# THE WHOLE GENOME SEQUENCE OF *VOLVARIELLA VOLVACEA* WILL FACILITATE RESOLUTION OF PROBLEMS LIMITING ITS COMMERCIAL EXPLOITATION

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## ABSTRACT

*Volvariella volvacea* is widely grown in southeastern Asia as a high quality human food source, and is one of most important cultivated mushrooms worldwide. However, developments in *V. volvacea* cultivation have been limited due to a low biological efficiency (i.e. conversion of growth substrate to mushroom fruit bodies), sensitivity to low temperatures, and an unclear sexuality pattern that has restricted the breeding of improved strains. The Institute of Edible Fungi, Shanghai Academy of Agricultural Sciences, has recently completed the sequencing of the whole genome of *V. volvacea* strain V23-1, a single-spore isolate derived from *V. volvacea* strain V23. The sequence, which has been deposited in the NCBI database, is assembled into 62 scaffolds with a total genome size of 35.7 megabases (Mb), and contains 11,084 predicted gene models. Comparative analyses based on the whole genome sequence have been made with other basidiomycete mushrooms with regard to the mating type system, polysaccharide degrading enzymes and lignin oxidizing enzymes. Transcriptional analysis of the loss of hyphal viability following exposure to low temperature (4 $^{o}$C) has also been undertaken. The *V. volvacea* genome contains numerous genes encoding enzymes involved in the degradation of cellulose, hemicellulose and pectin. Furthermore, the molecular structure of the mating type system is similar to that of the bipolar system of basidiomycetes, and indicates that *V. volvacea* has a secondary homothallic life cycle. The absence of genes encoding enzymes involved in initiating the biosynthesis of unsaturated fatty acids, trehalose and glycogen may be related in part to the sensitivity to low temperature exposure. Elucidation of the *V. volvacea* genome sequence will promote a deeper understanding at the molecular biological level of the mechanisms involved in substrate degradation and help to resolve problems limiting the industrial exploitation of this mushroom.

**Keywords:** *Volvariella volvacea,* whole genome sequence, sensitivity to low temperature exposure, mating type, polysaccharide degrading enzymes

## INTRODUCTION

*Volvariella volvacea,* also known as the Chinese mushroom and straw mushroom, is an edible fungus grown in tropical and subtropical regions. In the 18th Century, Buddhist monks of Nanhua Temple located in the Chinese Province of Guangdong, developed a primitive method of cultivating the straw mushroom to enrich their diet. Subsequently, *V. volvacea* was presented as a tribute to China's royalty [1, 2].

Although *V. volvacea* has been cultivated for approximately 300 years, there are still many associated production problems that greatly restrict the commercial exploitation of this mushroom. Mushroom yields in proportion to the dry weight of compost at spawning (biological efficiency) are only ~15% for straw substrates and 40% for cotton-waste 'composts' [3], which are very low values compared with many of the other major cultivated edible mushroom species such as *Agaricus bisporus, Lentinula edodes* and *Pleurotus* spp [4]. *V. volvacea* is also very sensitive to low temperatures, and the fungal mycelium will lose viability when exposed to temperatures below 15 $^{o}$C. Furthermore, mushroom fruit bodies suffer chilling damage and undergo autolysis when stored at low temperatures (4 $^{o}$C) [5], thereby restricting the shelf-life.

*V. volvacea* is generally recognized as a primary homothallic basidiomycete [1, 6, 7] although some single spore isolates are self-fertile (heterokaryotic) while others are self-sterile (homokaryotic) [8]. Moreover, *V. volvacea* hyphae are

multinucleate [9] and do not form clamp connections which, in other fungi, serve as morphological markers to distinguish self-fertile from self-sterile mycelia. These traits are problematic for the development of a simple and efficient method for *V. volvacea* cross-breeding.

Here, we report the complete genome sequence of the monokaryotic *V. volvacea* strain, V23-1. These data will facilitate analysis of the cellulase system and elucidate the sexual pattern of the mushroom. Furthermore, in order to help resolve a major problem restricting the industrial exploitation of *V. volvacea*, we have compared transcript profiles of mycelium exposed to low temperature with those of non-exposed mycelium in order to better understand the mechanism(s) underlying the mushroom's sensitivity to cold.

## MATERIALS AND METHODS

### Strains and culture conditions

*V. volvacea*, dikaryotic strain V23, was obtained from the Key Laboratory of Agricultural Genetics and Breeding of the Shanghai Institute of Edible Fungi, Shanghai Academy of Agricultural Sciences. Single spore isolates were obtained by separation of basidiospores of *V. volvacea* strains V23 and PY on potato dextrose agar (PDA) plates and identified as sterile homokaryons by the cultivation test.

### DNA extraction, genome sequencing, assembly and annotation

Genomic DNA of *V. volvacea* was extracted by an improved cetyl trimethyl ammonium bromide (CTAB) method and sequenced using the Roche 454 GSFLX (Roche, USA) and Illumina Solexa GAIIx (Illumina, USA) platforms. Following pre-processing, the Roche 454 reads were assembled into a primary assembly using Roche Newbler software and then scaffolded with Illumina paired-end and mate-pair reads using Velvet software. Gene model prediction was undertaken by combining four (GeneMark, Augustus, Fgenesh and Geneid) software types.

### RNA extraction

Fungal mycelium from cultures of *V. volvacea*, strain V23, was grown in 250 ml flasks containing 100 ml potato dextrose broth (PDB) at 32 $^{o}$C for 4 days, and the fungal mycelium exposed to 4 $^{o}$C at zero time and for 2 and 4 hours. After harvesting, mycelia were lyophilized and total RNA was extracted using the Trizol regent according to the manufacturer's instructions.

### Protein family classification

Carbohydrate-active enzymes (CAZy) were classified by local BLASTp searching against a library of catalytic and carbohydrate-binding module enzymes constructed from the CAZy database (http://www.cazy.org/). The distribution of sequences encoding putative lignin-modifying enzymes (LME) in the fungal genome was determined by comparison with LME-encoding sequences in the NCBI database. To build up the LME database, our custom program compiled by perl was used to choose the sequences based on the BLASTp result (BLASTp, cut-off e-value > $1e^{-50}$) from the obtained sequences. LME families were classified by BLASTp against the LME database.

## RESULTS AND DISCUSSION

### Genome sequencing and general features

The *V. volvacea* whole genome sequence was determined using Roche 454 GSFLX sequencing and Illumina Solexa sequencing, respectively. The combined sequencings generated 190×coverage of the genome, which was assembled into 62 scaffolds with an N50 of 388 kb and a total size of 35.7 Mb (Table 1). The size of the *V. volvacea* genome is similar to the genomes of several other species assigned to the Agaricaceae including *Schizophyllum commune* (38.5 M)[10], *Coprinopsis cinerea* (37 M) [11] and *P. ostreatus* (35 M) [12], but is bigger than that of another straw rotting fungus,

**Table 1.** Features of the *V. volvacea* genome

| General features | 0 |
|---|---|
| Size of assembled genome (Mb) | 36.45 |
| GC content (%) | 48.86% |
| Length of classified repeats (%) | 2.25 Mb (6.18%) |
| Number of predicted gene models | 11,084 |
| Average gene length (with intron) (bp) | 2,087 |
| Average transcript length (bp) | 1,572 |
| Number of single-exon genes | 1,066 |
| Average number of exons per multi-exon gene | 7 |
| Average exon size (bp) | 229 |
| Average intron size (bp) | 88 |

*Agaricus bisporus* (30.2 M) [13].

Annotation of the assembled genome sequence generated 11,084 gene models, 76.43% of which were supported by EST data. The average transcript length was 1,572 bp, and an average of six introns per multi-exon gene. The average exon and intron sizes were 252.3 bp and 83.2 bp, respectively (Table 1).

## Carbohydrate active enzymes

*V. volvacea* is a kind of straw rotting fungi and relies mainly on the degradation of cellulose to obtain the bio-energy. In the genome of *V. volvacea* a total of 357 CAZyme-coding gene homologs were identified. This value is higher than the average number of 305 of other basidiomycetes. The glycoside hydrolases (GH) superfamily in the genome of *V. volvacea* contained 224 homologs, belonging to 42 families. CE, GT, PL and CBM superfamilies had each 28, 66, 18 and 21 homologs, distributed in 8, 24, 3 and 6 families, respectively (Fig. 1). *V. volvacea* has the highest number of PL modules (18) compared to other basidiomycetes. We made a detailed comparative analysis of the number of CAZy families related to plant polysaccharide degradation in *V. volvacea* and other basidiomycete genomes (Fig. 2). This analysis shows *V. volvacea* has 47, 101 and 57 candidate CAZymes related to the degradation of cellulose, hemicellulose and pectin, respectively. These values are respectively higher than the average (36, 77 and 35) of other basidiomycetes. The *V. volvacea* genome also has a larger number (57) of enzymes for degradation of β-1,3-1,4-glucan, which widely distributed

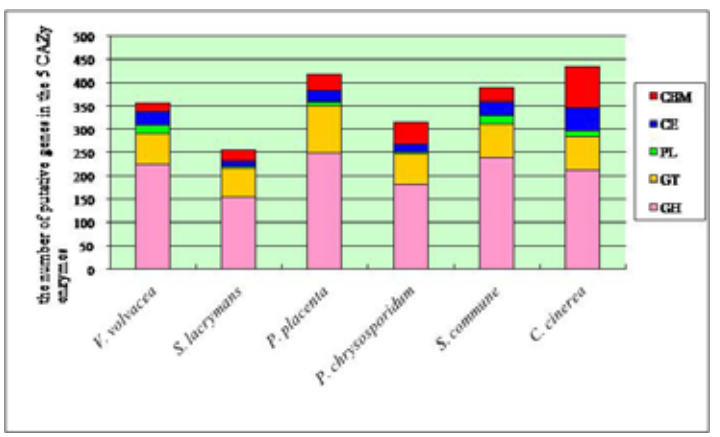


**Figure 1.** Comparison of the number of five CAZy gene groups in *V. volvacea* and other basidiomycetes fungi genomes. (From: Bao *et al.* [21])

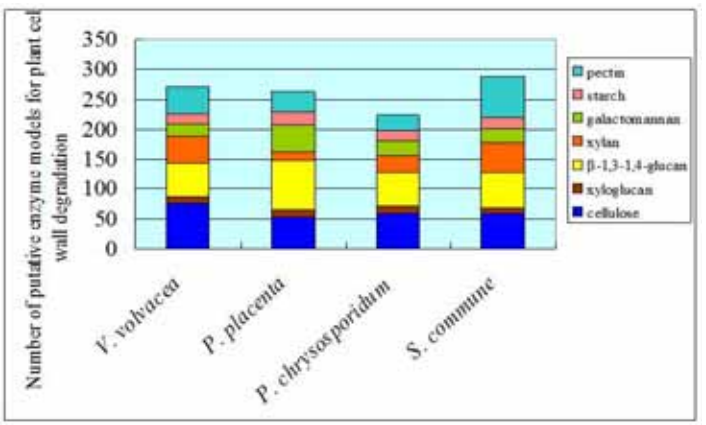


**Figure 2.** Number of putative enzyme models involved in plant cell wall degradation in *V. volvacea* and other basidiomycetes fungi genomes. (From: Bao *et al.* [21])

as non-cellulosic matrix phase polysaccharides in cell walls of grasses and cereal species. The set of CAZymes in the *V. volvacea* genome indicated the strong ability to digest the complex plant cell wall, especially for the pectin (57, average 37) and the xylan (56, average 31) (Fig. 2), suggesting *V. volvacea* mainly depend on pectin and xylan digestion to obtain the energy.

## Fungal oxidative lignin enzymes

*V. volvacea* is regarded as a straw-rotting fungus and appears to lack an effective ligninolytic enzyme system [14-16], which may account in part for its low biological conversion rate. Fungi degrade lignin by secreting multiple isoenzymes of three heme peroxidases (lignin peroxidases (LiPs), manganese peroxidases (MnPs), and hybrid enzymes known as versatile peroxidases (VPs)) and laccase. The *V. volvacea* genome contains two putative MnP and two putative VP gene encoding sequences, but lacks sequences encoding for LiP-encoding genes. Interestingly, the genome also contained 11 genes (*vv-lac1* to *vv-lac 11*) encoding laccase homologues, six of which were identical with the laccase genes obtained previously using PCR [17, 18]. Ten of the eleven were distributed within a 216 kb spanning region of scaffold 6 and arranged into a cluster supported by hypergeometric analysis. All the laccase genes except *vv-lac 11* were congregated in shorter chromosomal regions compared to *A. bisporous* and *C. cinerea* (Fig. 3), suggesting these genes may have been generated by duplication in recently evolutionary events. *VV-lac 11* is located at scaffold 8 and exhibits a high similarity with

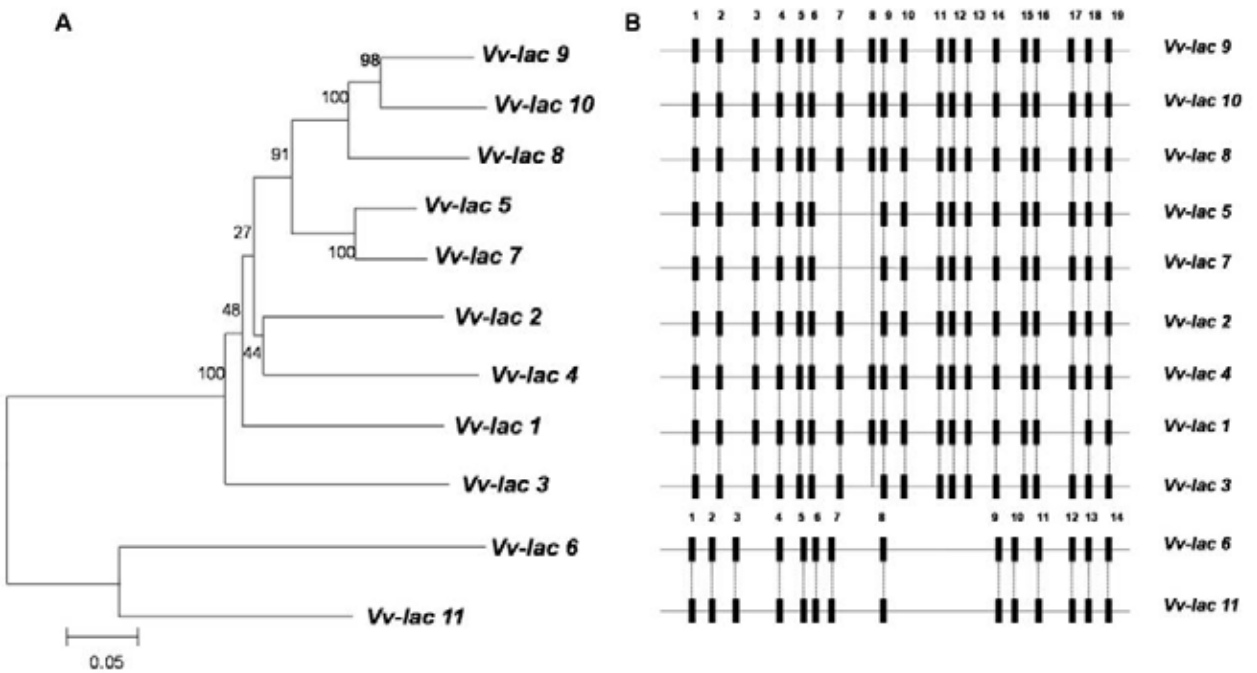


**Figure 3.** (A) Neighbor-joining tree of the deduced amino acid sequences of *V. volvacea*laccase genes. (B) Distribution of intron positions in *lac1-lac11*define two gene subfamilies. Black bars indicate intron positions. Dotted lines link the same introns. (From: Bao *et al.* [21])

*vv-lac6*, indicating their paralogous relationship (Fig. 3). Phylogenetic analysis revealed that nine *V. volvacea* laccase genes (*vv-lac1-5* and *vv-lac7-10*) are congregated within the same cluster (Fig. 3A), and share 17 (from a total of 19) conserved intron positions (Fig. 3B).

## Mating type loci and sexuality patterns

Scanning of the genome of the single spore-derived *V.volvacea*, strain V23-1, revealed two homeodomain (HD) genes, *VVO_04854* and *VVO_05004* (designated *vv-HD1*$^{-V23-1}$and *vv-HD2*$^{-V23-1}$) located 271 bp apart on scaffold 07. Both encoded HD proteins having a high similarity with homologues from the bipolar basidiomycetes, *A. bisporus* and *Pholiota nameko*, and the tetra polar basidiomycetes, *C. cinerea* and *S. commune*. We theorized that *V. volvacea* has one *A* mating type locus containing a pair of *HD* genes and accordingly, designed a pair of specific primers (VVmipF11:5'-

GTGACTGCTATGGAACACATTGGAC and VVmipR13: 5'- TCGGAGGAAGCGGGTCCACTACA) based on the DNA sequence surrounding the mating type *A* locus (designated *A*1) of strain V23-1 to amplify the *A* locus (designated *A*2) of another single spore-derived *V. volvacea* strain, V23-18. A pair of genes, *vv-HD1*$^{-V23\text{-}18}$ and *vv-HD2*$^{-V23\text{-}18}$ (Accession number: JX157875) was identified within the *A*2 locus, and the encoded HD1 and HD2 proteins from the spore monokaryons V23-1 and V23-18 showed 48% and 49% similarity, respectively.

In order to establish the efficacy of molecular marker-assisted cross-breeding techniques in generating improved *V. volvacea* cultivars, we designed four primer pairs based on the sequences of the *A* mating type genes (*HD1* and *HD2*) of the parental strains V23 (*A* mating type locus alleles *A1* + *A2*) and PY (*A* mating type locus alleles *A3* + *A4*), respectively. Of 124 single spore isolates obtained from strain V23, 101 were confirmed as homokaryons, 35 of which were mating type *A1* and 66 mating type *A2*. The remaining 23 were heterokaryons carrying both *A1* and *A2* mating type loci. Of 88 single spore isolates from strain PY, 72 were confirmed as homokaryons, of which 41 were mating type *A3* and 31 mating type *A4*. Sixteen were heterokaryons carrying both *A3* and *A4* mating type loci. Cross-breeding between 72 compatible pairs generated 58 hybrids, three of which were designated high quality hybrids based on agronomic traits determined by cultivation experiments.

**Transcriptional analyses of the 'cold shock response'**

In order to identify the cellular responses occurring during low temperature (4 °C) exposure of *V. volvacea*, we carried out high-throughput sequencing of mRNA expression in fungal mycelium exposed to 4 °C at zero time, and after 2 and 4 hours. Differences in gene expression levels between 0 h and 2 h, and 0 h and 4 h were then compared using | log 2 (fold-change)|≥ 0.5 and FDR <0.001 as the threshold for significant changes.

Previously, yeast genes involved in carbohydrate energy reserves, especially trehalose and glycogen biosynthesis, were reported to be induced after exposure to 4 °C [19,20], suggesting that biosynthesis and accumulation of these reserve carbohydrates may be necessary for cold tolerance and energy preservation [19]. Analysis of gene expression associated with starch and sucrose metabolism (PATH: ko00500) revealed that 32 genes were expressed at 0, 2 and 4 h (Fig. 4). However, no significant differences in the expression levels of the majority of these genes were observed and, while several genes were significantly under-expressed, none were significantly over-expressed. Among the former, expression of *VVO_02898* and *VVO_08127*, which respectively encode homologues of TPS1 (trehalose phosphate synthase) and

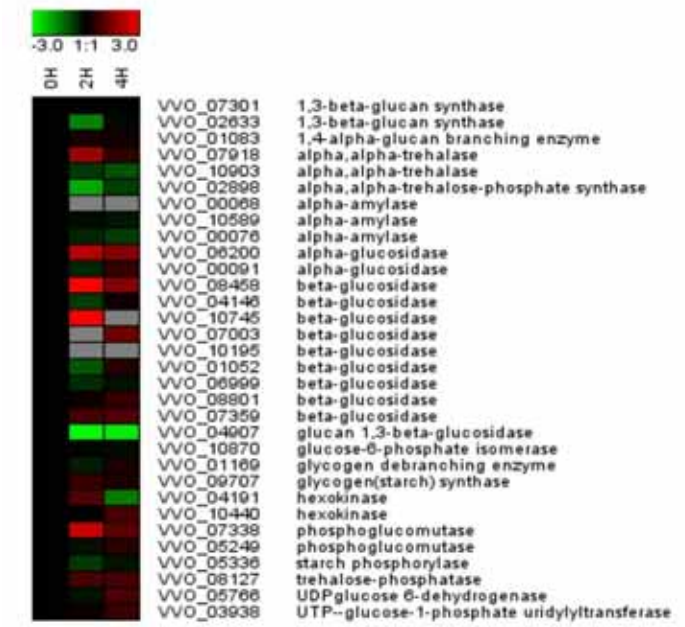


**Figure 4.** Heatmap showing expression levels of genes in starch and sucrose metabolism pathways after 0, 2 and 4 h exposure to 4 °C. (From: Bao *et al*. [21])

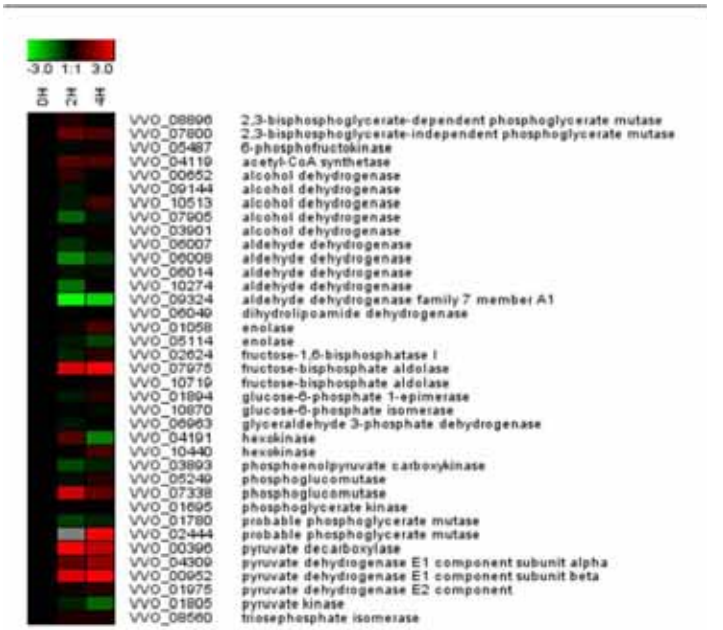


**Figure 5.** Heatmap showing expression levels of genes in glycolysis and gluconeogenesis pathways after 0, 2 and 4 h exposure to 4 °C (From: Bao *et al*. [21])

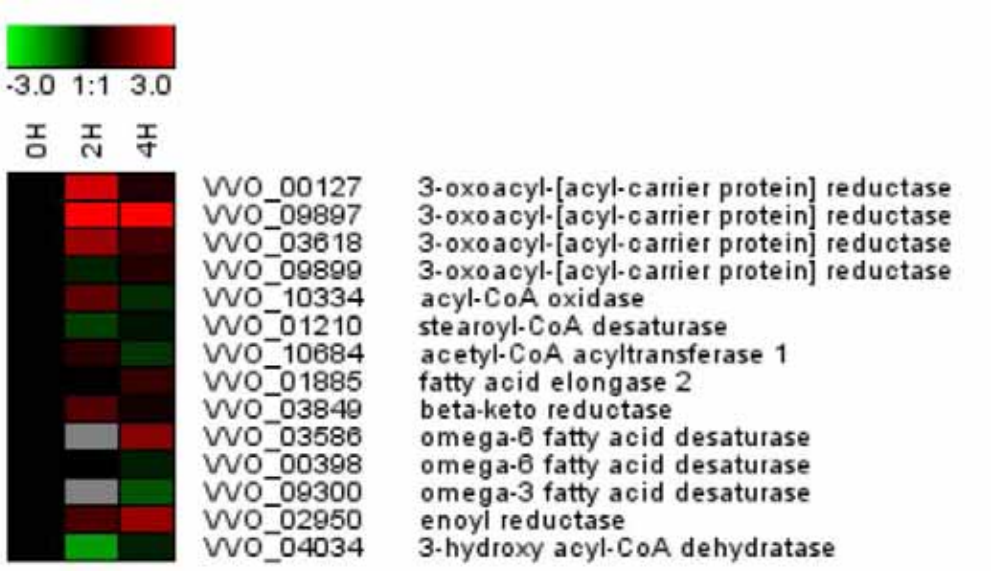


**Figure 6.** Heatmap showing expression levels of genes in unsaturated fatty acid biosynthesis after 0, 2 and 4 h exposure to 4 °C. (From: Bao *et al.* [21])

TPS2 (trehalose-6-phosphate phosphatase) in yeast, was either repressed (-2.032, $9.01E^{-8}$) or showed no significant change after 2 h exposure to 4 °C. Expression of genes associated with glycolysis and gluconeogenesis metabolism (PATH: ko00010), including *VVO_06963* (encoding glyceraldehyde 3-phosphate dehydrogenase), *VVO_03893* (encoding phosphoenol pyruvate carboxyl kinase) and *VVO_09324* (encoding aldehyde dehydrogenase family 7 member A1), was again either repressed or showed no significant change after 2 h and 4 h exposure to 4 °C (Fig. 5). Simiarly, three key genes involved in unsaturated fatty acid biosynthesis (PATH: ko01040): *VVO_01210* (encoding stearoyl-CoA desaturase), *VVO_00398* (encoding omega-6 fatty acid desaturase) and *VVO_10684* (encoding acetyl-CoA acyltransferase 1), exhibited no significant increase after exposure to 4°C (Fig. 6). However, expression of *VVO_00127* (encoding 3-oxoacyl-[acyl-carrier protein] reductase, FabG) was induced at 2 h (log2 (fold change) = 2.48, FDR=$7.22E^{-14}$) but showed significant change at 4 h (0.40, 0.56).

In summary, increased expression of genes encoding enzymes involved in unsaturated fatty acid, trehalose and glycogen biosynthesis recorded elsewhere was not evident in *V. volvacea*. However, no up-regulation of these genes was observed in our genome-wide expression analysis following low temperature exposure of *V. volvacea*. This may, in part at least, explain why the mushroom hyphae becomes non-viable at low temperatures although the underlying mechanism remains unclear.

## ACKNOWLEDGMENTS

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