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# The tree of life of copper-containing amine oxidases

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Copper-containing amine oxidases (CuAOs) catalyze the terminal oxidation of polyamines (PAs), producing ammonium, an aminoaldehyde, and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). Plant CuAOs are induced by stress-related hormones such as methyl-jasmonate (MeJA), abscisic acid (ABA), and salicylic acid (SA). Mammalian copper-containing amine oxidases (CAOs), encoded by four genes (AOC1-4) that catalyze the oxidation of primary amines to aldehydes, regulate various biological processes and are linked to diseases like inflammatory conditions and histamine intolerance. To understand the evolutionary history and functional divergence of CuAOs, we conducted phylogenetic and expression analyses of CuAOs in plants and animals. In this study, the *copper amine oxidase* (*CuAO*) genes were identified by HMMER and BLASTP, and verified by CDD/HMM/SMART. Multiple sequence alignment was performed using Muscle5, and the phylogenetic tree was constructed by IQ-TREE2. The syntenic relationship was analyzed by MCScanX and CIRCOS. Meanwhile, the expression data of *Arabidopsis thaliana* and human and other species were integrated for analysis. Here, 950 and 264 CuAO orthologues were identified in 188 plant and 79 animal genomes. Phylogenetic analyses indicate that CuAO originated in the common ancestor before the divergence of plants and animals. The copy numbers of CuAOs vary significantly across plant species, whereas they remain relatively stable in animal species, generally maintaining 3-4 copies per species. During the evolutionary process, plant CuAOs formed two clades (I and II), while animal CuAOs formed three clades (CAO-like, AOC1, AOC2-4). Interestingly, plant clade I CuAOs lacks the active site motif T/S-X<sub>1</sub>-X<sub>2</sub>-N-Y-D. The further differentiation of plant clade II CuAOs is related to the preference for X<sub>1</sub> and X<sub>2</sub> active sites. CAO-like and AOC1 are monophyletic branches. Mammalian AOC2-4 is separated from non-mammalian AOC2-4, and the differentiation of mammalian AOC3 and AOC4 occurs in a species-specific manner. Our study provides a comprehensive understanding of the evolutionary trajectory of the *CuAO* gene family in plants and animals at the genome-wide level. These findings lay a crucial foundation for future research to conduct in-depth functional characterization.

## KEYWORDS

CuAO, molecular evolution, origin, diversification, gene duplication

## Introduction

PAs are a class of small aliphatic nitrogen-containing compounds that are widely present in animals, plants, bacteria, and fungi (Wang et al., 2019). As important regulators of cell growth and differentiation, PAs are involved in basic cellular processes such as DNA replication and transcription, RNA modification, protein synthesis, ion channel regulation, free radical scavenging, cell cycle regulation, signal transduction, and programmed cell death (Tavladoraki et al., 2012; Murray-Stewart et al., 2016). In plants, PAs play crucial roles in flower development and differentiation, leaf development and aging, fruit ripening, and various stress responses (Wang et al., 2019). In mammalian cells, dysregulation of PA metabolism is associated with cancer development, making it an important target for anti-cancer therapy (Boomsma et al., 2003; Dunkel et al., 2008; Salmi and Jalkanen, 2019). The three most common PAs found in plants and animals are putrescine (Put), spermidine (Spd), and spermine (Spm) (Shukla et al., 2020). Their oxidative degradation is mainly catalyzed by copper amine oxidase (CuAO) and polyamine oxidase (PAO). CuAOs mainly catalyze the oxidation of putrescine to produce  $\text{NH}_4^+$ , 4-aminobutyrate, and  $\text{H}_2\text{O}_2$ , with 4-aminobutyrate further converting to  $\gamma$ -aminobutyric acid (Fortes et al., 2019). CuAOs not only play roles in plants, but also are widely distributed in mammalian tissues, and their activity changes are related to various diseases, such as histamine intolerance and rheumatism (Boomsma et al., 2003; Dunkel et al., 2008; Salmi and Jalkanen, 2019).

CuAOs are widely occurred enzymes that catalyze the oxidative deamination of primary amines and PAs such as diamines and histamine (Pietrangeli et al., 2003). Their molecular form is a dimer, with subunit molecular weights ranging from 70 to 95 kDa (Tavladoraki et al., 2016; Fraudentali et al., 2021). The enzymatic activity of CuAOs depends on two cofactors: covalently bound 2,4,5-trihydroxyphenylalanine quinone (TPQ) and a copper ion involved in the catalytic cycle (Brazeau et al., 2004). TPQ is produced by the modification of endogenous tyrosine residues, and copper ions play a key role in the biogenesis and catalytic reactions of TPQ-mediated substrate oxidation (Brazeau et al., 2004). Mammalian CuAOs have a highly conserved T/S-X<sub>1</sub>-X<sub>2</sub>-N-Y-D motif at the active site, where tyrosine is post-translationally modified to TPQ (Lopes de Carvalho et al., 2019). CuAOs primarily catalyze the terminal oxidation of diamines (such as Put) and, in some cases, the oxidation of higher PAs (such as Spd), thus playing a crucial role in the early steps of PA oxidative metabolism and regulating specific subcellular compartments of PA levels (Fraudentali et al., 2020a). CuAOs catalytic reactions involve three main processes: (i) TPQ biosynthesis, (ii) amine substrate oxidation to reduce TPQ, and (iii) molecular oxygen reduction via reduced TPQ (Brazeau et al., 2004). All of these reactions require the participation of molecular oxygen (Brazeau et al., 2004).

CuAOs have various biological functions in plants and animals. In *Arabidopsis thaliana*, there are 10 CuAO genes, of which *AtCuAOε1* (AT4G12270) and *AtCuAOε2* (AT4G12280) lack essential active residues and are considered to be fragment copies of *AtCuAOδ* (AT4G12290) (Qu et al., 2014; Tavladoraki et al., 2016; Fraudentali et al., 2020a). *AtCuAOα3* (AT1G31710) and *AtCuAOζ* (AT2G42490) play roles in regulating the stability of PA in peroxisomes (Planas-Portell et al., 2013). *AtCuAOγ1* (AT1G62810) primarily involves NO production mediated by PAs and/or abscisic acid (ABA), while *AtCuAOα2* (AT1G31690) regulates arginase activity to affect the availability of arginine and thus participates in NO production, revealing a new regulatory pathway for NO production in plants (Tavladoraki et al., 2016; Groß et al., 2017). *AtCuAOδ* plays a significant role in ABA-induced stomatal closure (Fraudentali et al., 2019), while *AtCuAOβ* (AT4G14940) is involved in the stomatal regulation process under mechanical damage conditions (Fraudentali et al., 2023). Furthermore, *AtCuAOβ* is involved in both MeJA-induced early root protoxylem differentiation and the response to wounding (Ghuge et al., 2015a; Ghuge et al., 2015b). The expression of *AtCuAOζ* in guard cells indicates that it is involved in ABA-mediated stomatal opening control (Qu et al., 2014). *AtCuAOβ*, *AtCuAOγ1*, and *AtCuAOγ2* are located in the apoplast (Fraudentali et al., 2020a; Fraudentali et al., 2020b). Additionally, *AtCuAOα2* and *AtCuAOα3* are located in peroxisomes (Fraudentali et al., 2020a). To date, the only vacuolar located CuAO isoform is *AtCuAOδ* (Fraudentali et al., 2019). There are 8 CuAO genes in *Solanum lycopersicum* genome, among which 6 CuAOs (*SICuAO1*, 2, 3, 4, 6, 7) are specifically expressed in the roots, *SICuAO5* is specifically expressed in the fruits, and *SICuAO8* is specifically expressed in the flowers (Upadhyay et al., 2024a). This indicates that the expression of amine oxidases is diverse in different tissues, and it is particularly prominent in the roots (Upadhyay et al., 2024a). In humans, there are four genes that encode CuAO: *AOC1* (diamine oxidase), *AOC2* (retinal-specific amine oxidase), *AOC3* (vascular adhesion protein-1, VAP-1), and *AOC4* (pseudogene) (Finney et al., 2014). *AOC1* is mainly expressed in the kidney, placenta, intestine, thymus, and seminal vesicle, and is the main enzyme for metabolizing histamine (Elmore et al., 2002; Maintz and Novak, 2007). Its activity is related to the risk of pregnancy (Maintz et al., 2008). *AOC2* was initially cloned from the retina and is also expressed in adipose tissue, playing a role in the process of adipocyte differentiation (Imamura et al., 1997; Heniquez et al., 2003; Bour et al., 2007). The substrates of *AOC2* include 2-phenylethylamine, tryptamine and p-tyramine, but it does not oxidize histamine (Finney et al., 2014). *AOC3* is highly expressed in adipocytes, smooth muscle cells, and endothelial cells, and is abundantly present in the lungs, aorta, liver, and ileum (Kurkijärvi et al., 1998).

While the roles of CuAOs are slowly being clarified, studies on these enzymes remain confined to a limited number of model organisms, including humans and *A. thaliana*. Currently, our understanding of the origin and evolution of CuAOs is sparse, and a comprehensive investigation into CuAOs across both plant and animal kingdoms is still missing. In this study, we explored the evolutionary

path of CuAOs by examining the complete genomic sequences from various plants and animals. As far as we are aware, this represents the most thorough phylogenetic analysis of CuAOs conducted so far. This research offers an in-depth view of the evolution of CuAOs, examining their origins, evolutionary processes, and functional diversity, while also establishing a strong basis for future studies on functional resolution and molecular evolution.

## Results

### Identification and distribution of *CuAO* genes

In order to comprehensively identify CuAO in plants and animals, we selected a diverse range of 188 plant species and 79 animal species, including rhodophytes, chlorophytes, charophytes, bryophytes (hornworts, mosses, liverworts), ferns, lycophytes, gymnosperms, basal angiosperms, angiosperms, invertebrates and vertebrates (Table 1, Supplementary Table S1). Through homology search and domain prediction, a total of 1214 candidate protein sequences of CuAO were retrieved, including 950 plant sequences and 264 animal sequences (Table 1, Supplementary Table S2). CuAO copy numbers are conserved among different animal lineages, while they vary significantly among different plant lineages (Table 1). The average count of CuAOs was 2 in rhodophytes, 1.6 in chlorophytes, 1.8 in charophytes, 1.29 in hornworts, 3.38 in mosses, 2.59 in liverworts, 3 in

ferns, 3.14 in lycophytes, 4.07 in gymnosperms, 2 in basal angiosperms, 2.36 in magnoliids, 5.57 in monocots, and 7.53 in eudicots. The copy numbers of CuAO were lower in algal species, higher in land plants, and significantly elevated in seed plants. This change may be closely related to the gradual increase in biological complexity or the whole genome duplication (WGD) events experienced during plant evolution. Interestingly, most animal lineages have a relatively stable number of CuAOs, typically ranging from 3 to 4.

### Evolutionary origin of CuAOs in plants and animals

To further explore the evolutionary origin of CuAOs, we selected representative species and constructed phylogenetic trees with IQ-TREE2 tool. The phylogenetic tree topologies distinctly categorized CuAOs from plants and animals into two separate clades, suggesting that the origin of CuAOs predates the divergence between plants and animals (Figure 1). Additionally, the phylogenetic tree shows that CuAOs underwent a distinct expansion in plants, forming two branches.

### Phylogenetic classification of plant CuAOs

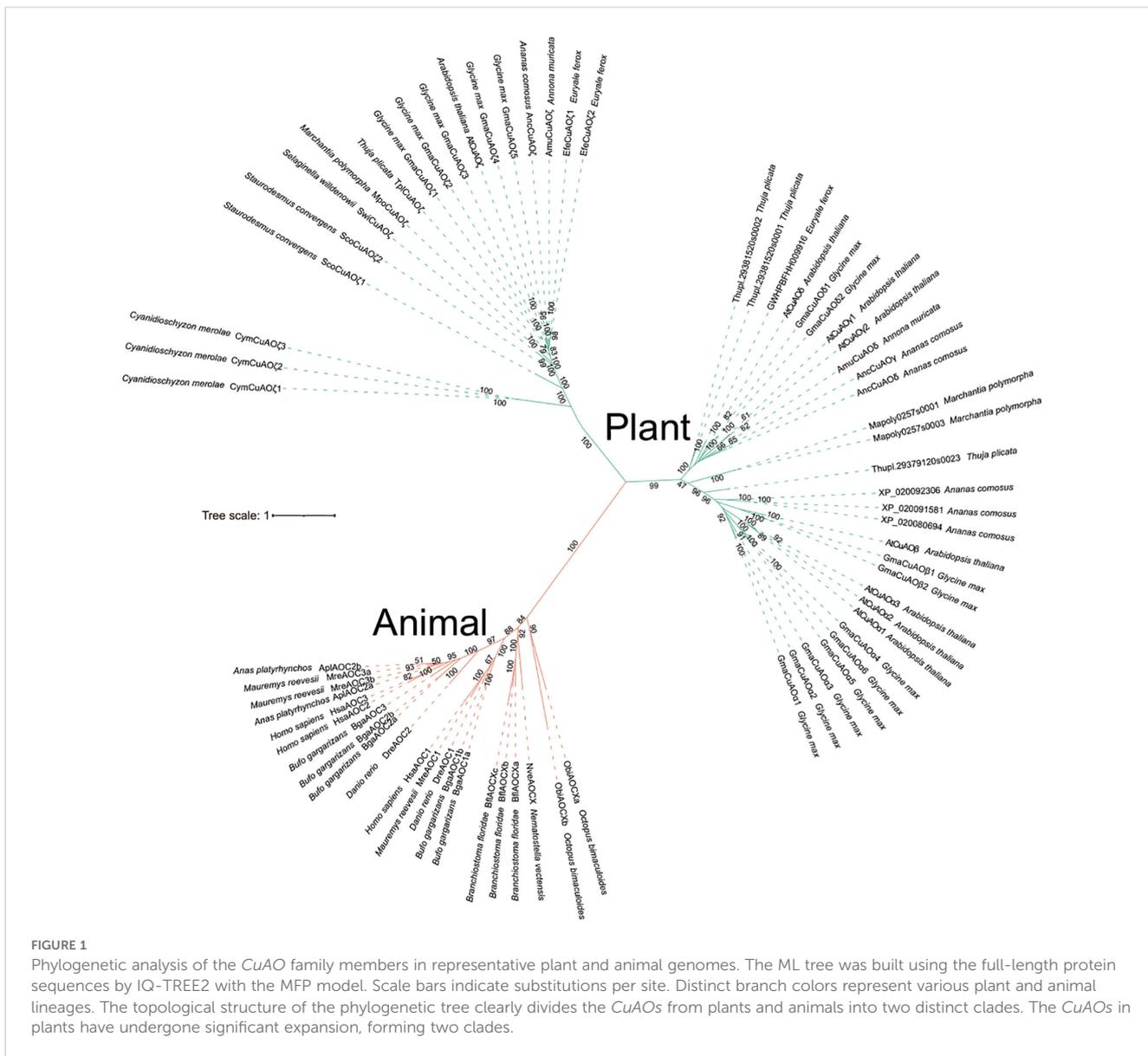
An IQ tree was constructed for CuAO proteins from plant lineages including rhodophytes, chlorophytes, charophytes, bryophytes (hornworts, mosses, liverworts), ferns, lycophytes, gymnosperms, basal angiosperms, angiosperms (Figure 2, Supplementary Figure S1). The phylogenetic tree shows that CuAO expanded and split into two branches in the most recent common ancestor of plants: clade I and clade II. Clade I is also known as the  $\zeta$  branch and is a monophyletic branch, indicating that its members share a common ancestor. They evolved independently during radiation of plants. Clade II expanded after the division of vascular plants into the lycophytes and ferns, forming the subbranches of  $\gamma + \delta$  and  $\alpha + \beta$ . The differentiation of  $\gamma$  and  $\delta$  occurred in the common ancestor of the mesangiosperms, while the differentiation of  $\alpha$  and  $\beta$  occurred in the common ancestor of the eudicots and magnoliids. Clade I lost the green algae, and Clade II lost hornwort.

### Phylogenetic classification of the animal CuAOs

To better understand the evolutionary relationships of animal CuAOs, we constructed an IQ tree using the CuAO sequences of 79 animals (15 invertebrates and 64 vertebrates) (Figure 3). In invertebrates, the CuAO from Coelenterata, Mollusca, and Leptocardii formed highly supported monophyletic clades. Vertebrates include mammals, birds, reptiles, amphibians and fish. The vertebrate CuAO can be divided into two main branches, AOC1 and AOC2-4. In AOC1, mammals and non-mammals form a monophyletic branch, while in AOC2-4, mammals and non-mammals are separated. Non-mammalian

TABLE 1 The number of *CuAO* in plant species and animal species.

Taxonomy	Number of species	Number of <i>CuAO</i>	Average number of <i>CuAO</i> per species
Rhodophytes	2	4	2.00
Chlorophytes	5	8	1.60
Charophytes	5	9	1.80
hornworts	7	9	1.29
mosses	8	27	3.38
liverworts	17	44	2.59
Lycophytes	7	22	3.14
Ferns	1	3	3.00
Gymnosperms	15	61	4.07
Basal angiosperms	3	6	2.00
Magnoliids	14	33	2.36
Monocots	30	167	5.57
Eudicots	74	557	7.53
Brassicaceae	36	273	7.58
invertebrates	15	53	3.53
vertebrates	64	211	3.30



**FIGURE 1** Phylogenetic analysis of the CuAO family members in representative plant and animal genomes. The ML tree was built using the full-length protein sequences by IQ-TREE2 with the MFP model. Scale bars indicate substitutions per site. Distinct branch colors represent various plant and animal lineages. The topological structure of the phylogenetic tree clearly divides the CuAOs from plants and animals into two distinct clades. The CuAOs in plants have undergone significant expansion, forming two clades.

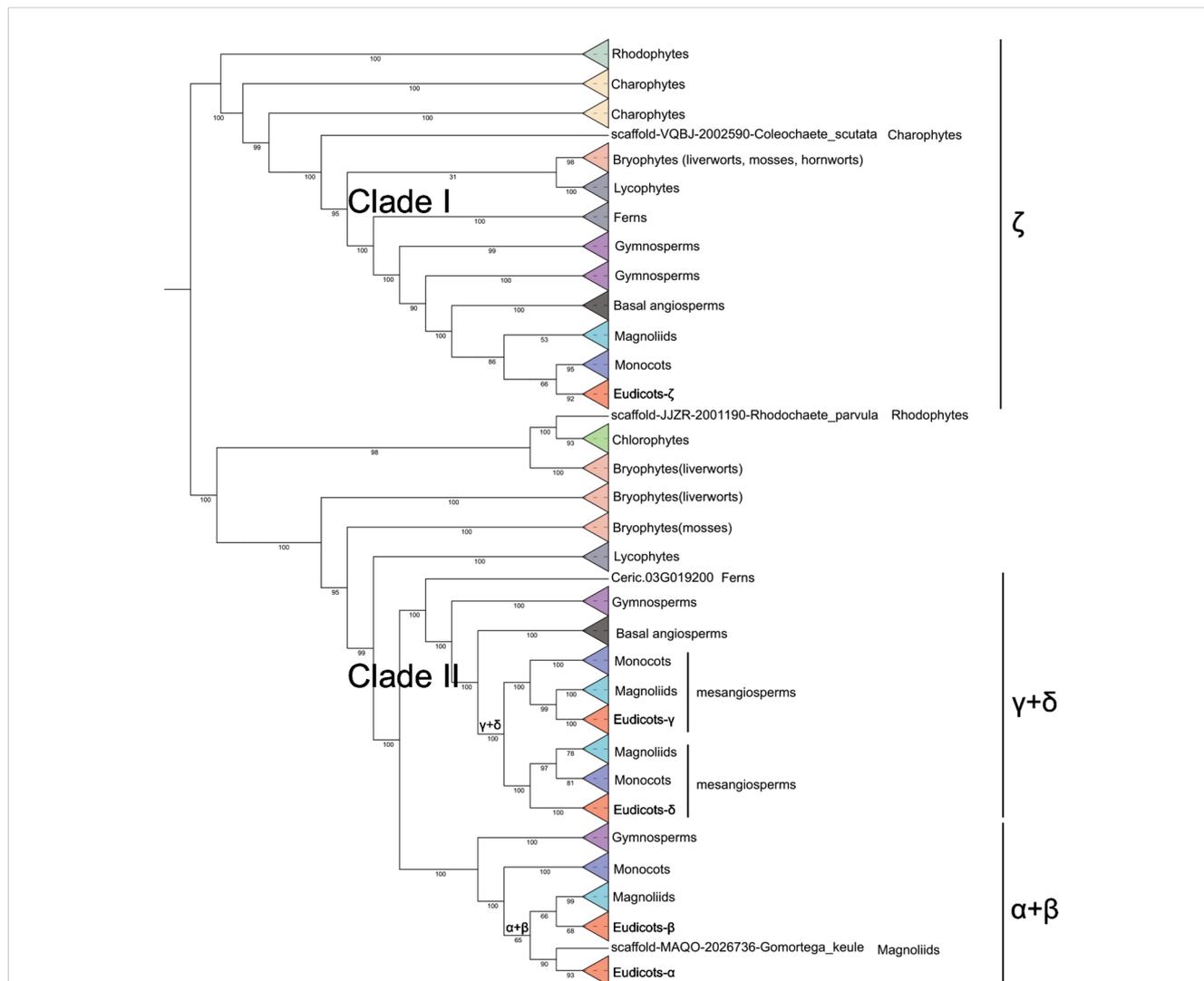
AOC2 and AOC3 form their own subbranches, while mammalian AOC2-4 further divide into the subbranches of AOC2 and AOC3/4. Interestingly, the differentiation of AOC3 and AOC4 in mammals occurs in species-specific ways, which may be due to different evolutionary pressures. AOC1 and AOC2 are present in all vertebrates, AOC3 is present in amphibians, reptiles, and mammals, and AOC4 is unique to mammals. CAO-like is present in invertebrates and reptiles.

### Phylogenetic classification of CuAOs in Brassicaceae

Certain species within the Brassicaceae family are widely cultivated and possess considerable economic importance. To further elucidate the systematic relationships within the Brassicaceae CuAO family, we constructed a phylogenetic tree

using 36 Brassicaceae plants and three sister groups (*Carica papaya*, *Tarenaya hassleriana*, *Cleome violacea*) with a total of 291 CuAOs (Figure 4). The five subbranches of the Brassicaceae are all monophyletic branches, and the  $\alpha$  branch has a significantly higher number of CuAOs than the other subbranches (Figure 4A).

Previous studies have shown that the variable residues X<sub>1</sub> and X<sub>2</sub> in the T/S-X<sub>1</sub>-X<sub>2</sub>-N-Y-D active site motif of proteases follow the substrate preference of mammalian CuAOs, which helps distinguish their subfamilies (Salminen et al., 1998; Kivi et al., 2009; Lopes de Carvalho et al., 2019). This leads us to believe that the residues at positions X<sub>1</sub> and X<sub>2</sub> may help distinguishing between the Brassicaceae CuAO subfamilies. The results show that the Brassicaceae CuAO $\zeta$  branch lacks the T/S-X<sub>1</sub>-X<sub>2</sub>-N-Y-D active site motif in its entirety, which may affect the function of the enzyme or its role in metabolic pathways, suggesting that this branch has different biological functions from other branches with active sites (Figure 4B). CuAO $\gamma$  and CuAO $\delta$  tend to have



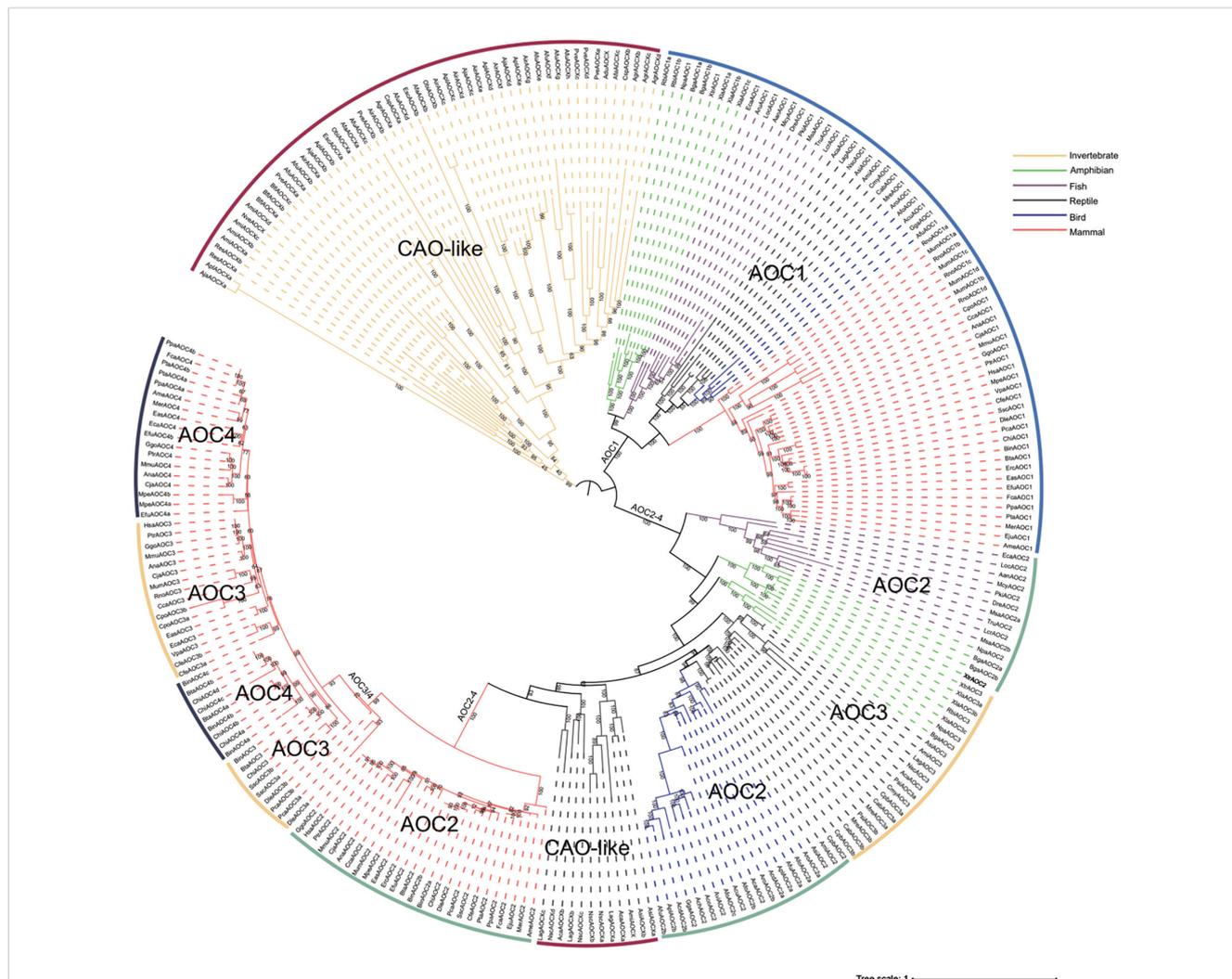
**FIGURE 2**  
 Phylogenetic relationship of *CuAO* genes in plants. The ML tree was built using the full-length protein sequences by IQ-TREE2 with the MFP model. The phylogenetic tree is simplified using the iTOL tool. Different colored triangles represent different plant lineages. *CuAO* expanded and split into two clades in the most recent common ancestor of plants: clade I and clade II. Clade I, also known as the ζ clade, is a monophyletic clade. Clade II expanded after the differentiation of vascular plants into lycophytes and ferns, forming the γ + δ and α + β sub-clades.

the first amino acid in the active site motif more biased towards serine (S), while *CuAOβ* and *CuAOα* are more biased towards threonine (T). The active site motifs of *CuAOγ* and *CuAOδ* are highly conserved, while those of *CuAOβ* and *CuAOα* differ. In *CuAOβ*, the residue at position  $X_1$  is always leucine (L), while in *CuAOα*, the residue is more inclined towards valine (V). Therefore, the specific bias of residue  $X_1$  corresponds with the phylogenetic classification of the Brassicaceae *CuAOα* and *CuAOβ*.

### Expansion of *CuAOs* during plant and animal evolution

In most species, tandem and segmental duplications serve as essential forces driving evolution (Peng et al., 2021). To explore the expansion of *CuAO* genes in plants and animals, we conducted a

synteny analysis (Figure 5, Supplementary Figure S2, Supplementary Table S3). 10, 12, 2, 4, 3, 2, 1, 16, 7, and 2 pairs of segmental duplication genes were identified in *B. napus*, *G. max*, *M. domestica*, *M. acuminata*, *P. somniferum*, *P. vulgaris*, *P. patens*, *T. aestivum*, *P. virgatum*, and *X. laevis*, respectively. Furthermore, tandem duplication resulted in 4, 3, 2, 2, 4, 2, 2, 2, 5, 2, 6, 2, 2, 6, 4, 5, 6, 3, 2, 6, 2, 5, 2, 2, and 2 additional *CuAO* genes in *A. comosus*, *A. thaliana*, *C. papaya*, *G. biloba*, *G. max*, *M. domestica*, *O. sativa*, *P. somniferum*, *P. vulgaris*, *S. tuberosum*, *T. cacao*, *V. vinifera*, *P. virgatum*, *B. indicus*, *B. taurus*, *B. gargarizans*, *C. hircus*, *G. gorilla*, *H. sapiens*, *M. musculus*, *N. parkeri*, *N. scutatus*, *R. bivittatum*, *X. laevis*, and *X. tropicalis*, respectively. Various mechanisms contribute to the expansion of *CuAO* genes in different plant species. Segmental duplication primarily drives this expansion in *G. max*, *P. somniferum*, and *P. virgatum*, while tandem duplication is the main mechanism for *P. vulgaris*. Segmental duplication occurs



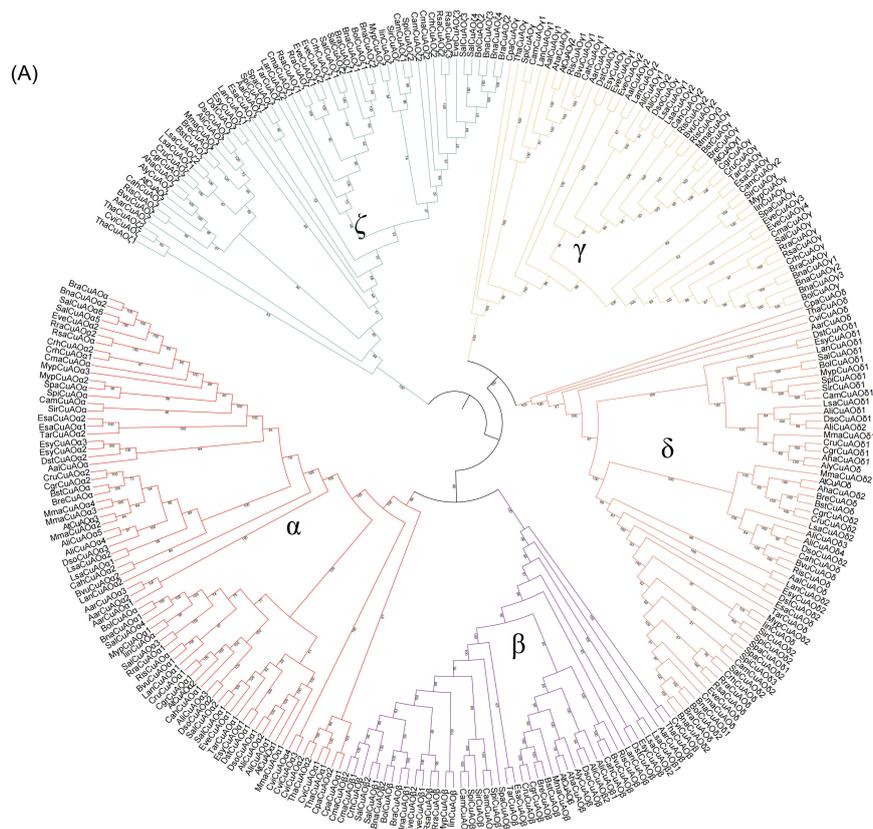
**FIGURE 3** Phylogenetic relationship of *CuAOs* in animals. The phylogenetic tree was built using IQ-TREE2. Distinct branch colors represent various animal lineages. Among invertebrates, *CuAOs* of the Coelenterata, Mollusca and Leptocardii form a highly supported monophyletic clade. In vertebrates, *CuAOs* can be divided into two major clades, namely *AOC1* and *AOC2-4*. Within *AOC1*, mammals and non-mammals form a monophyletic clade, while in *AOC2-4*, mammals and non-mammals are separated.

only in *B. napus*, *M. acuminata*, *P. patens*, and *T. aestivum*, whereas tandem duplication is exclusive to *A. thaliana*, *A. comosus*, *C. papaya*, *G. biloba*, *O. sativa*, *S. tuberosum*, *T. cacao*, and *V. vinifera*. Interestingly, in animal species, the vast majority only have tandem duplication. This emphasizes that different plant species adopt diverse evolutionary strategies to expand the *CuAO* genes, while animal species tend to have tandem duplication.

The analysis of Ka/Ks ratios (the rate of non-synonymous to synonymous substitutions) was performed to evaluate selection pressure on gene evolution (Zhang et al., 2024). A Ka/Ks ratio above 1 indicates positive selection, while a ratio below 1 suggests purifying or negative selection (Zhang et al., 2024). These results revealed that the Ka/Ks ratios for all *CuAO* paralogs were below 1, suggesting that they underwent purifying selection throughout their evolution (Supplementary Table S4).

To delve deeper into the evolutionary relationships of the *CuAO* gene, inter-species synteny analyses were conducted on 15 plant genomes and 12 animal genomes (Figure 6, Supplementary Table S5). The *CuAO* homologous genes of individual plants demonstrated one-to-one collinear relationships between *C. papaya* and *A. thaliana*, *R. sativus* and *L. sativa*, *O. sativa* and *M. acuminata*, *M. acuminata* and *D. cayenensis* (Figure 6A). In addition, either one-to-many or many-to-one homozygosity was identified between *A. trichopoda* and *P. somniferum*, *P. somniferum* and *C. papaya*, *A. thaliana* and *B. napus*, *B. napus* and *B. oleracea*, *B. oleracea* and *B. rapa*, *B. rapa* and *R. sativus*, *L. sativa* and *A. chinensis*, *A. chinensis* and *G. max*, *G. max* and *T. aestivum*, *T. aestivum* and *O. sativa* (Figure 6A). Interestingly, in vertebrates, *AOC1* shows interspecies synteny only with *AOC1*, while *AOC2*, *AOC3*, *AOC4*, and *AOCX* show interspecies synteny among themselves (Figure 6B).

(A)



(B)

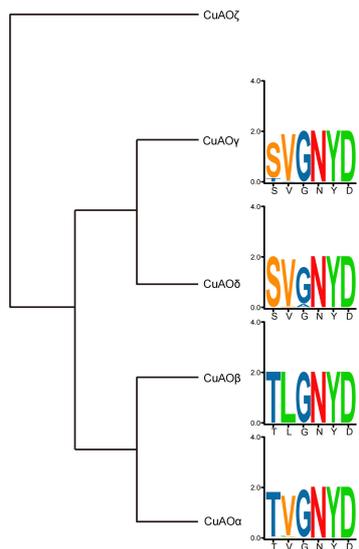


FIGURE 4

Phylogenetic classification of *CuAOs* in Brassicaceae. (A) The phylogenetic tree was built using IQ-TREE2. Distinct branch colors represent various plant lineages. All five subclades of Brassicaceae are monophyletic. (B) The Brassicaceae *CuAOzeta* clade completely lacks the T/S-X<sub>1</sub>-X<sub>2</sub>-N-Y-D active site motif. The specific preference of residue X<sub>1</sub> corresponds to the phylogenetic classification of Brassicaceae *CuAOalpha* and *CuAObeta*.

### Expression patterns of *CuAOs* in key evolutionary lineages of plants and animals

To investigate the expression of *CuAO* genes, we performed a gene expression analysis of *CuAOs* in eight representative species from

plants and animals: *A. thaliana*, *B. napus*, *B. oleracea*, *G. max*, *O. sativa*, *H. sapiens*, *M. musculus*, and *P. troglodytes* (Figure 7). In *A. thaliana*, we examined 23 different tissues and developmental stages (Figure 7A). *AtCuAOdelta*, *AtCuAOzeta*, *AtCuAOgamma1*, *AtCuAOalpha3*, *AtCuAObeta*, and *AtCuAOgamma2* are widely expressed in various tissues, with *AtCuAOzeta*

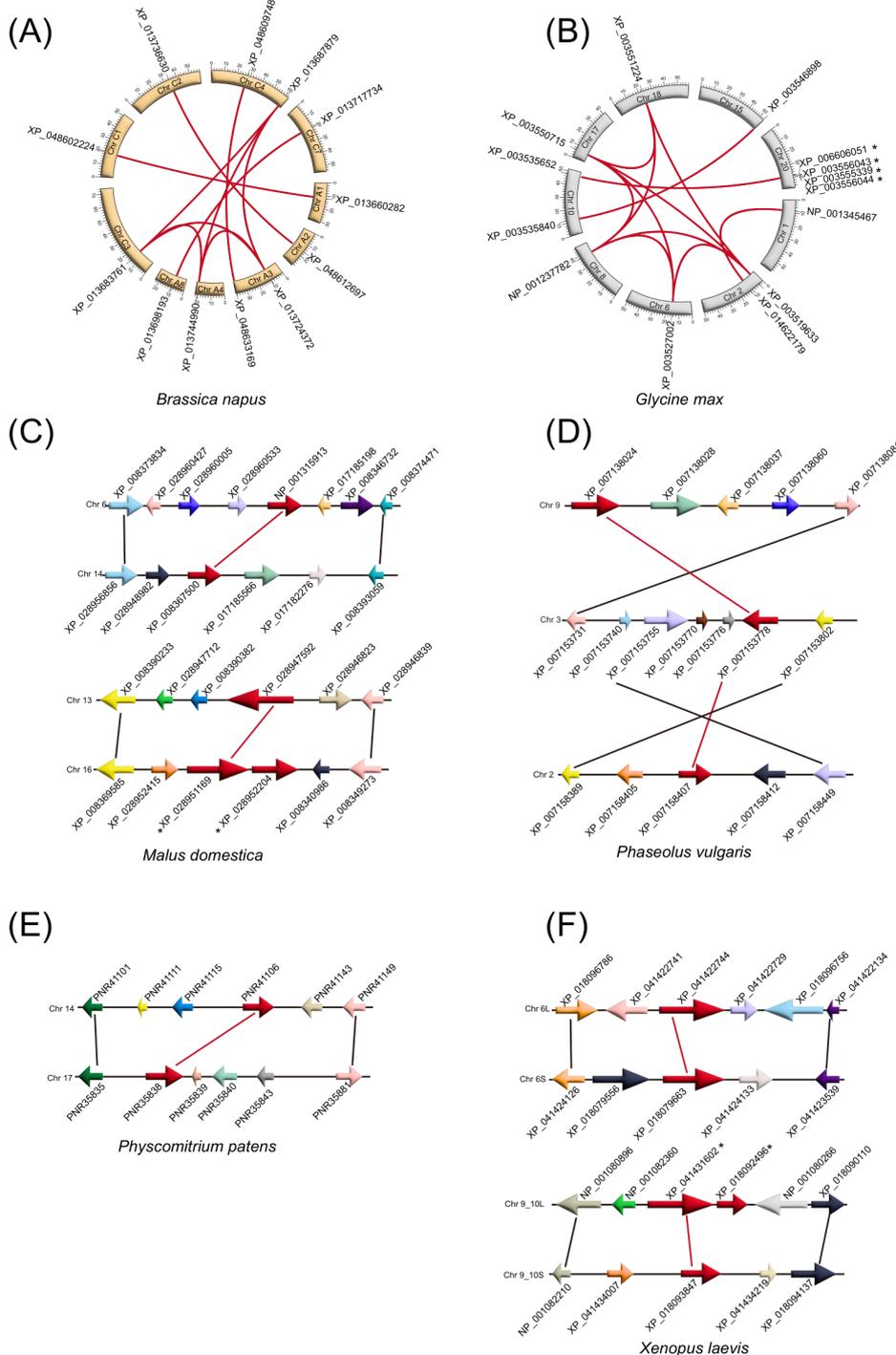
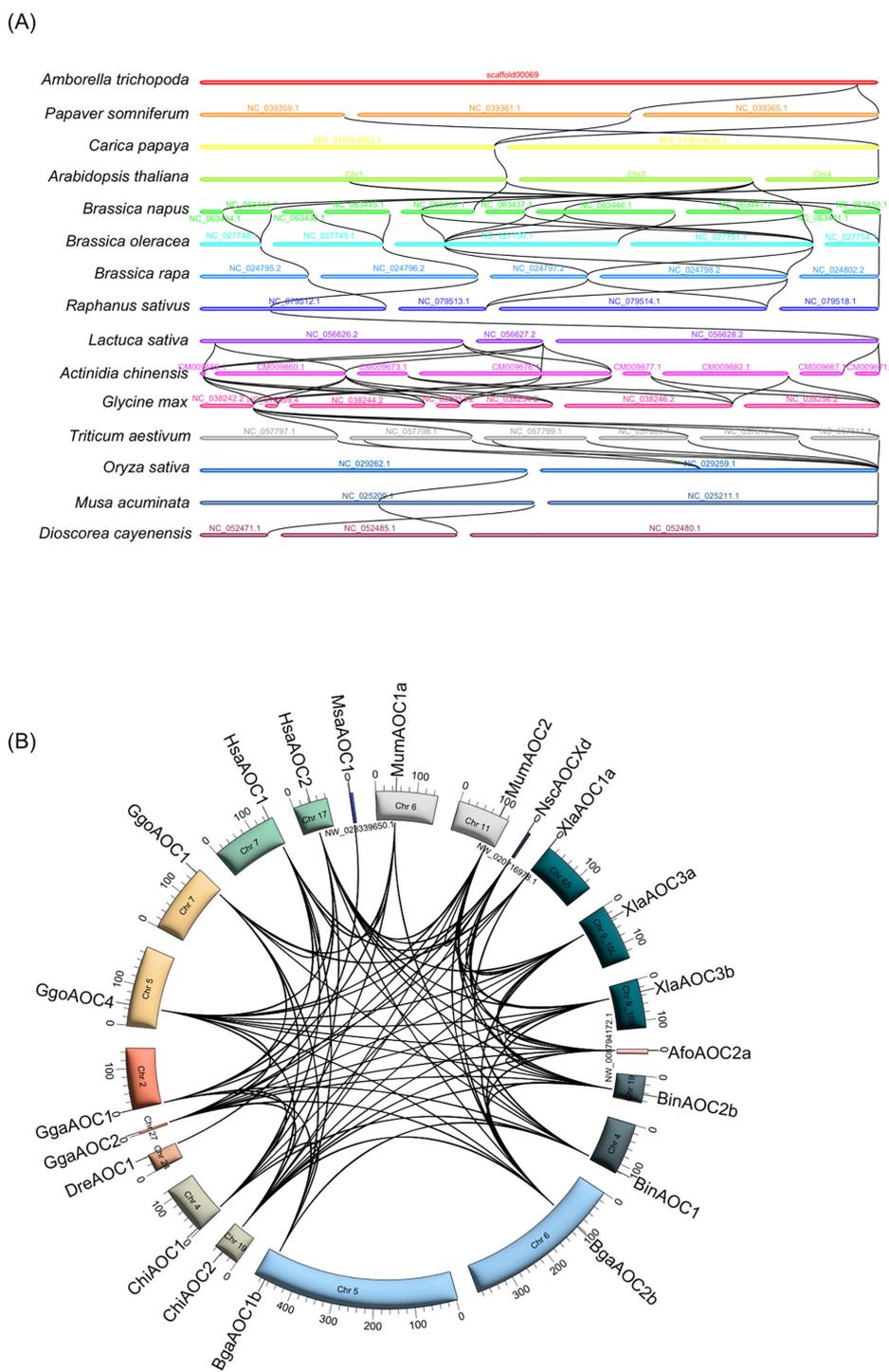


FIGURE 5

Intraspecies syntenic relationships of *CuAO* genes in representative plants and animals. The syntenic paralog of *CuAO* genes are connected by red lines. The asterisk indicates tandem duplicate pairs. The expansion of *CuAO* genes in different plant species is driven by multiple mechanisms acting in concert. In animal species, the vast majority are only tandem duplications. (A) *Brassica napus*; (B) *Glycine max*; (C) *Malus domestica*; (D) *Phaseolus vulgaris*; (E) *Physcomitrium patens*; (F) *Xenopus laevis*.

showing significantly higher expression than the other *CuAO* genes. Interestingly, in various tissues, *AtCuAOγ1* is generally expressed at higher levels than *AtCuAOγ2*, while *AtCuAOα3* shows significantly higher expression than both *AtCuAOα2* and *AtCuAOα1*. Notably, *AtCuAOα1* is highly expressed specifically in siliques, whereas

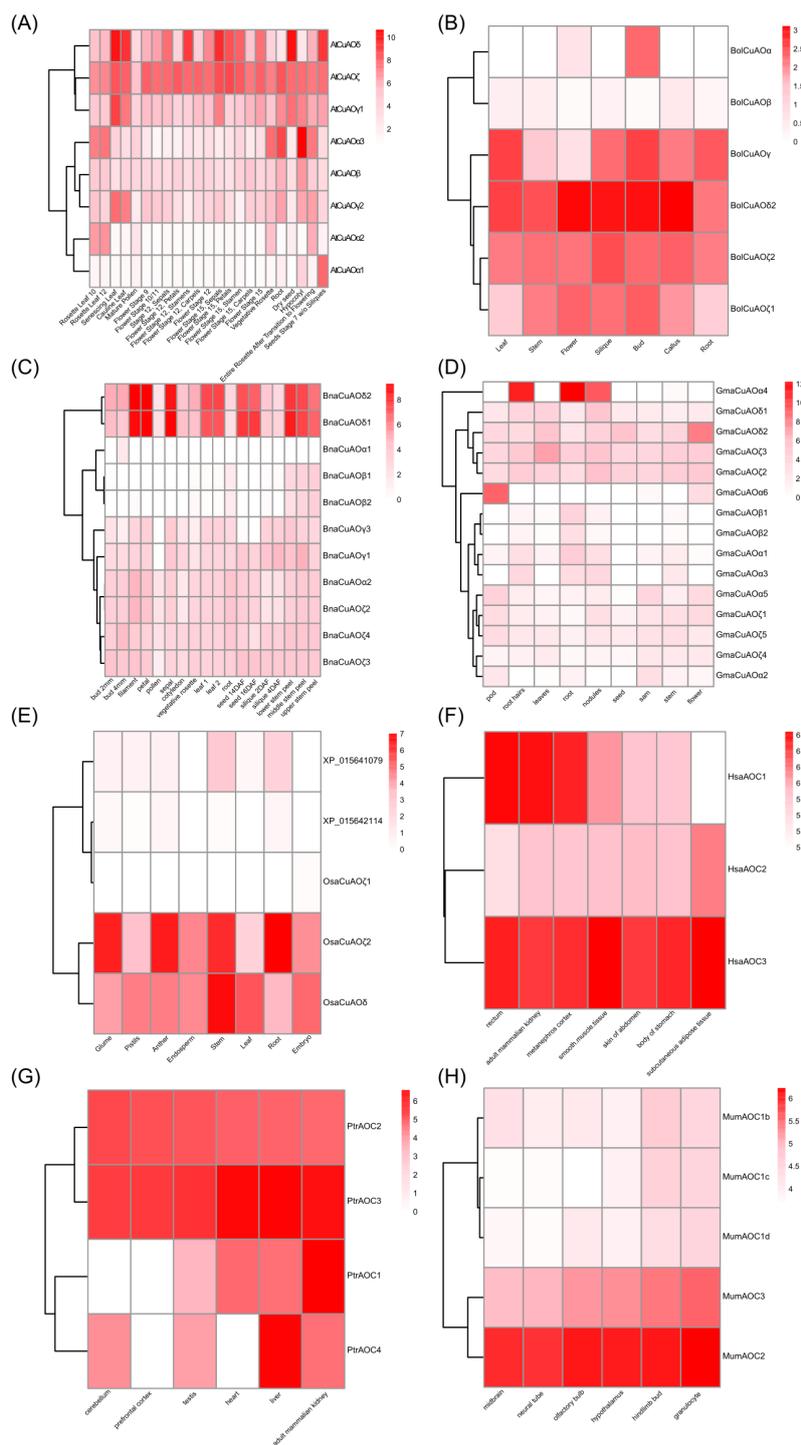
*AtCuAOα2* is predominantly expressed in rosette leaves. In *B. oleracea*, *BolCuAOδ2*, *BolCuAOζ2*, and *BolCuAOζ1* are widely expressed, with *BolCuAOδ2* showing higher expression levels (Figure 7B). In leaves, siliques, and callus, the expression of *BolCuAOζ2* exceeds that of *BolCuAOζ1*, while in flowers and buds,



**FIGURE 6** Interspecies syntenic relationships of *CuAO* genes in plants and animals. The syntenic paralog of *CuAO* genes are connected by black lines. (A) Plant *CuAO*s exhibit one-to-one or one-to-many homology. (B) In vertebrates, *AOC1* only shows interspecies orthology with *AOC1*, while *AOC2*, *AOC3*, *AOC4* and *AOCX* display interspecies orthology among themselves.

*BolCuAOζ1* is more highly expressed than *BolCuAOζ2*. Additionally, *BolCuAOα* is specifically highly expressed in buds. *B. napus* and *G. max* have higher number of *CuAO*s, but their expression levels are generally low across all detected tissues (Figures 7C, D). *BnaCuAOδ1* and *BnaCuAOδ2* exhibit highly similar expression patterns, showing

significantly higher specific expression than other genes in filaments, petals, sepals, leaves, seeds, and stem peels (Figure 7C). *GmaCuAOα4* is specifically expressed at high levels in root hairs, roots, and nodules, while *GmaCuAOα6* is specifically expressed at high levels in pods (Figure 7D). In rice, *OsaCuAOζ2* is specifically highly expressed in the



**FIGURE 7** Expression of *CuAOs* in plants and animals. **(A)**, *A. thaliana*; **(B)**, *B. oleracea*; **(C)**, *B. napus*; **(D)**, *G. max*; **(E)**, *O. sativa*; **(F)**, *H. sapiens*; **(G)**, *P. troglodytes*; **(H)**, *M. musculus*. The expression of duplicated *CuAO* genes in Brassicaceae varies in different tissues. In animals, *AOC2* and *AOC3* are widely expressed in most tissues.

glumes, anthers, stems, and roots, while *OsaCuAOδ* is specifically highly expressed in the stems (Figure 7E). In summary, duplicated *CuAO* genes in Brassicaceae exhibit differing expression across various tissues, indicating functional divergence among these paralogs. In soybean and rice, their specific expression suggests roles in different

growth stages or plant tissues, potentially involving development, growth, and nutrient metabolism.

Most animal lineages have a relatively few *CuAOs*, usually 3 to 4. *AOC2* and *AOC3* are widely expressed in most tissues (Figures 7F-H). In humans and chimpanzees, *AOC3* is expressed

significantly more than *AOC2* (Figures 7F, G), while in mice, *MumAOC2* is expressed significantly more than *MumAOC3* (Figure 7H). The expression of *HsaAOC1* is tissue-specific, with high specific expression in the rectum, adult mammalian kidney, and metanephros cortex in humans and in the adult mammalian kidney in chimpanzees (Figure 7F-G). Additionally, *PtrAOC4* is specifically highly expressed in the liver of chimpanzees.

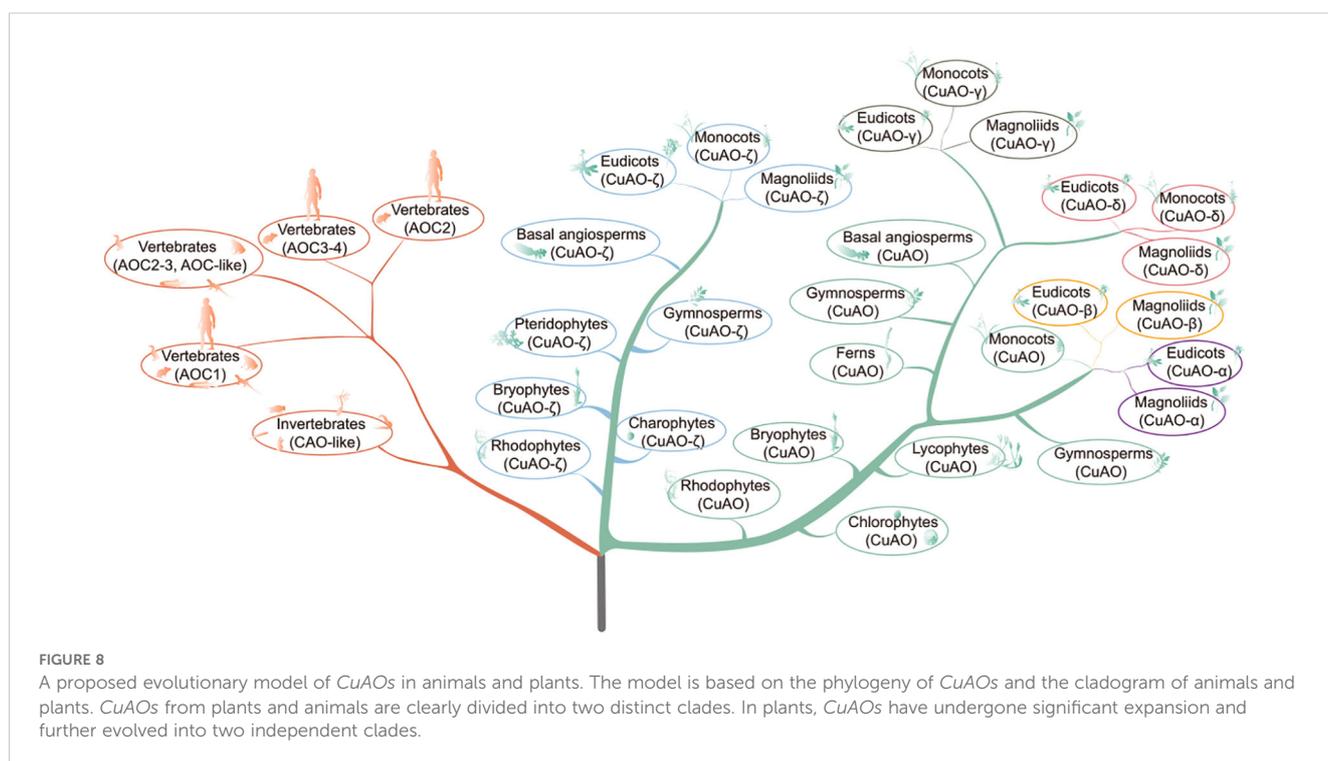
## Discussion

This study conducted phylogenetic classification and examination of the characteristics of thousands of *CuAOs* in hundreds of plant and animal species, deepening our understanding of *CuAOs* in plants and animals, particularly those in angiosperms and vertebrates. The phylogenetic perspective aids in uncovering the molecular and biological roles of different *CuAO* proteins (Figure 8).

### Phylogenetic relationship of *CuAOs* in plant and animal

Since genome-based identification of *CuAO* proteins might overlook certain proteins due to the quality of genome assembly and annotation accuracy, we incorporated numerous species within each phylogenetic group to mitigate the effects of any potentially missing proteins. Our search for the *CuAO* proteins revealed that the *CuAO* gene family is widely distributed across both plant and animal lineages, with varying copy numbers in different plant species and a more conservative distribution in animal species, with approximately 3-4 copies per species (Table 1). Our study suggests that the origin of

*CuAOs* predates the divergence of plants and animals, implying that the bioammonia degradation pathway was established in the common ancestor of plants and animals (Figure 1) (Brazeau et al., 2004; Murakawa et al., 2019). After the divergence of plant and animal species, *CuAOs* underwent significant expansion in plants, forming two branches (Clade I and Clade II) (Figure 2). It is worth noting that, consistent with previous research results, we identified a *CuAO* gene located in the  $\zeta$  branch in *Spirodela polyrhiza* (Upadhyay et al., 2024b). Interestingly, in the  $\zeta$  branch, most monocots have multiple copies of the *CuAO* gene, while a few monocots (*S. polyrhiza*, *A. americanus*, *K. littledalei*, *Z. mays*, *B. distachyon*) contain only a single copy (Supplementary Figure S1). Previous studies have shown that mammalian *CuAOs* can be classified based on the two residues  $X_1$  and  $X_2$  in the active site motif T/S- $X_1$ - $X_2$ -N-Y-D (Lopes de Carvalho et al., 2019). Our study shows that Clade I is a monophyletic branch that has lost the T/S- $X_1$ - $X_2$ -N-Y-D active site motif, which may affect the function of the enzyme or its role in the metabolic pathway, suggesting that Clade I is functionally diverse from Clade II (Figures 2, 4). In previous studies, the plant *CuAOs* were divided into three subfamilies (clades I-III), clade I consists of the  $\alpha$  and  $\beta$  subgroups, clade II consists of the  $\gamma$  and  $\delta$  subgroups, and clade III consists of the  $\zeta$  subgroup (Tavladoraki et al., 2016). In our study, Clade II further expanded during evolution and formed four major branches:  $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$ . *CuAO $\gamma$*  and *CuAO $\delta$*  tend to have serine (S) as the first amino acid in the active site motif, while *CuAO $\beta$*  and *CuAO $\alpha$*  are more inclined towards threonine (T) (Figure 4B). The active site motifs of *CuAO $\gamma$*  and *CuAO $\delta$*  are highly conserved, but those of *CuAO $\beta$*  and *CuAO $\alpha$*  differ. In *CuAO $\beta$* , the residue at position  $X_1$  is always leucine (L), while in *CuAO $\alpha$* , it is more likely to be valine (V). Thus, the specific bias of residue  $X_1$  correlates with the phylogenetic classification of *CuAO $\alpha$*  and *CuAO $\beta$*  (Figure 4).



In the phylogenetic analysis of vertebrate *CuAOs*, both mammalian and non-mammalian AOC1 proteins are grouped in the same clade, showing a robust bootstrap support of 100% (Figure 3). In contrast to AOC1s, non-mammalian AOC2-4 proteins deviate from their mammalian equivalents with a bootstrap value of 100% as well (Figure 3). Non-mammalian AOC2 and AOC3 proteins form distinct sub-branches, while mammalian AOC2-4 proteins are further classified into separate sub-branches for AOC2 and a combined group for AOC3/AOC4, both presenting high bootstrap values. These results are in line with previous studies (Lopes de Carvalho et al., 2019). Interestingly, the differentiation between mammalian AOC3 and AOC4 proteins occurs in a species-specific manner, perhaps due to different evolutionary pressures. Furthermore, unlike previous studies, our research showed that cartilaginous fish have not only AOC1 but also AOC2 (Lopes de Carvalho et al., 2019).

## Insights into *CuAO* evolution and functional diversification

To understand the evolutionary implications of the phylogenetic architecture of the *CuAO* family, it is optimal to cross-reference the phylogenetic relationships of *CuAOs* and their functions. Repeated events frequently cause the expansion and functional diversification of gene families (Peng et al., 2024). Generally, *CuAOs* within the same clade have similar and specific functions. *CuAO $\delta$*  is highly expressed in flowers, and this is the case in *A. thaliana*, *B. oleracea*, *B. napus*, *G. max* and *O. sativa* (Figure 7A-E). Studies have shown that *Malus domestica CuAO $\delta$*  (*MdAO2*) contributes to the formation of flower fragrance (Zarei et al., 2015). Furthermore, different members of the same clade might have functional specialization or redundancy. For example, the expression of *AtCuAO $\alpha$ 3* is significantly higher than that of *AtCuAO $\alpha$ 1* and *AtCuAO $\alpha$ 2*, and the expression of *AtCuAO $\gamma$ 1* is much higher than that of *AtCuAO $\gamma$ 2*; while the expression patterns of *BolCuAO $\zeta$ 1* and *BolCuAO $\zeta$ 2* are alike, and so are those of *BnaCuAO $\delta$ 1* and *BnaCuAO $\delta$ 2* (Figure 7A-E). *SlCuAO1* (*SlyCuAO $\beta$ 3*), *SlCuAO2* (*SlyCuAO $\zeta$ 1*), *SlCuAO3* (*SlyCuAO $\zeta$ 2*), *SlCuAO4* (*SlyCuAO $\zeta$ 3*), *SlCuAO6* (*SlyCuAO $\beta$ 2*) and *SlCuAO7* (*SlyCuAO $\beta$ 1*) in *S. lycopersicum* are mainly specifically expressed in roots, while *SlCuAO5* (*SlyCuAO $\delta$* ) is specifically expressed in fruits, and *SlCuAO8* (*SlyCuAO $\alpha$* ) is mainly expressed in flowers (Upadhyay et al., 2024a). The *CuAO* gene family in tomato shows obvious tissue-specific expression: the  $\beta$  and  $\zeta$  subtypes are mainly expressed in roots, the  $\delta$  subtype is enriched in fruits, and the  $\alpha$  subtype is highly expressed in flowers. This branch-specific expression pattern reflects the functional differentiation of the *CuAO* gene family.

Compared with *A. thaliana CuAO*, human, mouse, and chimpanzee *CuAO* isoforms show significant functional differences. For example, in humans and chimpanzees, the expression level of AOC3 is significantly higher than that of AOC2 and AOC1, while in mice, the expression level of AOC2 is significantly higher than that of AOC3 and AOC1 (Figure 7F-H). The expression of AOC1 is tissue-specific and is highly expressed in the kidneys (Schwelberger, 2006). AOC1 gene encodes diamine oxidase (Elmore et al., 2002), and human AOC1 is thought to have *in vivo* functions in cell

proliferation, inflammation, allergic reactions, and ischemia (McGrath et al., 2009). AOC2 gene encodes retinal-specific amine oxidase, and human AOC2 is thought to play a role in hereditary retinal diseases (Kaitaniemi et al., 2009). AOC3 gene encodes vascular adhesion protein-1, and human AOC3 expression is upregulated during inflammation and plays an important role in the migration of white blood cells from the blood into tissues (Salmi et al., 1993; Smith et al., 1998; Jalkanen et al., 2007). These difference suggest different functions of these three isoforms.

## Materials and methods

### Data sources and sequence acquisition

We selected 267 species and obtained their genome and proteome sequences from public databases like NCBI (<http://www.ncbi.nlm.nih.gov/>), Ensembl Plants (<http://plants.ensembl.org/index.html>), and BRAD (<http://brassicadb.cn/#/>) (Supplementary Table S1). To identify *CuAO* genes in animals and plants, we first performed an HMMER search (E-value = 1e-10) using the Hidden Markov Model profile of the *CuAO* domain (PF01179) in local databases (Potter et al., 2018; Chen et al., 2022). The HMMER parameters are set as follows: `hmmsearch -E 1e-10 PF01179.hmm protein.fa`. We also used BLASTP with *A. thaliana CuAO* amino acid sequences against the protein database, setting an E-value threshold of less than 1e-6. The putative *CuAOs* were further validated using online tools CDD, HMM, and SMART (Marchler-Bauer et al., 2015; Letunic et al., 2021).

### Multiple sequence alignment (MSA) and phylogenetic analysis

Multiple sequence alignments of *CuAO* were performed using Muscle5 (Edgar, 2022). Phylogenetic trees were generated from the full-length protein sequences of *CuAO*. The maximum likelihood (ML) tree was constructed with IQ-TREE2 using parameters ‘-m MFP -B 1000’ and 1000 bootstrap replicates (Minh et al., 2020). The consensus tree topology was visualized using iTOL (Letunic and Bork, 2024).

### Syntenic analysis

To identify homologous pairs in a specific species, we used the all-to-all BLASTP method. Syntenic blocks were inferred using MCScanX with default parameters, including an E-value threshold of 1e-10 and a minimum of 5 BLAST hits (Wang et al., 2012). The resulting gene arrangement was visualized with CIRCOS, linking potential duplicated genes to show their connections (Krzywinski et al., 2009). The duplicated *CuAO* gene pairs were subjected to TBtools software for calculation of synonymous (Ka) and non-synonymous (Ks) substitution rates (Chen et al., 2023).

## Expression analysis of CuAO genes

Expression data for ten species were downloaded from public databases, including *A. thaliana* (The Arabidopsis Information Resource), *B. napus* (<https://yanglab.hzau.edu.cn/BnIR>), *B. oleracea* (<http://bogdb.com/>), *G. max* (<https://www.soybase.org/>), *O. sativa* (<http://rice.uga.edu/index.shtml>), *H. sapiens* (<https://www.bgee.org/>), *M. musculus* (<https://www.bgee.org/>), and *P. troglodytes* (<https://www.bgee.org/>) (Supplementary Table S6). Heat map was established using the R package “pheatmap” (<https://github.com/raivokolde/pheatmap>).

## Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author/s.

## Author contributions

ZZ: Funding acquisition, Writing – original draft. TX: Software, Visualization, Writing – original draft. KL: Writing – original draft. KH: Writing – original draft. SW: Software, Visualization, Writing – review & editing. LW: Software, Visualization, Writing – review & editing.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fpls.2025.1544527/full#supplementary-material>

### SUPPLEMENTARY TABLE 1

List of complete predicted proteomes databases used in this study.

### SUPPLEMENTARY TABLE 2

The identified CuAO protein.

### SUPPLEMENTARY TABLE 3

Segmental duplication and tandem gene.

### SUPPLEMENTARY TABLE 4

The Ka/Ks analysis of *CuAO* genes.

### SUPPLEMENTARY TABLE 5

Segmental duplication gene pairs of *CuAO*s in interspecies.

### SUPPLEMENTARY TABLE 6

Source of expression data.

### SUPPLEMENTARY FIGURE 1

Phylogenetic relationship of representative *CuAO* genes from plants. The phylogenetic tree was built using IQ-TREE2. Distinct branch colors represent various plant lineages.

### SUPPLEMENTARY FIGURE 2

Intraspecies syntenic relationships of *CuAO* genes in representative plants. The syntenic paralogs of *CuAO* genes are connected by black lines. The asterisk indicates tandem duplicate pairs.

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