Journal of Proteomics 215 (2020) 103668

Contents lists available at ScienceDirect

Journal of Proteomics

journal homepage: www.elsevier.com/locate/jprot

Chilling stress reduced protein translation by the ubiquitination of ribosomal proteins in *Volvariella volvacea*

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ARTICLE INFO

Keywords:
Volvariella volvacea
Autolysis
Ubiquitination
Chilling stress
Ribosome

ABSTRACT

In *Volvariella volvacea*, an important edible mushroom species, cryogenic autolysis is a typical part of abnormal metabolism; however, the underlying mechanisms remain unclear. Ubiquitylome analysis revealed that chilling stress (CS) affected protein translation and degradation by ubiquitination. Comparative proteomics analysis showed that CS downregulated protein expression in *V. volvacea* V23 instead of VH3 (improved chilling stress resistance strain). The integrative ubiquitylome, proteomics, and transcriptome analyses indicated that CS reduced protein translation by the ubiquitination of ribosomal proteins. An activity assay of the 20S proteasome showed that CS decreased the degradation efficiency of the ubiquitin-proteasome system. UBEV2, one type of ubiquitin-conjugating enzyme E2 (UBE2) in *V. volvacea*, was upregulated after cold stress treatment using western blot analysis. GST pull-down experiments of UBEV2 provided evidence that CS affected protein translation by the ubiquitination of ribosomal proteins. Co-IP experiments confirmed that UBEV2 bound to the ubiquitinated SSB2, a ribosome-associated molecular chaperone. An anti-freezing experiment demonstrated that the UBE2 inhibitor could improve the cold stress resistance of *V. volvacea*. Our observations revealed that CS triggered ubiquitination-mediated autolysis associated with a decrease in protein translation and highlighted the mechanistic role of UBEV2 in facilitating cryogenic autolysis in *V. volvacea*.

Significance: *Volvariella volvacea*, the edible straw mushroom, is a highly nutritious food source widely cultivated on a commercial scale in tropical and subtropical regions. The challenges associated with the cryogenic autolysis preservation of *V. volvacea* have limited its marketability. This issue of cryogenic autolysis is both an interesting scientific problem to solve and a practical economic matter. Integrative ubiquitylome, proteomics, and transcriptome analyses, together with GST pulldown and Co-IP experiments, indicated that chilling stress reduced protein translation by the ubiquitination of ribosomal proteins in *V. volvacea*. This study significantly contributes to our understanding of ubiquitination-mediated autolysis associated with a decrease in protein translation in *V. volvacea*. Our data highlight the mechanistic role of UBEV2 in facilitating the cryogenic autolysis of *V. volvacea*. We provided a new idea for the preservation of *V. volvacea* by inhibiting UBEV2 to increase its marketability.

1. Introduction

Volvariella volvacea, known as the straw mushroom, is among the most extensively cultivated mushrooms in tropical and subtropical regions, and it requires relatively high temperatures (28–35 °C) for vegetative growth and fruiting [1–3]. Routine storage temperatures (4 °C) cause *V. volvacea* mycelia to undergo cryogenic autolysis, which is manifested by rapid softening, liquefaction, and even decay of the fruiting body [4]. For this reason, the short shelf life of this mushroom hampers its distribution over long distances and limits its cultivation and popularization. Because *V. volvacea* is one of the main varieties for the utilization of straw in biomass energy, cryogenic autolysis of *V. volvacea* indirectly affects the comprehensive utilization of straw resources and has significant economic consequences as the mushroom

Abbreviation: UBE2, ubiquitin-conjugating enzyme E2; UBEV2, one type of UBE2 in *V. volvacea*; CST, cold stress treatment; CS, chilling stress; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; WB, western blot; FDR, false discovery rate; ML, Maximum-likelihood; RFU, relative fluorescence units; UPS, ubiquitin-proteasome system; SSB2, ribosome-associated molecular chaperone.

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https://doi.org/10.1016/j.jprot.2020.103668
Received 16 September 2019; Received in revised form 14 January 2020; Accepted 22 January 2020
Available online 23 January 2020

ranks 5th in terms of annual worldwide production [5]. This issue of cryogenic autolysis is both an interesting scientific problem to solve and a practical economic matter [3].

Previous studies indicated that a type of ubiquitin-conjugating enzyme E2 (UBE2) in *V. volvacea* (UBEV2) participates in cryogenic autolysis [3]. UBE2 functions at the heart of the ubiquitin transfer pathway and is responsible for much of the diversity in ubiquitin cellular signaling [6], suggesting that ubiquitination may function in cryogenic autolysis. Ubiquitination, an essential posttranslational modification process, is first known for its role in the proteasome-dependent degradation of proteins [7]; however, growing evidence indicates that ubiquitination also regulates various cellular processes in many other manners [8]. Ubiquitination has been found to contribute to the stress response in many species. In plants, ubiquitination plays a crucial role in regulating the amount and activity of proteins required to adapt to abiotic stresses (e.g., adverse temperature, high salinity, and drought) [9]. In yeast, protein ubiquitination contributes to development, metabolism, and stress response [10]. Many studies have reported that cold temperatures affect the ubiquitin-proteasome system (UPS) in rice [11], protist *Trichomonas vaginalis* [12], banana [13], and Arabidopsis [14]. In these studies, E3-ubiquitin ligase has been reported to play a role in the modulation of chilling stress (CS) signaling [15–17]. Recently, ubiquitylome analysis indicated that cold stress triggered a large group of proteins with increased ubiquitination in yeast [18]. However, a comprehensive study of ubiquitination in the cold stress response is lacking in edible fungi, especially the mechanisms underlying the cryogenic autolysis of *V. volvacea*.

2. Material and methods

2.1. Sample preparation

The *Volvariella volvacea* V23 strain was obtained from the China General Microbiological Culture Collection Center (Beijing) (no. CGMCC5.289). The VH3 strain, obtained by composite mutagenesis of *V. volvacea* V23 strain [19], has an improved chilling stress resistance compared to V23 and is available from the Culture Collection Center at the Institute of Shanghai Academy of Agricultural Sciences. The mycelia of the fungus were first incubated on a potato dextrose agar plate at 32 °C for four days. They were then punched out and inoculated in 100 mL of potato dextrose broth (PDB) (Becton, Dickinson, Sparks, U.S.) in a 250 mL Erlenmeyer flask. The flasks were placed on a rotary shaker at 150 rpm and 32 °C for four days. The mycelia were harvested from the liquid cultures by filtration and then exposed to 4 °C for 0, 2, 4, 6, and 8 h. After being washed with sterile distilled water and patted dry with filter paper, the treated mycelia were immediately frozen in liquid nitrogen and stored at −70 °C before analysis.

2.2. qPCR assay and western blot analysis

Fungal mycelium from cultures of the *V. volvacea* VH3 strain was grown in a 250 mL flask containing 100 mL potato dextrose broth (PDB) at 32 °C for 4 days and then exposed to 4 °C for 4 h. The treated mycelia were then collected and lyophilized for RNA extraction. The details of real-time quantitative PCR (qPCR) and primers used for the qPCR were previously published [3].

The polypeptide of UBEV2 was designed and generated by Shanghai Qianchen Biotechnology Company. The peptide was conjugated to Keyhole Limpet Hemocyanin (KLH). The peptide-KLH was used for the immunization of the rabbit. The polyclonal antibody, with an ELISA titer higher than 1:4000, was used for western blot (WB) analysis of UBEV2.

The treated mycelia of *V. volvacea* V23 exposed to 4 °C for 0, 2, 4, 6, and 8 h were used for the total protein extraction. The pool of oocytes was lysed in Laemmli sample buffer with a protease inhibitor, heated at 100 °C for 5 min and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were transferred to polyvinylidene fluoride membranes. WB analysis was conducted as described previously [20].

2.3. Protein extraction for the ubiquitylome analysis

The treated mycelia of *V. volvacea* V23 or VH3 after cold stress treatment (CST) for 0 and 4 h with at least two independent biological replicates were used for the protein extraction. After digesting the extracted protein, the tryptic peptides were fractionated into fractions by high pH reverse-phase HPLC using a Thermo Betasil C18 column. The tryptic peptides dissolved in NETN buffer were incubated with prewashed antibody beads at 4 °C overnight with gentle shaking to enrich di-Gly-Lys modified peptides. Finally, the eluted fractions were combined and vacuum dried.

The peptides were dissolved by liquid chromatography with mobile phase A (0.1% (*v*/v) formic acid aqueous solution) and separated by EASY-nLC 1200 UPLC system. The separated peptides were subjected to an NSI source followed by tandem mass spectrometry (MS/MS) in Q Exactive™ HF-X. The resulting MS/MS data were processed using the MaxQuant search engine (v.1.5.2.8) [21]. Tandem mass spectra were searched against the transcriptome database of *V. volvacea* (9542) [2] concatenated with a reverse decoy database. The protein quantification was based on a label-free quantification. The false discovery rate (FDR) thresholds for proteins, peptides, and modification sites were set at 0.01. The minimum peptide length was set at 7, and a MaxQuant score was set at > 40. This work was performed by PTM Biolab Inc. (Hangzhou, China).

The criteria with fold-change value > 1.5 and p-value < 0.05 were used to determine differentially ubiquitinated proteins. Functional descriptions of identified protein domains were annotated by InterProScan. KEGG online service tool, KAAS [22], was used to annotate the protein's KEGG database descriptions. Then, the annotation results from the KEGG pathway database were mapped using the KEGG online service tool, KEGG Mapper. The online service on the STRING database (version 11.0) [23] was used for the enrichment analysis. WoLF PSORT was used for predicting the subcellular localization. Motif-x was used to analyze the model of sequences. Details about ubiquitylome analysis can be found in the supplementary file.

2.4. Protein extraction for proteomic analysis

Three independently grown mycelia of *V. volvacea* V23 or VH3 after CST for 0 and 4 h were used for protein extraction. One hundred micrograms of extracted protein was used for tryptic digestion, based on FASP [24] methods. For iTRAQ labeling, the lyophilized samples were resuspended in 100 μL 200 mM TEAB, and 40 μL of each sample was transferred into new tubes for labeling. The labeling peptide solutions were lyophilized and stored at −80 °C. Peptide separation was performed on an 1100 HPLC System (Agilent) using an Agilent Zorbax Extend RP column (5 μm, 150 mm × 2.1 mm). The separated peptides were lyophilized for MS detection using the Ekspert nanoLC 415 system (SCIEX, Concord, ON). IDA (information-dependent acquisition) mass spectrum techniques were used to acquire tandem MS data on a Triple TOF 6600 tandem mass spectrometer (Sciex, Concord, Ontario, Canada) fitted with a Nanospray III ion source.

The MS/MS data were analyzed for protein identification and quantification using ProteinPilot Software v.5.0 (Sciex Inc., USA). The local FDR was estimated to be 1.0% after searching against a transcriptome database of *V. volvacea* (9542) with the integrated PSPEP tool in the ProteinPilot Software. Only proteins identified at global FDR ≤ 1% and unique peptides ≥1 were considered for protein lists and further downstream analyses. For a protein to be determined as differentially expressed, it must have been identified and quantified with at least 1 significant peptide, and the p-value of the protein quantitation should be less than 0.05 and fold change > 1.5. The

OmicsBean cloud platform was used for the bioinformatics analysis of differentially expressed proteins, and these analyses included GO function annotation, enrichment analysis, and protein interaction analysis. R language was used for the heat map clustering of the expression pattern. This work was performed by Luming Biotechnology (Shanghai, China). Details about the proteomic analysis can be found in the supplementary file.

2.5. High-throughput sequencing of mRNAs

Cold-induced gene expression profiles were determined via high-throughput sequencing analysis of mRNAs expressed in the mycelia of *V. volvacea* (V23 strain) [2] and its mutagenic strain (VH3 strain) after cold exposure (4 ˚C) for 0, 2 and 4 h. The treated mycelia were collected and lyophilized, and total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. Double-stranded cDNA was synthesized from mRNA using the method of *Ng* et al. with modifications [25]. cDNA was size-calibrated and transformed into single-stranded template DNA libraries. Libraries were clonally amplified and sequenced with a 454 Genome Sequencer FLX system. The RNA extraction, sample preparation procedure, and high-throughput sequencing method were conducted according to the procedure by *Bao* et al. [2]. DEGseq software [26] was used to normalize the data and assess significant differences in gene expression, and $|\log_2(\text{fold-change})| \geq 1$ and FDR < 0.05 was used as thresholds to determine significantly differentially expressed genes.

2.6. Proteasome activity assay and transmission electron microscopy

For active proteasome extraction, the treated mycelia of *V. volvacea* V23 or VH3 after CST for 0 and 4 h were sampled, frozen in liquid nitrogen, and ground into a powder. Lysis buffer (500 μL for 300 mg powder, 50 mM HEPES pH 7.6, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 15% glycerol) was added to the powder, and the solution was mixed gently at 4 ˚C for 20 min and then centrifuged at 12,000 rpm at 4 ˚C for 10 min. The supernatants were used for the proteasome activity assay. In brief, 10 μL extracted active proteins was added to the reaction buffer (final concentration: 25 mM HEPES, pH 7.5, 0.5 mM EDTA, 0.05% NP-40 and 0.001% SDS (wt/vol)), and then the fluorogenic peptide substrate Suc-LLVY-AMC (Merck Millipore; reconstituted with dimethyl sulfoxide) was added to a final concentration of 1 mM. The reaction (total volume of 100 μL) was conducted in a 96-well fluorometer plate for 2 h at 37 ˚C or 45 ˚C. Fluorescence data were collected at 360 nm for excitation and 460 nm for emission. For the inhibition assay, 25 μM MG123 was preincubated with proteasome extracts for 15 min at room temperature, and then the Suc-LLVY-AMC substrate was added.

The grown mycelium of *V. volvacea* V23 or VH3 after CST for 0, 4, and 8 h were chopped into 1-mm blocks. The samples were prepared and then viewed with a transmission electron microscope, according to the procedures by *He* et al. [27].

2.7. GST pull-down experiment of UBEV2

The total gene of the GST-UBEV2 fusion sequence was synthesized and inserted into the expression vector pGEX-4 T-1 using the restriction enzymes BamHI and *Xho*I. The accuracy of the final expression vector was confirmed by enzymatic digestion and sequencing. Finally, the expression vector was transformed into the Top10 clone strain and the BL21 (DE3) expression strain. The GST-UBEV2 protein was expressed through IPTG induction and then purified by affinity chromatography (glutathione resin).

GST-UBEV2 was expressed in *Escherichia coli* cells and isolated as described above. The protein extract from the V23 mycelia after CST for 4 h was used for GST pull-down experiments. GST pull-down experiment was as previously described [28]. The GST tag protein was used as a negative control. The proteins bound to the beads were detected by SDS-PAGE and silver staining. The interacting proteins were identified by mass spectrometric analysis using a Q Exactive mass spectrometer (ThermoFisher) as described above. The protein interaction network was constructed on the STRING database with a confidence score of at least 0.7 (at the level of high confidence). Cell-PLoc 2.0 was used to predict the subcellular localization of proteins in different organisms [29].

2.8. Co-IP experiment of UBEV2

The UBEV2-FLAG and SSB2-His fusion protein sequences were inserted into the expression vector proEM5 by total gene synthesis and double enzyme digestion. The transfection-grade plasmid was extracted by the plasmid extraction kit and transformed into the DH5α clone strain. Transfection grade plasmids were extracted by a plasmid extraction kit, and then the plasmids were transfected into mammalian HEK293 cells for expression by a transfection reagent. Expression cells were collected and lysed with the lysates. The FLAG monoclonal antibody was used for the Co-IP experiments. Co-IP experiment was as previously described [30]. SDS-PAGE and WB experiments were used to analyze the experimental results.

2.9. Anti-freezing experiment of fruiting body

NSC697923 (Selleck) is a cell-selective UBE2 inhibitor, and it was added into H_2O to prepare a 300 μM NSC697923 solution for DMSO solubilization. The control treatment was carried out using 300 mL H_2O solution by adding an equal amount of DMSO. The egg-shaped fruiting bodies of *V. volvacea* V23 were soaked in the prepared solution for 30 min. The treated fruiting bodies were then exposed to 4 ˚C for 0, 2, 4, 6, 8, 10, 24, and 48 h.

3. Results

3.1. Ubiquitination affected protein translation and degradation

We first isolated the total protein from the mycelia of *V. volvacea* V23 and VH3 after CST for 0 and 4 h for the ubiquitylome analysis. The ubiquitylome study identified a total of 2692 ubiquitination sites on 1045 proteins, among which 2415 sites of 985 proteins contained quantitative information (Tables S1-S3). Although the mass error for the precursor ions was set to 5, 99.6% of all identified peptides had a mass error of less than 3 (Fig. 1A). Low values of relative standard deviation (RSD) in V23 and VH3 samples indicate their excellent repeatability (Fig. 1B). Ubiquitinated proteins contained different numbers of ubiquitination sites, ranging from 1 to 31. The length of the identified peptides ranged from 7 to 49 amino acids. A total of four conserved motifs for 996 unique sites were identified using Motif-X and accounted for 37% of the sites identified. These unique sites were named EK^{ub}, $K^{ub}E$, $K^{ub}D$, and $K^{ub}XXE$ (K^{ub} indicates the ubiquitinated lysine, and X indicates any amino acid), and they exhibit different abundances (Table S4). A survey of these motifs revealed acidic glutamic acid (E) and aspartic acid (D) upstream or downstream of the ubiquitinated lysine.

The ubiquitination levels at 238 sites were upregulated, and the ubiquitination levels at 327 sites were downregulated in V23-4 h/V23-0 h (Table S2 and Fig. 1C). The ubiquitination level at 434 sites increased, and the ubiquitination level at 231 sites decreased in VH3-4 h/VH3-0 h (Table S2). GO annotation of ubiquitinated proteins showed similar GO entries for the comparison between V23-4 h/V23-0 h and VH3-4 h/VH3-0 h (Table S5). The prediction of subcellular localization showed that CS affected the number of ubiquitinated proteins more in the cytoplasm, nucleus, plasma membrane, and mitochondria in V23 than those in VH3 (Table S6). String enrichment analysis showed that the upregulated ubiquitinated proteins were enriched in the ribosome

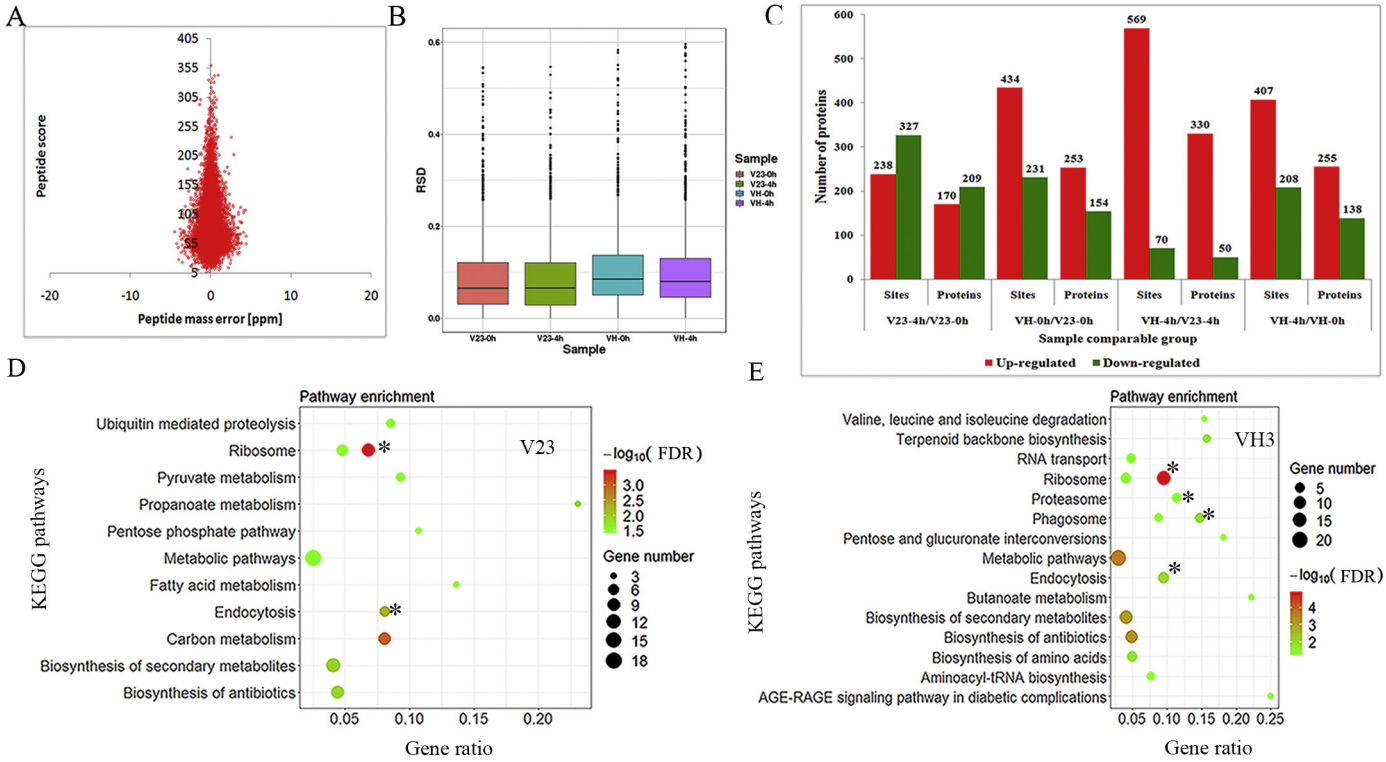

Fig. 1. Ubiquitylome analysis of *V. volvacea* V23 and VH3 after CST for 0 and 4 h. (A) Mass error distribution of all the identified peptides. (B) RSD analysis of the ubiquitinated sites. (C) Statistics of significantly ubiquitination modification level. The differentially ubiquitinated proteins were screened with fold change > 1.5 and *p*-value < 0.05 as the standard. (D) String enrichment analysis of the significantly differentially ubiquitinated proteins in V23. * Represents the KEGG pathway enriched from the significantly upregulated ubiquitinated proteins. (E) String enrichment analysis of the significantly differentially ubiquitinated proteins in VH3.

and endocytosis in V23 and VH3 (Fig. 1D and E). Enrichment analysis showed that the significantly downregulated ubiquitinated proteins were also enriched in the ribosome with a higher FDR in V23 and VH3 (Fig. 1E). The significantly downregulated ubiquitinated proteins were enriched in the pathway of biosynthesis of amino acids in VH3, and this did not occur in V23. These data indicated that CS affected protein translation and degradation by ubiquitination.

3.2. Chilling stress reduced protein translation and degradation

We performed proteomics analysis of the total protein from the mycelia of *V. volvacea* V23 and VH3 after CST for 0 and 4 h. There were 3688 credible proteins identified in all three groups (unused > 1.3 and peptides ≥1) (Tables S7-S9). Based on the selected credible proteins, the significantly differentially expressed proteins were screened with fold change > 1.5 and *p*-value < 0.05 as the standard (Fig. 2A and B). The results indicated that CS decreased the protein expression in V23 but not VH3 (Fig. 2C and D). GO annotation of the significantly downregulated proteins showed the biological processes, cell components, and molecular functions related to protein synthesis in V23 (Fig. S1A). Enrichment analysis showed that the significantly upregulated proteins were enriched in the biosynthesis of the amino acid pathway in VH3 (Fig. S1B), while the significantly downregulated proteins were enriched in RNA transport and mRNA surveillance in V23 (Fig. S1C). KEGG figures showed that mRNA surveillance and RNA transport pathways were related to protein translation and translation stalling (Figs. S2 and S3).

A Venn analysis diagram showed that nine upregulated ubiquitinated proteins in the enriched pathways of the ribosome (VVO_00248, VVO_03931, VVO_09672, and VVO_09980), endocytosis (VVO_02795) and phagosome (VVO_07273), as shown in Fig. 1D and E, had a significantly downregulated expression after CST in V23 instead of VH3 (Fig. 3A). Considering that ribosomes, endocytosis, and phagosomes are related to protein translation and degradation, these observations suggested that CS reduced protein translation and degradation in V23 by ubiquitination.

The high-throughput sequencing of the mRNAs expressed in the mycelia after CST for 2 h showed significantly upregulated mRNA genes in V23 that were higher than those in VH3 (Fig. 3B and Table S10). Transcriptome analysis indicated that CS triggered the upregulated mRNA expression in V23 compared to the downregulated mRNA expression in VH3 (Fig. 3B). Together with the downregulated proteins in V23 after CST (Fig. 2E), these data suggested that some upregulated mRNAs did not translate into the proteins. Venn analysis diagram of significantly expressed genes in V23 after CST showed that sixteen significantly upregulated mRNAs occurred in the significantly downregulated proteins (Fig. 3C) compared to one significantly upregulated mRNAs in VH3 after CST (Fig. S4A). Together with the similar Venn analysis diagram and enrichment results from the integrated analysis of mRNA and protein for 0–4 h after CST (Fig. S4B-D), these data indicated that some differential mRNAs did not translate into proteins in V23 after CST. For example, the interacted ribosomal protein RPS1A has upregulated mRNA expression after CST for 2 and 4 h (Fig. 3A, D, and S4D), and protein expression was downregulated with increased ubiquitination after CST for 4 h (Fig. 3A). String enrichment analysis showed that these differential genes were enriched in the translation pathway (Fig. 3D). These observations provided evidence that CS reduced protein translation by the ubiquitination of ribosomal proteins for degradation.

As the 20S proteasome was responsible for degrading ubiquitin-modified proteins [31,32], the activity of the 20S proteasome is positively correlated with protein degrading efficiency. The detection of 20S proteasome activity using the Suc-LLVY-AMC substrate showed decreased degradation efficiency in V23 after CST (Fig. 3E). These data confirmed that CS reduced protein degradation in V23. The increased degradation efficiency occurred in VH3 after CST (Fig. 3E), suggesting

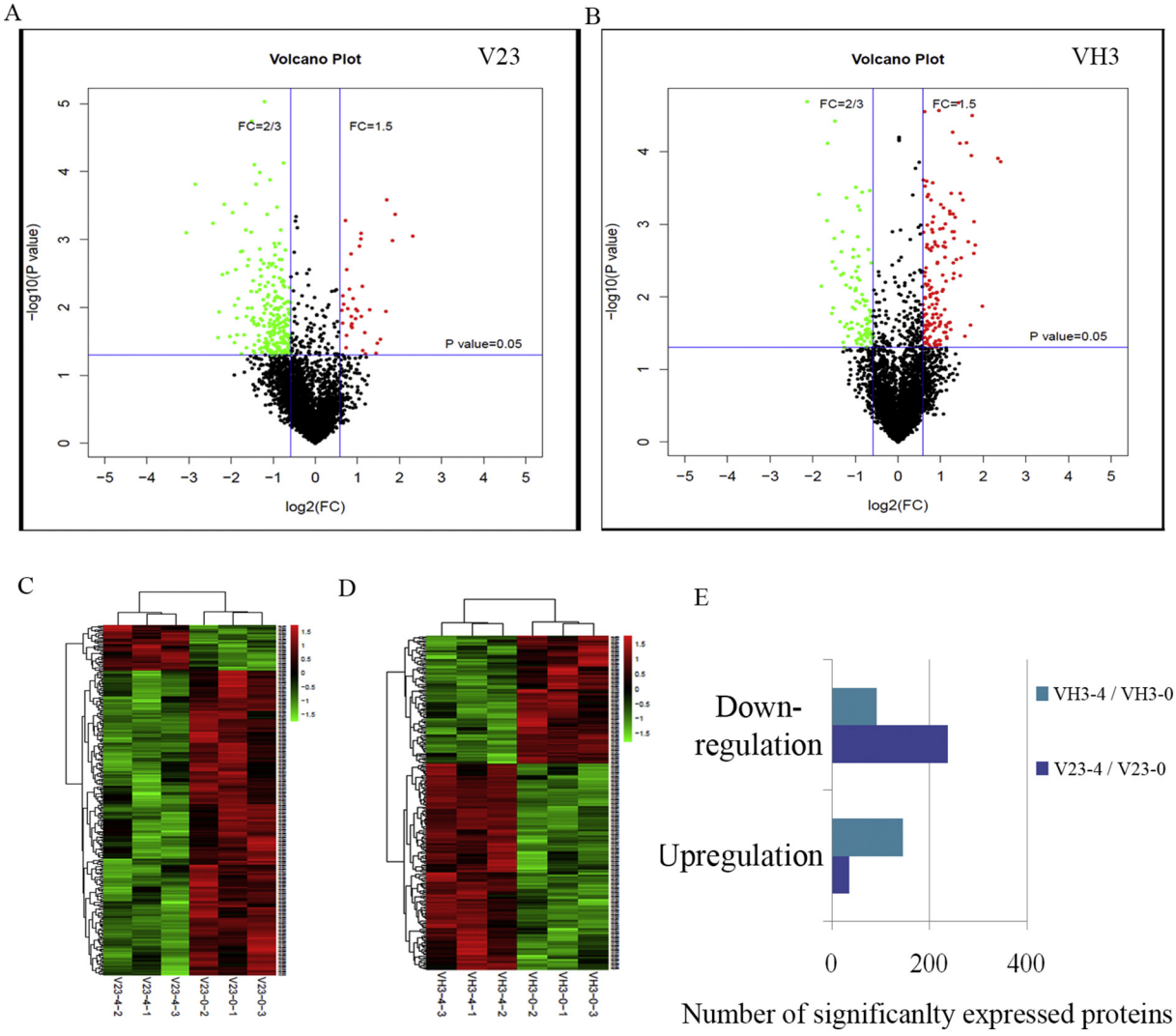

Fig. 2. Proteomics analysis of *V. volvacea* after CST for 0 and 4 h. (A) Volcanic map of protein expression in V23-4 h / V23-0 h. Green dots indicate significantly downregulated expressed proteins, red dots indicate significantly upregulated expressed proteins, and black dots indicate nonsignificantly differentially expressed proteins. (B) Volcanic map of protein expression in VH3-4 h / VH3-0 h. (C) Heatmap of the cluster analysis of the significantly differentially expressed proteins in V23. Green indicates downregulated expressed proteins; red indicates upregulated expressed proteins. (D) Heatmap of cluster analysis of the significantly differentially expressed proteins in VH3. (E) Statistics on the number of significantly differentially expressed proteins. Differentially expressed proteins were screened with fold change > 1.5 and p-value < 0.05 in the *t*-test as the standard. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

that UPS degradation efficiency is beneficial to cold stress resistance.

The ultrastructural features of the mycelia after CST for 0, 4, and 8 h were compared by transmission electron microscopy. At the 4 h stage, several autophagosomes containing recycling components were observed in V23 but not in VH3 (Fig. 4B). At the 8 h stage, plasma membrane blebbing was observed as well as the formation of large autophagic vacuoles in V23 (Fig. 4C). These observations confirmed that CS triggered autophagic formation for degradation in V23. The decreased protein translation followed by autophagic degradation might be mainly responsible for the significantly decreased protein expression after CST instead of UPS degradation.

3.3. Expression and interaction analyses of UBEV2 after cold treatment

Our data indicated that CS reduced degradation and protein translation by ubiquitination, shifting our attention to the role of UBEV2 in cryogenic autolysis. Previous studies showed the upregulated mRNA expression of UBEV2 after CST of *V. volvacea* V23 [3]. WB analysis further revealed the upregulated expression of UBEV2 after CST (Fig. 5A). qPCR analysis confirmed that the mRNA expression of UBEV2 was downregulated in *V. volvacea* VH3 after CST for 4 h (Fig. 5B). These data indicated the putative role of UBEV2 in facilitating cryogenic autolysis.

The control elution protein band is shown in lane 5, and the putative interacting proteins of GST-UBEV2 were captured in lane 10 (Fig. 5C). The detection of liquid mass spectrometry of the relevant eluted samples showed that 100 and 94 proteins were detected in lanes 5 and 10, respectively. Fifty-five putative interacting proteins were identified in lane 10 after eliminating the nonspecific binding of GST in lane 5 (Table S11). Enrichment of the interacting proteins showed the enriched pathway of the cytosolic large ribosomal subunit (GO: 0022625). Four ubiquitin-related proteins occurred in the interacting proteins (Table S11), and three proteins among them were located in the interaction networks with the support of many types of evidence based on sequence homology (Fig. 5D). String annotation showed that the interacting protein UBX3 (VVO_09914) is involved in CDC48-dependent protein degradation through the ubiquitin/proteasome pathway, and the interacting protein UBI4 (VVO_07448) is a ubiquitin marking protein for

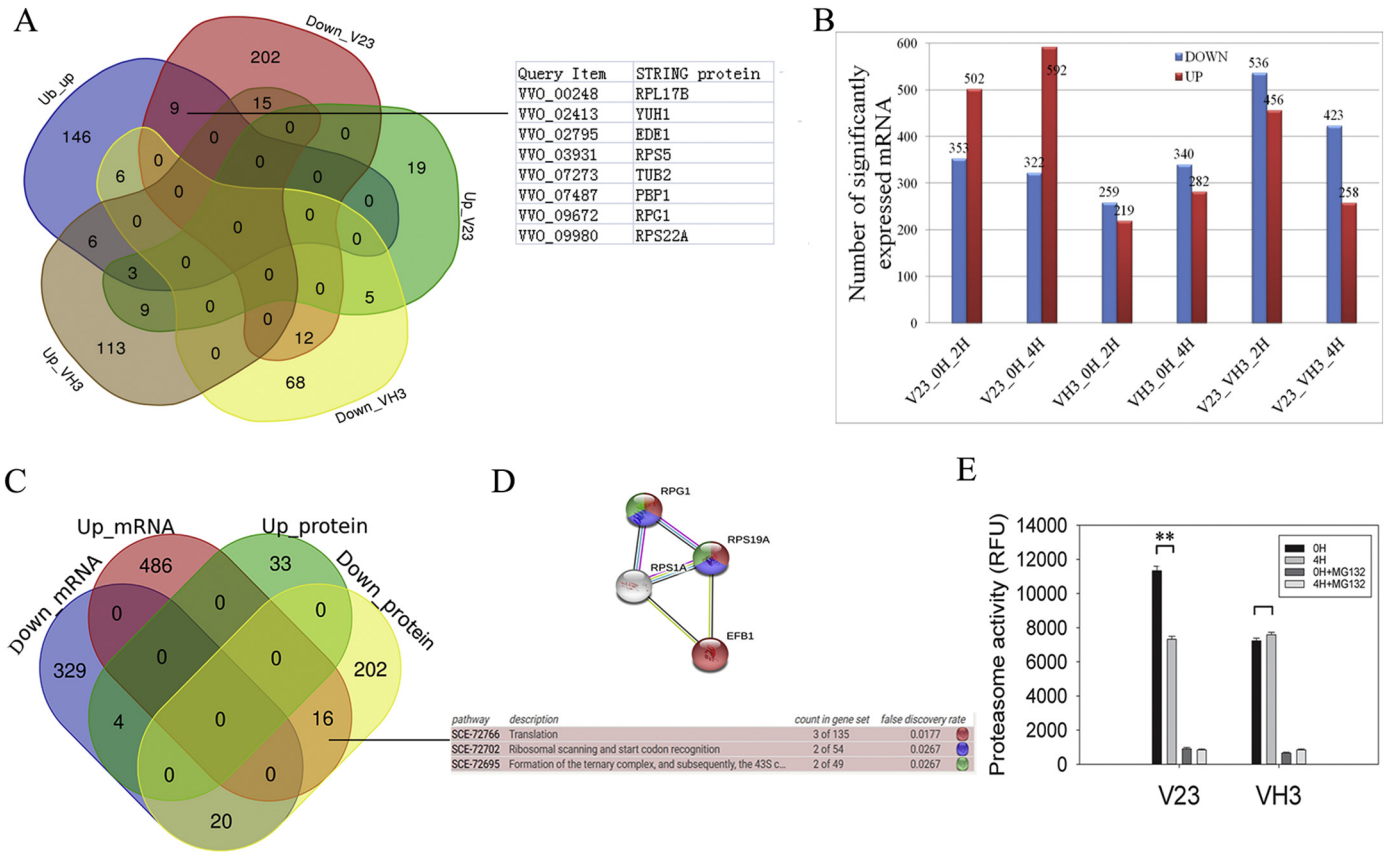

Fig. 3. Integrative omics analysis of *V. volvacea* after CST. (A) Venn analysis of the significantly upregulated ubiquitinated (Ub_up) proteins, the significantly differentially expressed (upregulated and downregulated) proteins in V23 and VH3. (B) Statistics on the number of significantly differentially expressed mRNAs. (C) Venn analysis of significantly differentially expressed mRNA of V23-2 h / V23-0 h and significantly differentially expressed proteins of V23-4 h / V23-0 h. (D) String analysis of 16 significantly upregulated genes. (E) Activity analysis of the 20S proteasome of *V. volvacea* after CST for 4 h. Activity analysis of the 20S proteasome in different genotypes using the Suc-LLVY-AMC substrate. RFU, relative fluorescence units. MG123 was used as an inhibitor of the 20S proteasome. Data represent the mean ± s.d. (n = 3). Significant differences were determined by Student's t-test for multiple pairwise comparisons. ** represents P < 0 .001.

selective degradation via the ubiquitin-26S proteasome system. These findings indicated that UBEV2 could function via ubiquitination for degradation.

Interestingly, ten proteins in the interacting proteins were found to be significantly ubiquitinated (Table S11), and four proteins in the interacting networks were found to be significantly ubiquitinated (Fig. 5D). These proteins are translation initiation factor eIF4A (TIF1), ribosomal protein L7a (RPL7A), ribosome-associated molecular chaperone (SSB2), and protein component of the large (60S) ribosomal subunit (RPL3), and all are involved in protein translation. These observations provided evidence that UBEV2 affected protein translation through ubiquitination of ribosomal proteins after CST. Co-IP experiments confirmed that UBEV2 bound a ribosome-associated molecular chaperone, SSB2 (Fig. 5E). The ubiquitinated SSB2 suggests that UBEV2 could weaken protein translation indirectly through binding SSB2 for ubiquitination.

3.4. Anti-freezing experiment of V. volvacea

An anti-freezing experiment showed that the egg-shaped fruiting body of *V. volvacea* soaked with the UBE2 inhibitor (e2i) was fuller in shape, brighter in color, and harder in hardness under CST, which was significantly better than that under the control treatment (Fig. 6). For example, the egg-shaped fruiting body of *V. volvacea* soaked by e2i after CST for 10 h maintained a full texture, bright color, and nonsticky surface (Fig. 6). For comparison, the egg-shaped fruiting body of *V. volvacea* under H_2O control treatment after CST for 10 h became soft, with a small part that was browning and sticky. This result indicated that the UBE2 inhibitor could inhibit the activity of UBEV2 and thus indirectly improve the cryogenic autolysis of *V. volvacea*.

4. Discussion

The UPS plays a vital role in chilling stress response signaling pathways, such as the modulation of chilling stress response in *Oryza sativa* L. [11], cold activation of the UPS in protist *Trichomonas vaginalis* [12], negative regulation of chilling stress response in banana [13], and regulation of flowering during vernalization in *Arabidopsis* [14]. The ubiquitylome analysis suggested that the increased ubiquitination affects protein synthesis and degradation after CST (Fig. 1D and E). As ubiquitination is utilized as a degradation signal by the UPS and autophagy-lysosome system [33], and this suggested that CS triggers the two systems by ubiquitination. Proteomics analysis showed that CS downregulated the expression of proteins in *V. volvacea* V23 after CST. The downregulated expressed proteins were enriched in ubiquitin-mediated proteolysis (Table S12), suggesting that CS weakened the UPS in *V. volvacea*. Our results confirmed the decreased UPS degradation efficiency in V23 after CST (Fig. 3E). UPS and autophagy are responsible for approximately 80–90% and 10–20%, respectively, of cellular proteolysis [34–36]. Therefore, the downregulated protein expression may be mostly due to the inhibition of protein translation rather than UPS degradation. Previous studies reported that decreased efficiency of UPS degradation triggered autophagic degradation [33], which was also supported by the result that the formation of large autophagic vacuoles after CST at 8 h occurred after the decreased UPS degradation at 4 h (Fig. 3). These observations indicated that the

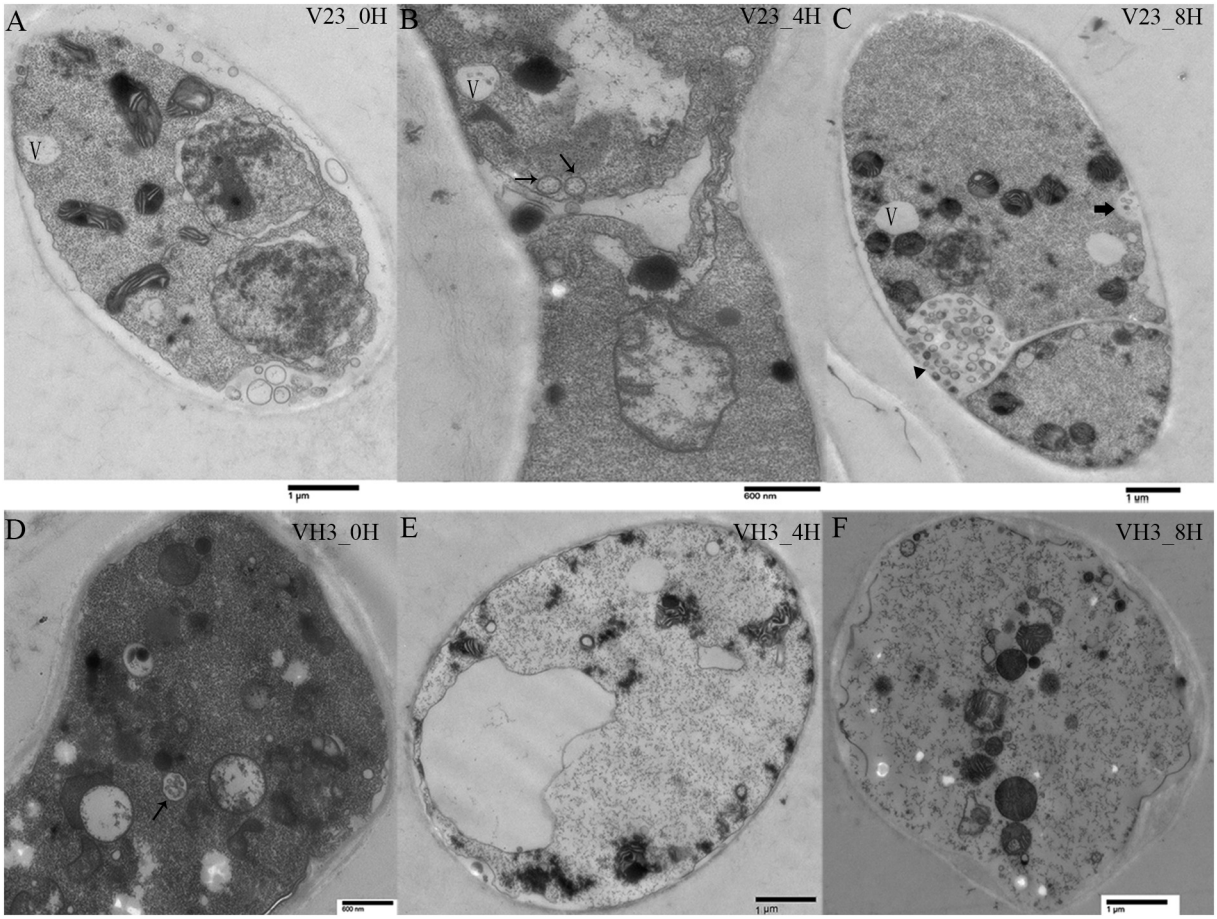

Fig. 4. Transmission electron microscopy images of ultrathin sections at the mycelium of *V. volvacea* V23 after CST for 0, 4, and 8 h (A, B, and C, respectively) and VH3 for 0, 4, and 8 h (D, E, and F). Phagosomes containing recycling components (Fig. 4B, arrows), plasma membrane blebbing (Fig. 4C, broad arrow), and large autophagic vacuoles (Fig. 4C, arrowhead) could be observed after CST. V = vacuole.

increased autophagic degradation might be responsible for the cellular constituents damaged by CS.

Previous studies reported that several UBE2 genes were involved in environmental stresses, and examples of this include a mung bean UBE2 that enhanced osmotic stress tolerance in *Arabidopsis* [37] and UBE2 genes from soybean or peanut that reacted to drought or salt stress in transgenic *Arabidopsis* [38–40]. Compared to many reports about the role of E3-ubiquitin ligase in the modulation of CS signaling [11,13,16,17], there are few reports about the role of UBE2 in the chilling stress response. Previous reports focused on the expression variation of UBE2 under cold pressure, and examples of this include the stable expression of UBE2 from both *Arabidopsis* and rice under chilling stress [39], differential expression of ZmUBCs under cold conditions [41] and the response of UBE2 genes to chilling stress in *Vitis vinifera* [42,43]. Some studies revealed expression divergence of the UBE2 gene family under multiple abiotic and phytohormone stresses in *Brassica rapa*, providing valuable clues for the critical role of UBE2 in development and multiple stress responses in *B. rapa* [44]. Our observations indicated that UBEV2 could function as a ubiquitination enzyme for degradation (Fig. 5). The increased UBEV2 protein levels might promote the modified cascade reaction and lead to the degradation of a large number of target proteins, such as ubiquitinated ribosomal proteins. Downregulated ribosomal proteins could damage protein translation, reduce the ability of cold stress resistance, and finally result in cryogenic autolysis. Our results indicated that UBEV2 played an essential role in facilitating the cryogenic autolysis of *V. volvacea*.

The chilling stress response of mammalian cells cultured in vitro showed that overall protein synthesis rates were gradually reduced to a temperature of 20 ˚C [45]. Additionally, the enhanced translation efficiency at low temperatures contributed to the cold adaptation in *Saccharomyces kudriavzevii* [46]. These previous data suggested that CS affects protein synthesis. Our data indicated that CS reduced protein translation by ubiquitination of the ribosomal protein. Co-IP experiments indicated that CS weakened protein translation by binding a ubiquitinated ribosome-associated molecular chaperone, SSB2. These observations indicated that CS triggered ubiquitination-mediated autolysis associated with a decrease in protein translation in *V. volvacea.*

In response to chilling stress, many proteins were molecular chaperones [45], and were also proteins that interacted with GST-UBEV2. Further detailed experiments revealed that the response to and recovery from CS was at least partially controlled by the modulation of p53, Grp75, and eIF3i levels [45]. Interestingly, a significantly ubiquitinated translation initiation factor, eIF4A (TIF1), was found to be a protein that interacted with UBEV2 (Fig. 5D and Table S11), implicating a role of TIF1 in regulating the chilling stress response. Several protein kinases found to interact with UBEV2 might transfer the produced signal and then trigger a series of response mechanisms.

Expression and interaction analyses of UBEV2 after CST suggested that the upregulated expression of UBEV2 could reduce protein translation of *V. volvacea* by ubiquitinating ribosomal proteins for degradation, finally resulting in cryogenic autolysis (Fig. 5). Previous studies showed that only UBEV2 was significantly upregulated among twenty-four UBE2 genes identified in the *V. volvacea* genome after CST [3]. Considering that UBEV2 has structural and functional sites similar to UBE2 [3], it was postulated that the UBE2 inhibitor could partially weaken the UBEV2-mediated ubiquitination process by inhibiting its

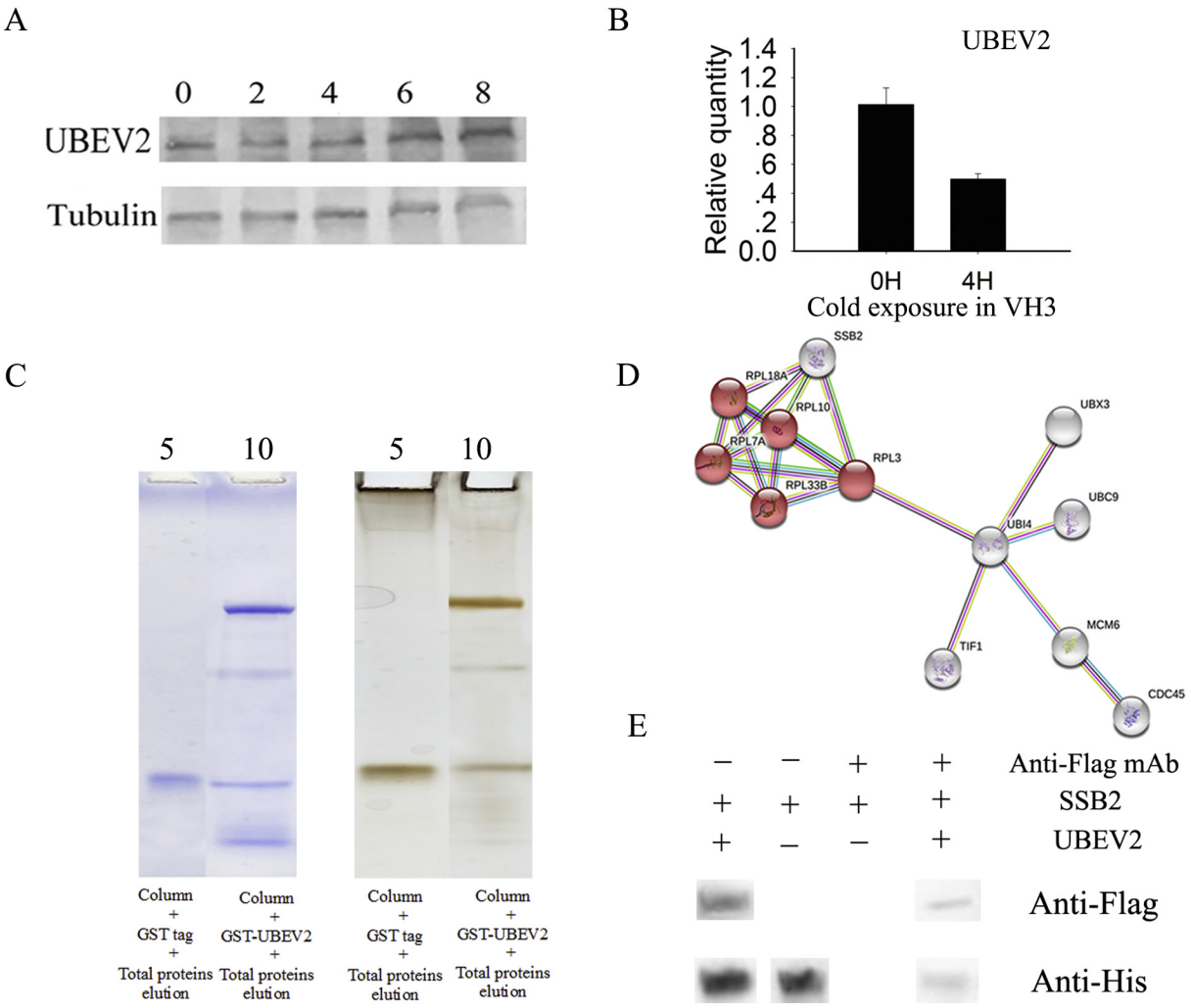

Fig. 5. Protein expression and interaction experiment of UBEV2 in *V. volvacea* after CST. (A) WB analysis of UBEV2 after CST in V23. (B) qPCR analysis of UBEV2 in *V. volvacea* VH3 after CST. Data represent the mean ± s.d. (n = 3). (C) GST pull-down results of GST-UBEV2 and total proteins in V23 after CST for 4 h were analyzed using SDS-PAGE and silver staining. Detailed results of GST pull-down are shown in Fig. S5. (D) Enrichment analysis of the interacting proteins of UBEV2. The minimum required interaction score is 0.7. (E) Co-IP analysis of Flag-UBEV2 and ubiquitinated ribosome-associated molecular chaperone SSB2.

upregulated expression after CST. Our anti-freezing experiment indicated that the UBE2 inhibitor could reduce the cryogenic autolysis of *V. volvacea* by inhibiting the activity of UBEV2 (Fig. 6).

Based on these observations, we proposed a putative UBEV2-mediated model highlighting the mechanistic role of UBEV2 in inhibiting protein translation by binding to a ribosome-associated molecular chaperone, SSB2, for ubiquitination (Fig. 7). This model demonstrated that UBEV2 might function with the ribosome for ubiquitination to inhibit protein translation after CST. Our proposed model displayed several putative UBEV2-mediated pathways in inhibiting protein synthesis, protein degradation, and signal regulation by ubiquitination (Fig. 7). These putative UBEV2-mediated pathways remain to be confirmed in future studies.

5. Conclusion

Our observations indicated that CS triggered ubiquitination-mediated autolysis associated with a decrease in protein translation in *V. volvacea*. UBEV2 might reduce protein translation by ubiquitinating ribosomal proteins. The proposed model highlights the mechanistic role of UBEV2 in facilitating the cryogenic autolysis of *V. volvacea*.

Author contributions

MG and YW designed research; MG, YW, ZPL, JNW, MJC, HW, JJS, SCZ, QT, and DPB performed research; MG analyzed data; MG, YW, and DPB wrote paper.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest with the contents of this article.

Acknowledgments

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://www.proteomexchange.org) via the PRIDE [47]. Project accession of proteomic of *V. volvacea* after CST is PXD013858. The data set of ubiquitylome analysis was uploaded in MS-Viewer. The search key for the saved data set is ybwd2vzzda. This study was sponsored by the Natural Science Foundation of Shanghai (Grant No.18ZR1416800) and Shanghai Agriculture Applied Technology Development Program (Grant No.2018-02-08-00-12-F01555), China.

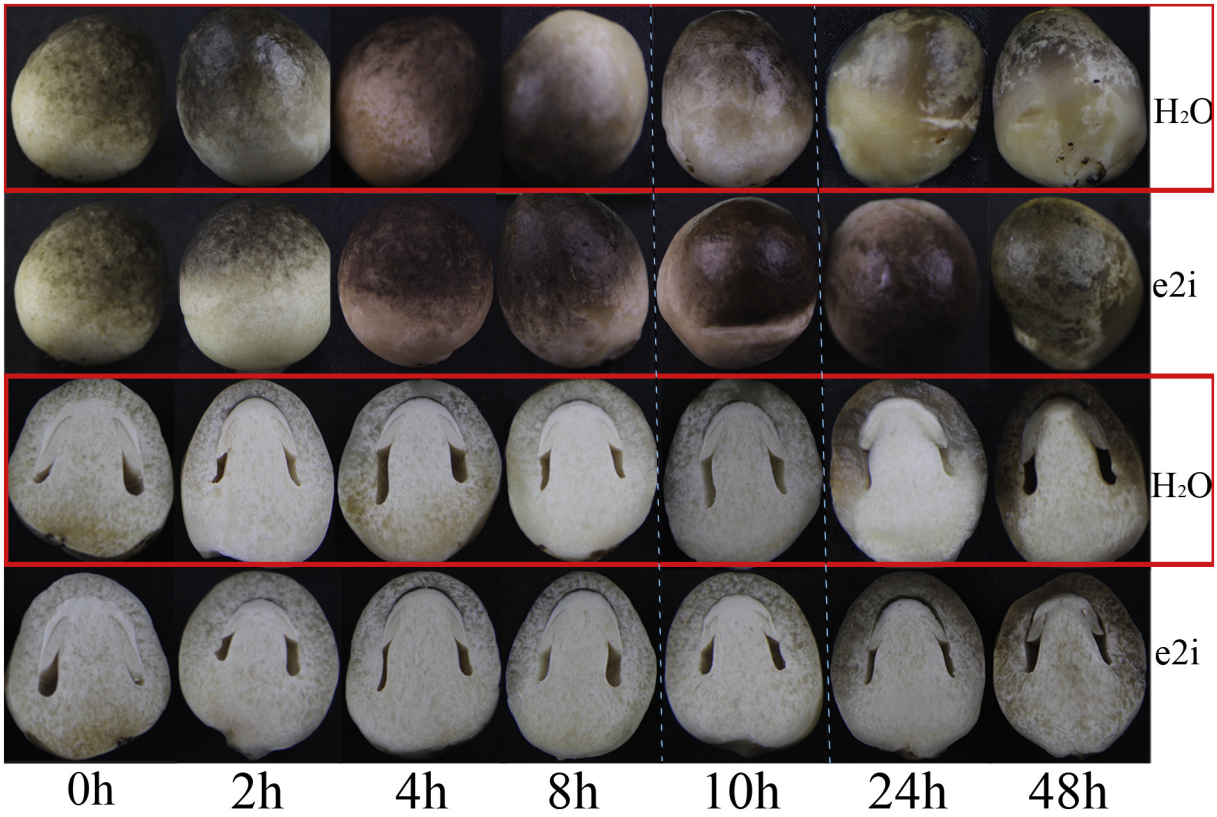

Fig. 6. Anti-freezing experiment of the fruiting body in *V. volvacea* V23. E2i represents the UBE2 inhibitor; H_2O represents the control treatment. Numbers represent hours for the CST. The blue dotted box represents CST for 10 h. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

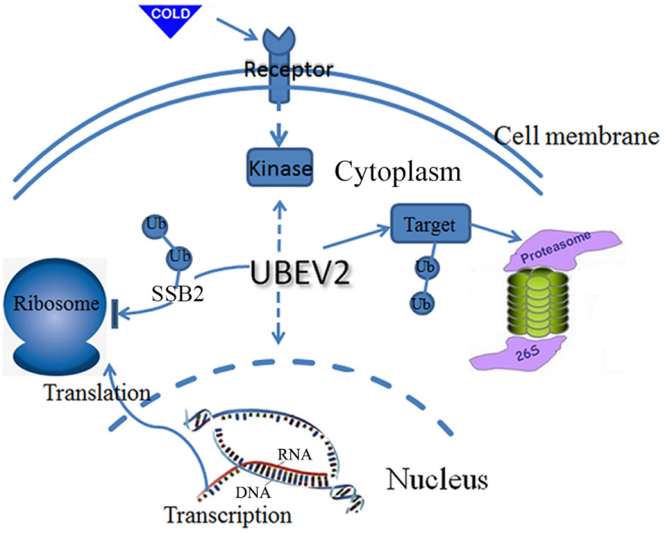

Fig. 7. The putative model in which UBEV2-mediated ubiquitination facilitates cryogenic autolysis. The model highlights the mechanistic role of UBEV2 in reducing protein translation through binding to SSB2, a ubiquitinated ribosome-associated molecular chaperone. UBEV2 might function with the ubiquitinated target protein, such as ribosomal proteins, for UPS degradation after CST. This model also displayed putative UBEV2-mediated pathways in regulating signal regulation by binding protein kinases after CST.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jprot.2020.103668.

References

[1] S. Chen, D. Ma, W. Ge, J.A. Buswell, Induction of laccase activity in the edible straw mushroom, Volvariella volvacea, FEMS Microbiol. Lett. 218 (1) (2003) 143–148.
[2] D. Bao, M. Gong, H. Zheng, M. Chen, L. Zhang, H. Wang, J. Jiang, L. Wu, Y. Zhu, G. Zhu, Y. Zhou, C. Li, S. Wang, Y. Zhao, G. Zhao, Q. Tan, Sequencing and comparative analysis of the straw mushroom (Volvariella volvacea) genome, PLoS One 8 (3) (2013) e58294.
[3] M. Gong, H. Wang, M. Chen, D. Bao, Q. Zhu, Q. Tan, A newly discovered ubiquitin-conjugating enzyme E2 correlated with the cryogenic autolysis of Volvariella volvacea, Gene 583 (1) (2016) 58–63.
[4] S.T. Chang, Volvariella volvacea, in: S.T. Chang, W.A. Hayes (Eds.), The Biology and Cultivation of Edible Mushrooms, Academic Press, London, UK, 1978, pp. 573–603.
[5] S.T. Chang, World production of cultivated edible and medicinal mushrooms in 1997 with particular emphasis on Lentinula edodes (Berk.) Sing. in China, Int. J. Med. Mushrooms 1 (1999) 291–300.
[6] D.M. Wenzel, K.E. Stoll, R.E. Klevit, E2s: structurally economical and functionally replete, Biochem. J. 433 (1) (2011) 31–42.
[7] M.H. Glickman, A. Ciechanover, The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction, Physiol. Rev. 82 (2) (2002) 373–428.
[8] J.D. Schnell, L. Hicke, Non-traditional functions of ubiquitin and ubiquitin-binding proteins, J. Biol. Chem. 278 (38) (2003) 35857–35860.
[9] W.J. Lyzenga, S.L. Stone, Abiotic stress tolerance mediated by protein ubiquitination, J. Exp. Bot. 63 (2) (2012) 599–616.
[10] M.D. Leach, A.J. Brown, Posttranslational modifications of proteins in the pathobiology of medically relevant fungi, Eukaryot. Cell 11 (2) (2012) 98–108.
[11] T. Lourenco, H. Sapeta, D.D. Figueiredo, M. Rodrigues, A. Cordeiro, I.A. Abreu, N.J. Saibo, M.M. Oliveira, Isolation and characterization of rice (*Oryza sativa* L.) E3-ubiquitin ligase OsHOS1 gene in the modulation of cold stress response, Plant Mol. Biol. 83 (4–5) (2013) 351–363.
[12] Y.K. Fang, K.Y. Huang, P.J. Huang, R. Lin, M. Chao, P. Tang, Gene-expression analysis of cold-stress response in the sexually transmitted protist trichomonas vaginalis, J. Microbiol. Immunol. Infect. 48 (6) (2015) 662–675.
[13] Z.Q. Fan, J.Y. Chen, J.F. Kuang, W.J. Lu, W. Shan, The banana fruit SINA ubiquitin ligase MaSINA1 regulates the stability of MaICE1 to be negatively involved in cold stress response, Front. Plant Sci. 8 (2017) 995.
[14] X. Hu, X. Kong, C. Wang, L. Ma, J. Zhao, J. Wei, X. Zhang, G.J. Loake, T. Zhang, J. Huang, Y. Yang, Proteasome-mediated degradation of FRIGIDA modulates flowering time in Arabidopsis during vernalization, Plant Cell 26 (12) (2014) 4763–4781.

[15] M.Y. Byun, L.H. Cui, T.K. Oh, Y.J. Jung, A. Lee, K.Y. Park, B.G. Kang, W.T. Kim, Homologous U-box E3 Ubiquitin Ligases OsPUB2 and OsPUB3 Are Involved in the Positive Regulation of Low Temperature Stress Response in Rice (*Oryza sativa* L.), Front. Plant Sci. 8 (2017) 16.
[16] H. Fang, Q. Meng, H. Zhang, J. Huang, Knock-down of a RING finger gene confers cold tolerance, Bioengineered 7 (1) (2016) 39–45.
[17] J.H. Jung, P.J. Seo, C.M. Park, The E3 ubiquitin ligase HOS1 regulates Arabidopsis flowering by mediating CONSTANS degradation under cold stress, J. Biol. Chem. 287 (52) (2012) 43277–43287.
[18] M. Isasa, C. Suner, M. Diaz, P. Puig-Sarries, A. Zuin, A. Bichman, S.P. Gygi, E. Rebollo, B. Crosas, Cold temperature induces the reprogramming of proteolytic pathways in yeast, J. Biol. Chem. 291 (4) (2016) 1664–1675.
[19] Y. Han, H. Cao, M. Chen, Y. Pan, Selection for cold-resistent strain by complex mutagenesis of Volvariella volvacea and identification of the strain, Mycosystema 23 (3) (2004) 417–422.
[20] D. Lu, M. Pava-Ripoll, Z. Li, C. Wang, Insecticidal evaluation of Beauveria bassiana engineered to express a scorpion neurotoxin and a cuticle degrading protease, Appl. Microbiol. Biotechnol. 81 (3) (2008) 515–522.
[21] S. Tyanova, T. Temu, J. Cox, The MaxQuant computational platform for mass spectrometry-based shotgun proteomics, Nat. Protoc. 11 (12) (2016) 2301–2319.
[22] Y. Moriya, M. Itoh, S. Okuda, A.C. Yoshizawa, M. Kanehisa, KAAS: an automatic genome annotation and pathway reconstruction server, Nucleic Acids Res. 35 (Web Server issue) (2007) W182–W185.
[23] D. Szklarczyk, A.L. Gable, D. Lyon, A. Junge, S. Wyder, J. Huerta-Cepas, M. Simonovic, N.T. Doncheva, J.H. Morris, P. Bork, L.J. Jensen, C.V. Mering, STRING v11: protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets, Nucleic Acids Res. 47 (D1) (2019) D607–D613.
[24] J.R. Wisniewski, A. Zougman, N. Nagaraj, M. Mann, Universal sample preparation method for proteome analysis, Nat. Methods 6 (5) (2009) 359–362.
[25] P. Ng, C.L. Wei, W.K. Sung, K.P. Chiu, L. Lipovich, C.C. Ang, S. Gupta, A. Shahab, A. Ridwan, C.H. Wong, E.T. Liu, Y. Ruan, Gene identification signature (GIS) analysis for transcriptome characterization and genome annotation, Nat. Methods 2 (2) (2005) 105–111.
[26] L. Wang, Z. Feng, X. Wang, X. Wang, X. Zhang, DEGseq: an R package for identifying differentially expressed genes from RNA-seq data, Bioinformatics (Oxford, Engl.) 26 (1) (2010) 136–138.
[27] P. He, Y. Cai, S. Liu, L. Han, L. Huang, W. Liu, Morphological and ultrastructural examination of senescence in Morchella elata, Micron 78 (2015) 79–84.
[28] J. Sambrook, D.W. Russell, Detection of protein-protein interactions using the GST fusion protein pulldown technique, Class. Protocols 2006 (1) (2006) 275–276.
[29] K. Chou, H. Shen, Cell-PLoc 2.0: an improved package of web-servers for predicting subcellular localization of proteins in various organisms, Nat. Sci. 2 (2010) 1090–1103.
[30] C. Lee, Coimmunoprecipitation assay, Methods Mol. Biol. 362 (2007) 401–406.
[31] S. Bhattacharyya, H. Yu, C. Mim, A. Matouschek, Regulated protein turnover: snapshots of the proteasome in action, Nat. Rev. Mol. Cell Biol. 15 (2) (2014) 122–133.
[32] A.M. Ruschak, T.L. Religa, S. Breuer, S. Witt, L.E. Kay, The proteasome antechamber maintains substrates in an unfolded state, Nature 467 (7317) (2010) 868–871.
[33] I. Dikic, Proteasomal and autophagic degradation systems, Annu. Rev. Biochem. 86 (2017) 193–224.
[34] A. Ciechanover, Y.T. Kwon, Degradation of misfolded proteins in neurodegenerative diseases: therapeutic targets and strategies, Exp. Mol. Med. 47 (2015) e147.
[35] A. Hershko, A. Ciechanover, The ubiquitin system, Annu. Rev. Biochem. 67 (1998) 425–479.
[36] K.L. Rock, C. Gramm, L. Rothstein, K. Clark, R. Stein, L. Dick, D. Hwang, A.L. Goldberg, Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules, Cell 78 (5) (1994) 761–771.
[37] E. Chung, C.W. Cho, H.A. So, J.S. Kang, Y.S. Chung, J.H. Lee, Overexpression of VrUBC1, a mung bean E2 ubiquitin-conjugating enzyme, enhances osmotic stress tolerance in arabidopsis, PLoS One 8 (6) (2013) e66056.
[38] G.A. Zhou, R.Z. Chang, L.J. Qiu, Overexpression of soybean ubiquitin-conjugating enzyme gene GmUBC2 confers enhanced drought and salt tolerance through modulating abiotic stress-responsive gene expression in Arabidopsis, Plant Mol. Biol. 72 (4–5) (2010) 357–367.
[39] X. Wan, A. Mo, S. Liu, L. Yang, L. Li, Constitutive expression of a peanut ubiquitin-conjugating enzyme gene in Arabidopsis confers improved water-stress tolerance through regulation of stress-responsive gene expression, J. Biosci. Bioeng. 111 (4) (2011) 478–484.
[40] C. Feng, L. Lijing, Z. Qingzhen, Z. Zhonghui, L. Qingliang, L. Baoying, W. Yaorong, T. Sanyuan, X. Qi, Arabidopsis ubiquitin conjugase UBC32 is an ERAD component that functions in brassinosteroid-mediated salt stress tolerance, Plant Cell 24 (1) (2012) 233–244.
[41] J. Dengwei, S. Xuelian, L. Shengqiao, D. Chen, Z. Qiufang, C. Hongliang, J. Liqiang, Genome-wide identification, phylogenetic and expression analyses of the ubiquitin-conjugating enzyme gene family in maize, PLoS One 10 (11) (2015) e0143488.
[42] Y. Gao, Y. Wang, H. Xin, S. Li, Z. Liang, Involvement of ubiquitin-conjugating enzyme (E2 gene family) in ripening process and response to cold and heat stress of Vitis vinifera, Sci. Rep. 7 (1) (2017) 13290.
[43] M.V. Poyurovsky, P. Christina, K. Alex, K.L.B. Borden, P. Zhen-Qiang, P. Nikola, P. Carol, The Mdm2 RING domain C-terminus is required for supramolecular assembly and ubiquitin ligase activity, EMBO J. 26 (1) (2014) 90–101.
[44] N. Khan, C.M. Hu, W. Amjad Khan, E. Naseri, H. Ke, D. Huijie, X. Hou, Evolution and expression divergence of E2 gene family under multiple abiotic and phytohormones stresses in Brassica rapa, Biomed. Res. Int. 2018 (2018) 5206758.
[45] A. Roobol, M.J. Carden, R.J. Newsam, C.M. Smales, Biochemical insights into the mechanisms central to the response of mammalian cells to cold stress and subsequent rewarming, FEBS J. 276 (1) (2010) 286–302.
[46] J. Tronchoni, V. Medina, J.M. Guillamon, A. Querol, R. Perez-Torrado, Transcriptomics of cryophilic Saccharomyces kudriavzevii reveals the key role of gene translation efficiency in cold stress adaptations, BMC Genomics 15 (2014) 432.
[47] Y. Perez-Riverol, A. Csordas, J. Bai, M. Bernal-Llinares, S. Hewapathirana, D.J. Kundu, A. Inuganti, J. Griss, G. Mayer, M. Eisenacher, E. Perez, J. Uszkoreit, J. Pfeuffer, T. Sachsenberg, S. Yilmaz, S. Tiwary, J. Cox, E. Audain, M. Walzer, A.F. Jarnuczak, T. Ternent, A. Brazma, J.A. Vizcaino, The PRIDE database and related tools and resources in 2019: improving support for quantification data, Nucleic Acids Res. 47 (D1) (2019) D442–D450.