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Chitin Synthases in *Cordyceps militaris*: Genome-Wide Gene Identification, Evolutionary Insights, and Life Cycle Transcript Profiling

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Abstract: *Cordyceps militaris* is one of the commercially cultivated mushrooms, valued for its medicinal and nutritional benefits. However, the fruiting body development mechanism has remained elusive. Chitin synthases (CHSs) are ubiquitous enzymes involved in the regulation of fungal growth, development and virulence. In this study, a total of eight *CmChs* genes were identified. Chromosomal localization analysis revealed an uneven distribution of CmCHSs across the *C. militaris* genome. Based on the phylogenetic analysis, 100 CHSs from Cordyceps sensu lato, encompassing *C. militaris*, were categorized into three divisions and seven classes, shedding light on their evolutionary relationships. There was no significant difference in the number of CHSs between ascomycetes and basidiomycetes in general ($p = 0.067$), as well as between pathogenic and saprotrophic fungi in general ($p = 0.151$ and 0.971 in Ascomycota and Basidiomycota fungi, respectively). This underscored the essential and conserved nature of these CHSs across various fungal lifestyles and ecological niches. The different transcript patterns of the eight *CmChs*s during key life cycle stages, such as conidia germination, infection, and fruiting body development, indicated that each CHS gene may have a distinct role during specific stages of the life cycle. In conclusion, these findings indeed lay the groundwork for a further exploration of the functional roles of CHSs in the regulatory mechanism of fruiting body development in *C. militaris*.

Keywords: chitin synthases; *Cordyceps militaris*; transcript profiling; life cycle

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1. Introduction

Cordyceps militaris, as an entomopathogenic fungi, can produce fruiting bodies on both host silkworm pupae and wheat medium, and has even achieved commercialization [1,2]. The fruiting bodies of *C. militaris* are rich in bioactive compounds and have been traditionally used in Eastern medicine for their various health benefits, including their anti-tumor and anti-inflammatory effects [3,4]. The recognition of *C. militaris* as a novel food by the Ministry of Health of the People's Republic of China in 2009 further underscores its potential as a safe and beneficial dietary addition [5].

Fungal cell walls are dynamic structures that are critical for various aspects of fungal life, including the maintenance of cell shape, protection against environmental stresses, and the mediation of interactions with other organisms [6,7]. Chitin is an important component

of the fungal cell wall, providing structural integrity and rigidity [8,9]. Chitin synthases (CHSs) (EC 3.2.1.14) are enzymes that catalyze the biosynthesis of chitin and are part of the glycosyltransferase-2 (GT-2) family (Carbohydrate-Active EnZymes) (http://www.cazy.org, accessed on 5 March 2024) [10–12]. The variation in the number of CHS genes among different fungal species reflects the diversity of life strategies and ecological niches occupied by fungi [10]. For example, *Saccharomyces cerevisiae*, a model organism with a relatively simple life cycle, contains three CHS genes [13]. In contrast, filamentous fungi, known for their complex morphologies and life cycles, tend to have a larger number of CHS genes. For instance, there were five CHSs in the filamentous fungus *Wangiella dermatitidis* [14], seven CHS genes (MaChsI-VII) in the insect pathogenic fungus *Metarhizium acridum* [15], and eight CHS genes in the plant pathogenic fungus *Trichoderma reesei* [10]. Various phylogenetic CHS classification systems have been proposed, each based on different assumptions regarding their origin, diversity, and evolution [9,10,16–18]. One common system involves grouping the fungal CHS genes into three divisions [14]. Division I encompasses classes I, II, and, III CHSs. These enzymes are characterized by the presence of type I (CS1, PF01644) and type II (CS2, PF03142) chitin synthase domains, as well as a chitin synthase N-terminal domain (CS1N, PF08407). Division II consists of classes IV, V, and VI CHSs, which also contain the CS2 domain but are distinguished by the presence of additional domains such as the cytochrome-b5-like domain (cyt-b5; PF00173). Division III comprises the class VII CHSs, characterized by having only the CS2 domain [17–19].

In the yeast *S. cerevisiae*, the three CHS genes play distinct functional roles in septum formation, cell wall expansion, and budding [13,20–22]. In the filamentous fungus *Metarhizium acridum*, the seven CHS genes have been found to influence various aspects of the fungus's biology. *MaChsIII*, *MaChsV*, and *MaChsVII* are specifically implicated in contributing to fungal virulence [15]. In the filamentous fungi *Magnaporthe oryzae*, *Metarhizium robertsii*, and *Neurospora crassa*, each of the seven CHS classes is represented by one gene [17]. The disruption of *ChsVII* in *Ma. oryzae*, *Me. robertsii*, and *N. crassa* results in growth defects under heat stress, with only *ChsV* and *ChsVII* contributing to fungal virulence, morphogenesis, and conidiation in *Me. Robertsii* [17]. In *Aspergillus fumigatus*, Muszkieta et al. [23] identified a total of eight CHS genes, with each CHS working cooperatively to synthesize chitin for vegetative growth, resistance to antifungal drugs, and the virulence of this filamentous fungus. The functional diversity of CHS genes across various fungal species underscores the importance of these enzymes in fungal physiology and their adaptation to different ecological niches and life strategies. Furthermore, CHSs have been considered promising targets for developing efficient antifungal agents to control pathogenic fungi, given the absence of chitin in plants and mammals [24–26].

Although the study of CHS gene families in mushrooms has been less extensive, the latest research by Chen et al. [27] on *Flammulina filiformis*, an edible mushroom species, expands our understanding of the role of these enzymes in the development of the mushroom fruiting body. The identification of nine CHS protein genes in *F. filiformis* and the analysis of their expression patterns during various stages of growth and development indicate that CHSs are involved in the morphogenesis of mushroom tissues and the formation of fruiting bodies. This suggests that CHSs could be key to understanding the mechanisms of mushroom fruiting body development and may have implications for mushroom cultivation and breeding.

In this study, eight CHS-encoding genes were identified from the *C. militaris* genome. The chromosomal locations of these eight members were analyzed. The phylogenetic analysis of 100 CHSs from 17 Ascomycota species was performed. The relationship of the number of CHSs in the genomes of 42 Ascomycota and 42 Basidiomycota fungi, in relation to their ecological strategies was revealed. The transcript patterns of the CHS protein genes during key developmental stages such as conidia germination, the infection of silkworm and fruiting body development were characterized to infer their potential roles. These results could provide useful information for further functional investigations of the CHS protein gene family.

2. Materials and Methods

2.1. Strains, Media and Growth Conditions

The *C. militaris* strain 633 (CGMCC 3.16323) (provided by Professor Caihong Dong from the Institute of Microbiology, Chinese Academy of Sciences), isolated from Hunan province in China, were maintained on potato dextrose agar (PDA) at 20 °C.

2.2. Identification of CHS Genes from Cordyceps militaris

The annotation of two *C. militaris* genomes were downloaded from the Sequence Read Archive database (accession number: SRA047932 and PRJNA323705) [28,29] and used to search for CHSs. Using the protein sequences from the genome annotations, a BLASTP (e-value = $1 \times e^{-10}$) search was conducted against the *C. militaris* genome to find all predicted CHS genes [30]. Then, the predicted CHS genes were further scanned for the Hidden Markov Model (HMM) profiles of the Chitin synthase 1 (PF01644), Chitin synthase 1N (PF08407), and Chitin synthase 2 domains (PF03142) in HMMERv3.2.1 using "hmmsearch", with an expected value (e-value) threshold of $<1 \times e^{-4}$ [31]. The online Gene structure display server 2.0 (http://gsds.gao-lab.org/, accessed on 5 March 2024) was utilized to analyze and visualize the exon–intron structure of the identified CHS genes. The molecular weight (MW) and theoretical isoelectric point (pI) of the CHS proteins were analyzed using tools available on the ExPASy server (http://www.expasy.org/tools/, accessed on 5 March 2024). Subcellular localization predictions for the CHS proteins were made using the WoLF PSORT tool (https://wolfpsort.hgc.jp/, accessed on 5 March 2024). Finally, the chromosomal positions of the CHS genes were displayed using TBtools [32].

2.3. Phylogenetic Analysis of CHS Proteins in Cordyceps Sensu Lato

Firstly, the 100 amino acid sequences of CHSs from Cordycipitaceae, Ophiocordycipitaceae, Clavicipitaceae, including *C. militaris*, as well as *S. cerevisiae* from Saccharomycetes and *Ustilago esculenta* from Ustilaginomycetes, were downloaded from GenBank (Table S1). The downloaded sequences were then aligned using the MAFFT program version 7 with default parameters [33,34]. The aligned sequences were used to construct a maximum likelihood (ML)-based phylogenetic tree using MEGA X based on the Nearest Neighbor Interchange (NNI) method and LG + G model [35].

2.4. CHSs Protein Structure Analysis

The SOPMA (Self-Optimized Prediction Method with Alignment) server (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa%20_sopma.html, accessed on 5 March 2024) (Number of conformational states: 4 (Helix, Sheet, Turn, Coil); Similarity threshold: 8; Window width: 17) was used to predict the second structure of the predicted CHS proteins in *C. militaris*. Homology modeling of the predicted CHS proteins in *C. militaris* was performed by the SWISS-MODEL server (https://swissmodel.expasy.org/, accessed on 5 March 2024) (SIB Swiss Institute of Bioinformatics, Lausanne, Switzerland) following the method described in a previous study [36].

2.5. Distribution of CHSs in Ascomycota and Basidiomycota Fungi with Different Life Styles

The CHS genes of 42 Ascomycete species and 42 Basidiomycete species were searched from the open genome at the National Center for Biotechnology Information (NCBI). The following procedures were used for each of the genomes: firstly, reference CHS protein sequences were blasted against each of the genomes (BlastP and tBlastn, threshold e-value = e^{-5}); second, proteins containing the specific Pfam domains associated with chitin synthases (PF01644, PF08407 and PF03142) were retrieved; finally, all the sequences that contained the relevant domains were re-blasted (BlastP, threshold e-value = $1 \times e^{-5}$) against each genome to refine the search and confirm the presence of these sequences in the genomes. Following these steps, a total of 688 CHSs were identified from the genomes of the Ascomycete and Basidiomycete species. The Ascomycete species were further categorized based on their ecological lifestyles: entomopathogenic, mycoparasitic, nematode parasitic,

plant pathogenic, and saprotrophic. The Basidiomycete species were categorized as non-phytopathogenic and phytopathogenic fungi. The significance of differences in the CHS distribution among different fungi was determined by IBM SPSS Statistics 23, including independent samples *t* tests or one-way analysis of variance (ANOVA). Tukey's test was employed for multiple comparisons when ANOVA indicated significant differences.

2.6. Conidia Germination Assays

The three different stages of conidia germination are as follows: the stage of conidia before germination (CG0), the early stage of conidia germination (CG1, with the length of the emerging mycelium being about 10 μm when cultured for 12 h) and the late stage of conidia germination (CG2, with the length of mycelium being about 100 μm when cultured for 48 h); these were observed under a microscope and collected for RNA extraction.

2.7. Fungal Virulence Assays

In the fungal virulence assays, 100 μL of a 10^7 conidia/mL suspension was injected into each silkworm pupa (Baoding Bocheng Trading Co., Ltd., Baoding, China) in three groups, with two pupae per group, for cuticle-bypassing infection. The infected pupae were monitored daily to observe the progression of the infection. The infected pupae were then collected at two different time points post infection: infected for 8 days (EST, early sclerotia) and 16 days (LST, late sclerotia) when the pupae were completely mummified, consistent with that used in a previous study by Li et al. [36].

2.8. Fruiting Body Production Assays

The *C. militaris* fruiting bodies were cultivated on wheat medium according to the method of Guo et al. [2]. The samples at different developmental stages included those at the stages of hyphae (HY), colored hyphae (CH), primordium (PR) and matured fruiting body (MF). The method of sample collection was consistent with that used in a previous study by Li et al. [36].

2.9. PCR Array and Data Analysis

Total RNA was extracted from various samples (conidia, germinated conidia, infected pupae, or fruiting bodies) using the E.Z.N.A.™ Plant RNA Kit from Omega (Stamford, CT, USA), referring to the manufacturer's protocol. The isolated RNA was then reverse transcribed into cDNA using the HiScript III 1st Strand cDNA Synthesis Kit (+gDNA wiper) from Vazyme (Biotech Co., Ltd., Beijing, China). To analyze the expression of CHS genes in *C. militaris*, real-time PCR was performed using LightCycler® 480 Multiwell Plate 96 from Roche Biosciences (Basel, Switzerland) with the RT^2 SYBR Green Mastermix from Qiagen. The expression values were normalized to a housekeeping gene (*rpb1* gene, CCM_05485). The relative fold changes in gene expression were determined using the $2^{-\Delta\Delta Ct}$ method [37]. All the experiments included three biological replicates. GraphPad Prism 10 software (Graphpad Software, Inc., La Jolla, CA, USA) was used to create graphs that visualize the expression data. IBM SPSS Statistics 23 was used for statistical analysis, including independent samples *t* tests or one-way analysis of variance (ANOVA) to determine the significance of differences in gene expression. Tukey's test was employed for multiple comparisons when ANOVA indicated significant differences.

3. Results

3.1. Identification of CHSs in Cordyceps militaris

A total of eight full-length genes encoding putative CHSs, designated as *CmChs1* through *CmChs8*, were identified from two *C. militaris* genomes (Table 1). The lengths of the *CmChs* genes were found to vary significantly, with the shortest being *CmChs7* at 2017 base pairs (bp) and the longest being *CmChs5* at 6153 bp (Supplementary Table S2). The gene structure analysis revealed that all of the *CmChs* genes contained introns, ranging from 2 to 4, except for *CmChs3* and *CmChs6*, which did not have any introns (Supplementary Figure S1). The

lengths of the CmCHS proteins ranged from 637 amino acids (aa) for CmCHS7 to 1936 aa for CmCHS5 (Supplementary Table S2). Correspondingly, the MWs of these proteins varied from 72.88 kilodaltons (kDa) to 215 kDa (Supplementary Table S2). The predicted pIs of the CmCHS proteins ranged from 5.66 to 9.04, indicating variability in the acidity or basicity of the proteins. Notably, only CmCHS5 had a pI value above 7, suggesting that it was more basic than the other seven members of the CmCHS protein family (Supplementary Table S2).

Table 1. CHSs identified in the genome of *Cordyceps militaris*.

Name	Protein ID [1]	Feature Domains	Subcellular Localization	Number of Transmembrane Structures
CmCHS1	CCM_00447/A9K55_001663	Chitin synthase 1 Chitin synthase N Chitin synthase C Chitin synthase 2	Plasma membrane	7
CmCHS2	CCM_01980/A9K55_006911	Chitin synthase 1 Chitin synthase N Chitin synthase C Chitin synthase 2	Plasma membrane	7
CmCHS3	CCM_02953/A9K55_006003	Chitin synthase 2	Plasma membrane	4
CmCHS4	CCM_02965/A9K55_005992	Chitin synthase C Cyt-b5-PF00173 MYSc_Myo17-cd14879 Chitin synthase 2	Plasma membrane	6
CmCHS5	CCM_02966/A9K55_005991	Chitin synthase C Cyt-b5-PF00173 MYSc_Myo17-cd14879 Chitin synthase 2	Plasma membrane	6
CmCHS6	CCM_06973/A9K55_007939	Chitin synthase 2 Chitin synthase C	Plasma membrane	4
CmCHS7	CCM_08096/A9K55_005325	Chitin synthase 2	Plasma membrane	5
CmCHS8	CCM_08511/A9K55_002055	Chitin synthase 1N Chitin synthase C Chitin synthase 1 Chitin synthase 2	Plasma membrane	10

[1] Protein ID represented the ID in the genomes SRA047932 and PRJNA323705 of *C. militaris*, respectively.

A protein feature domain analysis showed that the proteins CmCHS1, CmCHS2 and CmCHS8 possessed four conserved domains: Chitin synthase 1 (CS1, PF01644), CS1N (PF08407), CSC (cd04190), and CS2 (PF03142). CmCHS4, CmCHS5 and CmCHS6 all contained the CS2 and CSC domain. In addition, CmCHS4 and CmCHS5 also contained the Cyt-b5 domain (PF00173) and MYSc_Myo17 domain (cd14879). Meanwhile, CmCHS3 and CmCHS7 only had one conserved domain: CS2 (PF03142). In addition, all of the CmCHS proteins were predicted to be localized at the plasma membrane, which was consistent with their role in synthesizing chitin for the cell wall.

The amino acid sequence comparison among the eight CmCHS proteins reveals a low level of identities of 21.57–46.98% between them (Supplementary Table S3), indicating significant sequence divergence. CmCHS1 and CmCHS8 exhibited the highest identity at 46.98%, suggesting that these two proteins were more closely related to each other than to the rest of the CmCHS proteins. CmCHS4 and CmCHS6 had the second-highest identity at 44.00%. Despite both having the CS2 domain, CmCHS3 and CmCHS5 showed the lowest identity between them.

CmCHS3 and CmCHS6 had the fewest transmembrane structures, with four each, and CmCHS8 had the most transmembrane structures with eight (Table 1). The secondary

structure analysis of a subset of CHS sequences revealed that the proportions of alpha helices, extended strands, beta turns, and random coils were similar among the eight CHS proteins (Supplementary Table S3). Alpha helices and random coils made up a large portion of the secondary structure in the eight CmCHS proteins (Supplementary Table S4). The results of homology modeling were consistent with the secondary structure analysis, showing many alpha helices (Supplementary Figure S2) that likely contributed to forming the polymer-translocating and chitin-translocating channels.

3.2. Chromosome Distribution of CmCHS Family Genes

Eight CmChs genes were distributed unevenly across chromosomes III, V, VI and VII (Figure 1). Chromosome VI harbored the highest number of *CmChs*s, with four members, followed by two *CmChs*s on chromosome III. Chromosomes V and VII each contained only one *CmChs* gene. *CmChs2*, *CmChs3*, *CmChs4*, and *CmChs5* were all located on chromosome VI, with *CmChs3*, *CmChs4*, and *CmChs5* being adjacent to each other in the middle of the chromosome, similar to the position of *CmChs6* on chromosome VII. Meanwhile, *CmChs2* was situated 32 kb away from the cluster of *CmChs3*, *CmChs4*, and *CmChs5*, and was located in the 3′ region of chromosome VI, akin to the location of *CmChs7* on chromosome V. *CmChs1* and *CmChs8* were both found on chromosome III. They were separated by over 13 kb and located away from the chromosome ends, which was a different arrangement compared to the proximity of *CmChs3*, *CmChs4* and *CmChs5* on chromosome VI.

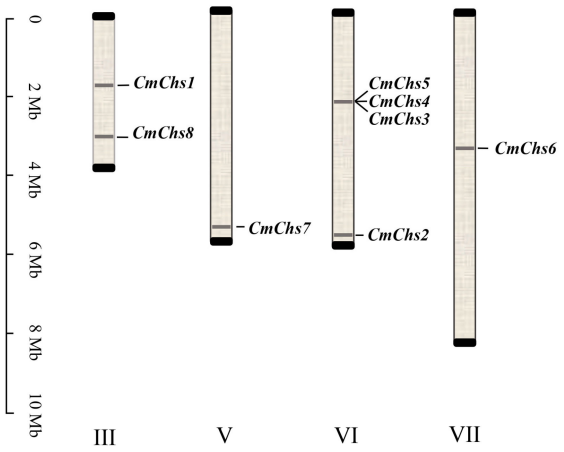

Figure 1. Chromosomal location of CHS genes in *Cordyceps militaris*.

3.3. Phylogenetic Analysis of CHS Proteins of Cordyceps s.l.

The ML phylogenetic tree was constructed using 100 CHS amino acid sequences from 13 Cordyceps s.l. species and 2 additional species in Ascomycota (Supplementary Table S1). The phylogenetic tree strongly supported three major divisions: division I, II, and III (Figure 2). Division I was subdivided into three classes: I, II, and III. Division II included classes IV, V, and VI. Meanwhile, division III contained only one class VII. Members from Cordyceps s.l. were more closely related to each other, indicating a common evolutionary lineage within this group. CHSs from *U. esculenta* (Ustilaginomycetes) and *S. cerevisiae* (Saccharomycetes) were distantly related to CHSs from Cordyceps s.l., such as ANG56500.1 in class I, NP_009594.1, ANG56502.1 and ANG56503.1 in class II, NP_014207.2 in class III, ANG56501.1, ANG56505.1, and NP_009579.1 in class V, and ANG56504.1 in class VII. These results were consistent with the species phylogeny.

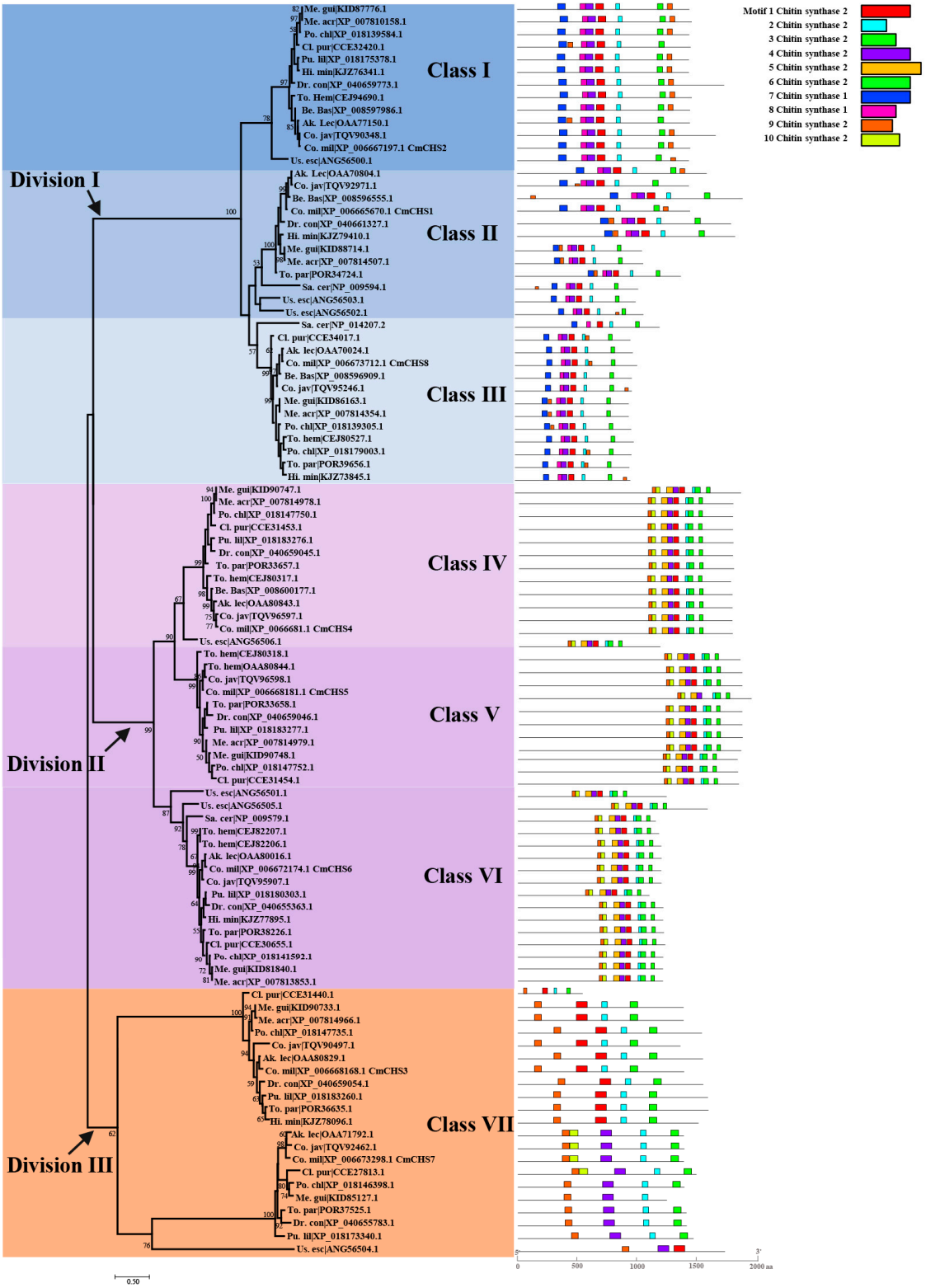

Figure 2. The phylogenetic analysis and conserved motif element distributions of CHS proteins from Cordyceps. s.l. The phylogenetic tree was constructed using MEGA X software. Fungal species were indicated with the following abbreviations: *Metarhizium acridum*: Me. acr, *Claviceps purpurea*: Cl. pur, *Pochonia chlamydosporia*: Po. chl, *Hirsutella minnesotensis*: Hi. min, *Drechmeria coniospora*: Dr. con, *Purpureocillium lilacinum*: Pu. lil, *Trichoderma harzianum*: Tr. har, *Metarhizium acridum*: Me. acr, *Metarhizium guizhouense*: Me. gui, *Tolypocladium paradoxum*: To. par, *Cordyceps javanica*: Co. jav, *Cordyceps militaris*: Co. mil, *Torrubiella hemipterigena*: To. hem, *Beauveria bassiana*: Be. bas, *Akanthomyces lecanii*: Ak. lec, *Saccharomyces cerevisiae*: Sa. cer, *Aspergillus fumigatus*: As. fum, *Ustilago esculenta*: Us. esc.

The distribution of conserved motifs in the CHSs was analyzed based on their phylogenetic relationships (Figure 2). A total of 10 distinct conserved motifs were identified in the CHS proteins. Motifs 7 and 8 were associated with the Chitin_synth_1 conserved domain, which was typically found in the CHS proteins of division I. The remaining eight motifs were associated with the Chitin_synth_2 conserved domain, which was present in the CHS proteins across divisions I, II, and III (Figure 2, Table 2, Supplementary Figure S3). Most members from divisions II and III included motifs 1, 2, 3, 4, and 9, which were the most conserved motifs in the CHS family, and motif 4 was only absent in class VI. Moreover, specific motifs were identified among different classes. For example, motif 7 and 8 (Chitin_synth_1) were unique to division I (classes I, II and III). Motif 10 (Chitin_synth_2) was found only in division II (classes IV, V, and VI) and division III (class VII). Motif 5 and 6 (Chitin_synth_2) were exclusive to division II (classes IV, V, and VI). The specificity of the motifs aligned with the observed characteristics of gene evolution within the CHS family. The presence of specific conserved motifs in different CHS classes suggested that these motifs were associated with the unique functions of each class.

Table 2. Conserved motifs of CmCHS proteins in *Cordyceps militaris*.

Motif	Length (aa)	Amino Acid Conserved Sequence	Motif Function
1	41	PJTAIQNFEYKISNILDKPFESVFGSVTCLPGAFSMYRIRA	Chitin_synth_2
2	20	TDVPDTWKVLLSQRRRWNGT	Chitin_synth_2
3	29	YLLLLPTYNFVLPVYAFWNTDDFSWGTTR	Chitin_synth_2
4	41	RPLNYEICLLVDADTKVGPBSLYHLVSAFDNDPKJGGACGE	Chitin_synth_2
5	50	SEQEKAKPGNRGKRDSQIJLMSFLNRVHFDERMTPLELEMFHQIWNIIGV	Chitin_synth_2
6	41	VHNLMELILVRDLCGFCCFSMRFVVFIDLLGTIVLPATIAY	Chitin_synth_2
7	41	HTELFIVITMYNEDEVLFARTMHGVMRNIRHICNRKKSKTW	Chitin_synth_1
8	28	VPVQMJFCLKEKNQKKINSHRWFFNAFG	Chitin_synth_1

Eight CmCHSs from *C. militaris* were classified into three divisions (Figure 2). CmCHS1, CmCHS2 and CmCHS8 were grouped into the clade of division I, while CmCHS4, CmCHS5 and CmCHS6 were grouped into the clade of division II. Additionally, CmCHS3 and CmCHS7 were grouped into the clade of division III, which was largely consistent with their domain structures. Despite CmCHS3 and CmCHS7 belonging to the same division (III) and the same class VII, they were not grouped within the same cluster. The other six CmCHS proteins (1, 2, 4, 5, 6, 8) were grouped into classes II, I, IV, V, VI, and III, respectively. This phenomenon also occurred in other fungal species, such as *Cordyceps javanica*, *Aspergillus fumigatus*, and *Pochonia chlamydosporia*. CHS proteins from *Me. Acridum* and *Me. Guizhouense*, as well as from *C. javanica* and *C. militaris*, generally grouped together, forming species-specific clades. Only CHS proteins from *U. esculenta* (ANG56502.1 and ANG56503.1) or *Torrubiella hemipterigena* (CEJ82206.1 and CEJ82207.1) grouped as a clade (Figure 2).

3.4. Distribution of CHS Proteins in Basidiomycota and Ascomycota Fungi with Different Lifestyles

The survey of the CHS protein distribution across fungal species within the two major fungal divisions revealed that Ascomycota fungi had an average of 7.9 CHS proteins per species, while Basidiomycota fungi exhibited a slightly higher average of 8.8 CHS proteins per species. The difference in the average number of CHS proteins between the two phyla was not statistically significant (p = 0.067), suggesting that both phyla had a similar complement of CHS genes (Figure 3a; Supplementary Tables S5 and S6). Within Ascomycota fungi, the number of CHS proteins did not significantly differ among the five lifestyles analyzed: entomopathogenic, mycoparasitic, nematode parasitic, plant pathogenic, and saprotrophic (Figure 3b; Supplementary Table S5). Entomopathogenic fungi had the lowest average number of CHS proteins at 8.42, while nematode parasitic fungi had the highest average at 9.57. Basidiomycota fungi with a pathogenic lifestyle

had a similar number of CHS-encoding genes to saprotrophic fungi, with no significant difference (p = 0.971) (Figure 3c; Supplementary Table S6).

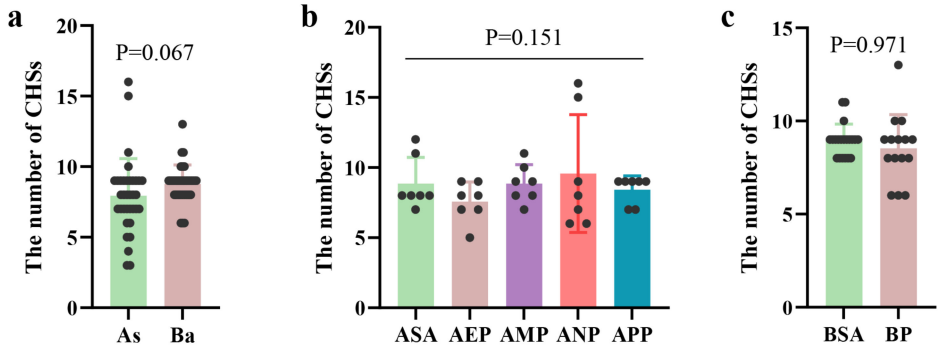

Figure 3. Distribution of CHS proteins in Ascomycota and Basidiomycota fungi with different lifestyles. (**a**) Number of CHS proteins in Basidiomycota and Ascomycota fungi; (**b**) Number of CHS proteins in Ascomycota fungi with different lifestyles; (**c**) Number of CHS proteins in Basidiomycota fungi with different lifestyles. As, Ascomycota fungi; Ba, Basidiomycota fungi; ASA, saprotrophic fungi in Ascomycota; AEP, entomopathogenic fungi in Ascomycota; AMP, mycoparasitic fungi in Ascomycota; ANP, nematode parasitic fungi in Ascomycota; APP, plant pathogenic fungi in Ascomycota; BSA, saprotrophic fungi in Basidiomycota; BP, pathogenic fungi in Basidiomycota. Error bars indicate standard errors of trials.

3.5. Transcript Analysis of CmChs Genes during Conidia Germination

During conidia germination, the transcript levels of *CmChs1* showed a significant increase, up-regulated by 13.5-fold at stage CG1 and 24.2-fold at stage CG2 when compared to stage CG0 (Figure 4, Supplementary Table S7). Conversely, the transcripts of *CmChs3*, *CmChs4*, *CmChs5*, *CmChs6*, and *CmChs8* were significantly lower at stages CG1 and CG2 compared to CG0 (Figure 4, Supplementary Table S7). The transcription levels of *CmChs2* and *CmChs7* remained unchanged during the conidia germination stages.

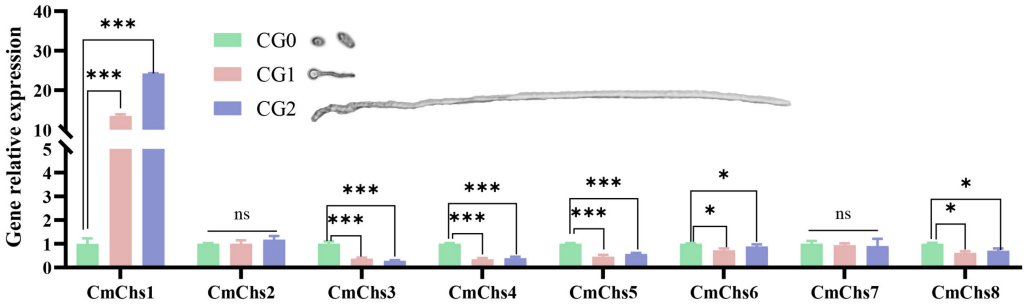

Figure 4. Transcript levels of CHS genes during conidia germination. CG0: conidia; CG1: early stage of conidia germination; CG2, late stage of conidia germination. A single asterisk above bars denoted significant difference, $p < 0.05$; a triple asterisk above bars denotes significant difference, $p < 0.001$; ns above bars denotes no significant difference, $p > 0.05$. Error bars indicate standard errors of three trials.

3.6. Transcript Analysis of CmChs Genes during Infecting Antheraea pernyi

The transcript levels of eight *CmChs*s were detected after the *C. militaris* conidia was injected into the pupae. The expressions of *CmChs1* and *CmChs3* consistently decreased throughout both infection stages (EST and LST). The expression of *CmChs7* showed a dynamic pattern, with a decrease to 0.50-fold at the EST stage, and an increase to 1.92-fold at the LST stage (Figure 5, Supplementary Table S8). For the other five *CmChs* genes, there were no significant changes in transcript levels during the infection of the pupae compared to the hyphal stage (HY) (Figure 5, Supplementary Table S8).

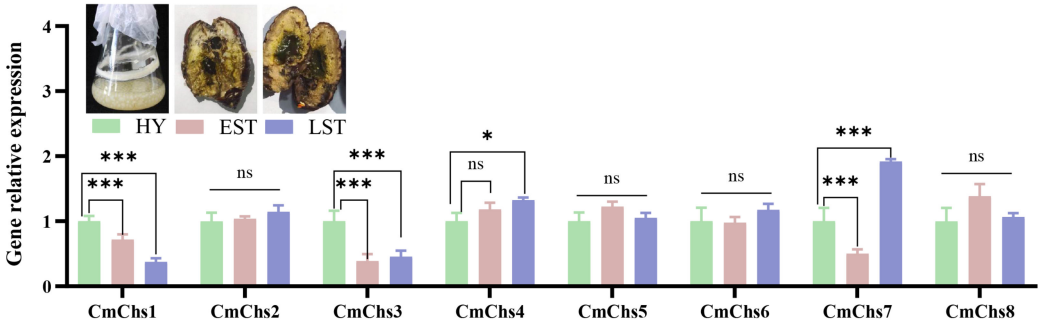

Figure 5. Transcript levels of CHS genes during the infection of silkworm pupae with conidia by the cuticle-bypassing method. HY: the hyphae cultured under darkness; EST: early of sclerotia; LST, late of sclerotia. A single asterisk above bars denotes significant difference, $p < 0.05$; a triple asterisk above bars denotes significant difference, $p < 0.001$; ns above bars denotes no significant difference, $p > 0.05$. Error bars indicate standard errors of three trials.

3.7. Transcript Analysis of CmChs Genes during Fruiting Body Development

The differential expressions of eight *CmChs* genes were observed during fruiting body development in *C. militaris* (Figure 6). *CmChs7* stands out, with a substantial increase in expression during the fruiting body development stages. At the primordium (PR) stage, its expression was up-regulated by 5.5-fold, and it continued to be elevated at the mature fruiting (MF) stage, with a 4.7-fold increase when compared to the hyphal (HY) stage (Figure 6, Supplementary Table S9). Additionally, *CmChs2* and *CmChs3* also showed increased expression at the PR stage, with 1.8-fold and 2.0-fold increases, respectively, indicating that they may be involved in the early stages of fruiting body development. However, their expression did not significantly change between the HY and MF stages. The other five *CmChs* genes did not exhibit significant changes in expression levels at the PR and MF stages compared to the HY stage. Additionally, the observation that all eight *CmChs* genes showed no differences in expression at the colored hyphae (CH) stage compared to the HY stage indicated that the transition to colored hyphae does not involve changes in the expression of these CHS genes.

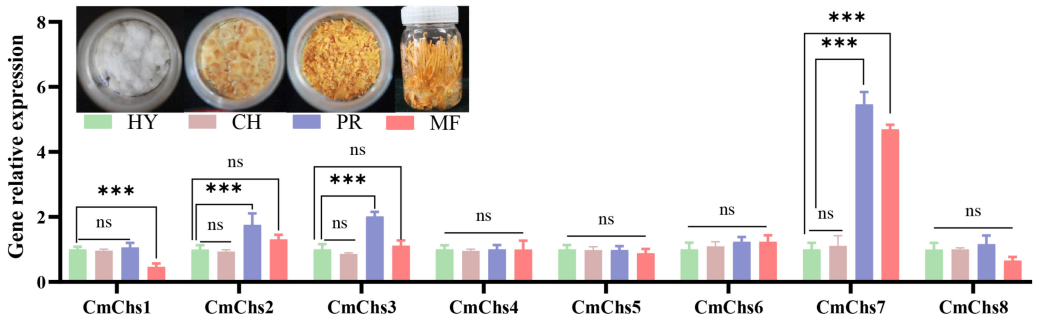

Figure 6. Transcript levels of CHS genes during fruiting body development. HY: the hyphae cultured under darkness; CH: the colored hyphae after light irradiation for 4 days; PR: sclerotium < 1 cm; MF: stroma > 7 cm. A triple asterisk above bars denotes significant difference, $p < 0.001$; ns above bars denotes no significant difference, $p > 0.05$. Error bars indicate standard errors of three trials.

4. Discussion

In the present study, the comprehensive identification and analysis of CHS genes in *C. militaris* were conducted. Eight CHS genes from *C. militaris* were systematically identified and analyzed. The phylogenetic analysis categorized the CHS gene family from Cordyceps s.l., which included *C. militaris*, into three distinct clades, indicating a level of evolutionary divergence within this gene family. Comparative analysis revealed no significant differences in the number of CHS genes among different fungal lineages with different lifestyles,

implying that the number of CHS genes was relatively conserved among these fungi, regardless of their ecological niches or life strategies. Different CHSs exhibited varied transcript levels during key biological processes such as conidia germination, infection and fruiting body development, suggesting that members of this family may have undergone functional diversification to a certain extent. These results provided a foundation for further function research on CHSs in *C. militaris*.

The chromosomal distribution of the eight *CmChs* genes, as depicted in Figure 1, provided important clues about their potential regulation and function. The fact that these genes were unevenly distributed across chromosomes III, V, VI, and VII was an interesting aspect of their genomic organization. This could explain the different roles that these genes play during the life cycle of *C. militaris*, as observed in their expression patterns (Figures 4–6). The observation that *CmChs3*, *CmChs4*, and *CmChs5* were located adjacent to each other on chromosome VI raised the possibility that they may have undergone subsequent functional diversification. The expression patterns of these genes during conidia germination, infection, and fruiting body development, as detailed in Figures 4–6, indicated that while *CmChs3*, *CmChs4*, and *CmChs5* shared a similar expression pattern during conidia germination (Figure 4), they may have distinct roles or regulatory mechanisms during the other two processes (Figures 5 and 6). This divergence could be attributed to the presence of different regulatory elements responding to specific developmental cues or environmental conditions.

In the context of the study on Cordyceps s.l. species, the CHS genes also formed three divisions, further categorized into seven classes with high bootstrap values (Figure 2), indicating strong statistical support for these groupings. A phylogenetic analysis of the fungal CHSs from nine fungal phyla, encompassing Ascomycota, Basidiomycota, Glomeromycota, Zygomycota, Blastocladiomycota, Chytridiomycota, Neocallimastigomycota, Microsporidia, and Cryptomycota, had been performed to clarify the evolution of fungal CHSs. They were designated as division 1, division 2, and division 3, as reported by Liu et al. [17]. The observation that all proteins within each clade shared the same domain structure (Figure 2), consistent with the findings of Li et al. [10] and Liu et al. [17], indicated that these domains had been preserved throughout the evolution of the CHS gene family, reinforcing the idea that domain architecture is a reliable indicator of the phylogenetic relationships among CHS genes.

The survey of the CHS protein distribution in Ascomycota and Basidiomycota fungi with different lifestyles showed that both two phyla had a similar complement of CHS genes, and that the fungi with different lifestyles in both two phyla also lacked a significant difference in CHS gene numbers (Figure 3). This finding, illustrated in Figure 3, supported the notion that a core set of CHS genes was fundamental to the basic biological functions of fungi, particularly in the construction and maintenance of the cell wall, as discussed in the works of Gow et al. [6] and Alcazar-Fuoli et al. [7]. The observation that there was no significant difference in the number of CHS genes between pathogenic and saprotrophic fungi within these two phyla suggested that the diversity of CHS genes was not directly linked to the lifestyle or pathogenic nature of the fungus. Instead, it implied that the conservation of CHS gene numbers was related to their essential role in fungal physiology. The study's findings indicated that factors other than pathogenicity were likely influencing the number and diversity of CHS genes, such as environmental adaptation and the complexity of interactions with hosts or substrates.

The role of CHS genes during conidia germination was critical, as these genes were responsible for producing chitin, a key structural component of the fungal cell wall, particularly during the formation of new hyphae. In the plant pathogenic fungus *Verticillium dahlia*, the deletion of *VdCHS5* resulted in a significant reduction in the germination rate, as shown by Qin et al. [38], underscoring the functional importance of this particular CHS gene in the early stages of conidia germination. Deletion mutants of *chs1* in the rice blast fungus *Magnaporthe oryzae* and *Fusarium asiaticum* also exhibited defects in conidial germination [39,40]. In the present study, the significant up-regulation of *CmChs1* during conidia germination (CG1 and CG2) compared to stage CG0 suggested that *CmChs1* likely played

a key role in this process. The down-regulation of other CHS genes (*CmChs3*, *CmChs4*, *CmChs5*, *CmChs6*, and *CmChs8*) during the same stages indicated that these genes may not be crucial for the initial germination phase or may serve different functions at later stages of development or under alternative environmental conditions. The steady expression levels of *CmChs2* and *CmChs7* throughout the conidia germination stages implied that these genes may not be directly involved in the germination process, or that their involvement did not require changes in transcript levels during these stages. CHS genes play a critical role in maintaining the structural integrity of the fungal cell wall and affect fungal pathogenicity. In the phytopathogenic fungus *F. asiaticum*, disrupting the chitin synthase gene *CHS1* led to a 58% reduction in chitin synthase activity, resulting in a 35% decrease in the chitin content and a significant decline in pathogenicity on wheat spikes and seedlings [40]. Similarly, Kim et al. [41] demonstrated that mutants lacking *GzCHS5* and *GzCHS7* in *Gibberella zeae* exhibited weaker cell wall rigidity and reduced pathogenicity compared to the wild-type strain. Among the eight *CmChs*s in *C. militaris*, only *CmChs7* appeared to be involved in the infection process, particularly during the late stage of sclerotia formation, indicating that *CmChs7* has a specialized function in adapting to the host environment during the later stages of infection. The expression patterns of the other *CmChs* genes in *C. militaris*, some being down-regulated or remaining steady during various infection stages, suggested potential functional diversification.

In edible mushrooms, chitin serves not only as a key structural component of the fungal cell wall, but also as a significant constituent of dietary fiber. Hassainia's report [42] emphasizes the significance of chitin in fungal structure, with varying concentrations in different tissues of the *Agaricus bisporus* mushroom, such as stipes, pileus, and gills. The biosynthesis and degradation of chitin have been verified to play a role in the development of the fruiting body in various mushrooms. For instance, in *Coprinellus komigratus*, the expression of chitinase 2 in autolyzed mature mushrooms was found to be 20 times or twice as high as that in the primordium or young mushrooms, respectively [43]. In *C. militaris*, two chitinase gene (*Chi1* and *Chi4*) deletions lead to a block in the differentiation of the primordium, and significant reductions in the number of fruiting bodies and the length of the single mature fruiting body compared to the WT strain [44]. Although research on CHS gene families in mushrooms has been limited, in *F. filiformis*, the differential expression of CHS genes at various growth and developmental stages, as mentioned by Chen et al. [27], suggested their roles in the morphogenesis of the fruiting body. Likewise, in *C. militaris*, the varied expression patterns of the eight *CmChs* genes throughout the life cycle indicated potential functional diversification in fruiting body development. The notable up-regulation of *CmChs7* at the PR and MF stages, with 5.5-fold and 4.7-fold increases, respectively, suggested that *CmChs7* could be a major regulator of fruiting body development. *CmChs2* and *CmChs3*, with their increased expression at the PR stage, may have specific roles related to the initiation of this process. The other five *CmChs* genes, which did not show significant expression changes during the PR and MF stages compared to the HY stage, may not be directly involved in the morphological changes observed in fruiting body development, or their roles may not require differential expression during these stages. The observation that the CHS gene family in *C. militaris* did not respond to light aligned with findings in other fungi, such as *Mucor circinelloides*, where López-Matas et al. [45] reported that visible light did not induce the accumulation of transcripts for the chitin synthase gene *Mcchs1*. This suggests that light may not universally regulate CHS gene expression in fungi.

Overall, these expression patterns suggested functional diversification within the *CmChs* gene family. Certain genes, like *CmChs1* and *CmChs7,* play critical roles in specific developmental processes such as conidia germination, infection, and fruiting body development. Further research, including functional genomics studies involving gene knockout or overexpression experiments, would be necessary to confirm the specific roles of these genes in *C. militaris*. Such studies would help elucidate the biological significance of the

observed expression patterns and the contribution of each CHS gene to the fruiting body development, adaptability, and pathogenicity of the fungus.

5. Conclusions

In this study, eight *CmChs* genes were identified in the genome of *C. militaris*, unevenly distributed across four chromosomes. Phylogenetic analysis revealed that the CHSs from Cordyceps s.l. were divided into three divisions and seven classes. The distribution of CHSs exhibited no significant difference in fungi with different lifestyles and phyla. The varied transcript patterns of the eight *CmChs*s during the life cycle suggested distinct functions for each gene at different developmental stages. These findings contribute comprehensive information on the CHS gene family in *C. militaris* and provide a well-established platform to explore the specific roles of these genes throughout the life cycle, especially in fruiting body development.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae10050494/s1, Figure S1: Gene structure of chitin synthase genes in *Cordyceps militaris*; Figure S2: Homology modeling of eight CmCHS proteins; Figure S3: Ten conserved motifs of chitin synthase proteins in *Cordyceps militaris*; Table S1: The CHS proteins from Cordyceps s.l. in Ascomycota used by phylogeny analysis; Table S2: List of 7 CHS genes identified in *Cordyceps militaris* with relevant information; Table S3: The percentage of identity between eight CmCHS proteins in *Cordyceps militaris*; Table S4: Analyses of secondary structure of CmCHS proteins in *Cordyceps militaris*; Table S5: The chitin synthase distribution with different life styles in Ascomycota; Table S6: The chitin synthase distribution with different life styles in Basidiomycota; Table S7: The relative expression levels of eight *CmChs*s during conidia germination; Table S8: The relative expression levels of eight *CmChs*s during the conidia of *Cordyceps militaris* infecting silkworm pupae; Table S9: The relative expression levels of eight *CmChs*s during fruiting body development in *Cordyceps militaris*.

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