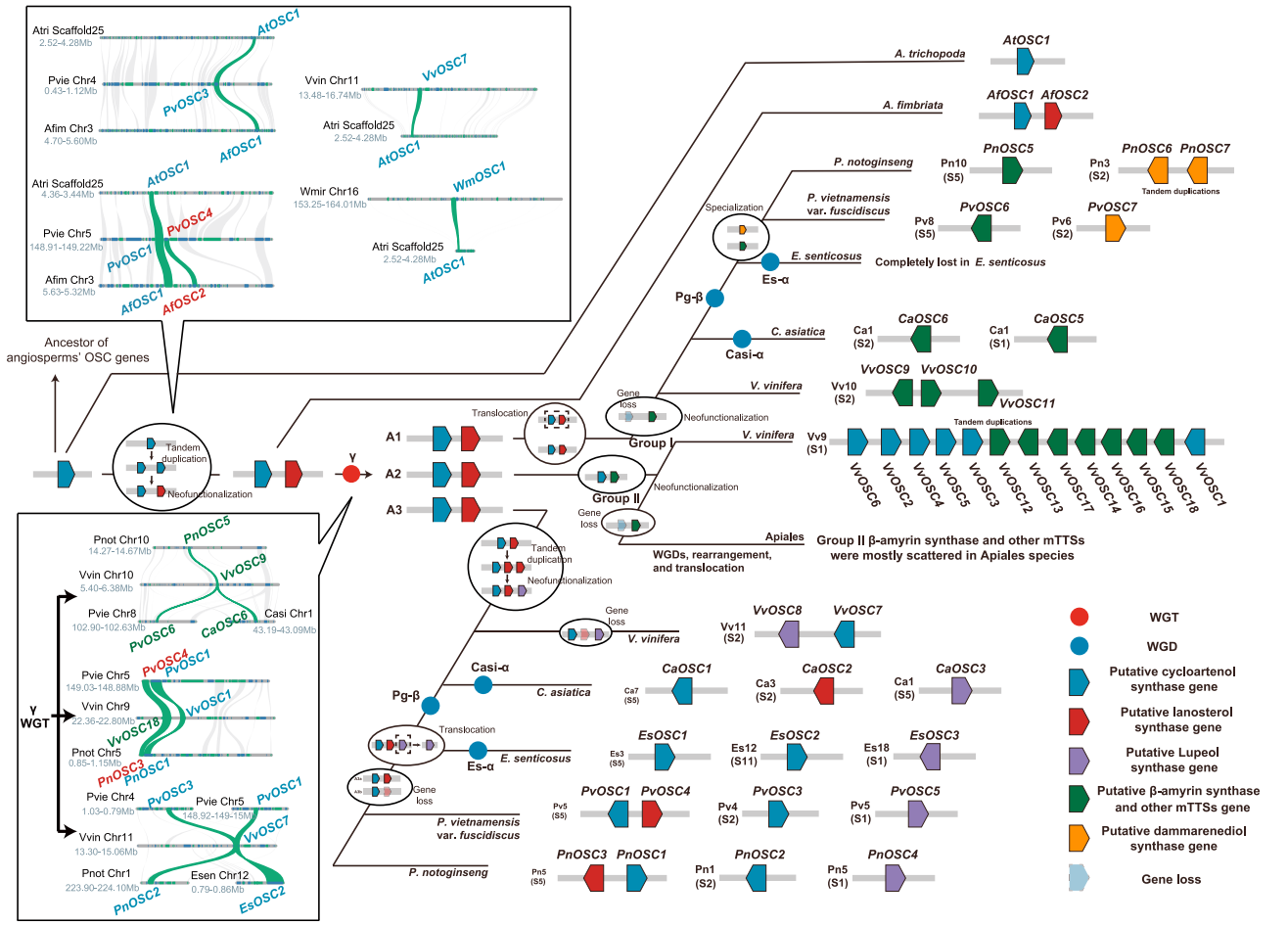

Figure 3. The inferred evolutionary trajectory of OSC genes in plants.

Polyploidizations are shown with blue and red circles. Pentagons with a solid border represent OSC genes. Gene loss is shown by pentagons with a dashed border. Interspecific micro-syntenic relationships of putative OSC genes are shown in boxes. Direct collinear relationships between putative OSC genes are highlighted in green (Vvin, *V. vinifera*; Pvie, *P. vietnamensis* var. *fuscidiscus*; Pnot, *P. notoginseng*; Atri, *A. trichopoda*; Afim, *A. fimbriata*; Wmir, *W. mirabilis*). Inferred gene duplication, neofunctionalization, translocation, and loss are highlighted in circles.

(Chen et al., 2021a). Functionally characterized DDSs from *Panax* species were nested within bAS and other mTTSs, indicating their close phylogenetic relationship (Tansakul et al., 2006; Wang et al., 2014). We also noticed that members of bAS and other mTTSs were recovered in two lineages (group I and group II) with high support, suggesting their distinct origins.

The distribution pattern of OSCs on the synteny-based species tree suggested that the expansion of OSCs was affected by WGDs (Figure 2B). In addition, the scarcity of OSCs on the lineage leading to *V. vinifera* S3 indicated that gene loss or translocation events had occurred before speciation. We next performed inter/intraspecific synteny analysis to investigate the evolutionary trajectory of OSCs with differentiation of paralogous and orthologous syntenic regions produced by polyploidizations and speciation. Intraspecific synteny comparisons revealed OSCs produced from recent WGDs (Casi-α and Pg-β WGD) in Apiales species (Supplemental Figure 18). In *P. vietnamensis* var. *fuscidiscus*, no direct syntenic relationship was found for *PvOSC7* (DDS gene) and *PvOSC6* (bAS and other mTTSs), yet both genes were located on highly syntenic chromosomal regions produced by the Pg-β WGD. The same phenomena were also observed in *P. notoginseng* between *PnOSC5* (bAS and other mTTSs) and the tandemly-duplicated *PnOSC6*/*PnOSC7* (DDS genes) (Supplemental Figure 18). Thus, we speculate that DDS in *Panax* species likely originated from neofunctionalization of a group I bAS and other mTTS copy produced from the Pg-β WGD. We observed only one syntenic relationship between *VvOSC12* (from chromosome [chr] 9) and *VvOSC9* (from chr 10) in *V. vinifera*. Considering the fact that OSCs in grape are found on only three chromosomes (chr 9, 10, 11) and that chr 9, chr 11, and a part of chr 4 were produced by the γ WGT, grape OSCs from chr 9, 10, and 11 are likely to share the same origin. After the γ WGT, a chromosomal region harboring OSCs in chr 4 was translocated to chr 10. This assumption was verified by interspecific synteny comparisons between *V. vinifera* and Apiales species, in which the grape CASs *VvOSC7* (from chr 11) and *VvOSC1* (from chr 9) showed syntenic relationships with the same *P. vietnamensis* var. *fuscidiscus* CAS, *PvOSC1* (Figure 3). We also compared OSC syntenic relationships of *P. vietnamensis* var. *fuscidiscus* and *V. vinifera* with species with non-duplicated genomes (*A. trichopoda* and *A. fimbriata*). The CASs produced by the Pg-β WGD (*PvOSC3* and *PvOSC1*)

and an LAS (*PvOSC4*) from *P. vietnamensis* var. *fuscidiscus* showed clear syntenic relationships with OSC genes from *A. trichopoda* (*AtOSC1*) and *A. fimbriata* (*AfOSC1* and *AfOSC2*). A syntenic relationship was also found between grape *VvOSC7* and *A. trichopoda AtOSC1* (Figure 3). Such conservation was even detected between *A. trichopoda* and the gymnosperm *Welwitschia mirabilis* (Figure 3), demonstrating that CAS genes are spatially conserved in higher plants.

With the above information, we deduced the evolutionary trajectory of OSCs (Figure 3). OSCs were conserved for sterol biosynthesis during the early stages of plant evolution, as only CASs were found in the genomes of lower plants (Xue et al., 2012). Following the emergence of angiosperms, one CAS duplicate (possibly produced by tandem duplication) may have diversified to give rise to LAS through neofunctionalization. The absence of LAS in *Amborella* suggests that the duplication probably occurred after *Amborella* speciation. The chromosomal region harboring CAS and LAS was triplicated into three copies by the γ WGT (A1–3). Several changes were inferred for the triplicated copies. Before the speciation of grape and Apiales, A1 experienced translocation followed by functional diversification of LAS to group I bAS and other mTTSs. The newly formed group I bAS and other mTTSs was duplicated by the Pg-β WGD, with one copy then neofunctionalizing to DDS in the ancestor of extant *Panax* species. For A2, neofunctionalization of LAS gave rise to group II bAS and other mTTSs. In the lineage leading to Apiales, CAS was lost, and the group II bAS and other mTTSs was likely affected by reshuffling, resulting in a non-syntenic distribution pattern. By contrast, CAS and group II bAS and other mTTSs were retained and proliferated through tandem duplications in grape. LUS may have been produced by neofunctionalization of a tandemly duplicated copy of LAS in A3 before the speciation of grape and Apiales. In the Apiales lineage, the LUS probably experienced translocation, as no syntenic relationships were found between LUS and other OSCs.

Functional characterization revealed the origin of ocotillol-type triterpenes in *Panax*

To verify the proposed Pg-β origin of DDSs in *Panax* species, we performed functional analysis to determine the catalytic activities of each tested OSC. Nine OSC genes (five from group I bAS and other mTTS clades and four from the DDS clade) were selected for the analysis (Figure 4B). The OSC genes were heterologously expressed in mutant yeast strain GIL77, which was engineered to accumulate the precursor oxidosqualene (Morita et al., 1997). The products were identified through GC–MS and NMR (Supplemental Figures 19–26; Supplemental Tables 20 and 21). Nine products were identified for every OSC from group I bAS and other mTTSs. For PvOSC6, PgOSC9, PnOSC5, and CaOSC5, α-amyrin, β-amyrin, ψ-taraxasterol, and 3-epicabraleadiol were identified as the main products, with trace amounts of δ-amyrin, taraxasterol, dammarenediol-II, ocotillol, and an unidentified product. The product profile of CaOSC6 was slightly different, with an increased proportion of ψ-taraxasterol and a decrease in α-amyrin content (Figures 4B, 4C, and 27; Supplemental Table 22). In a recent study, CaOSC5 was functionally characterized as a multifunctional OSC producing δ-amyrin, α-amyrin, β-amyrin, ψ-taraxasterol, taraxasterol, and an unidentified product in a ratio of 1:67:26:4:1:1 (Kim et al., 2018b). The previously reported catalytic activities of CaOSC5 are highly consistent with our results, except for the weak ability to produce dammarane-type triterpenes (dammarenediol-II, ocotillol, and 3-epicabraleadiol) identified in our study. Surprisingly, ocotillol and 3-epicabraleadiol were also detected in addition to dammarenediol-II as catalytic products of DDSs from *Panax* species (PgOSC11, PqDDS, PvOSC7, and PnOSC6) (Figure 4B and Supplemental Figure 27; Supplemental Table 22). This multifunctional nature of DDSs in *Panax* species has not previously been reported. We also noted that PgOSC11 produces mainly ocotillol, whereas the other DDSs predominantly produce dammarenediol-II. These findings validate our assumption that DDS in *Panax* species originated from a duplicate of a group I bAS and other mTTSs produced from a WGD. After the Pg-β WGD, one copy of group I bAS and other mTTSs retained its original function (similar to homologs in *C. asiatica*), whereas the other copy experienced neofunctionalization. Judging by the catalytic products, this neofunctionalization should be viewed as a specialization process: from a generalist ancestor to a more specialized state.

Selective forces underlying evolution of OSCs

According to their deduced evolutionary trajectory, OSC genes have experienced several rounds of independent neofunctionalization and specialization events. It is expected that the diversifications occurred under various selection pressures. To characterize the selective forces driving the evolution of OSC genes, we performed various branch-specific tests.

Branch-site unrestricted statistical test for episodic diversification (BUSTED) analysis found evidence (likelihood ratio test [LRT], $P < 0.05$) of gene-wide episodic diversifying selection on at least one site on at least one branch in the phylogeny (Supplemental Figure 28). Adaptive branch-site random effects likelihood (aBSREL) and mixed effects model of evolution (MEME) were then used to determine the exact lineages and sites that were under positive selection. With *a priori* knowledge that CAS genes serve as a blueprint for functional diversification of OSCs, CAS lineages were labeled as background in the branch-site analysis. Analysis with aBSREL found evidence of episodic diversifying selection on 20 out of 159 branches in the tested phylogeny (LRT, $P \leq 0.05$), with only four in the CAS and LAS clades and the rest distributed in lineages leading to LUS, bAS and other mTTSs, and DDS (Supplemental Figure 29). The fact that almost all of the CAS genes were under negative or neutral selection could be explained by the importance of their cycloartenol product, which is the precursor for almost all plant sterols and plays an essential role in plant developmental processes (Gas-Pascual et al., 2014). Notably, episodic diversifying selection was detected on internal branches leading to LUS and group I/II bAS and other mTTSs (nodes 3–5), in which the major neofunctionalization of OSCs occurred (Supplemental Figure 29). This indicates that the diversification of sterol biosynthesis toward triterpene biosynthesis in core eudicots was driven by episodic positive selection, possibly due to the better adaptability conferred by the triterpene products. Notably, the specialization of DDS from group I bAS and other mTTSs in *Panax* species was predicted to be under neutral or negative selection. This could be explained by trade-offs during specialization: for an enzyme in the generalist state, specialization toward certain functions requires a decrease

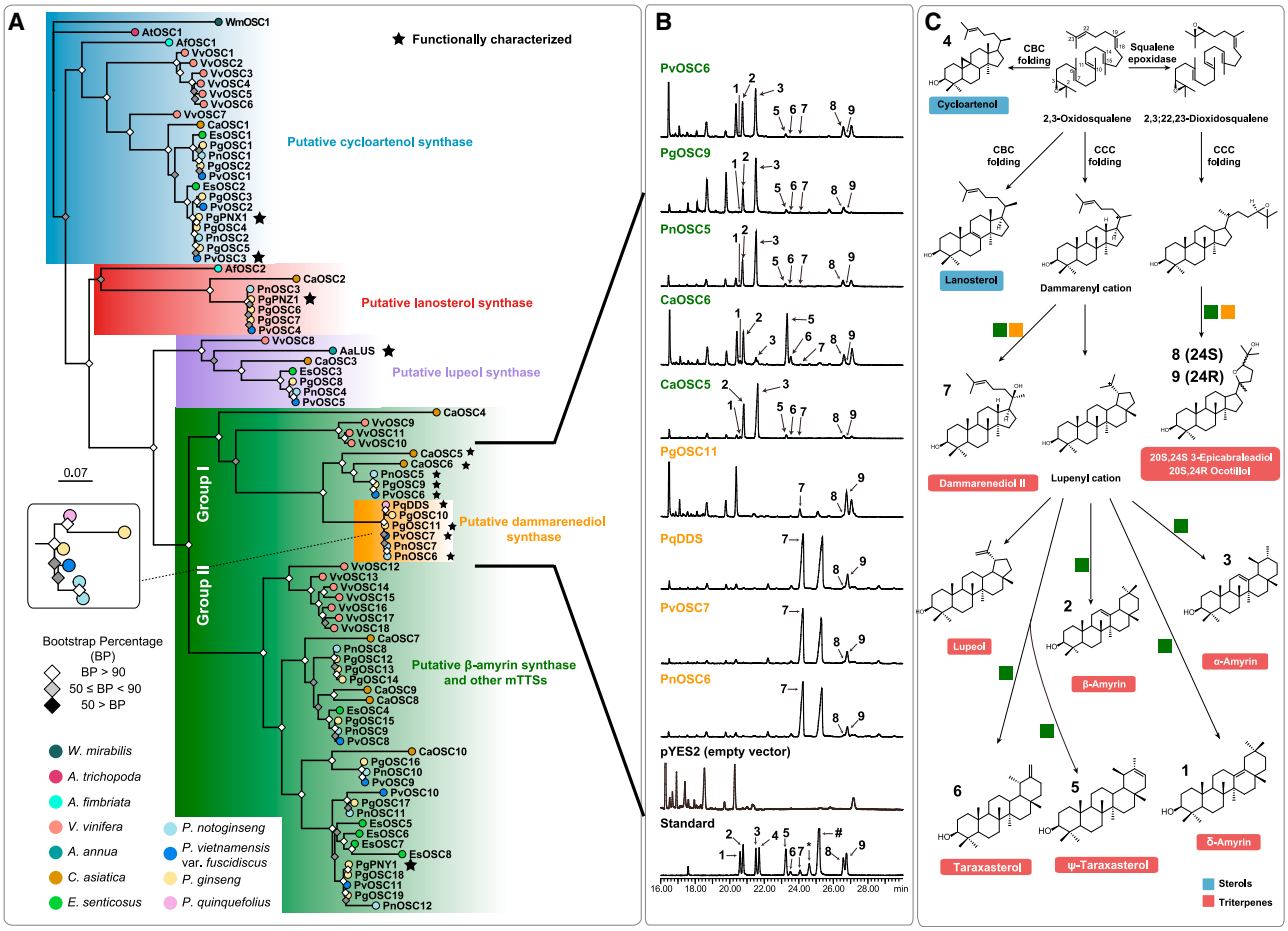

Figure 4. Phylogenetic analysis and functional characterization of OSCs.

(A) ML phylogenetic tree of OSCs based on codon alignments. Bootstraps are shown as colored squares at each node, and species are shown as colored circles at each terminal branch.

(B) Functional characterization of nine OSCs using heterologous expression. The asterisk (*) and hash (#) in the total ion chromatograms (TICs) represent the epoxydammaranes mono-trimethylsilyl ether and dammarenediol-II mono-trimethylsilyl ether, respectively. 1, δ-amyrin; 2, β-amyrin; 3, α-amyrin; 4, cycloartenol; 5, ψ-taraxasterol; 6, taraxasterol; 7, dammarenediol-II; 8, 20S,24S-3-epicabraleadiol; 9, 20S,24R-ocotillol.

(C) Schematic for triterpenoid biosynthesis with sterols highlighted in blue and triterpenes highlighted in red. Compound numbers correspond to the numbers in TICs from **(B)**. Colored squares indicate functions of enzymes in **(B)**.

in the rest. In most cases, reduced promiscuity was shaped by negative selection (Tokuriki et al., 2012; Noda-Garcia and Tawfik, 2020; Tawfik and Gruic-Sovulj, 2020).

MEME found evidence of episodic diversifying selection at 84 sites ($P < 0.05$) (Supplemental Figure 30A). Most of these sites were located near the N/C terminus and the putative active center (Supplemental Figure 30B). Residues from several function-related motifs were found to be under episodic positive selection. For the motif M(W/L)C(Y/H)CR, which has been proposed to stabilize tetracyclic or pentacyclic intermediates, the second site (W/L) was identified as being under episodic positive selection (Kushiro et al., 1999; Ito et al., 2016). Motif Y410, which has been proposed to play an important role in ceiling formation of the active center or in D-ring formation, was also under diversifying selection. The site is conserved as Y in CBC-folding OSCs and F in CCC-folding OSCs (Ma et al., 2016; Chen et al., 2021a). These results provide insights into the evolution of OSCs at the molecular level.

DISCUSSION

The discovered evolutionary trajectories for triterpenoid biosynthesis demonstrate the prominent role of gene duplication in creating a diverse array of triterpenoids in plants. WGDs and tandem duplications are the main forces driving the diversification of OSCs. An ancient tandem duplication of CAS during the early evolution of angiosperms may have given rise to LAS. This event possibly predates the emergence of Nymphaeales species, given the presence of LAS orthologs in *Nymphaea colorata* (Wang et al., 2022). The expansion and diversification of OSCs in core eudicots were attributed mainly to the γ WGT, with subsequent neofunctionalization occurring in the LAS triplicates. Specifically, triterpene synthases in core eudicots originated from independent neofunctionalization of LAS copies. This finding supports the hypothesis that eudicot triterpene biosynthesis

derives from LAS rather than CAS (Kolesnikova et al., 2006; Xue et al., 2012). Indeed, experimental evidence suggested that LAS can supplement the biosynthesis of phytosterols in plants. However, the methyl jasmonate/bacteria-induced regulatory mechanism and tissue-specific expression pattern of LAS suggest its similarity to triterpene synthases (Zimmermann et al., 2004; Kolesnikova et al. 2006). The altered regulation and expression pattern of LAS may represent the initial step toward triterpene biosynthesis. Major angiosperm lineages such as monocots also exhibit great potential for the synthesis of various triterpenes (Inagaki et al., 2011); thus, the revealed γ WGT origin of triterpene biosynthesis in core eudicots suggests convergent evolution in OSC diversification in addition to the prevalent divergent evolution. We also revealed that group I and group II bAS and other mTTSs in eudicots originated from different LAS copies, thus explaining their distant phylogenetic relationship. A recent study of the evolutionary path of OSC genes based on phylogenetic trees inferred several major duplication events for OSC genes, including one ancient duplication event generating the CAS and LAS lineages and another three separate duplication events generating triterpene synthase (LUS and bAS) (Wang et al., 2022). Our results show that the former ancient duplication event was likely caused by tandem duplication of the ancestral OSC gene, whereas the latter three duplication events actually resulted from a single duplication event, the γ WGT. This finding also demonstrates the limitations of using only phylogenetic trees when inferring the evolutionary paths of genes.

Dammarane-type and ocotillol-type triterpenes are abundant mainly in *Panax* species. Several *Panax* DDS genes have been functionally characterized as producing dammarenediol-II, but the genes responsible for synthesizing ocotillol-type triterpenes remain unclear. Our analysis revealed the origin of the DDS gene family in *Panax* species and its multifunctional nature. Future studies using site-directed mutagenesis and crystal structure analysis may provide insight into the reaction mechanism that underlies the shift in product profile of these OSCs.

In principle, reshaping of metabolite biosynthesis after gene duplication is strongly affected by selection. Our results suggest that most of the WGD-derived OSC gene copies were lost during evolution, possibly owing to accumulation of negative mutations under relaxed selection pressure. However, some OSC duplicates acquired altered functions through mutations, which were then fixed by fitting ecological opportunities. This scenario was illustrated by the effect of episodic diversifying selection on neofunctionalization of LAS copies toward triterpene synthases in core eudicots. Such functional innovations of duplicates are not always driven by positive selection. In ancestral lineages of *Panax* species, one group I bAS and mTTS copy (which mainly produced amyrin) that derived from the Pg-β WGD experienced functional specialization under negative/neutral selection, eventually giving rise to DDS. The absence of positive selection might be explained by the trade-off in catalytic activities between amyrin-type and dammarane-type triterpenes (Noda-Garcia and Tawfik, 2020). Accordingly, it should be noted that the absence of positive selection during the creation of novelties does not necessarily indicate a lack of improvement in adaptivity.

In summary, the revealed origin and evolutionary history of triterpenoid biosynthesis in angiosperms provide insight into how gene duplication can drive the diversification of metabolite biosynthesis. In plants, triterpenoids are often further modified by tailoring enzymes (e.g., cytochrome P450s, glycosyltransferases, and acyltransferases) (Thimmappa et al., 2014). Studies have suggested that genes for OSCs and tailoring enzymes are likely functionally co-opted by gene duplication, resulting in diversification in gene regulation and expression patterns for triterpenoid biosynthesis (Li et al., 2021b; Su et al., 2021). Future studies on the interactions between these tailoring enzymes and OSCs and their origins will deepen our understanding of the evolution of metabolite biosynthesis.

METHODS

Genome sequencing and assembly

Plant samples of *P. vietnamensis* var. *fuscidiscus* were collected from individuals cultivated in Jinping County, Yunnan, China. Fresh leaves, stems, and roots were stored in liquid nitrogen and sent to Novogene for sequencing (Beijing, China). High-molecular-weight genomic DNA was extracted from leaves using the cetyltrimethylammonium bromide (CTAB) method and purified with a QIAGEN Genomic Kit (Qiagen, USA). For long-read sequencing, 20-kb SMRTbell libraries were generated and sequenced on the PacBio Sequel platform. This produced ~67.7× PacBio long reads. We also generated ~132.6× Illumina short reads. Four libraries with an insert size of 300 bp were prepared and sequenced on the Illumina HiSeq 2000 platform (Illumina, San Diego, CA, USA). High-throughput chromosome conformation capture (Hi-C) libraries were prepared and sequenced on the Illumina HiSeq 4000 platform. In brief, chromatin was cross-linked with formaldehyde and digested with the restriction enzyme *DpnII* before sequencing. For the purpose of gene prediction, total RNA was isolated from leaves, stems, and roots using the RNAprep Pure Plant Kit (TIANGEN). RNA libraries with an insert size of 300 bp were generated and sequenced on the Illumina HiSeq 2000 platform (Supplemental Table 23).

The genome size of *P. vietnamensis* var. *fuscidiscus* was estimated using flow cytometry (BD FACSCalibur) and GenomeScope (v2.0) with kmer frequencies counted from 132.6× Illumina reads using Jellyfish (v2.2.10) (Marçais and Kingsford, 2011; Ranallo-Benavidez et al., 2020). The PacBio reads were assembled using NextDenovo (v2.4.0) (https://github.com/Nextomics/NextDenovo), followed by two rounds of polishing with NextPolish (v1.3.1) (Hu et al., 2020). After removing allelic contigs using Purge Haplotigs (v1.1.1) (Roach et al., 2018), we performed scaffolding using Juicer (v1.6.2) (Durand et al., 2016) and the three-dimensional (3D) *de novo* assembly (3D-DNA) pipeline (Dudchenko et al., 2017). Mis-joins were manually corrected on the basis of Hi-C contact signals. For transcriptome assembly, raw reads were trimmed with fastp (v0.20.1) (Chen et al., 2018) and assembled using Trinity (v2.11.0) (Grabherr et al., 2011).

The quality of the genome assemblies was evaluated using BUSCO (v5.1.2) (Manni et al., 2021) with dataset eudicots_odb10. We also mapped Illumina reads to the genome using BWA-MEM (v0.7.12) (Li and Durbin, 2009a) and calculated the mapping statistics using SAMtools (v1.9) (Li et al., 2009b).

Genome annotation

We used LTR_FINDER_parallel (v1.1) (Ou and Jiang, 2019) and LTRharvest (v1.0) (Ellinghaus et al., 2008) to predict long terminal repeat retrotransposons (LTR-RTs). The identified LTR-RT candidates were passed to LTR_retriever (v2.8) (Ou and Jiang, 2018b) to filter out the false positives and generate a genome LTR assembly index (LAI) (Ou et al., 2018a). Only intact LTRs were retained for insertion time estimation. The equation $T = K/2\mu$ was used for time estimation, where K is the LTR divergence rate and μ is the neutral mutation rate (1.3×10^{-8} mutations per site per year). We also used RepeatModeler (v2.0) (Flynn et al., 2020) to detect novel repeat sequences. Repetitive elements generated by LTR_retriever and RepeatModeler were fed to RepeatMasker (v4.0.9) (http://www.repeatmasker.org) for *de novo* prediction. For evidence-based methods, repetitive elements were predicted using RepeatMasker and RepeatProteinMask (v4.0.9) (http://www.repeatmasker.org) with Repbase (v24.06) (Bao et al., 2015) as the reference. The tandem repeats were annotated using Tandem Repeat Finder (v4.09) (Benson, 1999). The predicted LTR-RTs were further classified by TEsorter (v1.2.5) (Zhang et al., 2022) with REXdb Viridiplantae (v2.2) (Neumann et al., 2019).

Gene structures were predicted using a combination of *ab initio*-, homology-, and transcript-based methods. GenScan (v1.0) (Aggarwal and Ramaswamy, 2002), GlimmerHMM (v3.0.3) (Majoros et al., 2004), geneid (v1.4.4) (Alioto et al., 2018), Augustus (v3.2.2) (Stanke et al., 2008), and SNAP (v1.0) (Korf, 2004) were used for *ab initio* prediction of protein-coding genes. For the homology-based method, proteomes of *A. thaliana*, *V. vinifera*, *E. senticosus*, *D. carota*, and *P. ginseng* were searched against the genomes using TBLASTN (v2.2.29+) (Altschul et al., 1990) with $1e^{-5}$ as the cutoff e-value. Gene models were predicted by GenomeThreader (v1.7.3) (Gremme et al., 2005) using the above hits. For the transcript-based method, Program to Assemble Spliced Alignments (PASA) (v2.4.1) (Haas et al., 2003) was used for gene prediction by comparing Trinity transcripts with genomes. Finally, all gene models were integrated using EVidenceModeler (v1.1.1) (Haas et al., 2008) and updated with PASA. Functional annotation was performed with eggNOG-mapper (v2.1.7) (Cantalapiedra et al., 2021) by searching the eggNOG database (v5.0.2) (Huerta-Cepas et al., 2019) (Viridiplantae-33090) using DIAMOND (v2.0.14) (Buchfink et al., 2015).

Phylogenomics and evolutionary analysis

Orthogroups were identified using OrthoFinder (v2.5.4) (van Dongen, 2000; Emms and Kelly, 2019) based on protein sequences of 12 species (Supplemental Table 24). The Venn diagram was visualized using Evenn (Chen et al., 2021b).

Species trees were inferred based on single-copy orthogroups. Protein sequences from each single-copy orthogroup of 12 species were extracted and aligned using MAFFT (v7.475) (Katoh and Standley, 2013). Then, the protein alignments were converted to codon alignments using PAL2NAL (v14) (Suyama et al., 2006). Poorly aligned regions from codon alignments were trimmed using trimAl (v2.rev0) (Capella-Gutiérrez et al., 2009). For the concatenation-based method, an ML phylogenetic tree was built based on concatenated codon alignments using IQ-TREE (v2.0.3) (Nguyen et al., 2015) with the best-fit substitution model determined using ModelFinder (Kalyaanamoorthy et al., 2017). Branch supports were estimated using 1000 replicates with ultrafast bootstrap approximation (UFBoot2) (Hoang et al., 2018). For the coalescent-based method, a species tree was estimated using ASTRAL (v5.7.7) (Zhang et al., 2018) based on ML trees produced from IQ-TREE. We estimated species divergence times using MCMCTree from the PAML package (v4.9j) (Yang, 2007) with molecular clock and nucleotide substitution set as correlated rates and JC69 model. The MCMC process was run for 100 000 iterations with a burn-in of 50 000 and a sampling frequency of five. The tree was calibrated with the following constraints: divergence time of *D. carota* and *A. graveolens* (~22–37 Mya), divergence time of Araliaceae and Apiaceae (~45–70 Mya), and divergence time of *V. vinifera* and the other studied species (~111–131 Mya) (Kumar et al., 2017). Phylogenetic trees were visualized using FigTree (v1.4.4) (http://tree.bio.ed.ac.uk/software/figtree/).

Changes in gene family size during species evolution were estimated using CAFE (v5) (Mendes et al., 2020). Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses of gene families were performed using clusterProfiler (v4.2.2) (Wu et al., 2021) and TBtools (v1.098685) (Chen et al., 2020), respectively, with *P* values adjusted by the Benjamini and Hochberg method.

WGD and speciation analysis

The WGDI toolkit (v0.5.1) (Sun et al., 2022) was used to detect WGD and speciation events. First, BLASTP (v2.2.29+) was used to search for homologs with a $1e^{-5}$ cutoff e-value. Collinear genes were identified by WGDI on the basis of the identified homologs using the parameter -icl. *Ks* values of collinear gene pairs were then calculated using the YN00 program from the PAML package with the Nei-Gojobori method (Nei and Gojobori, 1986). The median *Ks* values of inter/intraspecific collinear blocks were fitted using Gaussian kernel density estimation with the parameter -pf. The intraspecific syntenic relationships within *P. vietnamensis* var. *fuscidiscus*, together with the GC content, TE density, and gene density, were visualized using Circos (v0.69-9) (Krzywinski et al., 2009).

Synteny-based phylogenetic analysis was used to infer the WGD and speciation history. On the basis of the similarity and completeness of inter/intraspecific syntenic blocks, syntenic regions were assigned to WGD-related putative sets for *V. vinifera*, *C. asiatica*, *E. senticosus*, *P. notoginseng*, and *P. vietnamensis* var. *fuscidiscus* with parameters -bi and -a. Collinear genes from the characterized sets were extracted and used to construct ML phylogenetic trees separately using IQ-TREE. Collinear gene pairs encompassing genes from all studied species were retained for ASTRAL analysis.

Inferring evolutionary trajectories of OSC genes

Putative OSCs were identified using HMMER (v3.1b2) (Eddy, 1998) by searching with the squalene-hopene cyclase N-terminal domain (PF13249) and C-terminal domain (PF13243) from Pfam (v35.0) (Mistry et al., 2021) with the parameter -cut_tc. Sequences that contained both domains were retained for analysis. For phylogenetic analysis, protein sequences of the putative OSCs

were aligned using MAFFT. The protein alignments were converted to codon alignments by PAL2NAL, followed by trimming with trimAl. IQ-TREE was used to construct an ML phylogenetic tree for the putative OSCs. The tree and motifs were visualized using the R packages ggtree (v2.4.1) (Yu et al., 2018) and ggmsa (v1.0.0) (http://yulab-smu.top/ggmsa/). To assist with classification of putative OSCs, sequences of functionally characterized OSCs were downloaded from NCBI and included in the analysis. One *P. vietnamensis* var. *fuscidiscus* OSC (PvOSC3) was also functionally characterized (Supplemental Figure 31). For synteny-based analysis, the syntenic relationships among putative OSC genes were identified with WGDI. We used JCVI utility libraries (v1.1.23) (Tang et al., 2008) to visualize the micro-synteny of OSCs.

Functional characterization of OSCs

Nucleotide coding sequences of putative OSC genes were synthesized and ligated into the yeast expression vector pYES2 (Invitrogen) under the control of the *GAL1* promotor by GeneCreate (Wuhan, China). Vectors carrying putative OSC genes were transformed into DH5α competent cells. The resulting plasmid DNAs were transformed into the mutant yeast strain GIL77 by the lithium acetate/single-stranded carrier DNA/PEG method (Gietz and Schiestl, 2007). Yeast strains transformed with the empty vector were used as controls. Yeast strains were incubated in synthetic medium containing ergosterol (20 μg ml^{-1}), hemin chloride (13 μg ml^{-1}), and Tween 80 (5 μg ml^{-1}) for 3 days followed by 48-h Gal induction and another 24-h incubation. Cells were harvested and refluxed in 20% KOH/50% EtOH for 10 min and extracted with petroleum ether three times. The organic phase was concentrated *in vacuo*. Gas chromatography–mass spectrometry (GC–MS) analysis was performed using an Agilent 7890A and Agilent 6540 Accurate-Mass Q-TOF (Santa Clara, USA). NMR analysis was performed on a Bruker AV 600 MHz spectrometer (Billerica, USA) (see supporting information Methods S1).

Selection analysis

The codon alignments and ML phylogenetic tree for putative OSCs from the previous step were used for selection analysis. We used HYPHY (v2.5.32) (http://hyphy.org/) to perform the BUSTED (Murrell et al., 2015), aBSREL (Smith et al., 2015), and MEME (Murrell et al., 2012) analyses. The 3D protein structure of *P. ginseng* CAS was downloaded from UniProt (UniProt Consortium, 2021) with identifier AF-O82139-F1 (predicted by AlphaFold [Jumper et al., 2021]). PyMOL was used for visualization of protein structures (The PyMOL Molecular Graphics System, Version 2.5, Schrödinger, LLC.).

ACCESSION NUMBERS

The raw sequencing data, assembly, and annotation files of *P. vietnamensis* var. *fuscidiscus* and *P. notoginseng* have been deposited at CNGBdb (https://db.cngb.org/) under project accession numbers CNP0002878 and CNP0003588.

SUPPLEMENTAL INFORMATION

Supplemental information is available at *Plant Communications Online*.

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AUTHOR CONTRIBUTIONS

S.Y. and Y.D. designed the research. Z.Y., X.L., L.Y., W.S., and G.Z. collected the data. Z.Y., X.L., L.Y., and S.P. performed the data and experimental analyses. Z.Y., W.C., S.Y., and Y.D. wrote the manuscript with contributions from all authors.

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