Monoamine Biosynthesis via a Noncanonical Calcium-Activatable Aromatic Amino Acid Decarboxylase in Psilocybin Mushroom

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ABSTRACT: Aromatic l-amino acid decarboxylases (AAADs) are a phylogenetically diverse group of enzymes responsible for the decarboxylation of aromatic amino acid substrates into their corresponding aromatic aryalkylamines. AAADs have been extensively studied in mammals and plants as they catalyze the first step in the production of neurotransmitters and bioactive phytochemicals, respectively. Unlike mammals and plants, the hallucinogenic psilocybin mushroom Psilocybe cubensis reportedly employs an unrelated phosphatidylserine-decarboxylase-like enzyme to catalyze l-tryptophan decarboxylation, the first step in psilocybin biosynthesis. To explore the origin of this chemistry in psilocybin mushroom, we generated the first de novo transcriptomes of P. cubensis and investigated several putative l-tryptophan-decarboxylase-like enzymes. We report the biochemical characterization of a noncanonical AAAD from P. cubensis (PcAAAD) that exhibits substrate permissiveness toward l-phenylalanine, l-tyrosine, and l-tryptophan, as well as chloro-tryptophan derivatives. The crystal structure of PcAAAD revealed the presence of a unique C-terminal appendage domain featuring a novel double-β-barrel fold. This domain is required for PcAAAD activity and regulates catalytic rate and thermal stability through calcium binding. PcAAAD likely plays a role in psilocybin production in P. cubensis and offers a new tool for metabolic engineering of aromatic-amino-acid-derived natural products.

Psilocybin is a psychoactive alkaloid produced by hallucinogenic mushrooms within the Psilocybe genus. Upon ingestion, psilocybin is dephosphorylated by alkaline phosphatases and other nonspecific esterases in intestine and kidneys to yield psilocin, the active compound responsible for euphoria, visual and mental hallucinations, changes in perception, and spiritual experiences.1−3 Although psilocybin and its naturally occurring analogs are currently registered as Schedule I substances by the United States Drug Enforcement Administration (DEA) with no approved medical use, recent research has led to renewed interest in exploring psilocybin as a therapeutic agent to treat severe psychiatric disorders, including addiction,4 obsessive–compulsive disorder5 and depression.6,7 Indeed, a total of 16 clinical trials are currently underway to establish clinical applications of psilocybin.8 The biosynthetic pathway of psilocybin has been proposed for decades;9 however, the requisite genes and the reaction order of their corresponding enzymes have only recently been elucidated.10 In this pathway, psilocybin is derived from l-tryptophan by a gene cluster encoding four biosynthetic enzymes, namely, l-tryptophan decarboxylase (TDC), PsiD; tryptamine 4-hydroxylase, PsiH; 4-hydroxytryptamine kinase, PsiK; and norbaeocystin N-methyltransferase, PsiM (Figure S1). Whereas PsiH, PsiK, and PsiM have apparently evolved from progenitors in the extensively studied cytochrome P450 (InterPro accession number IPR001128), kinase-like domain (IPR011009), and S-adenosyl-l-methionine-dependent methyltransferase (IPR029063) superfamilies, respectively, the evolutionary origin of PsiD remains enigmatic, as it represents a new class of fungal decarboxylases (AAADs, IPR003817).11,12 It appears that in psilocybin mushroom l-tryptophan decarboxylation is catalyzed by a neofunctionalized phosphatidylserine decarboxylase-like enzyme rather than the pyridoxal 5′-phosphate (PLP)-dependent aromatic l-amino acid decarboxylases (AAADs, IPR010977) typically responsible for aryalkylamine production in other kingdoms of life.13−16 AAADs have been extensively studied in mammals as l-3,4-dihydroxyphenylalanine (l-DOPA) decarboxylase, which catalyzes the first step in the biosynthesis of monoamine neurotransmitters.17 Unlike the singular mammalian gene, the...
AAAD family in plants has undergone extensive radiation to produce paralogous TDC, tyrosine decarboxylase (TyDC), and acetaldehyde synthase (AAS) genes.\textsuperscript{18–22} The resulting variations in substrate selectivity and catalytic mechanisms enable individual plant AAADs to operate at distinct positions on the interface between primary and specialized metabolism. Biochemical transformations catalyzed by AAADs commit amino acid substrates into the biosynthesis of numerous important natural products, including monoterpenoid indole alkaloids, benzylisoquinoline alkaloids and aromatic acetaldehyde derivatives. Unlike mammals and plants, AAADs in fungi produce paralogous TDC, tyrosine decarboxylase (TyDC), and acetaldehyde synthase (AAS) genes.\textsuperscript{18–22} The resulting variations in substrate selectivity and catalytic mechanisms enable individual plant AAADs to operate at distinct positions on the interface between primary and specialized metabolism. Biochemical transformations catalyzed by AAADs commit amino acid substrates into the biosynthesis of numerous important natural products, including monoterpenoid indole alkaloids, benzylisoquinoline alkaloids and aromatic acetaldehyde derivatives. Unlike mammals and plants, AAADs in fungi have received little attention despite their purported role in the biosynthesis of melanin and alkaloids such as bufotenine and communesin.\textsuperscript{23,24} To the best of our knowledge, Ceriporiopsis subvermispora TDC (CsTDC) is the only cloned and biochemically characterized fungal AAAD to date.\textsuperscript{25} Curiously, although AAAD homologues and even TDCs are present in fungi, \textit{Pilocybe cubensis} has achieved a parallel means of tryptophan decarboxylation through PsiD. In this study, we investigated several putative \textit{l}-tryptophan-decarboxylation-catalyzing enzymes in \textit{P. cubensis}, and identified a calcium-activatable noncanonical AAAD (PcncAAAD), which likely mediates an alternative route to psilocybin biosynthesis from \textit{l}-tryptophan in psilocybin mushroom.

\section*{RESULTS AND DISCUSSION}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{phylogeny.png}
\caption{Phylogeny of Agaricomycetes type II PLP-dependent decarboxylases and the biochemical reactions catalyzed by \textit{P. cubensis} AAAD enzymes characterized in this study. (A) A maximum likelihood (ML) phylogeny of Agaricomycetes type II PLP-dependent decarboxylases. Bootstrap values greater than 70 are displayed at the tree nodes. The three major clades are annotated as AAADs (green), ncAAADs (blue), and GADs (purple) based on the phylogenetic pattern and functional characterization. Four genes from \textit{P. cubensis} together with \textit{S. cerevisiae} GAD and \textit{C. subvermispora} TDC are shown in bold. Genes from \textit{P. cubensis} are noted with orange dots. \textit{C. subvermispora} TDC is the only characterized fungal AAAD with a known sequence prior to this study. \textit{S. cerevisiae} GAD does not belong to Agaricomycetes, but \textit{C. subvermispora} TDC is included here to help annotate the major clades. The average intrACLade pairwise protein sequence identity for the GAD, AAAD, and ncAAAD clades are 43.5%, 54.1%, and 38.0%, respectively, whereas the average pairwise protein sequence identity between two type II PLP decarboxylases from different clades is less than 23%. The scale bar measures evolutionary distances in substitutions per amino acid. (B) The catalytic functions of \textit{PcDHPAAS}, \textit{PcGAD1}, PsiD, and PcncAAAD. PcncAAAD can also decarboxylate \textit{l}-phenylalanine and \textit{l}-tyrosine.}
\end{figure}

Phylogenetic Analysis of Type II PLP Decarboxylase Family Proteins from \textit{P. cubensis}. To investigate potential \textit{l}-tryptophan-decarboxylation-catalyzing enzymes in psilocybin mushroom, we generated \textit{P. cubensis de novo} transcriptome data sets from both mycelia and fruiting body tissues. Four type II PLP decarboxylase-encoding genes were identified from the \textit{P. cubensis} transcriptomes. The evolutionary relationships of these \textit{P. cubensis} enzymes were investigated together with representative type II PLP decarboxylases from 22 sequenced agaricomycete species (Figure 1A). This analysis revealed that two \textit{P. cubensis} enzymes, \textit{PcGAD1} and \textit{PcGAD2}, belong to the \textit{l}-glutamate decarboxylase (GAD) family, whereas the other two fall into canonical and noncanonical clades of the AAAD family.

The GAD clade contains the previously characterized \textit{Sachcharomyces cerevisiae} GAD,\textsuperscript{26} to which \textit{PcGAD1} and \textit{PcGAD2} share 48% and 49% amino acid sequence identity, respectively. Like \textit{PcGAD1}, most of the sequenced agaricomycete species also contain two GAD-like genes, suggesting an ancestral gene duplication event before the agaricomycete class radiated. The second clade, annotated as the AAAD clade due to the inclusion of the characterized CsTDC,\textsuperscript{25} contains a single \textit{P. cubensis} enzyme with probable substrate selectivity toward \textit{l}-aromatic amino acid substrates. This enzyme could plausibly accommodate \textit{l}-tryptophan as a substrate; however, the substitution of a catalytic-loop tyrosine to phenylalanine at residue 329 suggests that this enzyme likely functions as an AAS rather than a canonical decarboxylation-catalyzing AAAD (Figure S2).\textsuperscript{27} Whereas both the AAAD and GAD clades contain genes with characterized enzymatic activities, proteins that fall into the noncanonical AAAD (ncAAAD) clade are much longer in sequence and have not been functionally characterized. Sequence analysis of the single \textit{P. cubensis} ncAAAD clade protein, revealed that the N-terminus (Met\textsuperscript{1–Val\textsuperscript{705}}) belongs to the PLP-dependent decarboxylase (IPR002129) family, whereas the C-terminus (Met\textsuperscript{706–Lys\textsuperscript{1013}}) shows no sequence homology to any characterized protein.

Tissue-Specific Transcript Abundance of \textit{P. cubensis} Type II PLP Decarboxylase-Encoding Genes. To assess the putative role of these newly identified \textit{P. cubensis} type II PLP decarboxylases in psilocybin biosynthesis, the abundance of the mycelia and fruiting-body transcripts were quantified and ranked by transcript-per-million (TPM) values using RSEM software and in-house scripts.\textsuperscript{28} PcncAAAD is among the most highly expressed genes with a TPM rank of 241 out of the 13 672 total fruiting body transcripts. As one of the top 2% most highly expressed genes, PcncAAAD surpassed the transcript abundance of the previously characterized psilocybin biosynthetic genes (Table S1 and Figure S3). In comparison, \textit{PcGAD1}, \textit{PcGAD2}, and \textit{PcAAS} are much less abundant with TPM rankings in fruiting body of 5029, 7196 and 9715, respectively. \textit{PcGAD1} and...
PcGAD2 show marginally higher transcript abundance in the mycelium (Figure S4), while PcAAS and PcnAAAD display tissue-specific expression patterns similar to those of the known psilocybin biosynthetic genes. Given the unusually high expression level of PcnAAAD and the fact that psilocybin is known to accumulate mostly in the fruiting body of psilocybin mushrooms, we hypothesized that PcnAAAD likely plays a role in psilocybin biosynthesis.

Biochemical Functions of Three P. cubensis Type II PLP Decarboxylase Family Proteins. To confirm their biochemical functions, the activities of the P. cubensis type II PLP decarboxylases were assessed in vitro with recombinant protein biochemistry or in vivo through heterologous expression in transgenic yeast followed by metabolic profiling. PcAAS was first investigated since it is most closely related to the previously described CstD. As predicted by the presence of the catalytic loop Tyr-to-Phe substitution at residue 329, recombinant PcAAS exhibits AAS activity with an apparent substrate selectivity toward L-DOPA (Figure S5). Accordingly, this enzyme was annotated as P. cubensis 3,4-dihydroxyphenylacetalddehyde synthase (PDHPAAS). Biochemically, PDHPAAS displays a similar activity to that of the insect chitin-based cuticle development α-methyl DOPA resistant (AMD-α) protein. Since chitin is a key structural component in both the exoskeleton of arthropods and the cell wall of fungi, PDHPAAS may serve a similar function as AMD-α in the production of dihydroxyphenylacetalddehyde from L-DOPA for crosslinking chitin.

We next characterized PcGAD1, as a representative enzyme of the GAD clade. Recombinant PcGAD1 was expressed, purified, and assayed against the full panel of proteinogenic L-amino acid substrates. Nevertheless, no measurable enzyme activity was detected. Inability to measure in vitro activity for recombinant fungal GADs has been previously reported, which was attributed to the absence of calcium and calmodulin required for the activation of GAD activity in vivo. To circumvent this caveat, we generated transgenic yeast expressing PcGAD1 and profiled the metabolome of the resultant cells by liquid chromatography-high-resolution accurate-mass mass spectrometry (LC-HRAM-MS). The PcGAD1-expressing yeast showed significantly higher accumulation of both γ-aminobutyric acid (GABA) and its downstream catecholamine succinate compared to the wild-type control (Figure S6), confirming the in vivo L-glutamate decarboxylation activity of PcGAD1. Although GADs are ubiquitous enzymes in both fungi and plants, their exact physiological functions remain to be investigated in their native hosts. In fungi, GABA was shown to serve as an alternative nitrogen source with potential roles in pathogenicity and development.

Finally, recombinant PcnAAAD, when challenged with L-amino acid substrates, demonstrated decarboxylation activity towards L-phenylalanine, L-tyrosine, and L-tryptophan (Figure S7). This broad substrate specificity observed in PcnAAAD is notable as plant AAADs typically display selectivity toward a single L-aromatic amino acid substrate. PcnAAAD was therefore selected for in-depth characterization as it represents a novel clade of fungal AAAD family enzymes capable of catalyzing L-tryptophan decarboxylation, the first committed step of psilocybin biosynthesis. The biochemical functions of PdDHPAAS, PcGAD1, and PcnAAAD are summarized in Figure 1B.

Enzyme Kinetics of PcnAAAD. Whereas previously characterized plant AAADs principally display selectivity towards an individual substrate, recombinant PcnAAAD exhibits L-aromatic amino acid substrate permissiveness. To assess the relative substrate preference of PcnAAAD towards L-phenylalanine, L-tyrosine, and L-tryptophan, we performed Michaelis–Menten kinetic assays using recombinant PcnAAAD against these three substrates in a generic NaCl-containing reaction buffer at pH 8.0 (Table 1 and Figure S8).

PcnAAAD exhibits $K_M$ values of 0.82 mM and 0.45 mM against L-phenylalanine and L-tryptophan, respectively, although the catalytic efficiency for L-phenylalanine (11.80 s$^{-1}$ M$^{-1}$) is greater than that of L-tryptophan (1.33 s$^{-1}$ M$^{-1}$). The turnover rate and Michaelis constant toward l-tyrosine was ultimately not determined as the maximum rate of the reaction was limited by the solubility of the substrate. The kinetic constants could not be measured, PcnAAAD readily decarboxylates l-tyrosine with a specific activity four times that of l-tryptophan and one-quarter that of l-phenylalanine at 2 mM substrate concentration.

**In Vivo Characterization of PcnAAAD Substrate Selectivity in Transgenic Yeast.** PcnAAAD catalyzes decarboxylation of multiple L-aromatic amino acids into their corresponding monoamine products in vitro; however, the role and abundance of these specialized metabolites in P. cubensis remain unclear. As psilocybin mushrooms reportedly contain multiple psilocybin-like biosynthetic gene clusters, PcnAAAD may operate within one of these gene clusters in the production of psilocybin-like specialized metabolites. Due to difficulties of genetic studies in P. cubensis, we compared the in vivo product formation of PcnAAAD against that of previously described AAADs operating in known biosynthetic pathways in the context of transgenic yeast. We generated transgenic yeast overexpressing the Catharanthus roseus TDC (GrTDC), Papaver somniferum TyDC (PsTyDC), PsiD, or PcnAAAD, and profiled the metabolome of these engineered yeast strains by LC-HRAM-MS (Figure 2 and Figure S9). Compared to the wild-type control, the PcnAAAD-expressing yeast ectopically accumulates tyramine, phenylethylamine, and tryptamine, confirming PcnAAAD’s broad in vivo substrate specificity. Tyramine accumulation in yeast expressing PcnAAAD was similar to that of yeast expressing PsTyDC, whereas ectopic tryptamine levels in the PcnAAAD, PsiD, and GrTDC-expressing strains are within 2-fold difference. Despite utilizing a number of L-aromatic amino acid substrates, the PcnAAAD-expressing yeast strain displayed accumulated levels of individual monoamines comparatively to those of the strains expressing substrate-specific decarboxylases.

**PcnAAAD Can Accommodate Chlorinated Tryptophans As Substrates.** Using halogenated L-tryptophan as a precursor for the biosynthesis of halogenated monoterpene indole alkaloid in C. roseus has been demonstrated as a viable approach to produce novel natural product analogs. However, the poor activity of the native GrTDC toward halogenated L-tryptophan acts as a metabolic bottleneck in the biosynthesis of downstream halogenated alkaloids. Consequently, the substrate-
permissive PncAAAD may demonstrate utility in metabolic engineering of natural products derived from halogenated L-tryptophan. To test whether PncAAAD can accommodate chlorinated substrates, we constructed transgenic yeast strains expressing two previously described halogenases, PyrH and RebH, that produce 5-chlorotryptophan and 7-chlorotryptophan, respectively, from L-tryptophan. The resultant chlorotryptophan then serves as the in vivo substrate for either PncAAAD, PsiD, or CrTDC in transgenic yeast (Figure 3A).

Whereas CrTDC displays no activity toward 5-chlorotryptophan and minimal activity toward 7-chlorotryptophan, PsiD demonstrated a level of substrate permissiveness toward both 5- and 7-chlorotryptophan (Figure 3B). PncAAAD also accommodates both 5- and 7-chlorotryptophan as substrates but displays higher 7-chlorotryptamine production than PsiD. In addition to monoterpene indole alkaloid engineering, PncAAAD may also aid in the production of halogenated psilocybin derivatives.

**Overall Structure of PncAAAD.** To understand the structural basis for substrate permissiveness and the function of the C-terminal appendage domain, we solved the X-ray crystal structure of PncAAAD at 1.97 Å resolution (PDB ID 6EBN, Table S2). Similar to other AAADs, PncAAAD purifies and crystallizes as a homodimer. N-terminal (Met1–Asp717), C-terminal (Gly985–Lys1013), and loop (Ile765–Arg770) residues were not modeled in the final structure due to poor electron density support. In the PncAAAD homodimer, the two lysine-pyridoxal-5′-phosphate (LLP 443) groups are symmetrically bound in the clefts of the monomer–monomer interface. Each individual chain is composed of the core type I aspartate aminotransferase superfamily domain (Val14–Asp717) and a novel double-β-barrel C-terminal appendage domain (Met718–Tyr984) (Figure 4A,B). The catalytic core domain, as described in other AAADs, is composed of the N-terminal, PLP-binding, and C-terminal segments (Figure S10).22,38 Superimposition of the core domain of PncAAAD with its most closely related crystal structure, Lactobacillus brevis TyDC (LbTyDC, PDB ID 5HSJ),39 demonstrates a high degree of conserved topology with a calculated RSMD of 0.34 Å (Figure S11A). The differences between LbTyDC and the catalytic domain of PncAAAD mainly occur on the exterior of the structure, with an additional two-stranded β-sheet (Asp211–Leu224) and a stretch of three inserted α-helices (Phe545–Gly594) in PncAAAD (Figure S11B). Additionally, a helix in the LbTyDC structure (Leu340–Thr363) adopts a loop configuration in the PncAAAD structure (Leu597–Val615), in which the chain has rotated outward toward the N-terminus and solvent shell (Figure S11C).

The PncAAAD catalytic loop (Trp607–Ile611), required for activity in type II PLP decarboxylases, is observed in an open conformation similar to that of the CrTDC structure (Figure S12).22 Although both the PncAAAD and CrTDC structures adopt open conformations, the short helix in CrTDC (Pro346–Lys359), postulated to “unlock” the initial closing motion of the...
catalytic loop, is displaced in the PencAAAD structure by a distal helix (Pro256−Ile267) not present in CrTDC.22 The dislocation, caused by the aforementioned insertion, protrudes the PencAAAD catalytic loop away from the active site to form new intermolecular interactions with the other PencAAAD monomer. The open conformations of the PencAAAD and CrTDC structures are in contrast to the closed configuration observed in PsTyDC and HmHDC in which the corresponding loop is sealed over the active site with the catalytic tyrosine positioned proximal to the substrate Cα.22,40 The alternative loop conformations revealed by PencAAAD and previously reported AAAD structures inform the dynamic nature of the loop pertinent to its role in the catalytic cycle.22

The C-terminal appendage domain consists of two perpendicularly stacked β-barrel repeats, each containing six β-strands followed by a downstream helix-containing loop (Figure 4C). Repeat 1 (Met718−Leu833) is distinguished from repeat 2 (Met834−Tyr945) as it lacks the loop insertions Ala891−Gln897, Tyr925−Lys939, and Ala970−His976 (Figure 4D). The sheet topology of each β-barrel repeat has the order of 6−3−2−1−5−6 with 3−2−1−4 being the Greek-key motif (Figure 4E,F). Fold similarity search using the DALI server41 failed to identify any known tertiary structures similar to the PencAAAD C-terminal appendage domain, suggesting a novel topology, which we named the Cuben fold after its source organism.

Each monomer of the PencAAAD homodimer contains two metal-binding sites occupied by sodium ions derived from the crystallization buffer. Both metal-binding sites are formed cooperatively from domain or repeat structural interactions and may regulate conformational changes and allostery in PencAAAD. The first metal-binding site, located at the boundary between domains, is composed of Gln629, Ser632, and Glu638 from the catalytic domain and Asp971 from repeat 2 of the C-terminal domain (Figure 5A). The second metal-binding site is composed of the C-terminal domain residues Glu824 and Asp825 from the Cuben-fold repeat 1 and Met930 of repeat 2 (Figure 5B). In addition to coordinating the sodium ion, the loop structure from the first metal-binding site also interacts with Gly478 of the catalytic loop via the carbonyl of Thr634. As the R-group of Thr634 in turn forms a hydrogen bond with one of the
metal-coordinating waters, this residue forms a link between the first metal-binding site and the catalytic loop (Figure 5C). Additionally, the C-terminal domain residues Lys1000 and Asp901 further engage hydrogen-bonding and electrostatic interactions with the catalytic loop residue Gln474. The specific interactions between the metal-binding sites, the C-terminal appendage domain, and the catalytic loop likely impact the dynamics of PcnAAAD. Since enzyme turnover requires conformational changes of the catalytic loop, metal binding could therefore influence catalysis.

**Molecular Docking and Active Site Composition.** To understand the catalytic mechanism, particularly substrate binding and recognition, AutoDock Vina42 was used to dock the ligands L-phenylalanine, L-tyrosine, or L-tryptophan to the PcnAAAD structure with the search area defined as the space between the LLP443 cofactor and the catalytic-loop Tyr471. Considering the binding configuration necessary for transaldimination and decarboxylation,43 as well as the ligand orientation of previously solved substrate-bound AAD structures,22,23,39,40 the most probable poses for the three amino acid substrates were determined (Figure S1A–C). In PLP carboxylases, the labile α-carbon-carboxyl bond of the ligand is required to be positioned perpendicular to the pyridine ring of the internal aldime LLP. In this orientation, the α-carbon of the quinonoid intermediate would be positioned out of the active site pocket and accessible to the catalytic Tyr471 responsible for carbanion intermediate protonation. Furthermore, since the first step of decarboxylation is a transaldimination reaction between the substrate and the PLP-bound Lys443, the α-N of the amino acid substrate and the 4′-C of PLP have to come into close proximity to initiate the reaction. The distances between α-N and 4′-C atoms in our models are 4.9 Å for L-phenylalanine, 5.3 Å for L-tyrosine, and 6.5 Å for L-tryptophan, suggesting a reasonable docking orientation capable of transaldimination. L-Phenylalanine, L-tyrosine, and L-tryptophan show similar orientations in the active site with their aromatic side chains buried deep inside the active-site cavity. The substrates are anchored at their amine and carboxyl groups to the side chains of His295 and Tyr296 by two to three hydrogen bonds. The hydrophobic active site environment created by Met108, Val131, Ala132, and Lys443 along with the π–π interaction of His107 and the aromatic substrate, likely engender the broad substrate selectivity of PcnAAAD. In the docking model, electrostatic bonds between PcnAAAD residues and the 4-hydroxyl group of L-tyrosine or the indole ring nitrogen of L-tryptophan appear to form additional stabilizing interactions.

**The C-Terminal Appendage Domain Modulates PcnAAAD Activity through Calcium Binding.** To test the function of the PcnAAAD appendage domain, we expressed the N-terminal (Met1–Asp717) and C-terminal (Arg718–Lys1013) domains, either alone or together in yeast. Metabolic profiling of monoamine production in the resultant yeast strains demonstrates that the N-terminal domain alone showed little to no in vivo decarboxylation activity in transgenic yeast, despite the presence of the entire type I aspartate aminotransferase folding structure (Figure 6A). Whereas the C-terminal appendage domain by itself has no enzymatic activity, coexpression of N- and C-terminal domains as separate polypeptides in transgenic yeast partially recovered decarboxylase activity toward all three proteinogenic aromatic amino acid substrates. These results suggest that the C-terminal appendage is indispensable for PcnAAAD activity.

To investigate whether metal binding affects PcnAAAD activity, we first measured the activity of PcnAAAD in a series of reaction buffers containing sodium, potassium, magnesium, calcium, zinc, or ferric ions (Figure S14). Among the various metal ions tested, only calcium enhanced PcnAAAD activity. Accordingly, we then determined the kinetic parameters of PcnAAAD against the three L-aromatic amino acid substrates in the presence of calcium. Notably, the catalytic rates of PcnAAAD against L-phenylalanine and L-tryptophan measured in calcium buffer are 300- and 500-fold higher than those measured in sodium buffer, respectively (Figure 6B, Tables 1 and 2, and Figure S15). Finally, ThermoFluor assays44 were used to measure the impact of calcium on PcnAAAD thermal stability (Table 3 and Figure 6C). The temperature-dependent PcnAAAD unfolding curves reveal two melting events around 51 and 67 °C in sodium or ion-free buffer. The presence of calcium significantly increases the melting temperatures of both events, suggesting that calcium binding imparts a stabilizing effect on the PcnAAAD protein. Altogether, these results suggest that the metal-binding sites observed in the PcnAAAD structure can positively regulate PcnAAAD activity through specific binding to calcium.

**CONCLUSION**

In this study, we characterized several type II PLP decarboxylase family enzymes identified through mining the *P. cubensis* transcriptomes. Among them, PcnAAAD was found to
represent a fungal-specific clade of AAADs that likely plays a role in psilocybin biosynthesis parallel to the psilocybin biosynthetic gene cluster previously reported by Fricke et al.\textsuperscript{10} In-depth biochemical characterization further shows that \textit{Pc}ncAAAD is a broad-spectrum AAAD capable of producing tryptamine from L-tryptophan both \textit{in vitro} and in transgenic yeast. \textit{Pc}ncAAAD features an unusual C-terminal metal-binding domain that modulates \textit{Pc}ncAAAD activity through calcium binding.

Although US federal regulation currently precludes our ability to study the function of \textit{Pc}ncAAAD in the context of its native host \textit{P. cubensis}, we postulate that \textit{Pc}ncAAAD may mediate de

Table 2. Kinetic Parameters of \textit{Pc}ncAAAD towards L-Phenylalanine and L-Tryptophan in 50 mM Tris, pH 8.0, and 30 mM Calcium Acetate Reaction Buffer

| Aromatic Amino Acid | $k_\text{cat}$ (s$^{-1}$) | $K_M$ (mM) | $k_{\text{cat}}/K_M$ (s$^{-1}$ mM$^{-1}$) |
|--------------------|---------------------------|-----------|---------------------------------|
| L-Phenylalanine    | 3044.00 ± 263.80          | 0.78 ± 0.21 | 3902.56                         |
| L-Tryptophan       | 255.40 ± 16.23            | 0.36 ± 0.09 | 709.44                          |

Table 3. Melting Temperatures, $T_{m1}$ and $T_{m2}$ of \textit{Pc}ncAAAD in Ion-Free, Sodium or Calcium Containing Buffers Measured in Sextuplicate

| Buffer          | $T_{m1}$ (°C) | $T_{m2}$ (°C) |
|-----------------|---------------|---------------|
| Ion-free buffer | 50.94 ± 0.42  | 67.30 ± 0.39  |
| 10 mM Na$^+$    | 50.71 ± 0.99  | 66.29 ± 0.24  |
| 10 mM Ca$^{2+}$ | 54.27 ± 0.19  | 69.41 ± 0.03  |

Figure 6. Role of C-terminal appendage domain and calcium binding in modulating \textit{Pc}ncAAAD activity and thermostability. (A) \textit{In vivo} monoamine production in transgenic yeast strains expressing full-length \textit{Pc}ncAAAD, \textit{Pc}ncAAAD catalytic domain, \textit{Pc}ncAAAD C-terminal domain, or \textit{Pc}ncAAAD catalytic and C-terminal domains as separate polypeptides. The extracted ion chromatograms (XICs) for monoamine products derived from three L-aromatic amino acids are displayed. (B) Relative \textit{in vitro} production of monoamines by \textit{Pc}ncAAAD in sodium buffer (50 mM Tris, pH 8.0, and 30 mM NaCl) or calcium buffer (50 mM Tris, pH 8.0, and 30 mM calcium acetate). (C) ThermoFluor assay demonstrates the effect of metal binding on the thermostability of \textit{Pc}ncAAAD. The first derivative of fluorescence versus temperature plot is shown. The melting temperature differences between samples measured in sodium and calcium buffers are shaded in blue.
novis lysergic acid diethylamide (lysergic acid N,N-diethylamide, or LSD). It was first identified in a fungus called Claviceps purpurea by Albert Hofmann in 1938. LSD is known for its strong hallucinogenic and psychoactive effects. It has been studied extensively for its potential therapeutic applications, particularly in treating conditions such as anxiety and obsessive-compulsive disorder (OCD). However, due to concerns about misuse, it is currently not approved for medical use in most countries. In this study, we aimed to discover new genes that could potentially contribute to the biosynthesis of these compounds, focusing on the alternative microbial host strategy.

**Methods**

Reagents. 1-Amino acids, tyramine, phenylethylamine, tryptamine, γ-aminobutyric acid, succinate, and PLP were purchased from Sigma-Aldrich.

RNA Isolation, Library Preparation, Transcriptome Assembly, cDNA Production, and Molecular Cloning. Total RNA was extracted from the fungus and the yeast M. nulli with the RNeasy Mini Kit (Qiagen). RNA quality was assessed by Bioanalyzer (Agilent Technologies). For the RNA-seq experiment, strand-specific mRNA libraries were prepared using total RNA prepared separately from the fungus and the yeast M. nulli using the TrueSeq Stranded mRNA Library Prep Kit (Illumina) and sequenced on an Hiseq 2000 sequencer (Illumina) in paired-end mode (PE100). Sequence FASTQ files were trimmed for sequencing adapters using Trimmomatic. 47 and assembled into de novo transcriptomes using Trinity in strand-specific mode. Gene expression statistics (TPM values) were determined by RSEM. 48 Combining all raw sequencing reads for both tissues, we assembled a total of 39350 unique transcripts. The combined transcriptome was evaluated by the metric of Benchmarking Universal Single-Copy Orthologs (BUSCO). 49 It was set as 97.6% complete. When using BUSCO, the fun sp_odb9 set was as lineage and “ustilago” was set as model species. Putative coding regions were predicted using Transdecoder. 50 Transcripts and predicted protein sequences were annotated with TPM values and lowest BLAST hits using in-house scripts. The version described in this paper is the first version, GGMK01000000. Transcriptome mining was performed on a local BLAST server. First-strand cDNAs were synthesized by RT-PCR using total RNA sample as template and the Invertigo SuperScript III kit (Invitrogen) with the oligo( dT) 20 primer. The coding sequences (CDS) of candidate genes were amplified from first-strand cDNAs by PCR using gene-specific primers (Table S1). RebH, RebF, and PyrH were codon optimized for expression in S. cerevisiae, and ordered as IDT gBlocks. Gibson assembly was used to ligate PCR amplicons into several base vectors. These include pHs8-4, a bacterial expression vector containing an N-terminal 8XHis tag followed by a tobacco etch virus (TEV) cleavage site for recombinant protein production in E. coli, and p423TEF, a 2-μm plasmid 51 containing the HIS3 auxotrophic growth markers for constitutive expression in S. cerevisiae. The tryptophan halogenation multigene vectors containing RebH/RebF or PyrH/RebF were constructed using the Promega Promoter UAS regulator. These vectors were transformed into the yeast BY4743 and the bacterial BL21 strains using CaCl2 transformation. The L-tryptophan halogenation multigene vectors containing the desired pHis8-4 expression vector were grown at 37 °C in terrif broth to OD600 of 0.9, induced with 0.15 mM isopropyl-β-D-thiogalactoside (IPTG), and allowed to grow for an additional 20 h at 18 °C. Cells were harvested by centrifugation, washed with PBS (137 mM NaCl, 2.7 mM KCl, 10 mM NaH2PO4, and 1.8 mM KH2PO4), resuspended in 150 μL of lysis buffer (50 mM Tris, pH 8.0, 50 mM NaCl, 20 mM imidazole, and 0.5 mM DTT), and lysed with five passes through an M-110L microfluidizer (Microfluidics). The resulting crude protein lysate was clarified by centrifugation prior to Qiagen nickel-nitritrotiacetic acid (Ni-NTA) gravity flow chromatographic purification. After loading the clarified lysate, the polyhistidine-tagged recombinant protein-bound Ni-NTA resin was washed with 20 column volumes of lysis buffer and eluted with 2 column volumes of elution buffer (50 mM Tris, pH 8.0, 0.5 M NaCl, 250 mM imidazole, and 0.5 mM DTT). One milligram of polyhistidine-tagged TEV protease was added to the eluted protein, followed by dialysis at 4 °C for 16 h in dialysis buffer (50 mM Tris, pH 8.0, 0.1 M NaCl, 20 mM imidazole, and 2 mM DTT). After dialysis, protein solution was then passed through Ni-NTA resin to remove uncleaved protein and TEV. The recombinant protein was further purified by gel filtration on a Fastprotein liquid chromatography (FPLC) system (GE Healthcare Life Sciences). The purified protein was either flash frozen in storage buffer (20 mM Tris, pH 8.0, 25 mM NaCl, and 0.5 mM DTT) or further processed to remove metal ions. To do so, the buffer of the purified enzyme was exchanged with a chelating buffer (50 mM imidazole, pH 8.0, 5 mM crown ether, 5 mM EDTA, and 2 mM DTT) and then a metal-ion-free storage buffer (25 mM Tris, pH 7.5, 0.2 mM PLP, and 2 mM DTT) using an Amicon Ultra-S15 centrifugal filter unit with Ultracel-30 membrane (EMD Millipore). After flash freezing, the concentration of the recombinant protein was subsequently evaluated by image densitometry against a standard curve of bovine serum albumin standards. 52

**Enzyme Assays.** DHPAAS was measured through the detection of the hydrogen peroxide coproduct using the Pierce Quantitative Peroxide Assay Kit (Pierce) against a standard curve of hydrogen peroxide. PcnAAD enzyme was initially challenged in 200 μL reactions against 0.2 mM L-phenylalanine, L-tyrosine, or L-tryptophan in 20 mM Tris, pH 8.0, 25 mM NaCl, and 0.5 mM DTT. Reactions were started with addition of 10 μg of recombinant enzyme, incubated at 30 °C for 10 min, and quenched with addition of 200 μL of methanol. The reaction mixture was centrifuged and the supernatant was analyzed by LC-MS. Ten microliters of reaction mixture was analyzed on an Ultimate 3000 liquid chromatography system ( Dionex) equipped with a 150 mm C18 Column (Kinexet 2.6 μm silica core—shell C18 100 Å pore, Phenomenex) and coupled to an UltiMate 3000 diode-array...
detector (DAD) in-line UV–vis spectrophotometer (Dionex) and a TQS Quantum Access MAX triple-quadrupole mass spectrometer (Thermo-Scientific). Aromatic amino acids and their corresponding monoamine products were separated through gradient elution of water (0.1% formic acid) and acetonitrile (0.1% formic acid). L-Tryptophan, tryptamine, L-phenylalanine, and phenylethylamine were detected by selected reaction monitoring (SRM) method. Alternative means of separation and detection were employed to measure the decarboxylation of L-tryptophan. L-Tyrosine and tyramine elute at the same retention time using the aforementioned method, and L-tyrosine displays in-source fragmentation to produce tyramine. Consequently, L-tyrosine and tyramine were detected by UV absorbance at 280 nm using ion-pairing isocratic LC-UV method as previously described.20

Analytical standards were used to confirm the formation of products. When examining the PcncAAAD metal-ion dependence, aliquots of recombinant protein washed with chelators (crown ether and EDTA) and stored in salt free buffer were incubated with substrate and 10 mM NaCl, CaCl2, KI, MgSO4, ZnCl2, or Fe(NO3)3.

Kinetic Characterization. Kinetic characterization of PcncAAAD was conducted in 100 μL reaction volume of either sodium buffer (50 mM Tris, pH 8.0, and 30 mM NaCl) or calcium buffer (50 mM Tris, pH 8.0, and 30 mM calcium acetate) containing substrate concentrations from 0.3 μM to 5 mM. Reactions in sodium buffer were started with the addition of 30 μg of metal-ion-free recombinant enzyme, whereas 2 μg of metal-ion-free recombinant enzyme was used for reactions in calcium buffer. Reactions were incubated at 25 °C for 10 min and quenched with the addition of 100 μL of methanol. After clarification, samples were analyzed by LC-HRAM-MS using the previously described ZIC-PhIIIC method.20 Product formation was calculated against a standard and stored in salt free buffer were incubated with substrate and 10 mM NaCl, CaCl2, KI, MgSO4, ZnCl2, or Fe(NO3)3.

ThermoFluor Assays. In individual wells of a 384-well plate, 20 μg of enzyme premixed with 150X SYPRO Orange dye was mixed with buffer (25 mM Tris, pH 7.5, containing 8 mM NaCl, 8 mM CaCl2, or no additional salt as a control) to reach a final reaction volume of 10 μL. LightCycler 480 was used to measure fluorescence intensities using excitation at 480 nm and emission at 575 nm over a temperature ramp of 20 to 85 °C at a rate of 0.06 °C/s. The protein melting temperatures (Tm and Tml) were determined from the peaks of the negative first derivative curve of the fluorescence signal, conducted and measured in sextuplicate.

Protein Crystallization. PcncAAAD crystals were grown by hanging drop vapor diffusion at 4 °C. The initial drop contained 2 μL of 10 mg/mL protein mixed with 1 μL of reservoir solution of 3.5 M sodium formate, pH 7.0. Crystals were cryoprotected by transferring to a reservoir solution plus an additional 25% glycerol. Single crystals were mounted in a cryoloop and flash-frozen in liquid nitrogen.

X-ray Diffraction and Structure Determination. X-ray diffraction data were collected at beamlines 24-ID-C of the Advanced Photon Source at Argonne National Laboratory for PcncAAAD crystals. Diffraction intensities were indexed and integrated with iMosflm and scaled with Scala under CCP4. The molecular replacement model of PcncAAAD was generated using Robetta67 using LfTyDC (PDB ID 5HSJ) as the template.39 The molecular replacement solution of PcncAAAD was determined using Molrep.26 The C-terminal domain was subsequently built both manually in Coot and automatically using phenix autobuild.16 Structural refinement was performed with Refmac 5.2,60 and other Phenix programs.61

Molecular Docking. All docking calculations were accomplished with AutoDock Vina 1.1.2.41 The active site was defined by the space between PLP and the catalytic tyrosine. The parameters used to set the grid box dimensions and center were: center_x = 28.97; center_y = 22.00; center_z = 26.51; size_x = 25; size_y = 25; size_z = 25.

Accession Codes

The sequences of P. cubensis genes reported in this article have been deposited in NCBI GenBank under the following accession numbers: PsiD (P0PDPA6), PsiH (P0PDPA7), PsiK (P0PDPA8), PsiM (P0PDPA9), PcGAD1 (MH710579), PcGAD2 (MH710580), PcncAAAD (MH710581), and PdhPAAS (MH710582). The raw RNA-Seq reads have been deposited in NCBI SRA (SRR7028478 and SRR7028479). The de novo transcriptomes assembled from the raw reads have been deposited in NCBI TSA (GGMK00000000). The atomic coordinates and structure factors of PcncAAAD have been deposited in the Protein Data Bank (6EBN).

Author Contributions

M.P.T.S. and C.-T.L. are co-first authors. M.P.T.S. and J.K.W. designed the research. M.P.T.S., C.-T.L., and Y.K.C. performed all experiments. T.P. assisted with transcriptome assembly and metabolomics analysis. M.P.T.S. and C.-T.L. analyzed and interpreted data. M.P.T.S., C.-T.L., and J.K.W. wrote the paper.
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Notes
The Weng Lab of the Whitehead Institute holds Schedule I permits from the DEA and Massachusetts Controlled Substances Registration (MCSR) to carry out experiments involving psilocybin, psilocin, and N,N-dimethyltryptamine. The authors declare the following competing financial interest(s): J.K.W. is a cofounder of the member of the Scientific Advisory Board, and a shareholder of DoubleRainbow Biosciences, which develops biotechnologies related to natural products.

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