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Molecular cloning and characterization of copper amine oxidase from *Huperzia serrata*

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ABSTRACT

A cDNA encoding a novel copper amine oxidase (CAO) was cloned and sequenced from the Chinese club moss *Huperzia serrata* (Huperziaceae), which produces the Lycopodium alkaloid huperzine A. A 2043-bp open reading frame encoded an *M*r 76,854 protein with 681 amino acids. The deduced amino acid sequence shared 44–56% identity with the known CAOs of plant origin, and contained the active site consensus sequence of Asn-Tyr-Asp/Glu. The phylogenetic tree analysis revealed that HsCAO from the primitive vascular plant *H. serrata* is closely related to *Physcomitrella patens* subsp CAO. The recombinant enzyme, heterologously expressed in *Escherichia coli*, catalyzed the oxidative deamination of aliphatic and aromatic amines. Among them, the enzyme accepted cadaverine as the best substrate to catalyze the oxidative deamination to Δ^1-piperideine, which is the precursor of the Lycopodium alkaloids. Furthermore, a homology modeling and site-directed mutagenesis studies predicted the active site architecture, which suggested the crucial active site residues for the observed substrate preference. This is the first report of the cloning and characterization of a CAO enzyme from the primitive *Lycopodium* plant.

Copper amine oxidases (CAOs) (EC 1.4.3.21) are quinoenzymes, and are widely distributed in bacteria, yeasts, fungi, plants and animals.[1–6] The enzymes catalyze the oxidative deamination of primary amines to the corresponding aldehydes, with the concomitant reduction of dioxygen to hydrogen peroxide via a ping-pong mechanism involving a covalently bound redox cofactor, 2,4,5-trihydroxyphenylalanine quinone (TPQ), and a copper ion Cu^{2+} (Fig. 1A). The CAOs are homodimeric proteins consisting of 70–90 kDa subunits, each of containing a single copper ion and the covalently bound cofactor TPQ, formed by the post-translational modification of the CAO's conserved tyrosine side chain within the consensus active site sequence of Asn-Tyr-Asp/Glu (Fig. 2).[7] Among the members of the Cu-TPQ class CAOs, the plant enzymes are generally catalytically more active than those from animals.[8]

The Lycopodium alkaloids are quinolizine, or pyridine and α-pyridone type alkaloids, and a number of the alkaloids have been isolated from 54 species of the *Lycopodium* plant.[9] For example, *Huperzia serrata* (Thunb.) Trev. (Huperziaceae, recently reclassified by taxonomists, formerly *Lycopodium serrata*), produces the Lycopodium alkaloid huperzine A, which is a potent inhibitor of acetylcholinesterase and thus a promising drug for Alzheimer's disease.[10,11] Although the biosynthesis of the Lycopodium alkaloids remains poorly understood, various studies, including an EST analysis,[12,13] suggested that it is initiated by the decarboxylation of lysine to form cadaverine, with the subsequent formation of Δ^1-piperideine, which is catalyzed by CAO (Fig. 1B).[9] However, CAO genes involved in the biosynthesis of the Lycopodium alkaloids have been still uncovered. In order to shed light on the biosynthesis of the Lycopodium alkaloids in *H. serrata*, we performed PCR screening of CAO enzymes, by using primers based on the conserved sequences of the known CAOs. Here we report the cloning and characterization of a novel CAO from the primitive vascular plant *H. serrata*. The enzyme accepted cadaverine as the best substrate to catalyze the oxidative deamination to Δ^1-piperideine, which is the precursor of the Lycopodium alkaloids. This is the first report describing a CAO enzyme from a primitive *Lycopodium* plant.

The cDNA encoding a novel CAO (HsCAO) was cloned and sequenced from the roots of *H. serrata* by RT-PCR, using degenerate primers based on the conserved sequences of the known plant CAO enzymes.[14] The terminal sequences of the cDNA were determined by the 3′- and 5′-RACE methods. The full-length cDNA contained a 2043 bp ORF encoding an *M*r 76,854 protein with 681 amino acids (Fig. 2). The nucleotide sequence has been deposited in the GenBank database (GenBank ID: JN247732). No additional cDNAs encoding CAO isomers were obtained in this study. The deduced amino acid sequence shared 44–56% identity with those of the known plant CAOs: 56.5% identity to *Solanum lycopersicum*

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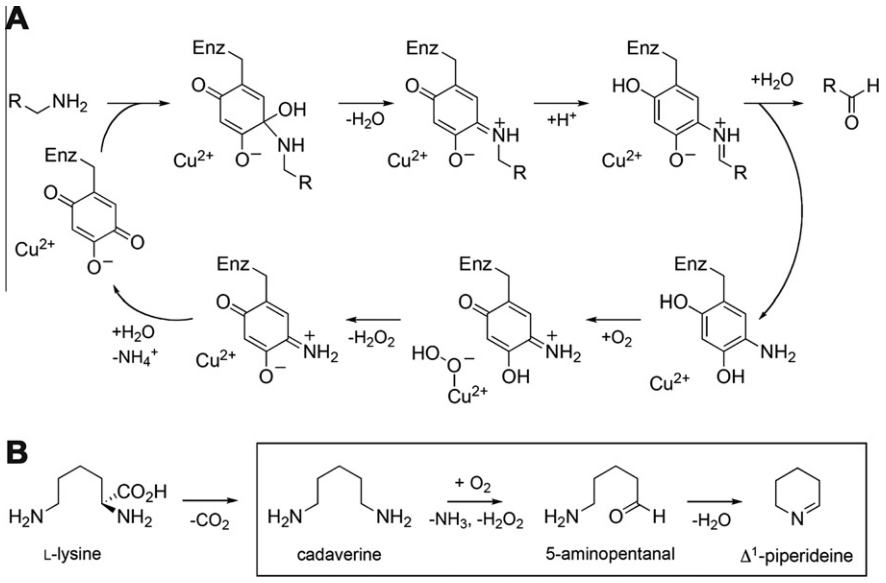


Figure 1. Proposed mechanism of CAOs. (A) Overall proposed reaction mechanism from primary amine to aldehyde, catalyzed by CAOs. (B) Proposed biosynthetic pathway from lysine to Δ^1-piperideine.

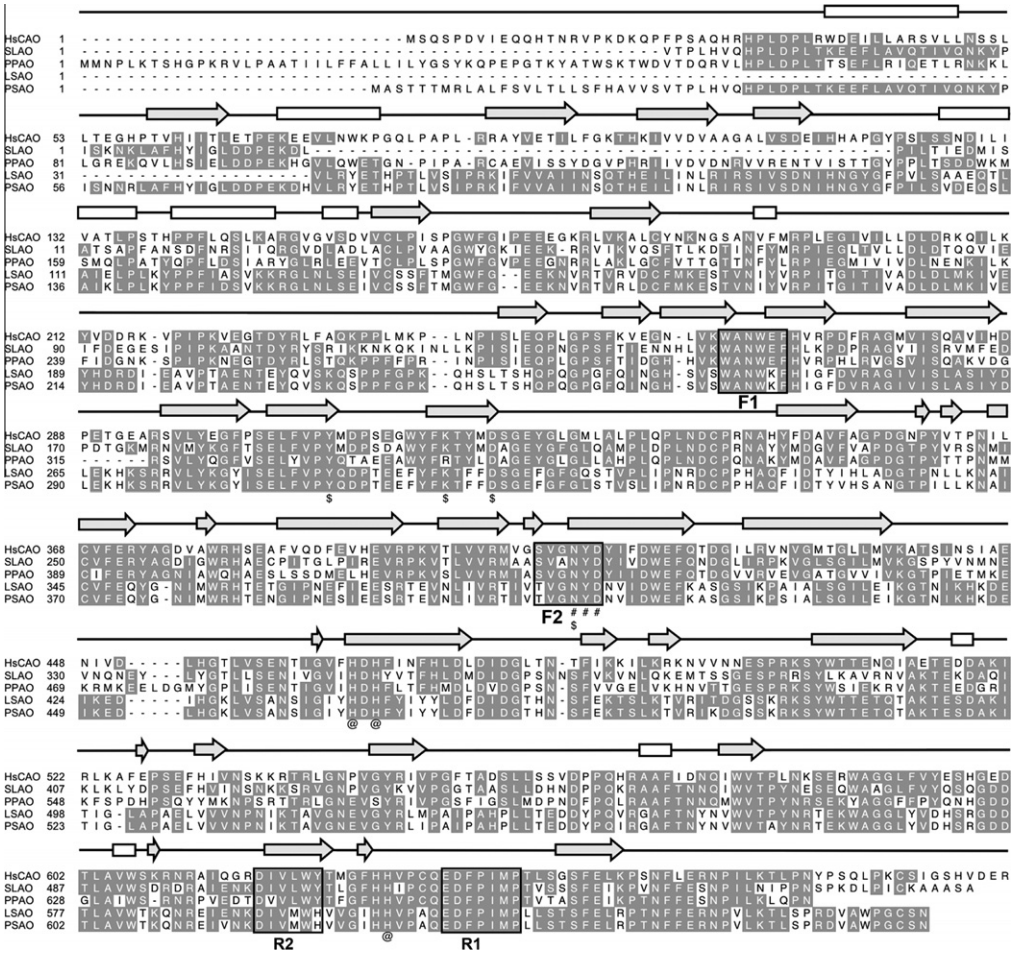


Figure 2. Comparison of the primary sequences of HsCAO and other plant CAOs. The predicted secondary structures, α-helices (rectangles), β-strands (arrows), and loops (bold lines) of HsCAO are also diagrammed. The secondary structure was predicted by the PredictProtein site (http://www.predictprotein.org). The CAO's conserved consensus sequence Asn-Tyr-Asp/Glu, and the copper binding histidine residues are marked with # and @, respectively. The Tyr, Lys, Asp, and Asn residues, which are thought to play a crucial role in the activation of catalytic center TPQ, are marked with $. The regions that employed to design the degenerated primers F1, F2, R1 and R2 were indicated with closed squares. Abbreviations (GenBank accession numbers): HsCAO, *Huperzia serrata* CAO; SLAO, *Solanum lycopersicum* CAO (CAI39243); PPAO, *Physcomitrella patens* CAO (XP_001772536); LSAO, *Lathyrus sativus* CAO (CAH10210); PSAO, *Pisum sativum* CAO (Q43077).

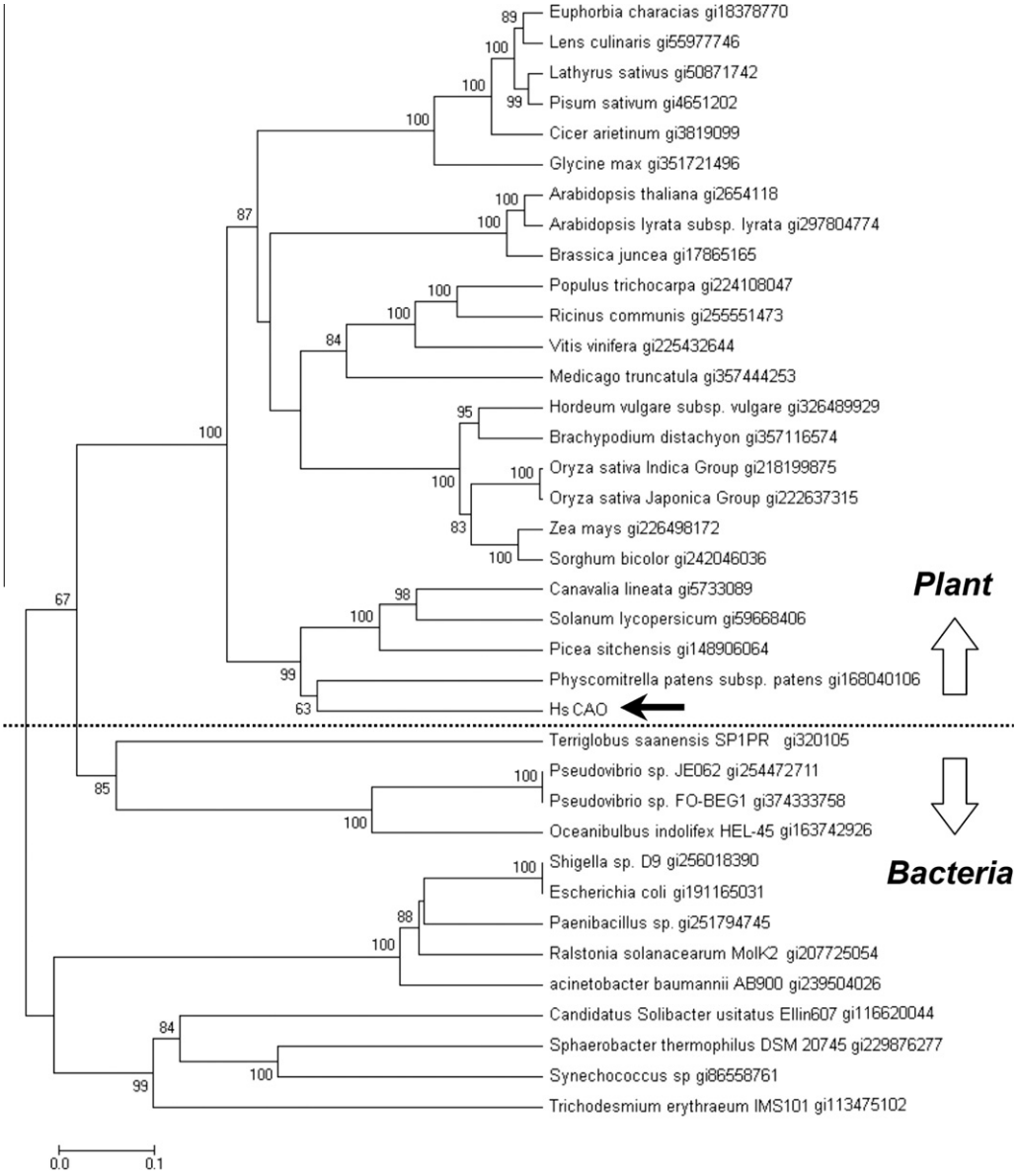


Figure 3. Phylogenetic analysis of plant and bacterial CAO enzymes. HsCAO is highlighted by an arrow. The optimal tree with the sum of branch length = 7.18335546 is shown. Bootstrap values (%) out of 1000 resamplings are at each node. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances are in the units of number of amino acid substitutions per site. The indicated scale represents 0.1 amino acid substitutions per site.

CAO (SLAO), 54.6% identity to *Physcomitrella patens* CAO (PPAO), 45.4% to *Lathyrus sativus* CAO (LSAO), and 44.2% to *Pisum sativum* CAO (PSAO). A sequence analysis revealed that HsCAO contains the conserved active site consensus sequence, Asn-Tyr-Asp/Glu.[7] The active site tyrosine side chain within the Asn-Tyr-Asp/Glu sequence is the post-translationally modified to form the covalently-bound co-factor, TPQ. In addition, HsCAO contains the three histidine residues (His465, His467 and His627) for copper ion binding, as well as the two active-site residues (Tyr308 and Asp322) conserved in the CAO family enzymes. Furthermore, a phylogenetic tree analysis revealed that HsCAO, from the primitive vascular plant *H. serrate*, is closely related to *Physcomitrella patens* subsp CAO (Fig. 3).[15]

The full-length cDNA of HsCAO was sub-cloned into the pET22b(+) vector, and the recombinant HsCAO protein, with a hexahistidine-tag at the C-terminus, was expressed in *Escherichia coli* BLR and BL21(DE3)pLysS, and purified to homogeneity by Ni-chelating affinity column chromatography.[16,17] The purified protein migrated as a single band with an *M*r of 78 kDa, in good agreement with the calculated value of 77,676 Da (Fig. 4). In contrast, a gel-filtration experiment yielded an *M*r of 162 kDa, suggesting that the recombinant HsCAO is a homodimeric enzyme, as in the case of the known CAOs.[1,2,7]

As previously reported for the copper-containing amine oxidases (phenethylamine oxidase and histamine oxidase from *Arthrobacter globiformis*),[18,19] the catalytically active, recombinant HsCAO protein was obtained only in the presence of $CuSO_4$, and was present in the soluble fraction when expressed in *E. coli* BLR. In fact, when the catalytically active recombinant HsCAO was reacted with phenylhydrazine,[20] which is the conventional method to detect the covalently bound Cu-TPQ cofactor at the active site, a UV spectra analysis revealed a peak with maximal absorption at 448 nm, corresponding to the phenylhydrazone, as previously observed for the known CAOs.[1,2,5,7,21] In contrast, the absence of $CuSO_4$, the inactive, Cu^{2+}-free recombinant HsCAO protein did not afford the peak in the phenylhydrazine reaction. These

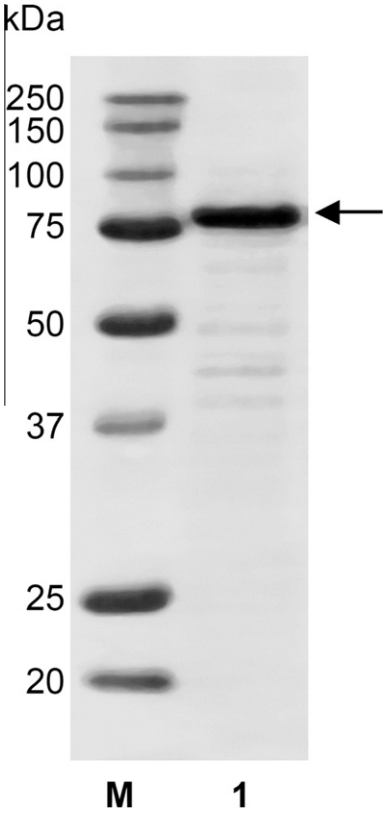
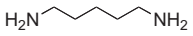
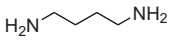
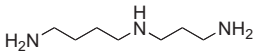
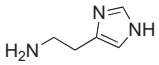
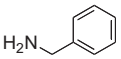
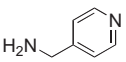
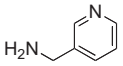
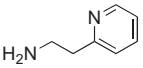


Figure 4. SDS–PAGE of recombinant HsCAO purified by Ni-chelating affinity column chromatography. The arrow indicates the recombinant HsCAO, at 78 kDa. M, protein molecular weight markers; lane 1, protein solution after Ni-chelating affinity column purifications.

Table 1
Steady-state kinetic parameters of recombinant HsCAO

Substrate	K_M (mM)	K_{cat} (s^{-1})	K_{cat}/K_M (s^{-1} mM^{-1})
 cadaverine	0.3	1.0	3.3
 putrescine	1.2	1.9	1.6
 spermidine	0.8	2.3	2.9
 histamine	0.7	1.8	2.6
 benzylamine	0.5	1.4	2.8
 4-aminomethylpyridine	1.9	3.1	1.6
 3-aminomethylpyridine	1.2	1.9	1.6
 2-aminoethylpyridine	1.7	2.9	1.7

observations suggested that the catalytically active, recombinant HsCAO is a copper/TPQ-containing homodimeric enzyme, and the active site tyrosine side chain within the Asn-Tyr-Asp/Glu sequence is post-translationally modified to form the covalently-bound cofactor TPQ.

An LC–ESIMS analysis[22] revealed that the catalytically active, recombinant HsCAO accepts cadaverine as the amine substrate to produce Δ^1-piperideine, which is the putative biosynthetic precursor of the Lycopodium alkaloids[9] (Fig. 5). The piperideine-forming activity was maximal at pH 8.0, within the range of pH 6.8–8.5. In contrast, when EDTA or the inactive form of HsCAO was added to the enzyme reaction mixture, the piperideine-forming activity was undetectable. Furthermore, since the CAO family enzymes

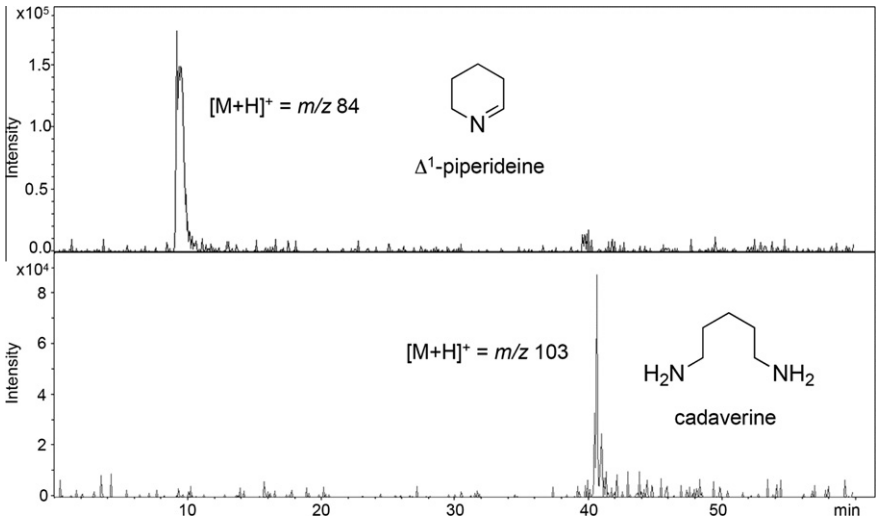


Figure 5. LC–ESIMS analysis of the enzymatic formation of Δ^1-piperideine from cadaverine by HsCAO. Ion chromatograms extracted with m/z 84 and m/z 103 are shown.

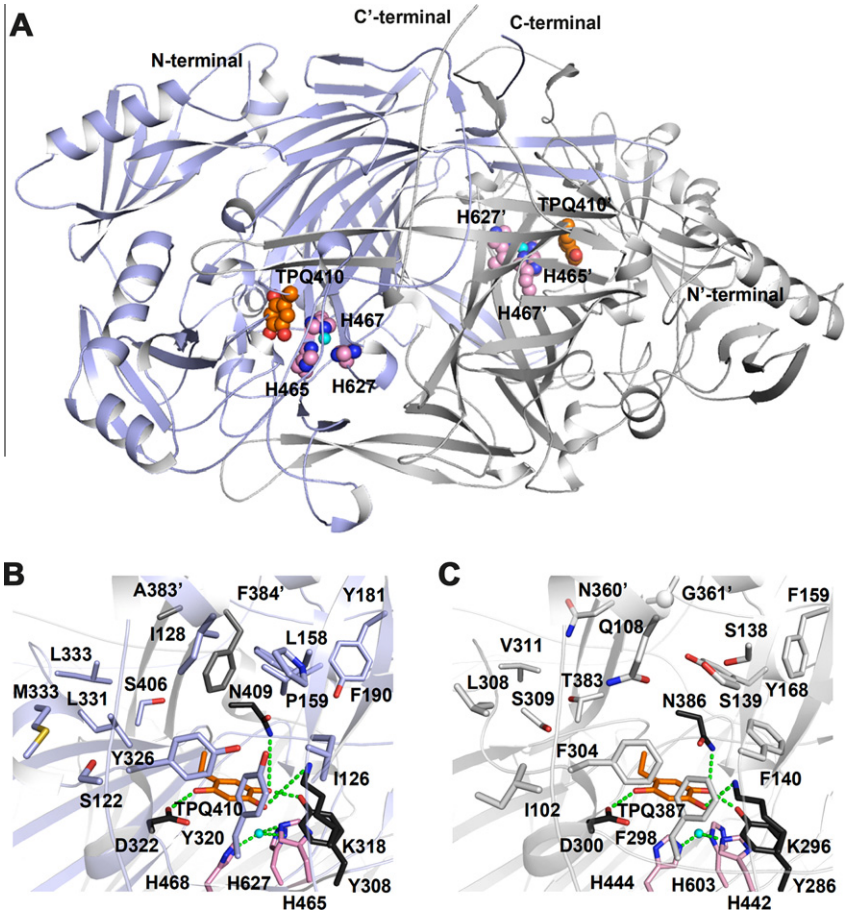


Figure 6. Overall structure of the homology model of HsCAO. (A) Both monomer A (blue) and B (silver) are represented by ribbon models. The catalytic center TPQ410 is represented with an orange CPK model. The copper ion binding histidine residues His465, His467, and His627, are indicated with pink CPK models. (B and C) Comparison of the homology model of (B) HsCAO and the crystal structure of (C) PASO. The catalytic center, TPQ410 is represented with an orange stick model. The copper ion binding histidine residues, His465, His467, and His627, are indicated by pink stick models. The four residues, Tyr308, Lys318, Asp322, and Asn409, in HsCAO, and the equivalent four residues in PSAO, are shown with black stick models. The copper ion molecule and the hydrogen bonds are indicated by a light-blue sphere and green dotted lines, respectively.

are known to exhibit broad substrate specificities,[1,2,23] we also tested the aliphatic (putrescine and spermidine) and aromatic (histamine, benzylamine, tyramine, 4-aminomethylpyridine, 3-aminomethylpyridine, and 2-aminoethylpyridine) primary amines as substrates,[24] and measured the steady-state kinetic parameters (Table 1).[25] These analyses demonstrated that HsCAO also accepts all of the tested substrates, excepted for tyramine. Interestingly, the steady-state kinetics analyses of HsCAO revealed a K_M = 0.3 mM and a k_{cat} = 1.0 s^{-1} for cadaverine, with respect to the hydrogen peroxide formation activity, representing the best catalytic efficiency (k_{cat}/K_M) among the substrates tested. This is in sharp contrast to the previously reported *L. sativus* CAO (LSAO) and *P. sativum* CAO (PSAO), as the former even accepts tyramine, while latter exhibits better catalytic efficiency (k_{cat}/K_M) for putrescine. These observations indicated that HsCAO is functionally different from LSAO and PSAO, and suggested that HsCAO is involved in the biosynthesis of the Lycopodium alkaloids. To test this hypothesis, further metabolomic and proteomic analyses, such as developmental or inducible variation of HsCAO in the alkaloid biosynthesis, are required.

To clarify the structural basis for the HsCAO enzyme reaction, a homology model was constructed,[26] based on the X-ray crystal structure of *P. sativum* CAO (PSAO).[27] The model consists of residues 28–671 of both monomers A and B (Fig. 6A). In the Ramachandran plot calculated for the model, a total of 84.9% of the residues in the model are in the most favored regions, and 14.4% are in the additional allowed regions. The homology model predicted that HsCAO shares the same overall fold, consisting of a large β-sandwich domain and two α/β domains, as in the case of PSAO. Upon dimerization, residues 383–386 are linked to the other monomer to complete the wall of the active-site cavity in each monomer. The Tyr in the catalytic center (corresponding to TPQ) and the copper ion binding site, consisting of three histidine residues, are sterically conserved within each monomer (Tyr410 for the catalytic center, and His465, His467 and His627 for the copper ion binding site), and sit at the bottom of the internal active-site cavity (Fig. 6B). In addition, Tyr, Lys, Asp and Asn, which are thought to play crucial roles in the activation of the TPQ catalytic center of PSAO, are present within each monomer (Tyr308, Lys318, Asp322, and Asn409) (Fig. 2 and 7B). Therefore, HsCAO may also catalyze the oxidative deamination of primary amines to the corresponding aldehydes *via* a ping-pong mechanism, by exploiting TPQ and Cu^{2+}, as in the cases of the other CAOs. On the other hand, the homology modeling study suggested that 14 residues lining the active-site cavity and entrance of PSAO are uniquely altered in these regions of HsCAO. Especially, the internal active-site residues Phe140, Tyr168, Phe298, Phe304, Thr383, and Gly351′ in PSAO are substituted with Ile160, Phe190, Tyr320, Tyr326, Ser406 and Phe384′ in HsCAO, respectively (Fig. 6B and C), which may account for the different substrate specificities

and catalytic activities between these enzymes, as shown in the steady-state kinetics studies.

To test this hypothesis, we constructed a set of point mutants of HsCAO (I160F, F190Y, Y320F, Y326F, S406T and F384G), and investigated the mechanistic consequences of the mutagenesis.[28] The mutants were thus expressed in *E. coli*, at levels comparable to that of the wild type enzyme, and purified to homogeneity by a Ni-chelate affinity column. The enzymatic activities of the mutants were evaluated with the hydrogen peroxide assay by using the substrates used for wild type HsCAO. All of the mutants lost the activity within the range of pH 6.8–8.5, suggesting that the residues are crucial for the catalytic activities of HsCAO. Presumably, the single substitution of the residues resulted in conformational changes in the active site, which lead to the loss of the enzyme activity.

This is the first report of the cloning and characterization of a novel CAO enzyme from the primitive club moss *H. serrata*, which produces the Lycopodium alkaloid huperzine A, a potent inhibitor of acetylcholinesterase. The deduced amino acid sequence shares 44–56% identity with the known plant CAOs, and contains the active site consensus sequence of Asn-Tyr-Asp/Glu. Furthermore, functional analyses demonstrated that HsCAO exhibits the best substrate specificity for cadaverine, which is the proposed biosynthetic precursor of the Lycopodium alkaloids. Although further metabolomic and proteomic studies are needed, this report contributes to the clarification of the pooly understood biosynthetic machinery of the Lycopodium alkaloids.

Acknowledgments

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14. The *H. serrata* plant used in this study was obtained from Guoshen Chen (Zhejiang Academy of Medical Science, Hangzhou, China). Total RNA was extracted from roots of *H. serrata* by using an RNeasy plant mini kit (Qiagen), and was reverse-transcribed by using the SuperScript First-Strand Synthesis System Kit for RT-PCR (Invitrogen). The single strand cDNA thus obtained was used as the template for the PCR reactions with degenerate oligonucleotide primers, which were designed based on the conserved sequences of the known plant copper amine oxidase, as follows: AOF1 = 5′-TGGGC(A/C/T)(A/G)A(C/T)TGGAA(A/G)TT(C/T)C-3′, AOF2 = 5′-AC(T/C/A/G)GT(T/C/A/G)GG(T/C/A/G)AA (T/C)TA(T/C)GA(T/C/G)T-3′, AOR1 = 5′-GGCAT(T/C/A)A(T/C)(T/C/A/G)GG(C/A/G)(C/A)A(T/G)TC(T/C)TC-3′, AOR2 = 5′-(T/C)GT(A/G)CCACA(T/C/A/G)(C/A)AC(T/A/G)AT(A/G)TC-3′. Nested PCR was performed with the AOF1 and AOR1 primer sets, and then with AOF2 and AOR2, to amplify the core fragment. The 3′-RACE, using two gene-specific primers (SRT-AOF1: 5′-TGGGAGTTTCAGACGGATGGCA-3′, SRT-AOF2: 5′-ACAAGAGTGAACGATGGGCTGGTG-3′) and an oligo-dT primer (race32: 5′-GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTT-3′) was used to amplify a 642 bp DNA fragment. The 5′-RACE was performed using the 5′ RACE System for Rapid Amplification of cDNA Ends (Invitrogen) and three specific primers, SRT-AOR1 (5′-ACCCTACAGGATTCCCGAGCC-3′), SRT-AOR2 (5′-ACAAGCCAGTCATTCCACATTCA-3′) and SRT-AOR3 (5′-TGCCATCCGTCTGAAA CTCCCA-3′), to amplify an approximately 1.6 kb DNA fragment. The nucleotide sequence was determined by using an ABI Prism 310 Genetic Analyzer (Applied Biosystems). The active site was investigated via the PDB site (http://www.pdb.org/).
15. Amino acid sequences were aligned using ClustalW (Thompson, J. D.; Higgins, D. G.; Gibson, T. J. *Nucleic Acids Res.* **1994**, *22*, 4673), and phylogenetic tree was constructed using MEGA5 (Tamura, K.; Peterson, D.; Peterson, N.; Stecher, G.; Nei, M.; Kumar, S. *Methods. Mol. Biol. Evol.* **2011**, *28*, 27319) with UPGMA method. Evolutionary distances were computed with the Poisson correction method. In total, 37 amino acid sequences of HsCAO and other amine oxidases from different species were assessed. All positions containing gaps and missing data were eliminated. There were a total of 467 positions in the final dataset. Bootstrap tests with 1000 replications were conducted to examine the reliability of the interior branches and the validity of the trees obtained.
16. The full-length cDNA encoding HsCAO was amplified using the 1st strand cDNA as the template, with a forward (5′-ATTACATATGAGCCAATCCCCAGACG TAATAG-3′) primer (the *Nde* I site is underlined) and a reverse (5′-ATAAC TCGAGCCTCTCGTCAACATGGCTGC-3′) primer (the *Xho* I site is underlined). The amplified DNA fragment was digested with *Nde* I/*Xho* I and cloned into the *Nde* I/*Xho* I sites of the pET22b(+) expression vector (Novagen), for expression as a fusion protein with a hexahistidine-tag at the C-terminus. After confirmation of the sequence, the resultant expression plasmid was transformed into *E. coli* BLR and BL21(DE3)pLysS. The cells harboring the plasmid were cultured to an OD_{600} of 0.6 in LB medium containing 100 μg/ml of ampicillin at 30 °C. Isopropylthio-β-D-galactopyranoside (IPTG; final, 1.0 mM) and $CuSO_4$ (final, 50 μM) were then added to the culture medium, to induce gene expression and to activate the expressed recombinant HsCAO, respectively, and the culture was incubated for a further for 16 h at 16 °C.
17. All of the following procedures were performed at 4 °C. The *E. coli* cells were harvested by centrifugation at 5000 g and resuspended in 50 mM Tris–HCl pH 8.0. The cells were disrupted by sonication, and the lysate was centrifuged at 12,000 g for 30 min. The supernatant thus obtained was loaded onto a Ni Sepharose 6 Fast Flow affinity column (GE Healthcare) equilibrated with the buffer A (40 mM potassium phosphate (KPB), pH 7.9, containing 0.1 M NaCl and 5 mM imidazole). After washing the column with buffer B1 (20 mM KPB, pH 7.9, containing 0.5 M NaCl) and buffer B2 (20 mM KPB, pH 7.9, containing 0.2 M NaCl and 40 mM imidazole), the recombinant HsCAO protein was eluted with 15 mM KPB buffer pH 7.5, containing 0.5 M imidazole and 10% glycerol. Finally, the protein solution was dialyzed against 50 mM Tris–HCl buffer pH 8.0, containing 10% glycerol. The protein concentration was determined by the Bradford method (Protein Assay Dc, Bio-Rad) using bovine gamma globulin as the standard.
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20. The phenylhydrazine assay was performed, according to the described method.[4] The purified recombinant HsCAO (50 μM) was dissolved in 1 ml of 0.1 M potassium phosphate buffer, pH 7.2, and then two molar equivalents of phenylhydrazine hydrochloride (Wako Pure Chemical Industries) were added to the enzyme solution. The mixture was incubated at 37 °C for 30 min, and phenylhydrazone formation was then monitored in the range of 300–700 nm, by using a UV-1700 UV–VIS Spectrophotometer (SHIMADZU).
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22. The reaction mixture (final volume of 50 μl) contained 100 nmol cadaverine and 10 μg of the purified recombinant HsCAO in 50 mM Tris–HCl buffer, pH 8.0. Incubations were performed at 25 °C for 16 h. The products were then extracted with methanol, and separated by reverse-phase HPLC on a Shiseido ODS C8 column (4.6 × 150 mm), at a flow rate of 0.2 ml/min. Gradient elution was performed with H_2O and acetonitrile, both containing 1% acetic acid: 0-20 min, linear gradient from 40 to 70% acetonitrile; 20–40 min, 70% acetonitrile; 40-60 min, linear gradient from 70 to 100% acetonitrile. Online LC–ESIMS spectra were measured with an Agilent Technologies HPLC 1100 series HPLC coupled to a Bruker Daltonics Esquire4000 ion trap mass spectrometer fitted with an ESI source. The ESI capillary temperature and the capillary voltage were 320 °C and 4.0 V, respectively. The tube lens offset was set to 20.0 V. All spectra were obtained in the positive mode, over a mass range of *m*/*z* 50–600, and at a rate of one scan every 0.2 s. The collision gas was helium, and the relative collision energy scale was set at 30.0% (1.5 eV).
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24. Tyramine, spermidine trihydrochloride, benzylamine and histamine dihydro chloride were purchased from Nacalai Tesque, Inc. Cadaverine dihydrochloride and putrescine were purchased from Sigma. 2-Aminoethylpyridine, 4-ami nomethylpyridine and 3-aminomethylpyridine were purchased from Wako Pure Chemical Industries.
25. Steady-state kinetic parameters were determined by using Hydrogen Peroxide Assay kit (Biovision Incorporated). The experiments were performed using six concentrations of substrate (0.1, 0.2, 0.5, 1, 2, 4 mM) in the assay mixture, containing 10 μg of the purified enzyme, in a final volume of 50 μl of 50 mM Tris–HCl pH 8.0. The reactions were incubated at 25 °C for 10 min. The reactions with boiled enzyme were used as the negative control. The Reaction Mix Solution (50 μl/test) was then added into the reaction mixture, and the H_2O_2 concentration was measured at OD_{570}, according to manufacturer's protocol. Lineweaver-Burk plots of data were employed to derive the apparent K_M and k_{cat} values, using the Microsoft Excel program (Microsoft).

26. The model of HsCAO was generated by the SWISS-MODEL package (http://expasy.ch/spdpv/), based on the crystal structure of PSAO (PDB ID: 1KSI). The model quality was assessed with PROCHECK (Laskowski, R.A.; MacArthur, M.W.; Moss, D.S.; Thornton, J.M. *J. Appl. Crystallogr.* **1993**, *26*, 283). All crystallo graphic figures were prepared with PyMOL (DeLano Scientific, http://www.pymol.org).
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28. The plasmids expressing the HsCAO mutants (I160F, F190Y, Y320F, Y326F, F384G, and S406T) were constructed with a QuikChange Site-Directed Mutagenesis Kit (Stratagene), according to the manufacturer's protocol, using the following pairs of primers (mutated codons are underlined): I160F (5′-ATGTCGTCTGCTTGC CATTCTCTCCAGGATGGTTTG-3′ and 5′-CAAACCATCCTGGAGAGAATGGCAAGCAG ACGACAT-3′), F190Y (5′-TGGATCTGCGAATGTTTACATGAGGCCACTGGAGG-3′ and 5′-CCTCCAGTGGCCTCATGTAAACATTCGCAGATCCA-3′), Y320F (5′-GGATGGTATT TCAAAACATTCATGGATTCTGGAGAG-3′ and 5′-CTCTCCAGAATCCATGAATGTTTTG AAATACCATCC-3′), Y326F (5′-CATGGATTCTGGAGAGTTTGGATTAGGAATGCTGG-3′ and 5′-CCAGCATTCCTAATCCAAACTCTCCAGAATCCATG-3′), F384G (5′-GGAGAC ACTCAGAGGCCGGTGTCCAAGATTTTGAG-3′ and 5′-CTCAAAATCTTGGACACCGGC CTCTGAGTGTCTCC-3′), and S406T (5′-TGGTCCGAATGGTTGGAACAGTGGGGAATT ATGAC-3′ and 5′-GTCATAATTCCCCACTGTTCCAACCATTCGGACCA-3′). The mutant enzymes were expressed and purified with the same procedures as described for the wild type, and used for the Hydrogen Peroxide Assay.