

# SCIENTIFIC REPORTS



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## Expression and functional analysis of the lysine decarboxylase and copper amine oxidase genes from the endophytic fungus *Colletotrichum gloeosporioides* ES026

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Huperzine A (HupA) isolated from *Huperzia serrata* is an important compound used to treat Alzheimer's disease (AD). Recently, HupA was reported in various endophytic fungi, with *Colletotrichum gloeosporioides* ES026 previously isolated from *H. serrata* shown to produce HupA. In this study, we performed next-generation sequencing and *de novo* RNA sequencing of *C. gloeosporioides* ES026 to elucidate the molecular functions, biological processes, and biochemical pathways of these unique sequences. Gene ontology and Kyoto Encyclopedia of Genes and Genomes assignments allowed annotation of lysine decarboxylase (LDC) and copper amine oxidase (CAO) for their conversion of L-lysine to 5-aminopentanal during HupA biosynthesis. Additionally, we constructed a stable, high-yielding HupA-expression system resulting from the overexpression of *CgLDC* and *CgCAO* from the HupA-producing endophytic fungus *C. gloeosporioides* ES026 in *Escherichia coli*. Quantitative reverse transcription polymerase chain reaction analysis confirmed *CgLDC* and *CgCAO* expression, and quantitative determination of HupA levels was assessed by liquid chromatography high-resolution mass spectrometry, which revealed that elevated expression of *CgLDC* and *CgCAO* produced higher yields of HupA than those derived from *C. gloeosporioides* ES026. These results revealed *CgLDC* and *CgCAO* involvement in HupA biosynthesis and their key role in regulating HupA content in *C. gloeosporioides* ES026.

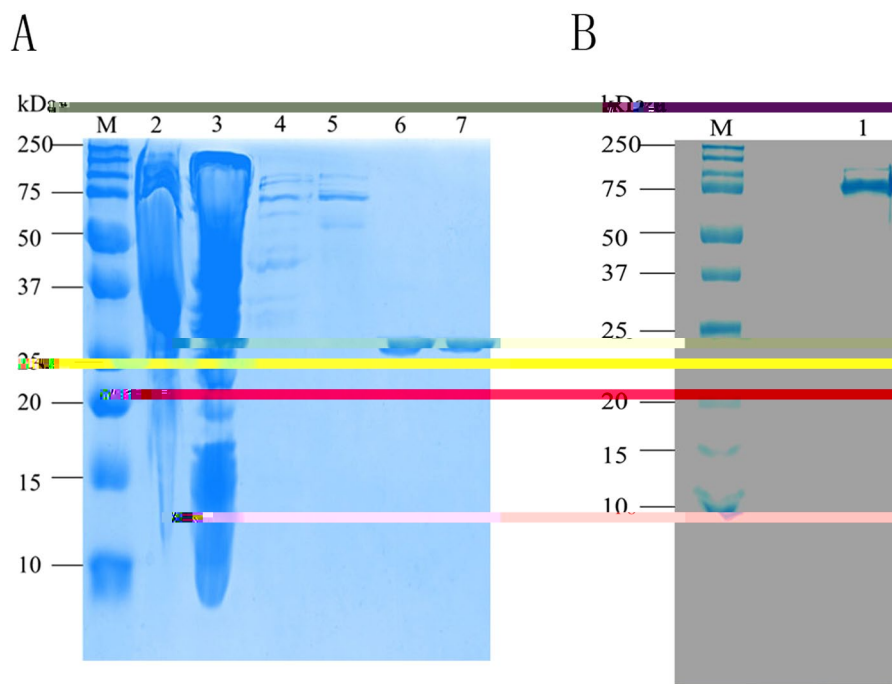
Huperzine A (HupA) is a pyridine-type alkaloid derived from *Huperzia serrata*<sup>1,2</sup> and constitutes a highly active acetylcholinesterase inhibitor, making it a valuable therapeutic option for the treatment of Alzheimer's disease (AD)<sup>3,4</sup>. Currently, >46 million people are affected with dementia, with this number predicted to increase to 131.5 million by 2050<sup>5</sup>. HupA is highly selective and exhibits low toxicity, reversibility, and a long duration time relative to other drugs used to treat AD<sup>6</sup>. Furthermore, HupA also exhibits anti-inflammatory activity and appears effective in the treatment of cerebrovascular-type dementia and benign senescent forgetfulness<sup>7,8</sup>.

Currently, HupA is a compound used in herbal supplements mainly extracted from the Chinese club moss *Huperzia serrata*; however, it has a limited distribution and slow growth rate<sup>9</sup>. Furthermore, the complex extraction process from plants and the high costs of downstream purification have impeded HupA utility<sup>10,11</sup>. Consequently, for successful commercial production of HupA, large volumes of *H. serrata* are required. Therefore, in order to protect plant resources from over-harvesting and reduce the cost of HupA-containing medicine, alternative methods for mass producing HupA are needed. The chemical synthesis of HupA was attempted, but the resulting synthesized HupA constituted a racemic mixture exhibiting much less potency than natural HupA. Alternatively,

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some endophytic fungi associated with *Trichostema* are capable of producing HupA<sup>12–14</sup> with *Trichostema* ES026 yielding 45 µg/g dried mycelium according to our previous study<sup>15</sup>. However, HupA production by these endophytes is hindered by low yields and the loss of biosynthetic capability after several generations. Therefore, methods involving overexpression of the enzymes associated with HupA biosynthesis need to be developed in a heterologous host if stable and efficient production is to be achieved<sup>15–17</sup>.

Although HupA biosynthesis remains poorly understood, previous studies revealed its initiation by the decarboxylation of L-lysine to generate cadaverine, with the subsequent formation of 5-aminopentanal. Conversion of L-lysine to cadaverine and cadaverine to 5-aminopentanal is catalyzed by lysine decarboxylase (LDC) and copper amine oxidase (CAO), respectively [TJ 0 0 1 scn /GS0 gs 0.050 Tw 5.85 0 0 5.85387–



**Figure 2.** SDS-PAGE analysis of recombinant CgLDC and CgCAO purified by Ni-affinity chromatography. (A) SDS-PAGE analysis of recombinant CgLDC. Molecular mass marker (M), supernatant (lane 2), precipitant (lane 3), cell lysate of BL21(DE3)-pET28a-CgLDC (lanes 4 and 5), and purified CgLDC (lanes 6 and 7). (B) SDS-PAGE analysis of recombinant CgCAO. Molecular mass marker (M), purified CgCAO (lane 1).

precursor of HupA (Fig. 4). By contrast, no catalytic activity was detected from the inactive forms of CgLDC or CgCAO. These results suggested possible CgLDC and CgCAO involvement in HupA biosynthesis.

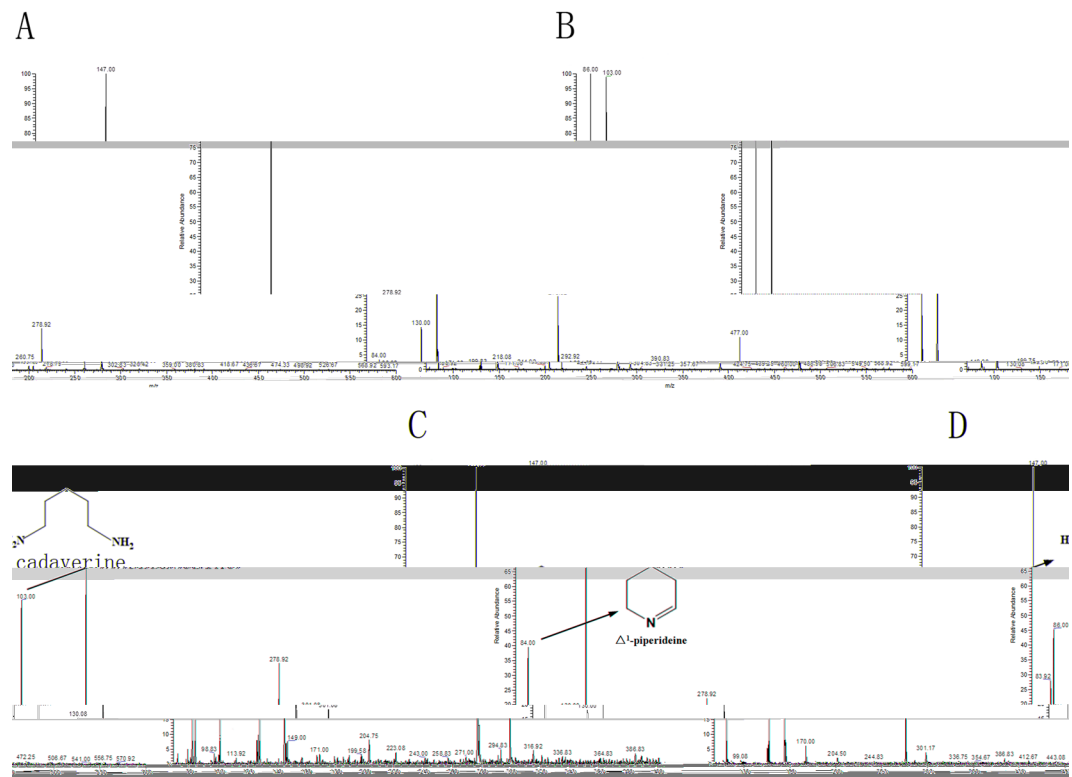
**Transformation of *C. gloeosporioides* ES026 and qRT-PCR analysis.** To validate the relationship between *hupA* and *hupA* expression and HupA production, 10 *hupA* and *hupA* overexpressing plasmids containing different promoters were constructed (Fig. 5). According to methods used for *hupA* transformation, *C. gloeosporioides* ES026 was transformed using the 10 plasmids, and a randomly selected transformant was confirmed by PCR. Our results indicated amplification of appropriately sized DNA fragments (769 bp and 2072 bp, Fig. 6), verifying *C. gloeosporioides* ES026 genetic transformation. Quantification by qRT-PCR of *hupA* and *hupA* expression during fermentation indicated that the *PagdA*-CgLDC and *Palca*-CgCAO transformants exhibited the highest expression levels (Fig. 7).

**Measurement of HupA production.** To investigate transformant effects on HupA production, HupA yield associated with all mutants was analyzed by LC-HRMS. Our results showed that different expression levels of *hupA* and *hupA* produced different HupA yields; however, high levels of *hupA* and *hupA* expression resulted in higher yields of HupA, although transformants exhibiting the highest expression levels did not produce the highest yields of HupA. Two genetically altered strains (*Polic*-CgLDC and *PgpdA*-CgCAO) yielded stable, high-yielding HupA production (Fig. 8). Our findings revealed that CgLDC and CgCAO were involved in HupA biosynthesis, but that the HupA-synthesis pathway was regulated by separate enzymes.

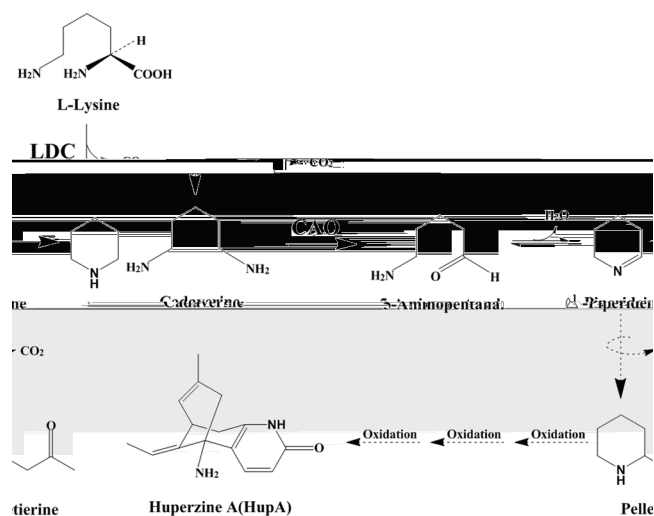
**Physicochemical properties of CgLDC and CgCAO.** *C. gloeosporioides* ES026 produced a 28-kDa CgLDC protein containing 256 amino acids, with a predicted formula of  $C_{266}H_{444}N_{88}O_{111}S_{23}$ . The theoretical pI of CgLDC was 5.06, and the instability index (II) was 48.51, indicating a potentially unstable protein. *C. gloeosporioides* ES026 produced a 76-kDa CgCAO protein containing 672 amino acids, with a predicted formula of  $C_{341}H_{527}N_{92}O_{101}S_{21}$ . The theoretical pI of CgCAO was 5.60, and the instability index (II) was 39.05, indicating a stable protein.

## Discussion

AD affects millions of people worldwide and is among the four principal death-causing diseases, including heart disease, cancer, and stroke. HupA isolated from *C. gloeosporioides* is a natural acetylcholinesterase inhibitor used to treat AD. As mentioned in the introduction, very few biosynthetic studies have been performed with HupA, although no investigations have been reported that have attempted to identify the biosynthetic pathway leading directly to HupA, two enzymes (LDC and CAO) have been proposed as the entry point enzymes into the pathway to the HupA<sup>22, 23</sup>. However, work on that enzymes have only been performed in nonrelated taxa<sup>24</sup>. Nevertheless, the feeding that catalyze key transformations in the biosynthesis of HupA and other Lycopodium alkaloids. In this study, next-generation sequencing and RNA sequencing of *C. gloeosporioides* ES026 was performed, and

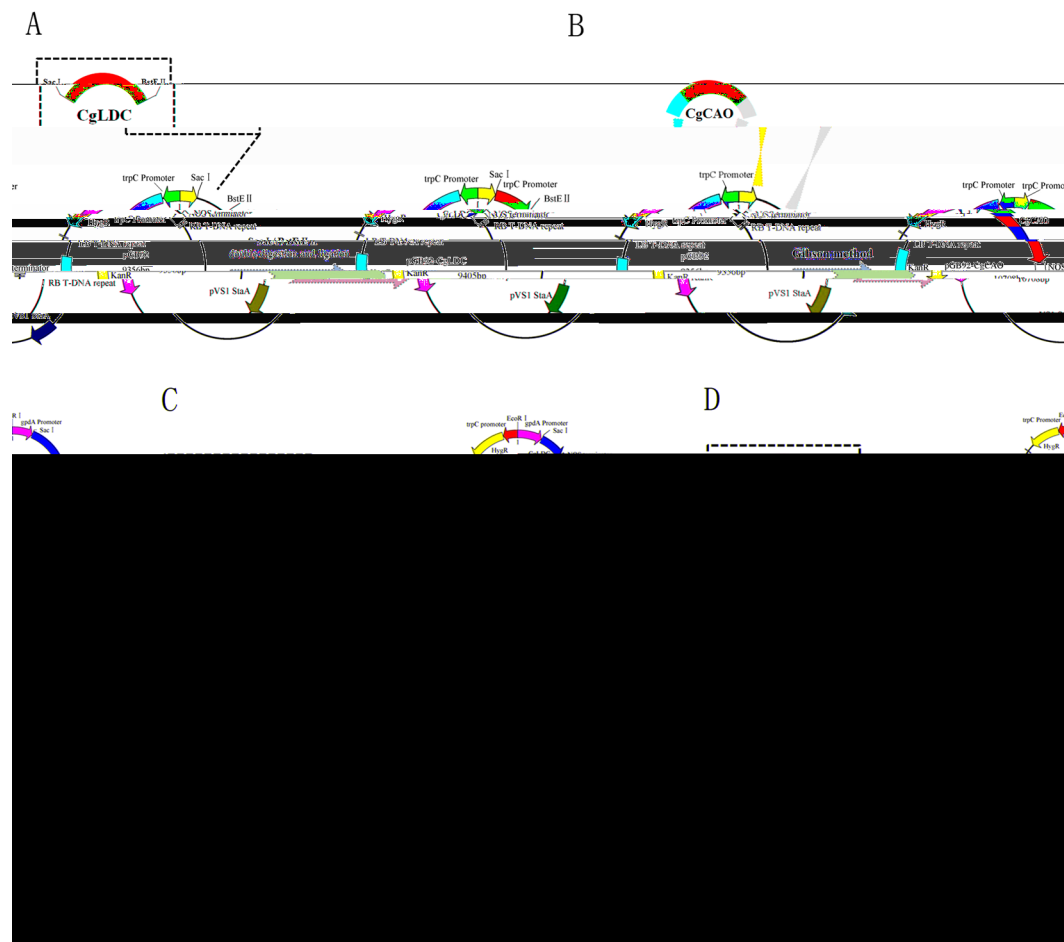


**Fig. 3.** LC-MS analysis of products. (A) LC-MS analysis of L-lysine standard. (B) LC-MS analysis of cadaverine standard. (C) LC-MS analysis of enzymatic formation of cadaverine from L-lysine by CgLDC. Ion chromatograms extracted with  $m/z$  103. (D) LC-MS analysis of enzymatic formation of  $\Delta^1$ -piperidine from cadaverine by CgCAO. Ion chromatograms extracted with  $m/z$  84.

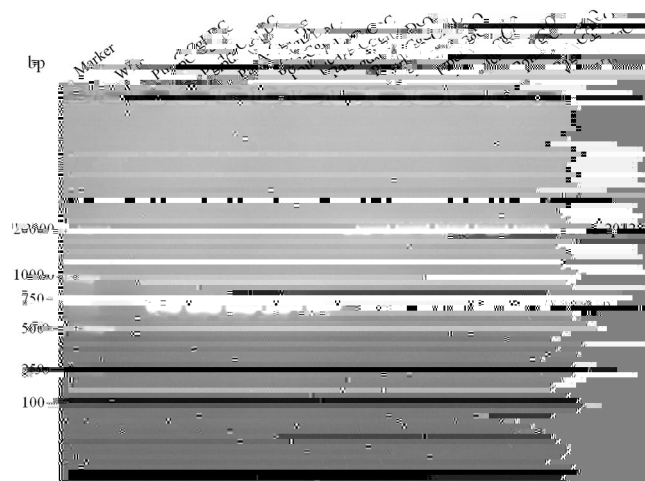


**Fig. 4.** The proposed biosynthetic pathway from L-lysine to  $\Delta^1$ -piperidine. LDC: Lysine decarboxylase; CAO: Copper amine oxidase.

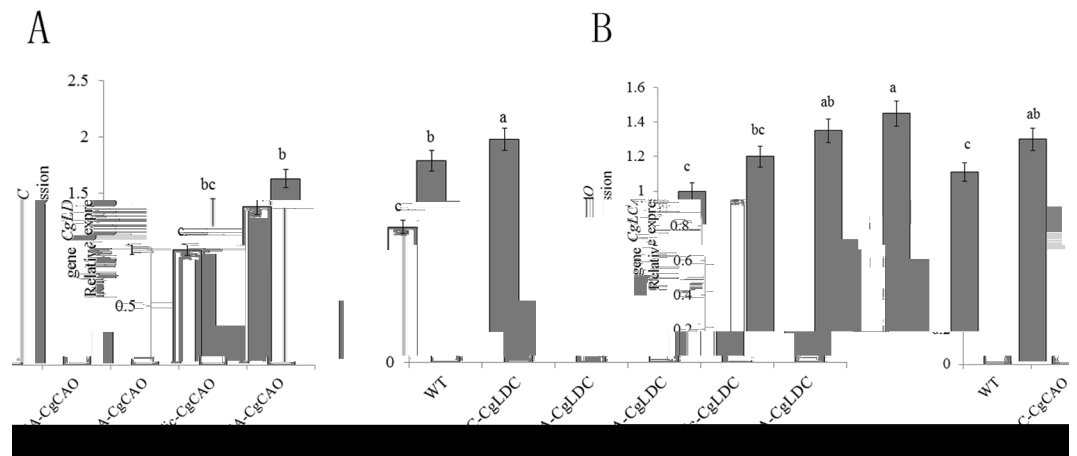
based on transcriptome analyses by Ma *et al.* and Luo *et al.*<sup>22, 25, 26</sup>, the HupA biosynthetic pathway was investigated. HupA biosynthesis involves primary and secondary enzyme conversion, initiating with acetyl-CoA and biotin and ending with the development of L-lysine, followed by secondary metabolism involving the development of cadaverine. LDC converts L-lysine to cadaverine, and CAO converts cadaverine to 5-aminopentanal and piperidine. Sun *et al.*<sup>27</sup> cloned genes from *Lycopodium obscurum*, and Du Zhu *et al.*<sup>28</sup> cloned genes into the endophytic fungus *Aspergillus nidulans* sp. Sif14 from *Lycopodium obscurum*, enabling verification of specific characteristics related to LDC and CAO biosynthesis of lycopodium alkaloids. Pelletierine, which is a precursor, is also converted, resulting in HupA synthesis. HupA biosynthesis involves LDC as the first enzyme and CAO as the second enzyme, with LDC



**Figure 5.** Construction of plasmids overexpressing *gpdA* and *alcA*. (A) *gpdA* was cloned into the pGB92 vector with *trpC* promoter between restriction sites *SacI* and *BstEI* to obtain the recombinant plasmid pGB92-CgLDC. (B) The Gibson method was used to construct the recombinant plasmid pGB92-CgCAO. (C) The *gpdA*, *alcA*, *olic*, and *agdA* promoters were ligated into the recombinant plasmid pGB92-CgLDC to produce pGB93-CgLDC, pGB94-CgLDC, pGB95-CgLDC, and pGB96-CgLDC. (D) The *gpdA*, *alcA*, *olic*, and *agdA* promoters were ligated into the recombinant plasmid pGB92-CgCAO to produce pGB93-CgCAO, pGB94-CgCAO, pGB95-CgCAO, and pGB96-CgCAO.



**Figure 6.** Identification of transformants by PCR.



**Figure 7.** Relative gene expression levels in *ES026* and transformed *ES026* after a 5-day culture. **(A)** Relative expression in *ES026* and in PtrpC-CgLDC, PgpdA-CgLDC, PalcA-CgLDC, Polic-CgLDC, and Pagda-CgLDC transformants using different promoters after 5 days. **(B)** Relative expression of in *ES026* and PtrpC-CgCAO, PgpdA-CgCAO, PalcA-CgCAO, Polic-CgCAO, and Pagda-CgCAO transformants using different promoters after 5 days. \*Duncan's multiple range test;  $< 0.01$ .

transforming L-lysine to cadaverine, and CAO transforming cadaverine to 5-aminopentanal in lycopodium alkaloid biosynthesis. According to Kyoto Encyclopedia of Genes and Genomes analysis, there is only one pathway involved in synthesizing 5-aminopentanal catalyzed by LDC and CAO.

Recombinant plasmids using different promoters to overexpress and in *ES026* were constructed, and their expression was determined by qRT-PCR. Additionally, the differential expression of key enzymes involved in HupA biosynthesis and associated metabolic pathways were analyzed, with results indicating that elevated expression of CgLDC and CgCAO produced increased levels of HupA as compared with wild-type *ES026*.

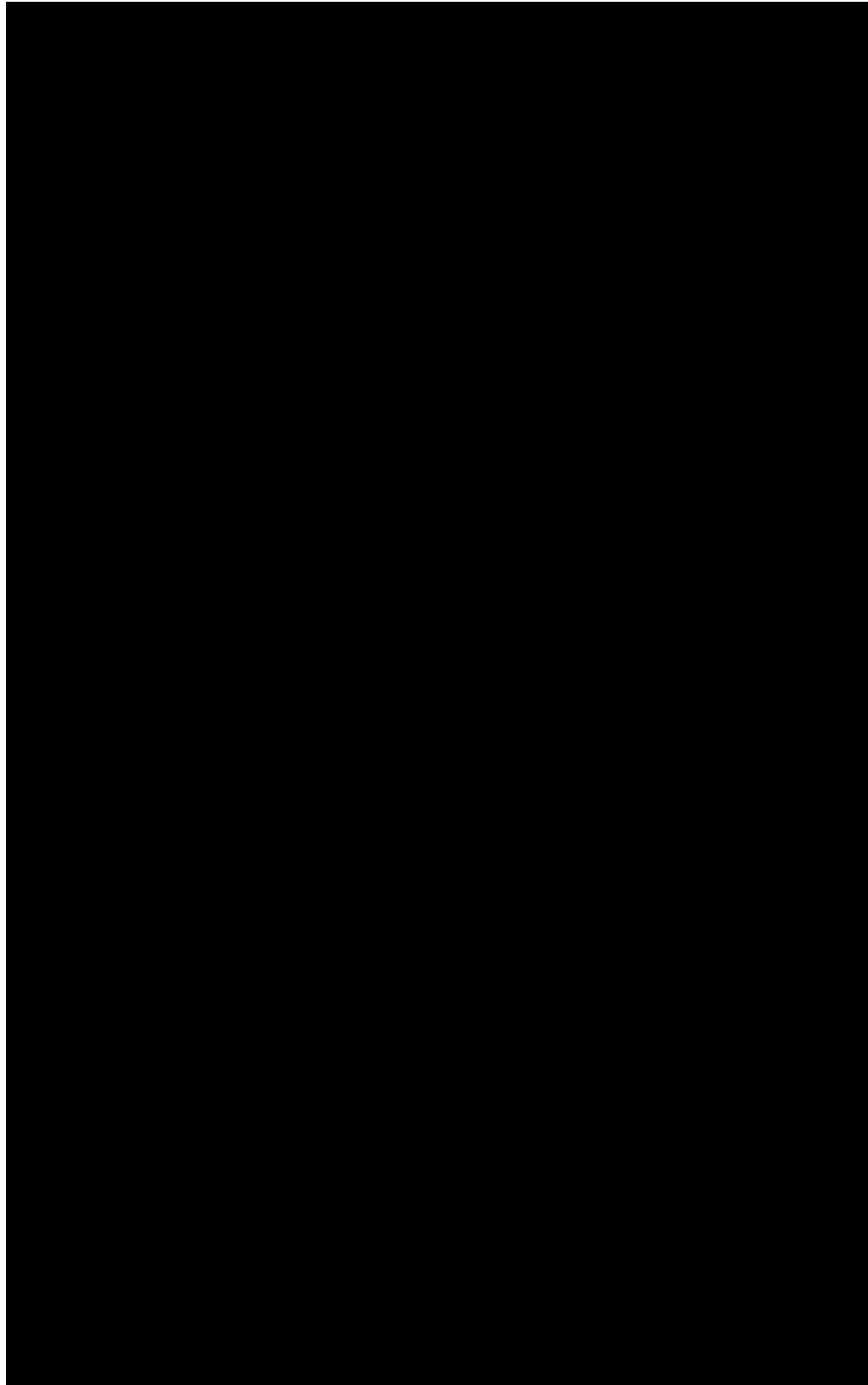
In this study, according to the *ES026* genome analysis, CgLDC and CgCAO from *ES026* are unique genes, were investigated for their conversion of L-lysine to 5-aminopentanal in HupA biosynthesis. CgCAO is different from HsCAO<sup>18</sup>, which can produce 5-aminopentanal. Our results indicated that these enzymes could be efficiently expressed in *ES026*, that the resulting CgLDC was capable of cadaverine production, and the resulting CgCAO was capable of 5-aminopentanal production, both of which are HupA biosynthetic intermediates, but *ES026*, this reaction may be weak, experiments are needed to investigate whether CgLDC and CgCAO have similar properties to other LDCs and CAOs. These findings revealed that genetic modification of *ES026* resulted in a variant capable of stable, high-yield production of HupA. Furthermore, we observed that transformants yielding the highest expression of LDC and CAO did not produce the highest yields of HupA, which might have been due to interference by other enzymes involving in HupA biosynthesis. Further investigation is required to elucidate additional details regarding the pathways involved in HupA biosynthesis.

## Materials and Methods

**Fungal strains and plasmids.** The strain *ES026*, which produced the highest amount of HupA, was isolated from *ES026* and preserved at the China Center for Type Culture Collection (CCTCC No. 2011046; Wuhan, China). *BL21* and *DH10B* cells were cultured in Luria broth (LB) at 37 °C. The plasmid pET28a containing the kanamycin-resistance gene was used as an assisting plasmid for the transformation of *BL21* cells. Plasmids pGB92, pGB93, pGB94, pGB95, and pGB96 containing the hygromycin B resistance were used as assisting plasmids for the transformation of *ES026*.

**CgLDC and CgCAO expression in *E. coli* BL21 (DE3) cells and protein purification.** According to the *ES026* genome sequence and transcriptome analysis<sup>21</sup>, the coding regions of and were amplified by polymerase chain reaction (PCR) from *ES026* genomic DNA. PCR products were purified using a gel-extraction kit (Omega Bio-tek, Norcross, GA, USA) and cloned into the pET28a vector between the I and I restriction sites to create plasmids pET28a-CgLDC and pET28a-CgCAO for production and purification of the target proteins. The plasmids expressed recombinant proteins containing a hexahistidine-tag at the C-terminus. Subsequently, pET28a-LDC and pET28a-CAO were transformed into *BL21* cells via heat shock, and transformants were verified by PCR and restriction-enzyme digestion.

Cells were cultured to an OD<sub>600</sub> of between 0.6 and 0.8 in LB medium containing 100 µg/mL kanamycin at 37 °C and shaking at 200 rpm. Isopropyl β-D-1-thiogalactopyranoside and CuSO<sub>4</sub> were added to the culture medium to a final concentration of 0.1 mM and 50 µM, respectively, to induce the expression of recombinant CgLDC and CgCAO. The induced broth was maintained at 16 °C with shaking at 200 rpm for an additional 16 h. Cells were collected by centrifugation at 4 °C at 5000 g for 5 min, resuspended in buffer A [50 mM Tris-HCl, 300 mM NaCl, and 4 mM 2-mercaptoethanol (pH 7.6)], and lysed by sonication. Lysates were then centrifuged



**Figure 8.** HupA yield from wild-type *ES026* and *ES026* transformants. (A) LC-HRMS analysis results of wild-type *ES026* and PtpC-CgLDC, PgpA-CgLDC, PalcA-CgLDC, Polic-CgLDC, and PagdA-CgLDC transformants. (B) LC-HRMS analysis results of HupA yields from wild-type *ES026* and PtpC-CgCAO, PgpA-CgCAO, PalcA-CgCAO, Polic-CgCAO, and PagdA-CgCAO transformants. (C) HupA yields from wild-type *ES026* and PtpC-CgLDC, PgpA-CgLDC, PalcA-CgLDC, Polic-CgLDC, and PagdA-CgLDC transformants. (D) HupA yields from wild-type *ES026* and PtpC-CgCAO, PgpA-CgCAO, PalcA-CgCAO, Polic-CgCAO, and PagdA-CgCAO transformants.

at 12,000 g for 30 min, and the supernatant was loaded onto a Ni-NTA resin column. Recombinant CgLDC and CgCAO proteins were eluted with buffer B [50 mM Tris-HCl, 300 mM NaCl, 4 mM 2-mercaptoethanol, and 500 mM imidazole (pH 7.6)], and the sizes of the purified proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)<sup>18</sup>.

**Detection of CgLDC and CgCAO activity.** The CgLDC reaction mixture was prepared according to methods reported by Qiao *et al.*<sup>29</sup>. The reaction contained 1.46 mg L-lysine, 1 mg/mL purified recombinant CgLDC, and 40 µg pyridoxal phosphate in 0.1 mM potassium phosphate buffer (pH 8.0). The mixture was incubated at 37°C for 45 min prior to adding 30 µL HCl to stop the reaction. The same reaction containing boiled (inactive) CgLDC was used as the negative control. Reaction products were extracted with chloroform and analyzed by liquid chromatography mass spectrometry (LC-MS). Column: Thermo Hypersil GOLD aQ column, 150 mm × 2.1 mm, operation of the mass spectrometer was in electrospray positive ion mode. The MS source and chamber conditions were optimised to give maximum analyte signal intensity as follows: Spray voltage: +3500 V; Capillary Temperature: 320°C; Sheath Gas: 30.0 psi; Aux Gas: 5.0 psi. Probe Heater Temperature: 300°C; Scan Range: 50–600 m/z; Scan Rate: 1 Hz), gradient conditions with mobile phases of H<sub>2</sub>O and acetonitrile, both containing 1% acetic acid: 0–2



**Measurement of HupA production.** Transfer of transformant solution was performed according to the method of Zhao<sup>33, 34</sup> with minor modifications. Fermented mycelia were collected by centrifugation at 12,000 g or 10 min, followed by drying at 40 °C overnight and grinding into powder. For chemical extraction, each sample of raw material (1 g) was produced using 0.5% HCl [(30 mL (w/v))] overnight, followed by ultrasonication in a water bath at 40 °C for 1 h. The ingredients were then filtered, and the filtrates were rendered with ammonia solution to pH 9.0. After 1 h, the water phase was extracted three times with chloroform, and the combined chloroform extracts were evaporated to dryness. The dry residue was mixed with 1 mL methanol, passed through a 0.45- $\mu$ m polytetrafluoroethylene syringe filter, and analyzed by LC-HRMS (Agilent Zorbax SB-C18; 150 mm  $\times$  4.6 mm, 5- $\mu$ m diameter, operation of the mass spectrometer (MS) was in electrospray positive ion mode. The MS source and chamber conditions were optimised to give maximum analyte signal intensity as follows: Spray voltage: +3500 V; Capillary Temperature: 320 °C; Sheath Gas: 30.0 psi; Aux Gas: 5.0 psi. Probe Heater Temperature: 300 °C; Scan Range: 50–600 m/z; Scan Rate: 1 Hz). The mobile phases consisted of H<sub>2</sub>O and 5% acetonitrile or 100% acetonitrile (65%: 35%) at a flow rate of 0.6 mL/min. Quantification was performed using the standard curve generated from the HupA standard over a concentration range of between 0.5 and 8.0 mg/L, where the peak area and height showed linear correlations with the absorbance ( $R^2 = 0.9991$ ).

**Bioinformatics analysis of CgLDC and CgCAO.** Physicochemical properties were predicted using the ExpASY-ProtParam tool (<http://web.expasy.org/protparam/>), and hydrophobic/hydrophilic analysis was performed by ExpASY-ProtScale (<http://web.expasy.org/protscale/>). Protein signal peptides were predicted using the SignalP 4.1 server (<http://www.cbs.dtu.dk/services/SignalP/>), and transmembrane regions were predicted using the TMHMM server version 2.0 (<http://www.cbs.dtu.dk/services/TMHMM/>). Protein subcellular localization was predicted by ProtComp version 9.0 (<http://linux1.sobery.com/berry.com/berry.html?topic=protcompan&group=programs&subgroup=proloc>).

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## Acknowledgements

This work was supported by the Ministry of Science and Technology of the People's Republic of China [the Project of International scientific and technological cooperation between China and South Korea (Grant No. 2011DFA31290)] and thanks are due to professor Tiangang Liu (Wuhan University) for assistance with the experiments and valuable discussion.

## Author Contributions

Mo Wang and Xiangmei Zhang conceived and designed the experiments. Xiangmei Zhang and Zhangqian Wang performed the experiments. Xiangmei Zhang and Qian Yang analyzed the data. Xiangmei Zhang and Saad Jan wrote the manuscript. All authors have read and approved the manuscript for publication.

## Additional Information

**Competing Interests:** The authors declare that they have no competing interests.

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