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Colletotrichum gloeosporioides ES026

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Huperzia serrata *Colletotrichum gloeosporioides* **ES026 previously isolated from** *H serrata* ***de novo*** *C gloeosporioides* **ES026 to L-lysine to 5-aminopentanal during HupA biosynthesis. Additionally, we constructed a stable, high-** *CgLDC* *CgCAO* *C gloeosporioides* **ES026 in** *Escherichia coli* **transcription polymerase chain reaction analysis confirmed** *CgLDC* *CgCAO* *C gloeosporioides* **ES026. These results revealed CgLDC and CgCAO** *C gloeosporioides* **ES026.**

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

Currently, HupA is a compound used in herbal supplements mainly extracted from the Chinese club moss ; however, it has a limited distribution and slow growth rate[9]. Furthermore, the complex extraction process from plants and the high costs of downstream puri cation have impeded HupA utility[10,11]. Consequently, for successful commercial production of HupA, large volumes of are required. erefore, 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. e 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 [illegible] are capable of producing HupA[12–14] with [illegible] [illegible] ES026 yielding 45 μg/g dried mycelium according to our previous study[15]. 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[15–17].

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

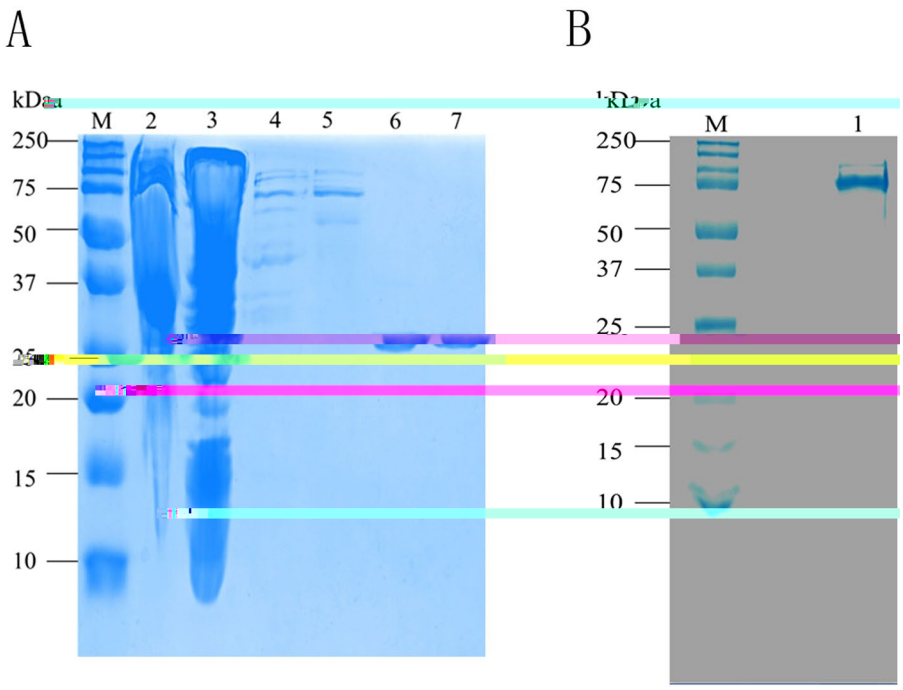

Fig e 2. SDS-PAGE analysis of recombinant CgLDC and CgCAO puri ed by Ni- a nity chromatography. (**A**) SDS-PAGE analysis of recombinant CgLDC. Molecular mass marker (M), supernatant (lane 2), precipitant (lane 3), cell lysate of BL21(DE3)-pET28a-CgLDC (lanes 4 and 5), and puri ed CgLDC (lanes 6 and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

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

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

To investigate transformant e ects on HupA production, HupA yield associated with all mutants was analyzed by LC-HRMS. Our results showed that di erent expression levels of and produced di erent HupA yields; however, high levels of and 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 ndings revealed that CgLDC and CgCAO were involved in HupA biosynthesis, but that the HupA-synthesis pathway was regulated by separate enzymes.

ES026 produced a 28-kDa CgLDC protein containing 256 amino acids, with a predicted formula of $C_{2662}H_{4441}N_{885}O_{1112}S_{230}$. e theoretical pI of CgLDC was 5.06, and the instability index (II) was 48.51, indicating a potentially unstable protein. ES026 produced a 76-kDa CgCAO protein containing 672 amino acids, with a predicted formula of $C_{3416}H_{5274}N_{920}O_{1019}S_{21}$. e theoretical pI of CgCAO was 5.60, and the instability index (II) was 39.05, indicating a stable protein.

AD a ects millions of people worldwide and is among the four principal death-causing diseases, including heart disease, cancer, and stroke. HupA isolated from 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[22,23]. However, work on that enzymes have only been performed in nonrelated taxa[24]. 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 ES026 was performed, and

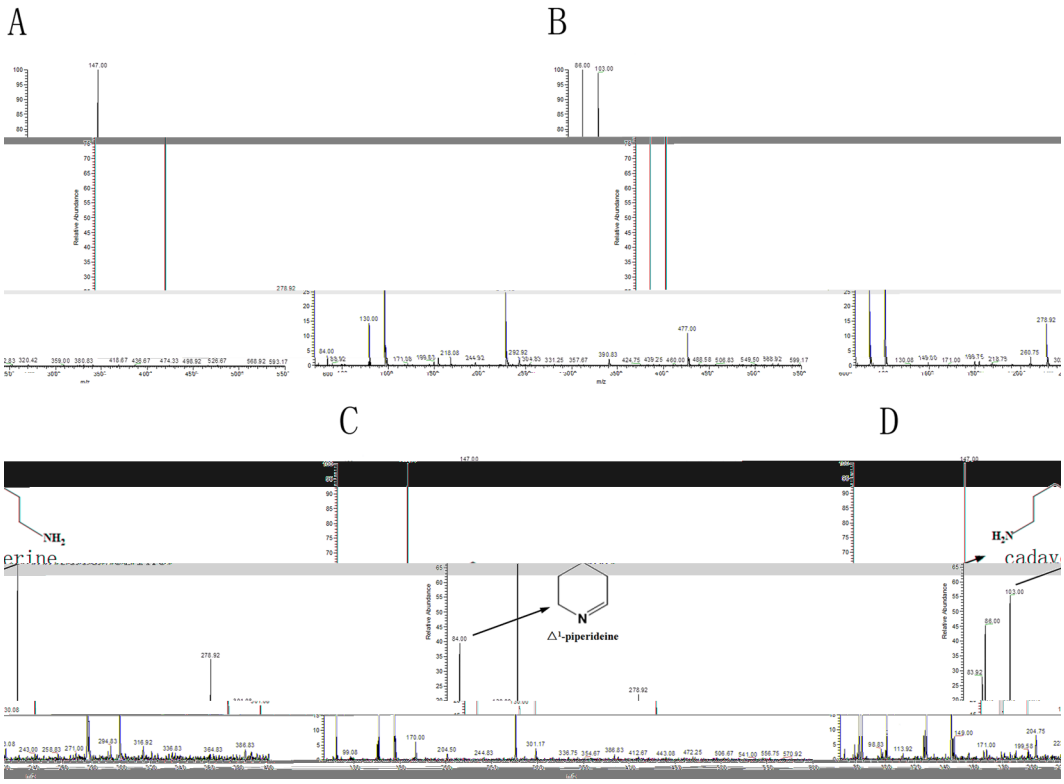

Fig e 3. LC-MS analysis of products. (**A**) LC-MS analysis of L-lysine standard. (**B**) LC-MS analysis of cadaverine standard. (**C**) LC-MS analysis of enzymatic formation of cadaverine from L-lysine by CgLDC. Ion chromatograms extracted with 103. (**D**) LC-MS analysis of enzymatic formation of Δ^1-piperideine from cadaverine by CgCAO. Ion chromatograms extracted with 84.

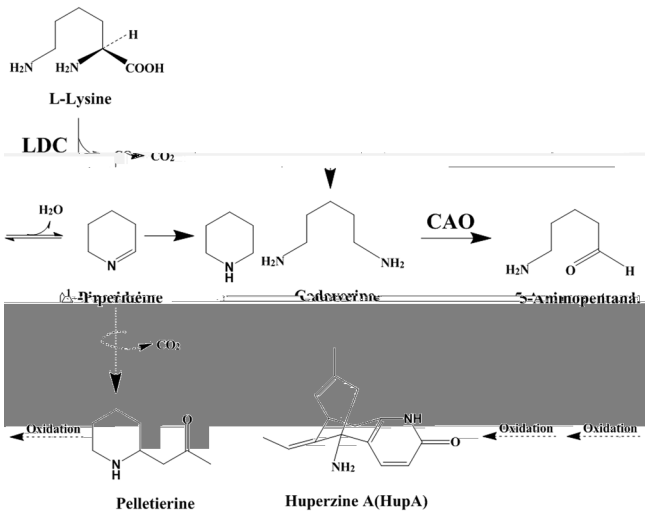

Fig e 4. e proposed biosynthetic pathway from L-lysine to Δ^1-piperideine. LDC: Lysine decarboxylase; CAO: Copper amine oxidase.

based on transcriptome analyses by Ma . and Luo .[22,25,26], 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 piperideine. Sun .[27] cloned genes from , and Du Zhu .[28] cloned genes into the endophytic fungus sp. Slf14 from , enabling veri cation of speci c 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 rst enzyme and CAO as the second enzyme, with LDC

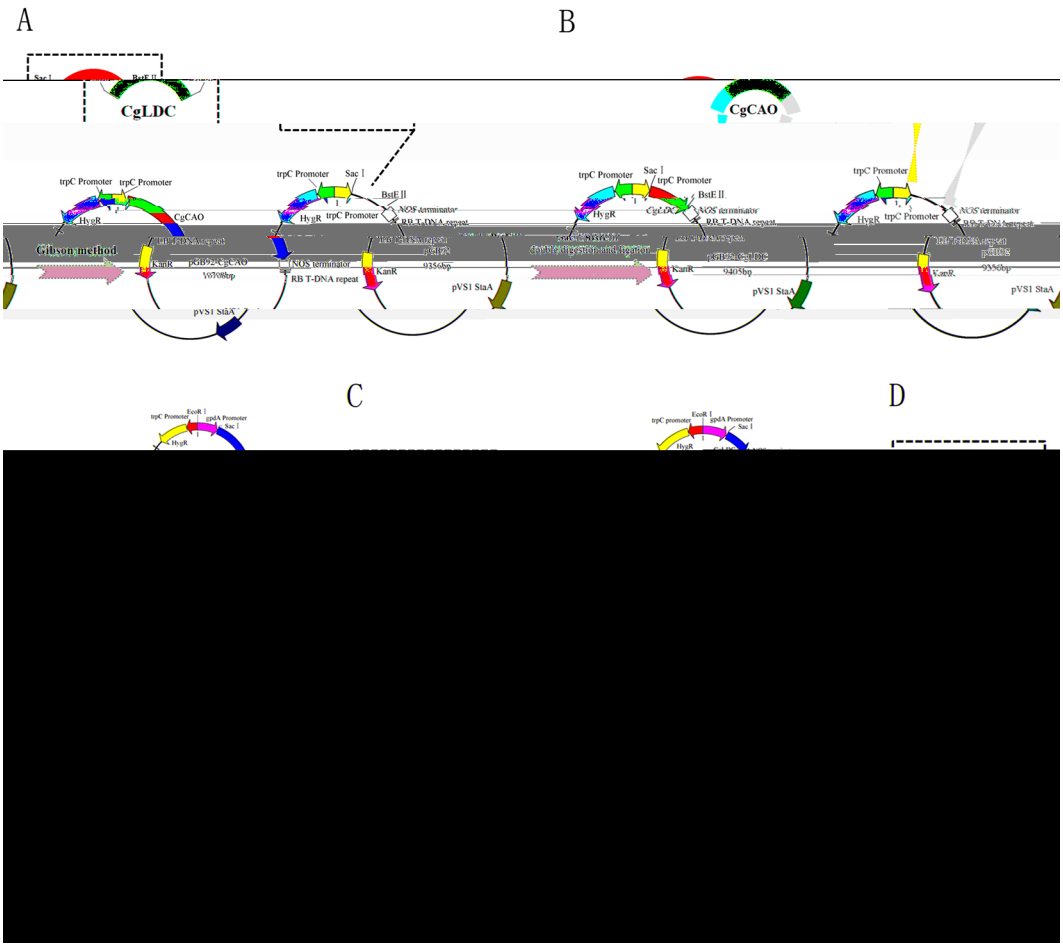

Fig e 5. Construction of plasmids overexpressing . and . . . (**A**) . was cloned into the pGB92 vector with trpC promoter between restriction sites SacI and BstEI to obtain the recombinant plasmid pGB92-CgLDC. (**B**) e Gibson method was used to construct the recombinant plasmid pGB92-CgCAO. (**C**) e 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**) e 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.

Fig e 6. Identi cation of transformants by PCR.

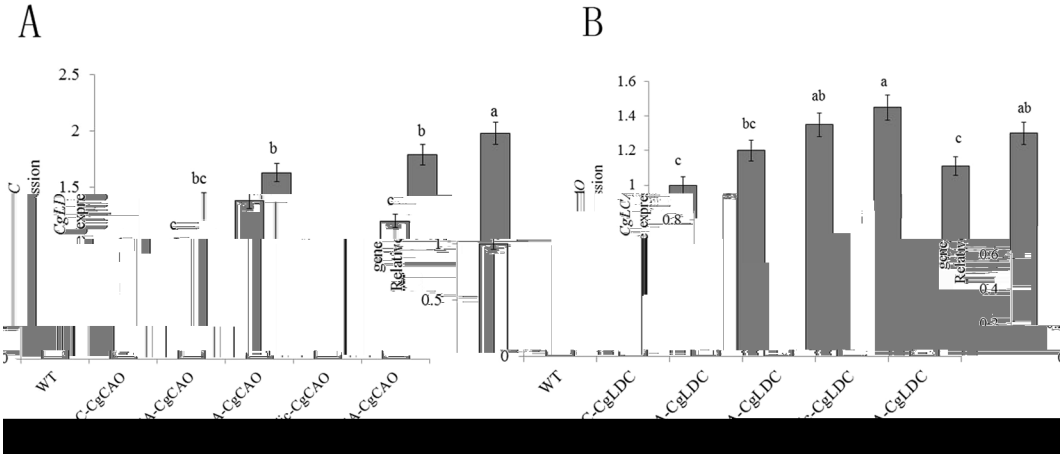

Fig e 7. Relative gene expression levels in . . . . . ES026 and transformed . . . . . ES026 a er a 5-day culture. (**A**) Relative expression . . in . . . . . ES026 and in PtrpC-CgLDC, PgpdA-CgLDC, PalcA-CgLDC, Polic-CgLDC, and PagdA-CgLDC transformants using di erent promotors a er 5 days. (**B**) Relative expression of . . . in . . . . . ES026 and PtrpC-CgCAO, PgpdA-CgCAO, PalcA-CgCAO, Polic-CgCAO, and PagdA-CgCAO transformants using di erent promotor a er 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 di erent promoters to overexpress . . and . . in . . . . . ES026 were constructed, and their expression was determined by qRT-PCR. Additionally, the di erential 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 di erent from HsCAO[18], which can produce 5-aminopentanal. Our results indicated that these enzymes could be e ciently expressed in . . . . . , 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 . . . . , this reaction may be weak, experiments are needed to investigate whether CgLDC and CgCAO have similar properties to other LDCs and CAOs. ese ndings revealed that genetic modi cation 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.

e strain . . . . . ES026, which produced the highest amount of HupA, was isolated from . . . . 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. e 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 CgCAO E coli* BL21 (DE3) cells and protein purification.** According to the . . . . . ES026 genome sequence and transcriptome analysis[21], the coding regions of . . and . . . were ampli ed by polymerase chain reaction (PCR) from . . . . . ES026 genomic DNA. PCR products were puri ed using a gel-extraction kit (Omega Bio-tek, Norcross, GA, USA) and cloned into the pET-28a vector between the . I and . I restriction sites to create plasmids pET28a-CgLDC and pET28a-CgCAO for production and puri cation of the target proteins. e 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 veri ed by PCR and restriction-enzyme digestion.

Cells were cultured to an OD_{600} 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 $CuSO_4$ were added to the culture medium to a nal concentration of 0.1 mM and 50 μM, respectively, to induce the expression of recombinant CgLDC and CgCAO. e 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 bu er A [50 mM Tris-HCl, 300 mM NaCl, and 4 mM 2-mercaptoethanol (pH 7.6)], and lysed by sonication. Lysates were then centrifuged

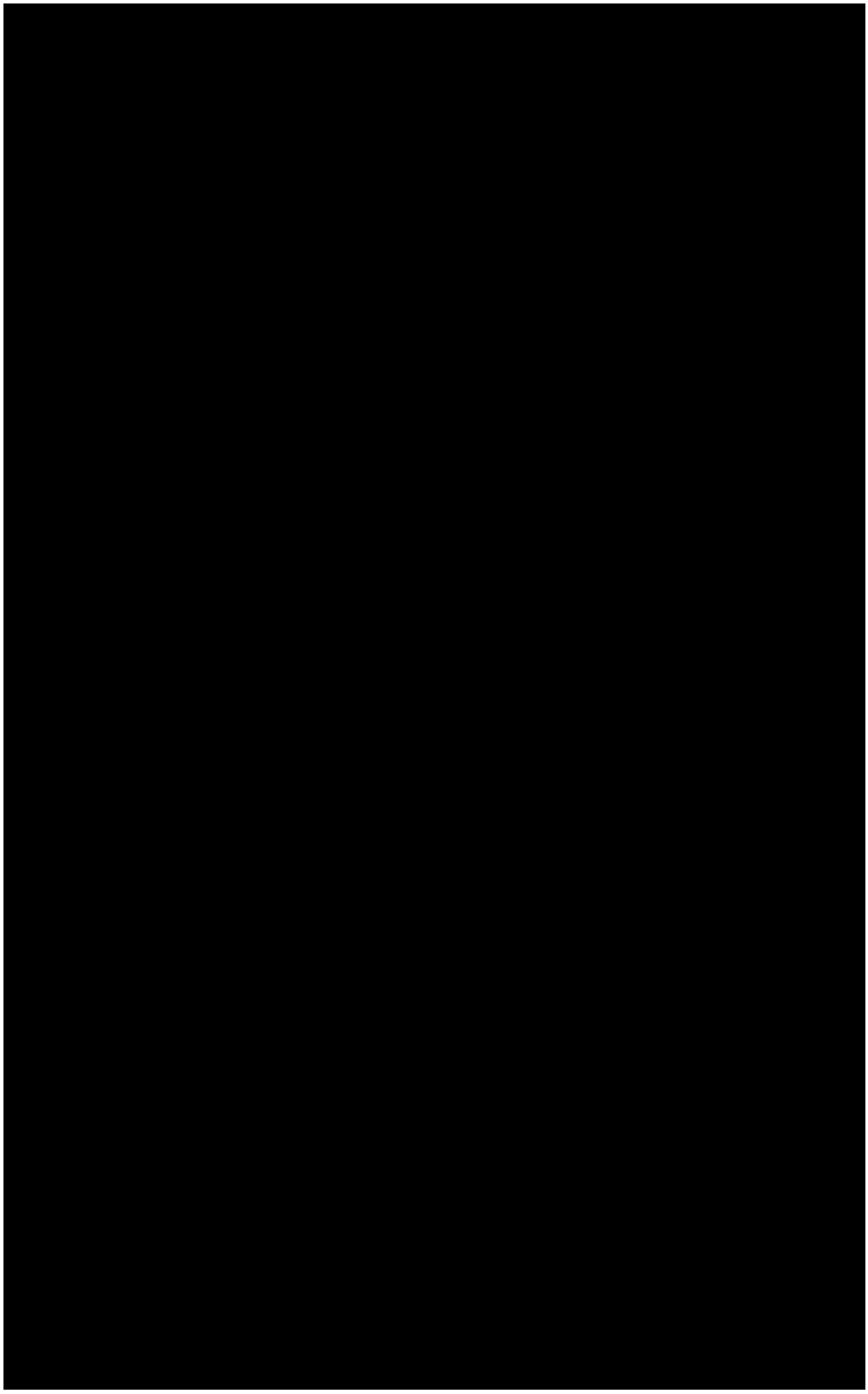

Figure 8. HupA yield from [illegible] ES026 and [illegible] ES026 transformants. (**A**) LC-HRMS analysis results of wild-type [illegible] ES026 and PtrpC-CgLDC, PgpdA-CgLDC, PalcA-CgLDC, Polic-CgLDC, and PagdA-CgLDC transformants. (**B**) LC-HRMS analysis results of HupA yields from wild-type [illegible] ES026 and PtrpC-CgCAO, PgpdA-CgCAO, PalcA-CgCAO, Polic-CgCAO, and PagdA-CgCAO transformants. (**C**) HupA yields from wild-type [illegible] ES026 and PtrpC-CgLDC, PgpdA-CgLDC, PalcA-CgLDC, Polic-CgLDC, and PagdA-CgLDC transformants. (**D**) HupA yields from wild-type [illegible] ES026 and PtrpC-CgCAO, PgpdA-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 bu er B [50 mM Tris–HCl, 300 mM NaCl, 4 mM 2-mercaptoethanol, and 500 mM imidazole (pH 7.6)], and the sizes of the puri ed proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)[18].

e CgLDC reaction mixture was prepared according to methods reported by Qiao . .[29]. e reaction contained 1.46 mg L-lysine, 1 mg/mL puri ed recombinant CgLDC, and 40 µg pyridoxal phosphate in 0.1 mM potassium phosphate bu er (pH 8.0). e mixture was incubated at 37 °C for 45 min prior to adding 30 µL HCl to stop the reaction. e 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: ermo Hypersil GOLD aQ column, 150 mm × 2.1 mm, operation of the mass spectrometer was in electrospray positive ion mode. e 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_2O and acetonitrile, both containing 1% acetic acid: 0–2

Transfer of transformant solution was performed according to the method of Zhao [33, 34] with minor modifications. Fermented mycelia were collected by centrifugation at 12,000 g or 10 min, followed by drying at 40 °C overnight and grounding 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-μm polytetrafluoroethylene syringe filter, and analyzed by LC-HRMS (Agilent Zorbax SB-C18; 150 mm × 4.6 mm, 5-μm 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_2O 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 ($R^2 = 0.9991$).

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.softberry.com/berry.phtml?topic=protcompan&group=programs&subgroup=proloc).

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C . e i g I e e : e authors declare that they have no competing interests.

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