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H_2S is synthesized *via* CBS/CSE pathway and triggered by cold conditions during *Agaricus bisporus* storage

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ABSTRACT

Background: H_2S is proved to be functioning as a signaling molecule in an array of physiological processes in the plant and animal kingdom. However, the H_2S synthesis pathway and the responses to cold conditions remain unclear in postharvest mushroom.

Results: The biosynthesis of H_2S in the *Agaricus bisporus* mushroom tissues exhibited an increasing tendency during postharvest storage and was significantly triggered by cold treatment. The cystathionine γ-lyase (*AbCSE*) and cystathionine β-synthase (*AbCBS*) genes were cloned and proved responsible for H_2S biosynthesis. Furthermore, transcriptional and posttranscriptional regulation of *AbCSE* and *AbCBS* were crucial for the enzyme activities and subsequent H_2S levels. However, the *AbMST* was not involved in this process. Moreover, the *AbCSE* and *AbCBS* genes displayed low identity to the characterized genes, but typical catalytic domains, activity sites, subunit interface sites, and cofactor binding sites were conserved in the respective protein sequences, as revealed by molecular modeling and docking study. The potential transcription factors responsible for the H_2S biosynthesis in cold conditions were also provided.

Conclusions: The H_2S biosynthetic pathway in postharvest mushroom was unique and distinct to that of other horticultural products.

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1. Introduction

Traditionally, exposure under H_2S is considered highly toxic, especially at high levels. Nevertheless, mounting results *in vivo* imply that H_2S is prevalent in organisms, cells, and different intracellular organelles [1]. Notably, it is proved that H_2S plays an essential role in physiological processes as the gasotransmitter, like nitric oxide (NO) and carbon monoxide (CO) [2]. The endogenous H_2S, generated from L- and D-cysteine, involves in signal transduction processes in the mammalian diverse organs and exerts powerful effects on regulating apoptosis, cell cycle, inflammation, and oxidative stress [3]. Moreover, in green plants, H_2S acts not only as an antioxidant in response to environmental stress, but also as a signal molecular for plant growth and development by altering the phytohormone signaling equilibrium [4].

In mammalian cells, H_2S is generated *via* the enzymatic actions of cystathionine β-synthase (CBS, EC 4.2.1.22), cystathionine γ-lyase (CSE, EC 4.4.1.1), and 3-mercaptopyruvate sulfurtransferase (MST, EC 2.8.1.2) [3]. These enzymes are involved in the transsulfuration and reverse transsulfuration pathways. The L-Cysteine, homocysteine, and their derivatives are the common substrates of these H_2S-generating enzymes [3]. There are six biosynthetic pathways to generate H_2S in green plants, including D-cysteine desulfhydrase (DCD), L-cysteine desulfhydrase (LCD), cyanoalanine synthase (CAS), sulfite reductase (SiR), and cysteine synthase (CS)

Abbreviations: CSE, cystathionine γ-lyase; CBS, cystathionine β-synthase; MST, 3-mercaptopyruvate sulfurtransferase; NO, nitric oxide; CO, carbon monoxide; ROS, reactive oxygen species; DCD, D-cysteine desulfhydrase; LCD, L-cysteine desulfhydrase; CAS, cyanoalanine synthase; SiR, sulfite reductase; CS, cysteine synthase; PLP, pyridoxal 5′-phosphate.

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[5], while LCD is considered as the main H_2S biosynthesis enzyme during fruit and vegetable postharvest storage [6]. Meanwhile, H_2S produced by yeast during wine fermentation is well understood, which mainly originates from the sulfate assimilation pathway and glutathione reductase pathway. However, organic sulfur source, such as cysteine, is presumed to involve in the action of CBS, CSE in fungi kingdom, although little research in this regard is currently available [7]. Particularly, the H_2S biosynthetic pathway in postharvest mushroom, a unique form of filamentous fungi, remains unclear.

Like other horticulture products, the postharvest mushroom is subject to high respiration rate, browning, microbial spoilage, and quality deterioration, while this process is hindered under cold conditions [8]. Nevertheless, the H_2S proves effective as signal molecular for postharvest fruits and vegetables to maintain energy charge, ameliorate chilling injury, regulate color metabolism, delay the outburst of reactive oxygen species (ROS), reduce respiration rate, ethylene production, and decay [6]. Thereafter, the application of H_2S in controlling horticultural products postharvest senescence is promising. However, much remains to be learned about whether cold treatment can affect the endogenesis of H_2S biosynthesis.

In the present study, the H_2S production during white button mushroom *Agaricus bisporus* postharvest storage was monitored. The key enzymes involved in H_2S production were screened *via* homologous comparison and PCR cloning. The catalyzing and regulatory mechanisms of the enzymes were revealed *in silico*.

2. Materials and methods

2.1. Materials and treatments

The button mushroom *A. bisporus* was harvested from a local farm in Chengyang (Qingdao, China) and screened for even levels of maturity, uniform size (average diameter at 3.5 cm), and the absence of mechanical damage. The mushrooms were randomly divided into two groups and stored in darkness under 95% humidity. One group was stored at 20°C in an artificial climate chamber (Shanghai Yiheng Technical CO., LTD), while the other group was kept at 4°C in a refrigeration house. Each treatment was replicated three times, and each replicate contained 70 sporophores. Ten sporophores from each replicate were sampled at 2 d intervals.

2.2. Cloning and transcription analysis of the AbCBS, AbCSE, and AbMST

The total RNA was extracted from *A. bisporus* tissue using TRNzol Reagents according to the manufacturer's instruction (Transgen, China), and treated using DNase I, subsequently. The first-strand cDNA was synthesized following the protocol of PrimeScript 1st strand cDNA Synthesis Kit (TaKaRa, Japan). The PCR products were amplified using pfu polymerase using the respective gene-specific primers as displayed in Table S1 and ligated to pEASY-blunt vector (Transgen, China) for sequencing. Moreover, qRT-PCR was performed on Real-Time PCR System (ABI Applied Biosystems, USA), and the relative target gene expression was determined using the $2^{-\Delta\Delta Ct}$ method, with *AbEFα* as the reference gene [9].

2.3. In silico analysis of the proteins and respective genes

The multiple sequence alignment was conducted using Clustal X, and the phylogenetic tree was constructed with the MEGA 6 program using the neighbor-joining method. The tertiary structure of the protein was predicted using I-TASSER (https://zhanglab.ccmb.med.umich.edu/I-TASSER/), and the result was evaluated based on a Ramachandran plot and Profile 3D results. The theoretical isoelectric point (pI), mass values, protein subcellular localization, putative signal peptides, and the transmembrane domain were also predicted [10]. The transcription factor binding sites (TFBS) were analyzed using the online sever JASPAR (http://jaspar.genereg.net/).

2.4. Determination of endogenous H_2S content

The quantification of the *A. bisporus* endogenous H_2S was conducted according to Christou et al., method with minor modifications [11]. About 1.0 g of mushroom sample was ground in liquid nitrogen and resuspended in 1 mL 100 mM potassium phosphate buffer (pH 7.0, 10 mM EDTA). After centrifugation at 6000*g* at 4°C for 15 min, 200 μL of supernatant was added to 4 mL of 100 mM potassium phosphate buffer (pH 7.0), containing 10 mM EDTA, 0.2 mM 5,5′-dithiobis (2-nitrobenzoic acid). After incubation at 37°C for 2 min, the absorbance was determined at 412 nm. The H_2S quantity was deduced from a standard curve obtained with known NaHS concentrations.

2.5. Assay of AbCBS, AbCSE, and AbMST enzyme activities

The CBS and CSE activity was determined according to the method of Asimakopoulou et al., with minor modifications [12]. About 1.0 g of frozen mushroom cap tissues was homogenized with 5 mL of 50 mM sodium phosphate buffer (pH 8.2) on ice and centrifuged at 12,000*g* for 10 min. For CSE activity assay, 460 mL of extracted enzyme solution was added with 20 μL 10 mM L-cysteine and 20 μL 2 mM pyridoxal 5′-phosphate (PLP). After 60 min of incubation at 37°C, the reaction was terminated by adding 1% ZnAc and 10% trichloroacetic acid. Afterward, 133 μL of N,N-dimethyl-p-phenylenediamine-sulfate (30 mM in 7.2 M HCl) and 133 μL of $FeCl_3$ (30 mM in 1.2 M HCl) solutions were added immediately, and the absorbance of the resulting solution was measured at 670 nm after incubation in room temperature for 30 min. The H_2S content was calculated against a calibration curve of standard H_2S solutions. For the CSE enzyme activity, the procedure was the same with 1 mM L-cysteine replaced by homocysteine. The MST activity was determined according to the method described by Wróbel et al [13].

2.6. Data analysis

All experiments were conducted in triplicate for each treatment in a completely randomized design, and significant differences were determined with t-tests. All the figures were drawn using Origin 9.0 software (OriginLab, Massachusetts, USA), and the data are expressed as the mean ± standard deviation. Differences were considered significant if $P < 0.05$.

3. Results and discussion

3.1. Production of H_2S in different A. bisporus tissue under different conditions

As demonstrated in Fig. 1A, high levels of H_2S were detectable in the pileus, gill, and stipe of *A. bisporus* without significant difference ($P > 0.05$). The *A. bisporus* pileus, the main edible tissue, could be sampled as an indicator to evaluate the H_2S level during postharvest storage. The endogenous H_2S level rose continuously during room temperature storage (20°C), as indicated in Fig. 1B. After a 6 day storage period at this temperature, the *A. bisporus* lost edibility and commodity value. However, the cold storage dramat-

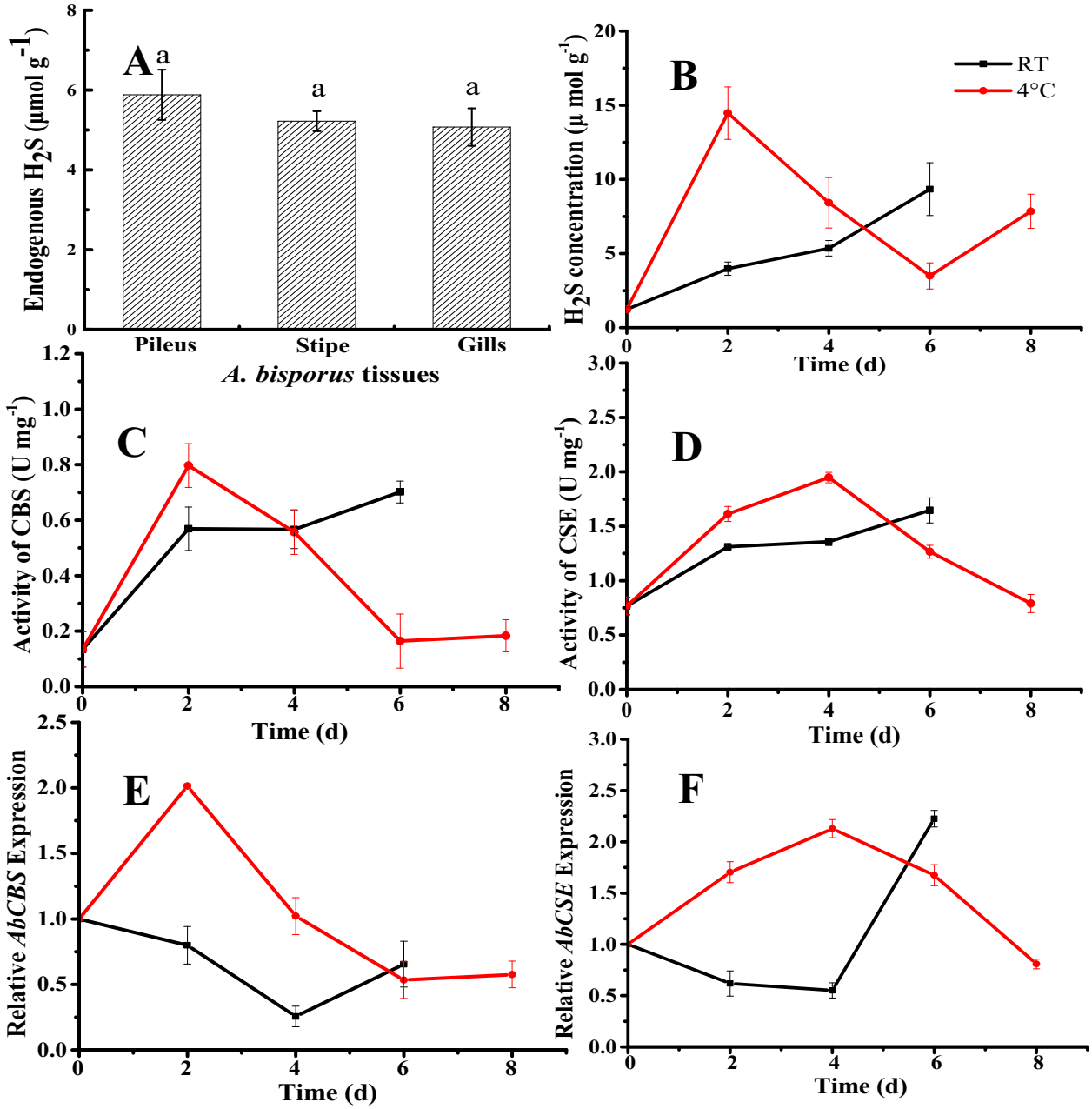

Fig. 1. Production of H_2S, the enzyme activities and gene expression under different conditions. (A) Distribution of H_2S in different *A. bisporus* tissues; (B) production of H_2S under different storage temperatures; (C) the AbCBS activities; (D) the AbCSE activities; (E) *AbCBS* expressions change, (F) *AbCSE* expression change. Data were represented by the means ± standard deviations, n = 3.

ically stimulates the endogenous H_2S generation and reached a peak after 2 d, with a downturn thereafter. The cold treatment is also reported to induce the endogenous H_2S accumulation in bermudagrass (*Cynodon dactylon* (L). Pers.), which functions as an activation of the antioxidant response [14]. The above results indicated the potential roles of H_2S burst in regulating the postharvest senescence of mushroom under cold conditions.

3.2. Cloning of the H_2S synthesis genes in A. bisporus

The H_2S synthesis gene sequences, including DCD, LCD, CAS, SiR, CS, MST, CBS, and CSE of plant, animal, and yeast sources were retrieved from UniProt and GenBank. However, homologous sequence alignment results suggested that DCD, LCD, CAS, SiR, and CS genes were absent from the *A. bisporus* genome and transcriptome data. Whereas, three proteins exhibited much higher identity to the CBS, CSE, and MST protein sequence from *Homo sapiens*, *Mus musculus*, and *Saccharomyces cerevisiae* ranging from 61.4-88.5%. The three proteins were all annotated as hypothetical protein in NCBI, and the functions were uncovered.

The putative *AbCBS* and *AbCSE* were cloned using reverstranscribed PCR (Fig. S1). The *AbCBS* mRNA sequence composed of 5 exons, 1137 bp open reading frame (ORF), and encoding 378 amino acids. Whereas the 1467 bp mRNA sequence of *AbCSE* was composed of 3 exons, and the ORF was 1332 bp, encoding a 443 amino acid protein. According to the homologous alignment analysis, the putative *AbMST* is composed of 6 exons and the ORF was 1002 bp, encoding 333 amino acids. However, during the button mushroom postharvest storage, the mRNA sequence of *AbMST* was not successfully cloned, which resulted from the fact that this gene was not expressed (Section 3.5). The MST was reported as the third H_2S-producing enzyme, but exclusively located in the

mammalian brain and kidney, while the functions of MST in plant, bacteria, and fungi kingdom remain uncovered [15]. The above results indicated that the H_2S synthesis pathway in *A. bisporus* was distinct to that of green plants, and CBS/CSE were the main enzymes during button mushroom postharvest storage.

3.3. Response of gene expressions and activities of AbCBS, AbCSE during storage

As demonstrated in Fig. 1, the AbCBS and AbCSE activity and the respective gene expressions were remarkably stimulated in cold storage conditions during the first period of storage. Nevertheless, the activity and the gene expression of AbMST were not detected in all the samples. The AbCBS and AbCSE activity exhibited an "up and down" trend, while the *AbCBS* and *AbCSE* expression level reached the respective peak after 2 and 4 d. The data regarding the transcript levels of the two enzymes supported the hypothesis of the differential contribution of the CBS and CSE enzyme to H_2S production in different mushroom storage conditions. Notably, the AbCBS and AbCSE activity rose continuously and positively correlated with the endogenous H_2S concentration at 20°C. In contrast, the expression level of *AbCBS* and *AbCSE* was downregulated and followed by a significant improvement (Fig. 1). This discrepancy that the expression of the CBS and CSE enzyme may not directly correspond to its protein level and/or enzyme activity was also discovered in murine [16] and human tissues [17]. The potential mechanism of this phenomenon may result from constitutively active H_2S synthetic enzymes and posttranslational modification of enzymes by endogenous cofactor. This assumption was supported by the evidence that the apo-CSE was formed during the catalysis of H_2S production, and the rate of H_2S production was increased with higher exogenous PLP concentrations [18].

3.4. In silico analysis of the AbCBS proteins

In silico analysis of AbCBS and AbCSE were conducted to reveal the catalytic and posttranslational regulation mechanism. As summarized in Table S2, the putative AbCBS protein had a predicted molecular weight of 42.96 kDa and a theoretical pI of 5.77. The instability index was 22.41, which indicated that the protein was stable. The coding for AbCBS protein, lack of signal peptide, and transmembrane domain was predicted to be located in the cytoplasm. Moreover, the coding protein sequence exhibited the highest phylogenetic similarity of 78.27% with the corresponding enzymes in *Leucoagaricus sp.*

The AbCBS protein shared the typical conserved domain of other CBSs, including cysta_beta superfamily (cl36831) and CBS_like (cd01561) (Fig. S3A). Multiple alignment analysis (Fig. 2A) with the CBS from human and yeast indicated that the putative AbCBS had six highly conserved signature regions related to the cofactor PLP binding site (marked using asterisk), including the 47GSVKDRI53 motif, where 50 K is predicted as catalytic residue and created a Schiff base with PLP [19]. Structurally, the AbCBS protein (Fig. 2C) generated high similarity to the yCBS from *S. cerevisiae* (PDB: 6C2H), which is a homodimer consisting of two monomers [20]. The monomer AbCBS contained 12 α-helices and 10 β-strands, while the dimer interface was composed of the hydrophobic side chains of the residues 20LIR22, 37L, 43F, 45A, 88L, 91A, 108EK109, 112 L, 115VL116, 174EA175, 286EG287, 320N, 330R, and 333M. All these residues were located on the surface of the monomer structure and lacked catalytic pockets. According to the docking analysis, the PLP engaged in numerous hydrogen-bonding interactions with the surrounding residues (50K, 81N, 199GTGGT203, 249G, 293S, and 326PD327), many of which were conserved among the PLP-dependent enzymes (Fig. 2C). These traits suggested that the protein encoded for the functional enzyme.

As revealed in Fig. 2A and Fig. S2A, the AbCBS lacked a heme-binding domain in N-terminal naturally, which was consistent with the yCBS, and distinct from the *H. sapiens* CBS (hCBS) [20]. The heme in the hCBS-bound gaseous molecules like CO and NO, as well as cyanide and isonitriles, suggested that the heme played a regulatory role. In addition, the C-terminal domain of hCBS has been proven to exert intrasteric inhibition, which alleviated with the binding of S-adenosylmethionine (SAM) [21]. Proteolytic removal of the C-terminal region also activated hCBS and yCBS. However, distinct from the hCBS, SAM does not bind the C-terminal domain to activate the yCBS. Notably, the truncation of this C-terminal regulatory domain was accompanied by a change in the oligomeric state from mixed oligomers to the dimer [22]. However, in the present study, the C-terminal domain of AbCBS was naturally absent from this regulatory domain as revealed by the conserved domain analysis and multiple sequence alignment, which indicated that the AbCBS functioned as dimer but was regulated differently.

3.5. In silico analysis of the AbCSE proteins

The AbCSE was predicted with a molecular weight of 47.91 kDa and a theoretical pI of 6.02, respectively. Moreover, the AbCSE protein was predicted to be unstable and located in the cytoplasm, without signal peptide and transmembrane domain (Table S2). A phylogenetic tree was constructed with the CSE from other organisms, and the AbCSE shared 84.9%, 50.1%, and 48.9% homology to the CSE from *Leucoagaricus* sp., humans, and yeast (Fig. S2B).

The typical conserved Beta_elim_lyase superfamily (cl18945) domain was also found to be present in the AbCSE protein, with lysine (230K) as the active residue (Fig. S3B and Fig. 2B) [23]. Putative substrate-cofactor binding sites (101SG102, 126Y, 174E, 205D, 208F, 227S, 229TK230, and 239V) were located in several conserved domains and were responsible for PLP binding (Fig. 2B) [24]. Additionally, the conserved 129T and 207T were implicated in the PLP binding of AbCSE, while the hydrophobicity amino acid 381E was crucial in determining the specificity of the enzyme for the catalysis of the α, γ-elimination or α, β-elimination reaction [18]. The AbCSE protein model was generated using the highest similarity structure of human CSE (PDB: 5eig). As demonstrated in Fig. 2D, the predicted conserved hydrophobic residues, including 57T, 71Y, 73R, 101S, 126Y, 131R, 229T, 237D, 239V, and 273D, were located on the surface of the protein, which indicated the AbCSE formed a tetrameric structure like yCSE and hCSE, with the four catalytic sites at the interface between the subunits [24].

3.6. TFBS analysis of AbCBS and AbCSE

The transcription factors involved in H_2S biosynthesis are limited in the fungi kingdom. So as to illustrate the regulatory mechanism of *AbCBS* and *AbCSE* gene under cold conditions, the respective promoter regions were submitted to JASPER sever for potential TFBS analysis. As revealed in Table S3, the transcription factors mainly belonged to the C6 Zn clusters, C2H2-like Zn fingers family. Transcription factors involved in the sulfur or sulfur amino acid metabolism frequently existed in the promoter region of AbCBS and AbCSE, such as FZF1, MET28, MET31, and MET32 [25] with the stress response transcription factors, including CRZ1, ASG1, HAL9, MSN2, and MSN4 [26]. Notably, the CRZ1, MSN2, and MSN4 have been reported to be involved in coordinating the regulation of low-temperature response [27,28]. All these transcription factors might play potential roles in transcriptional regulation of hydrogen sulfide-producing enzymes under

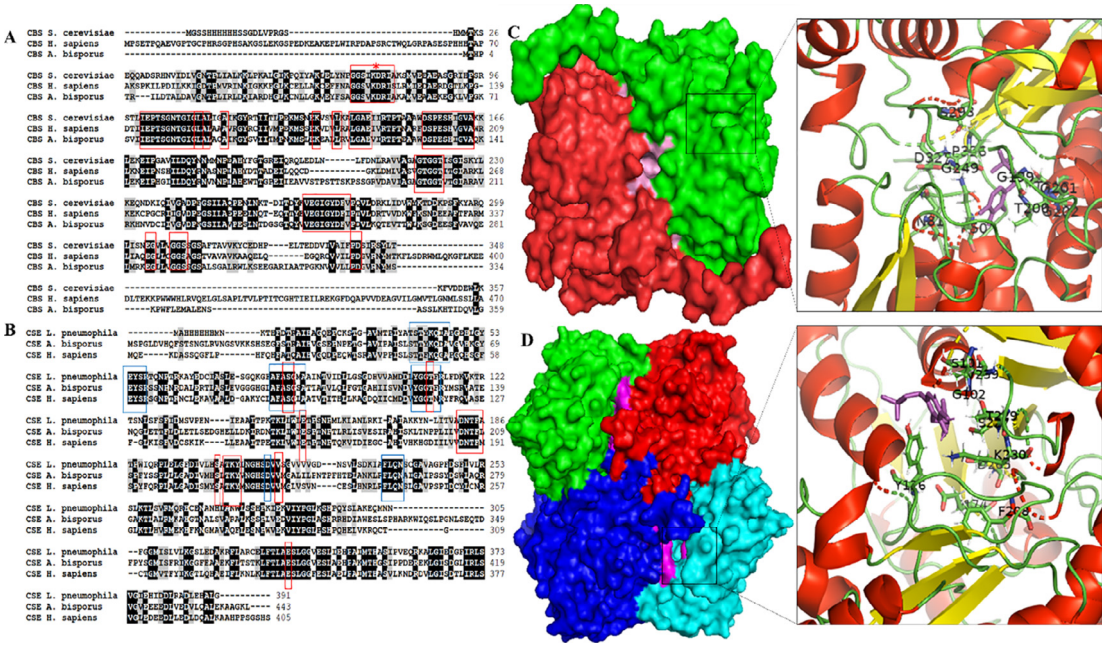

Fig. 2. Multialignment of the protein sequences and predicted tertiary structure. (A) Multialignment of AbCBS (A) and AbCSE (B), identical residues are shaded, and predicted active sites or conserved domains are marked with rectangles; tertiary structure of AbCBS (C) and AbCSE (D), different subunits were denoted by different colors, and interfaces were colored pink. Cofactors and substrate were labeled and denoted in magenta stick.

low-temperature conditions and require further experimental verification.

4. Conclusion

The present study discovered that the endogenesis of H_2S, *AbCSE* and *AbCBS* expression level and enzyme activity are significantly triggered by cold treatment, while no *AbMST* expression nor AbMST activity are detectable. The above results imply that the H_2S can be synthesized through the CBS/CSE pathways in *A. bisporus* in postharvest duration, and the posttranscriptional regulation of AbCSE and AbCBS are crucial for the enzyme activity and subsequent H_2S levels. *In silico* study verified that the AbCBS and AbCSE, respectively, exhibited low identity to the known genes, but typical catalytic domains, activity sites, and cofactor binding sites. The analysis of transcription factors in the *AbCBS* and *AbCSE* promoter regionhas also elucidated the possibility of transcriptional regulation under cold conditions. The present study provides evidence for the possible mechanism of H_2S biosynthesis during the mushroom postharvest storage.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary material

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