Eur J Plant Pathol (2024) 168:15–28
https://doi.org/10.1007/s10658-023-02724-w

Proteomic analysis of the responses to chelerythrine in the fungal pathogen *Ustilaginoidea virens*

Qing-Hui Wei · Xue-Feng Liu · Yang-Yang Chai · Ming Liang · Bao-Jiang Zheng · Min Zhao

Accepted: 29 June 2023 / Published online: 17 July 2023

Abstract Chelerythrine (CHE) is a natural benzophenanthridine alkaloid with broad pharmacological activities. Herein, we investigated the responses to CHE of the fungal pathogen *Ustilaginoidea virens* using a proteomics approach. At 7.5×10^{-3} mg/mL, the *U. virens* inhibition rate reached 56.1%, significantly more potent than another two isoquinoline alkaloids and the commercial fungicide validamycin. The disease control effect on plants in the field was > 50% after spraying with 500× liquid containing 20% CHE, significantly better than that of validamycin. A total of 4734 proteins were identified in CHE-treated *U. virens* using tandem mass tag (TMT)-based quantitative proteomics, and 823 were differentially expressed proteins (DEPs). Gene Ontology (GO) of DEPs yielded 107 biological process (BP) subcategories, 45 cellular component (CC) subcategories and 43 molecular function (MF) subcategories. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were employed, and seven proteins were selected for subsequent parallel reaction monitoring (PRM) analysis. The results indicate that CHE might lead to apoptosis, but further studies are necessary.

Keywords Chelerythrine · *Ustilaginoidea virens* · Antifungal activity · Apoptosis · Bioinformatics · TMT-based quantitative proteomics

Qing-Hui Wei, Xue-Feng Liu and Yang-Yang Chai contributed equally to this work.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10658-023-02724-w.

Q.-H. Wei · X.-F. Liu · Y.-Y. Chai · M. Liang · B.-J. Zheng (✉) · M. Zhao (✉)
Northeast Forestry University, No. 26, Hexing Road, Harbin 150040, China
e-mail: zbjnefu@126.com

M. Zhao
e-mail: 82191513@163.com

Q.-H. Wei
Institute of Plant Protection, Heilongjiang Academy of Agricultural Sciences, No. 368, Xuefu Road, Harbin 150086, China

Introduction

Rice is consumed worldwide and is a staple food in many countries. More than 750 million tons are typically produced annually worldwide (FAO, 2018). Due to rapid human population growth, rice diseases threaten global food security. *Ustilaginoidea virens* (*U. virens*) is a fungus that infects flowers and forms false smut balls in rice ears. Due to various factors such as the large-scale promotion of hybrid rice, excessive application of nitrogen fertilizer in rice fields, and climate change, the occurrence of rice false smut has become increasingly serious, and has become one of the main fungal diseases of rice in China. Rice false smut not only reduces rice production, but also produces toxins harmful to humans and livestock, directly

affecting the edible safety of rice (Huang et al., 2019; Li et al., 2013). Chemical fungicides are the most effective method for controlling crop diseases. However, their repeated use over a long period of time may result in pesticide resistance and accumulation of harmful residues in the environment (Marei et al., 2012). Therefore, there is an urgent need to develop environmentally compatible methods to control crop diseases (Zhao et al., 2019), and botanical pesticides are attractive in this regard (Wei et al., 2017).

Chelerythrine (CHE) is a benzo[c]phenanthridine alkaloid present in members of the Papaveraceae family with antitumor, antibiotic and anti-inflammatory effects (Lin et al., 2017; Pencikova et al., 2012). CHE exhibits broad antifungal activity and may have potential applications for biological control of fungal pathogens. CHE inhibits fungal growth of *Fusarium oxysporum* f. sp. melonis, *Verticillium dahlia* and *Vermicularia capsica* (Fan et al., 2010). Various pharmacological studies have reported inhibition of mitochondrial energy coupling and accumulation of reactive oxygen species (ROS), suggesting these compounds are potent inducers of apoptosis in a variety of cell lines (Fan et al., 2010; Kumar, 2014; Tang et al., 2017). However, proteins inhibited by CHE in *U. virens* have not been investigated.

In the present work, the effects of CHE on five species of rice phytopathogens were investigated *in vitro*, and the mitochondrial membrane potential (MMP) of *U. virens* was measured. Proteins differentially expressed in *U. virens* cells treated with CHE were identified using tandem mass tag (TMT)-based quantitative proteomics. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were employed to explore the molecular mechanisms of differentially expressed proteins (DEPs). Seven proteins were selected for parallel reaction monitoring (PRM) analysis. These findings lay a foundation for the targeted protein control of fungal diseases by botanical pesticides.

Materials and methods

Reagents, media and strains

We extracted CHE as described previously (Wei et al., 2017). Standard CHE samples (plant extract, ≥ 97% purity), chelidonidine (plant extract, ≥ 98% purity) and sanguinarine (plant extract, ≥ 98% purity) were purchased from Tong Tian Biotechnology Corp (Shenzhen, China). Validamycin was purchased from Shanghai Hu Zheng Biological Technology Co. Ltd. (Shanghai, China) and used as a reference fungicide. Dimethylsulphoxide (DMSO) was purchased from Tianjin Guang Fu Technology Development Co. Ltd (Tianjin, China). A JC-1 mitochondrial membrane potential assay kit was purchased from Beyotime Institute of Biotechnology Co. Ltd (Shanghai, China).

Magnaporthe oryzae and *Cochliobolus miyabeanus* strains were kindly provided by the Northeast Agricultural University (Harbin, China) and the Xinyang College of Agriculture and Forestry (Xinyang, China), respectively. *Nigrospora oryzae* and *Aspergillus ruber* strains used in this study were preserved strains from the Northeast Forestry University Microbial Preservation Center (Harbin, China). *U. virens* strains were kindly provided by the Liaoning Academy of Agricultural Sciences (Shenyang, China).

The *M. oryzae*, *C. miyabeanus*, *N. oryzae* and *A. ruber* strains were incubated in potato dextrose agar (PDA) medium. *U. virens* strains were cultured in potato sucrose agar (PSA) or potato sucrose (PS) medium. All fungi were cultured at 28 °C.

Antifungal activity *in vitro*

Antifungal activity of CHE was examined by testing its effects on the growth of five fungal species using previously described methods (Fan et al., 2010; Wei et al., 2017; Yang et al., 2015). In brief, the CHE solution (5×10^{-3}, 7.5×10^{-3} and 1×10^{-2} mg/mL) was added to sterile PSA (or PDA) medium to obtain drug-containing medium, and medium without CHE served as a control. Each treatment was repeated three times. After culturing the fungi for 7 days at 28 °C, the antifungal activity of CHE was assessed by measuring colony diameters (cm) and calculating the mean diameter. The rate of fungal growth inhibition was calculated using a formula as described previously (Egan et al., 2016):

$$\text{Inhibition rate}(\%) = \frac{[(\text{dc} - 0.8) - (\text{ds} - 0.8)]}{(\text{dc} - 0.8)} \times 100$$

where 0.8 is the diameter of the phytopathogenic fungi disk, and dc and ds are the diameters of fungal colonies in control and CHE-treated groups.

The antifungal effects of CHE and another two alkaloids (sanguinarine and chelidonidine) on *U. virens* were compared with the commercial fungicide validamycin as a positive control. The drug concentration in all drug-containing PSA media was 7.5×10^{-3} mg/mL.

Evaluation of chelerythrine against *U. virens* in field experiments

A pesticide solution containing 20% CHE was prepared. Limin Pesticide Factory (Harbin, China) was commissioned to prepare the dispersible oil pesticide suspension with CHE as the active component. The formula was 20% CHE as the active ingredient, 15% methyloleate emulsifier (SP-OF3462), 2% silica, 2% organic bentonite, and methyl oleate to 100%. Quality control indices were pH 5 − 7, suspension rate > 95%, 12 mL after one min of continuous foaming, 99% fineness, qualified dispersibility, qualified stability at low temperature, and dumping property = 0.9 mL.

Four groups were set up; a water control group CK (without drug), drug group A (1000× liquid, 750 mL/ha dosage of suspension agent (12 mL/ha active ingredient concentration)), drug group B (500× liquid, 1500 mL/ha dosage of suspension agent (24 mL/ha active ingredient concentration)), and drug group C (validamycin positive control). The experiment was repeated three times. Each group covered an area of 60 m^2, and each block was interleaved, with a ridge built between each block.

About 7 days before the rupture period of rice, the first spraying was performed, and the second spraying was carried out after 10 days. The effect of control was investigated at the milk-ripe stage. Each experimental group was investigated using the five-point sampling method, with five clumps investigated at each point, and 25 clumps of rice investigated for each treatment. The disease index of rice false smut was calculated, and the prevention and treatment effects were determined. Reference to the agricultural industry standard of the People's Republic of China "Guidelines for Pesticide Field Efficacy Trials" NY/T 1464.54 -Part 54 of 2014. The disease index was calculated using the new classification standard (Per panicle): grade 0, no disease; Grade 1, 1 false smut ball; Grade 3, 2 false smut balls; Grade 5, 3 − 5 false smut balls; Grade 7, 6 − 9 false smut balls; Grade 9, more than 10 false smut balls (Wang et al., 2023). The specific calculation formula was as follows:

$$\text{Disease index} = \frac{\Sigma\ (\text{various disease strains} \times \text{corresponding level number})}{(\text{total number of strains} \times 9)} \times 100$$

$$\text{Control effect}(\%) = \frac{\text{disease index of control area} - \text{disease index of treatment area}}{\text{disease index of control area}} \times 100$$

Measuring MMP

A PS medium with mycelium of *U. virens* was centrifuged at 8000 g for 10 min to remove the supernatant. Mycelium was mixed with liquid nitrogen and ground until homogeneous. Mitochondria extraction was carried out as previously described (Yakovlev et al., 2002). Treated groups were incubated with CHE (5×10^{-3} mg/mL, 7.5×10^{-3} mg/mL, 1.5×10^{-2} mg/mL). A JC-1 kit was employed and CCCP treatment served as a positive control for MMP (Jiang et al., 2018). The effect on the MMP of *U. virens* was analysed by flow cytometry with an excitation wavelength of 488 nm, and fluorescence intensity was detected by fluorescence channels FL1 (green) and FL2 (red).

Protein preparation and TMT labelling

U. virens treatment groups were cultivated in PS medium at 28 °C for 10 days followed by incubation with 7.5×10^{-3} mg/mL of CHE for 24 h. No CHE was added to control groups. Samples were centrifuged,

supernatants were discarded, and pellets were resuspended in Lysis Buffer (Roche Ltd. Basel, Switzerland) containing 1% sodium dodecyl sulphate (SDS), 8 M urea, and 1 × Protease Inhibitor Cocktail (Sigma Ltd. USA). Samples were agitated, ground in liquid nitrogen three times for 7 min each time, incubated on ice for 30 min, and the precipitate was removed by centrifugation at 15,000 g for 15 min at 4 °C.

The protein concentrations in the supernatant was determined using a BCA protein assay kit (GlpBio, Montclair,CA, USA). Protein (100 µg) was adjusted to 100 µL with urea (8 mol/L), 2 µL of tris(2-carboxyethyl)phosphine (TCEP, AmyJet Scientific Inc, Wuhan, China) (0.5 M) was added and incubated at 37 °C for 1 h, followed by 4 µL of iodoacetamide (1 M). Samples were incubated at 25 °C for 40 min in the dark. Subsequently, five volumes of pre-chilled acetone (-20 °C) was added and incubated overnight. Samples were then centrifuged at 12,000 rpm for 20 min at 4 °C and supernatants were removed. After washing twice with pre-chilled acetone solution (90%), samples were dried at room temperature until any acetone on the surface was completely volatilised. Finally, the samples were dissolved in 100 µL of Triethylammonium bicarbonate buffer (TEAB, Hefei TNJ Chemical Industry Co., Ltd., China) (100 mM) and digested with trypsin (Promega, Madison, WI) at an enzyme:protein mass ratio of 1:50 at 37 °C overnight. Next, C18 Zip-Tips were used to desalt the peptide mixture, which was subsequently quantified by Pierce Quantitative Colorimetric Peptide Assay (23,275). TMT labelling was performed using a TMT-10plex Isobaric Mass Tag Labeling Kit (Thermo Fisher Scientific, MA, USA) (Liu, 2018).

High pH reversed-phase separation and nano-high performance liquid chromatography tandem mass spectrometry (HPLC–MS/MS) analysis

Peptides were separated on a C18 column (4.6 mm × 250 mm, 5 µm, Waters Corporation, MA, USA) attached to an Ultimate 3000 System (Thermo Fisher scientific, MA, USA). The peptide mixture was dissolved in buffer A (20 mM $HCOONH_4$ solution, pH 10.0). Peptides were eluted at a flow rate of 1 mL/min with a linear gradient of 5% to 45% buffer B (20 mM $HCOONH_4$ in 80% acetonitrile, pH 10.0) over 40 min (Baghdady and Schug, 2019).

A total of 12 vacuum-dried fractions, which were analysed by on-line nanospray LC–MS/MS using an Orbitrap Fusion (Thermo Fisher Scientific) coupled to a nanoACQUITY UPLC (Waters Corporation) equipped with a C18 column (75 µm × 25 cm, Waters nano Ease M/Z HSS), were dissolved in solvent A (0.1% HCOOH aqueous solution). Injections were 4 µL and the gradient run was from 4 to 32% buffer B (0.1% HCOOH-acetonitrile) over 1.5 h with a flow rate at 400 nL/min (Xiao et al., 2019).

Validation by PRM

Peptides were re-dissolved in solvent A (0.1% formic acid in water) and analysed by on-line nanospray LC–MS/MS using an Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific) coupled to an EASY-nanoLC 1200 system (Thermo Fisher Scientific). Peptide (9 µL) was loaded onto an Acclaim PepMap C18 analytical column (75 µm × 25 cm) and separated over a 120 min gradient. The column flow rate was maintained at 400 nL/min with a column temperature of 40 °C. The electrospray voltage was 2 kV versus the inlet of the mass spectrometer. Data-independent acquisition mode was employed for the main spectra, and automatically switched between MS and MS/MS mode (Zhang et al., 2022).

Bioinformatics analysis

Peaks Studio (Bioinformatics Solutions Inc., Waterloo, Canada) data base was employed with trypsin as the digestive enzyme to explore the *U. virens* databases (8666 entries). Peptides were identified using a 1% false discovery rate (FDR) and at least one unique peptide. Analysis of variance (ANOVA) was carried out for statistical analysis. The abundance of all peptides was averaged and results are presented as medians. DEPs were considered when fold-change (FC) > 1.5 and there were at least two unique peptides with significance > 13 ($p < 0.05$).

PRM data were analysed using the default parameters of SpectroDive 10.0, and the q-value of peptide identification reliability was ≤ 0.01. Protein expression was normalised against the total ion current for each sample extracted by Peaks Studio.

Table 1 Inhibitory effect of CHE extract on 5 kinds of fungal pathogens of rice grown on specific media

Pathogens	5×10^{-3} mg•mL^{-1}	7.5×10^{-3} mg•mL^{-1}	1×10^{-2} mg•mL^{-1}
U.virens	25.2% ± 0.01[a]	56.1% ± 0.013[b]	69.2% ± 0.02[c]
A.ruber	28.9% ± 0.015[a]	32.8% ± 0.018[b]	36.8% ± 0.02[c]
M.oryzae	12% ± 0.016[a]	17.3% ± 0.025[b]	26.7% ± 0.02[c]
N.oryzae	59.4% ± 0.01[a]	73.7% ± 0.019[b]	80.5% ± 0.01[c]
C.miyabeanus	34.7% ± 0.027[a]	55.3% ± 0.022[b]	58.8% ± 0.01[c]

Means ± SD of three independent experiments, followed by the different letters within a row are significantly different according to ANOVA test with $P \leq 0.05$

Statistical analysis

All assays were carried out using biological triplicates. Results are expressed as means ± standard error of the mean (SEM). All statistical analyses were performed with SPSS 19.0 software (Chicago, IL, USA). Statistically significant differences were tested using t-tests and ANOVA ($p < 0.05$).

Results

Antifungal effects of CHE and other isoquinoline alkaloids on fungal growth

CHE exhibited significant antifungal activity against five fungal pathogens of rice (Table 1). With increasing CHE concentration, inhibition was significantly increased. At 7.5×10^{-3} mg/mL, the inhibition rate was > 50% against *U. virens, N. oryzae* and *C. miyabeanus.* When the concentration was 1×10^{-2} mg/mL, inhibition rates were 69.2% against *U. virens*, 26.7% against *M. oryzae* and 36.8% against *A. ruber*. Compared with chelidonidine, sanguinarine and validamycin (7.5×10^{-3} mg/mL), inhibition of the mycelia of *U. virens* was greater with CHE. The inhibition rate of validamycin (28.7%) was only half that of CHE (Fig. 1). Overall, CHE exhibited significant inhibition at a low dosage of 7.5×10^{-3} mg/mL.

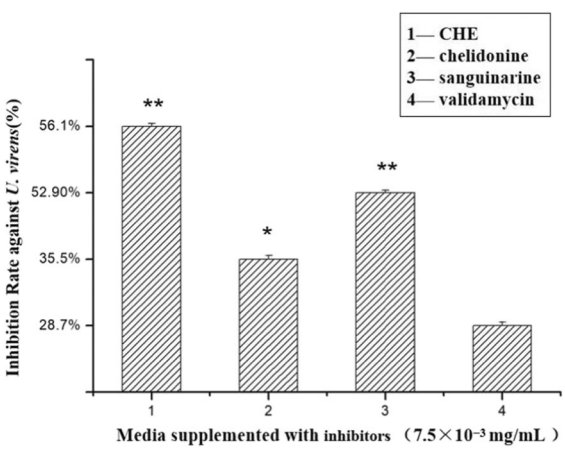

Fig. 1 Inhibition of mycelium of *U.virens* by CHE, chelidonine, sanguinarine and validamycin at the concentration of 7.5×10^{-3} mg/mL. Values are means ± SD (n = 3), $*p < 0.05$, $**p < 0.01$.

Field control effect of *U. virens*

After spraying twice in the rice field, the CK disease index of the control group was 2.12. For group A (1000 × liquid) the disease index was 1.68, and the prevention and treatment effect was 20.75%. The disease index for group B (500 × liquid) was 0.99, and the prevention and treatment effect was 53.30%. The

Table 2 Field control of *U. virens* with plant fungicide

Treatment	Disease index	control effect(%)
CK	2.12	—
A	1.68	20.75 ± 0.12
B	0.99	53.30 ± 0.52[a]
C	1.05	50.47 ± 0.23[b]

Means ± SD of three independent experiments, followed by the different letters within a column are significantly different according to ANOVA test with $P \leq 0.05$. A, group A (1000 × liquid), 750 mL/ha dosage of suspension agent (12 mL/ha active ingredient concentration). B, group B (500 × liquid), 1500 mL/ha dosage of suspension agent (24 mL/ha active ingredient concentration). C, group C (validamycin)

disease index for group C (validamycin) was 1.05, and the prevention and treatment effect was 50.47%. Based on these indices, all fungicides exerted a control effect on rice false smut in the field (a complex and changeable environment), and the control effect after spraying with 500× insecticide liquid was > 50%, better than for the commercial fungicide validamycin (Table 2).

Effect of CHE on the MMP of *U. virens*

MMP is generated due to the electrochemical gradient of protons across the inner mitochondrial membrane, which is accompanied by proton movement during mitochondrial electron transport. Normal MMP is a prerequisite for maintaining oxidative phosphorylation of mitochondria and producing ATP, and the stability of MMP is conducive to maintaining normal physiological functions of cells. In recent years, it has been found that the apoptosis of various cells is accompanied by the decrease of MMP under the action of different factors (Jia et al., 2022). Flow cytometry results showed that the proportion of damaged cells was increased by 31% with increasing CHE concentration (5×10^{-3} to 7.5×10^{-3} mg/mL). However, damage was not worse when the concentration was 1.5×10^{-2} mg/mL (Fig. 2), maybe apoptosis has maxed out. This result implies a decrease in MMP, a hallmark of apoptosis.

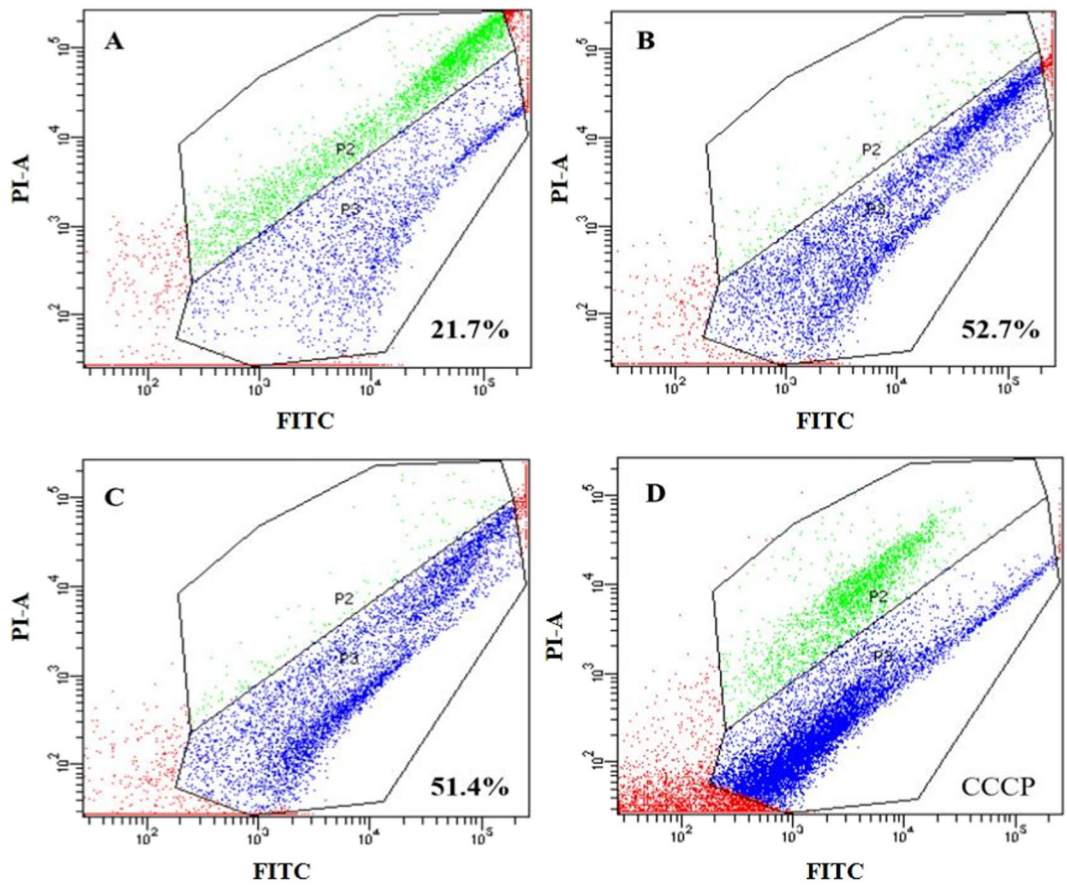

Fig. 2 Effect of CHE on the MMP of *U. virens*. A.5×10^{-3} mg/mL; B.7.5×10^{-3} mg/mL; C. 1.5×10^{-2} mg/mL; D. Positive control. Phylum P2 represents cells with high mitochondrial membrane potential (normal cells), and phylum P3 represents a population with low mitochondrial membrane potential (damaged or apoptotic cells). FITC is fluorescence intensity. With the increase of drug concentration, the proportion of cells in P3 phylum increased significantly, that is, the proportion of cell damage increased. Thus, the higher the drug concentration, the more obvious the cell damage

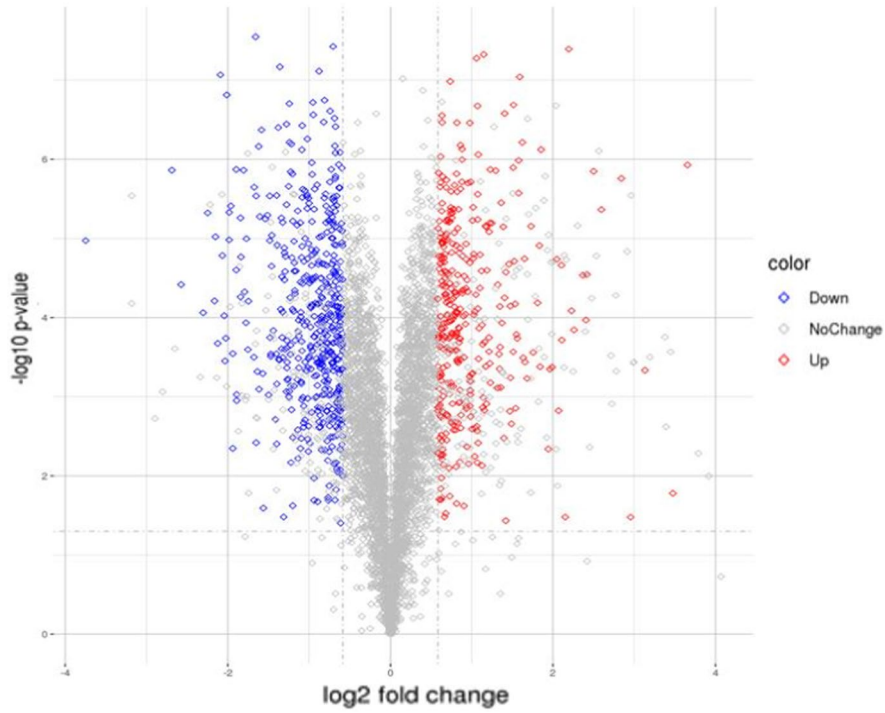

Fig. 3 Differentially expressed genes analyzed. Volcano plot for comparison between CHE treatment and control. The percentage of up-regulated and down-regulated genes for each group of genes shown. Red clusters indicate up-regulation; blue clusters indicate down-regulation

Proteomic analysis of the response of *U. virens* response to CHE

Statistical variation of DEPs was assessed by Fisher's exact test in BLAST2GO (Conesa et al., 2005; Klopfenstein et al., 2018). Through KEGG pathway analysis, functional information of proteins related to metabolic processes was obtained and specific biological pathways were predicted. A total of 4734 proteins were identified, including 823 significant DEPs with $p < 0.05$ and FC > 1.5 (346 upregulated and 477 downregulated). DEPs are listed in Fig. 3.

As shown in the GO enrichment analysis (Fig. 4), all DEPs were divided into cell component (CC), molecular function (MF) and biological process (BP) categories. GO analysis of DEPs classified 107 subcategories for BP, 45 subcategories for CC and 43 subcategories for MF. The top 20 categories in BP, CC and MF are shown in Fig. 4A.

The top 20 BP terms included nine subcategories, with metabolic process and cellular process accounting for the highest percentage, followed by cellular metabolic process (Fig. 4B). The top 20 CC terms included seven subcategories, with membrane part accounting for the highest percentage, followed by cell part, intracellular part, and protein-containing complex (Fig. 4C). These findings indicate that changes in cell membrane and cytoplasmic composition were consistent with our SEM and TEM results (Wei et al., 2020). The top 20 MF terms included eight subcategories, with many linked to catalytic activity (Fig. 4D). Overall, these proteins were mainly involved in metabolism and genetic information processing.

A total of 91 KEGG enrichment pathways were identified, of which metabolic pathways included 90 proteins and biosynthesis of secondary metabolites included 39 proteins. Two specific pathways ($p < 0.05$) were classified (Fig. 5A), including metabolism (A) and genetic information processing (B).

The KEGG enrichment results were sorted by *p*-value ($p < 0.1$), and the number of proteins enriched in the first 20 items with the smallest *p*-value were selected. A pie chart was constructed to show the proportion of proteins for each item (Fig. 5B). Soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs), involved in vesicle docking and fusion, mediate endoplasmic reticulum (ER)-Golgi transport.

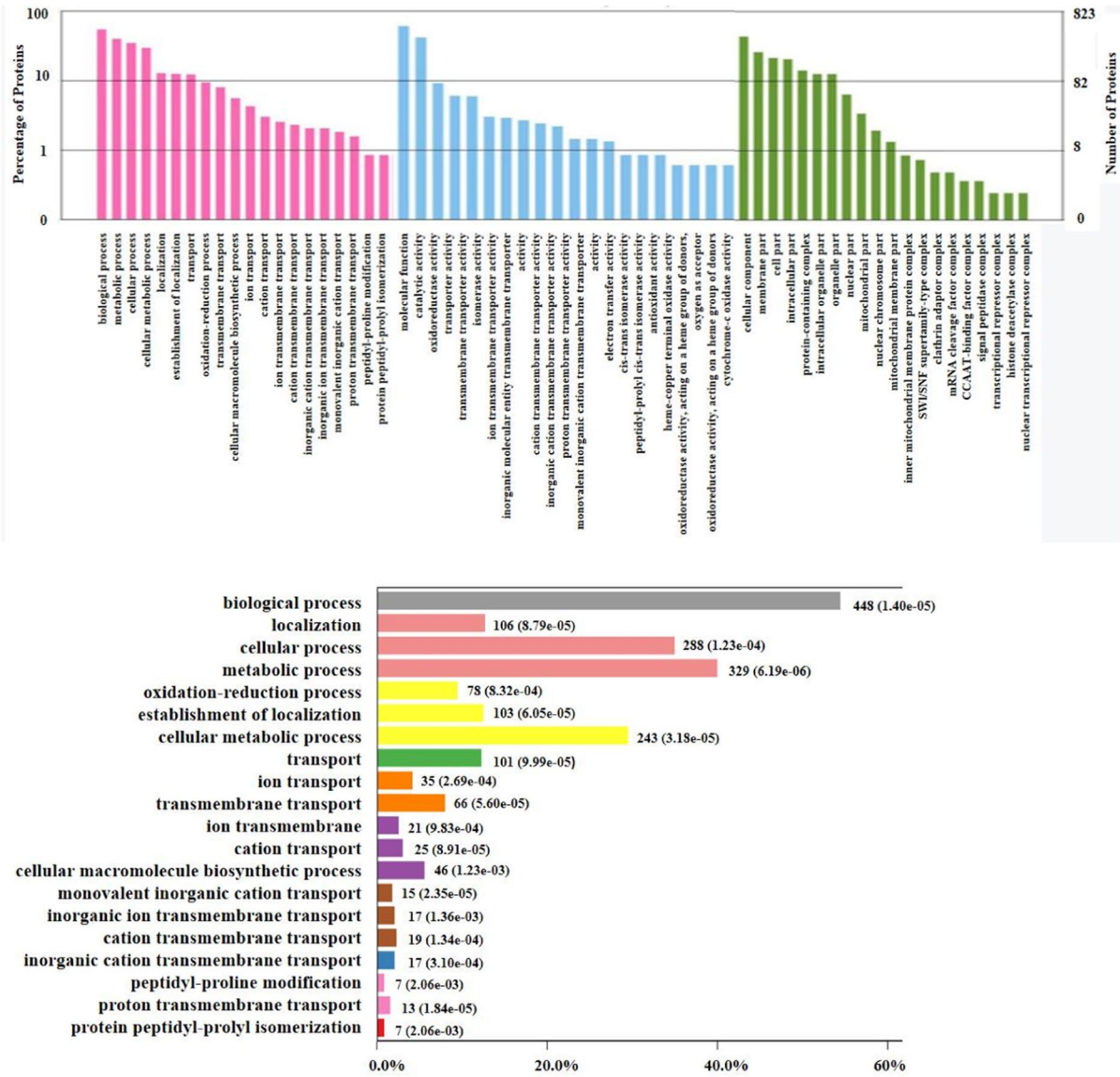

Fig. 4 GO of DEPs in control group and CHE-treatment group (Top 20). **A** GO enriched BP, CC and MF. **B** GO of BP. **C** GO of CC. **D** GO of MF

Oxidative phosphorylation accounted for 38% of the enriched pathways, and SNARE interactions and the vesicle transport pathway accounted for 10%.

Differences in the expression levels of seven proteins were significant, all of which are important for oxidative phosphorylation and SNARE interactions mediating vesicle transport pathways. FIP1 affects cell division and proliferation in response to different abiotic stresses, whereas acylcarrier protein (ACP1) has antioxidant properties (Téllez-Robledo et al., 2019). Cytochrome C oxidase (COX) is the terminal enzyme complex of the mitochondrial electron transport chain, which plays a very important regulatory role in oxidative phosphorylation (Belevich and Verkhovsky, 2008; Cheng and Zhuang, 2018). MAM2 is linked to vesicle-associated membrane protein 2, and BET1 is involved in membrane vesicle transport between the ER and the Golgi (Parlati et al., 2000). SED5 is a phosphoprotein and a syntaxin family member, and cycling

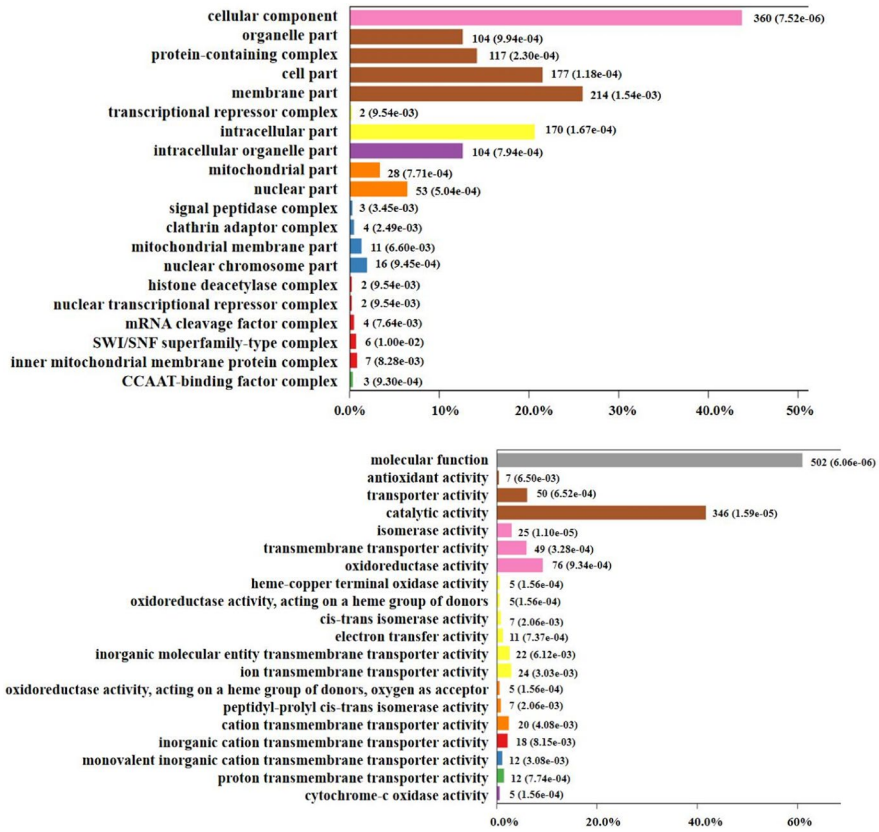

Fig. 4 (continued)

of SED5 phosphorylation and dephosphorylation is required for normal t-SNARE function, and may choreograph Golgi ordering and dispersal (Adina et al., 2005).

After treatment of *U. virens* with CHE, in oxidative phosphorylation, expression of ACP1, FIP1, COX6 and COX5B was downregulated. In the SNARE vesicle transport pathway, expression of MAM2, SED5 and BET1 was downregulated. The expression levels of the above genes were altered, which disrupts oxidative phosphorylation and the SNARE vesicle transport pathway (supplement Table 1).

Validation by PRM

Based on the TMT quantitative proteomic results, seven of 823 DEPs in normal and CHE-treated groups were selected for further validation. Expression levels of ACP1 (A0A063BUG2), FIP1 (A0A063C1A3), COX5B (A0A063BV13), COX6 (A0A063BS89), BET1 (A0A063BV15), MAM2 (A0A063BZR0) and SED5 (A0A063BQL8) proteins were measured via PRM (Fig. 6). Expression levels of all seven protein were decreased in CHE-treated *U. virens* cells. The PRM statistical analysis showed that the protein fold change ratios were consistent with the TMT proteomic results (Figs. 7 and 8).

Discussion

CHE exhibits antimicrobial activity and induces cell apoptosis

Chelidonidine has been widely studied in the medical field, and has been shown to noticeably inhibit the proliferation of KB cell lines is (Tao and Ran, 2018). Sanguinarine has a variety of pharmacological effects, such as antibacterial, anti-tumor, and insecticidal effects, and can affect livestock and poultry

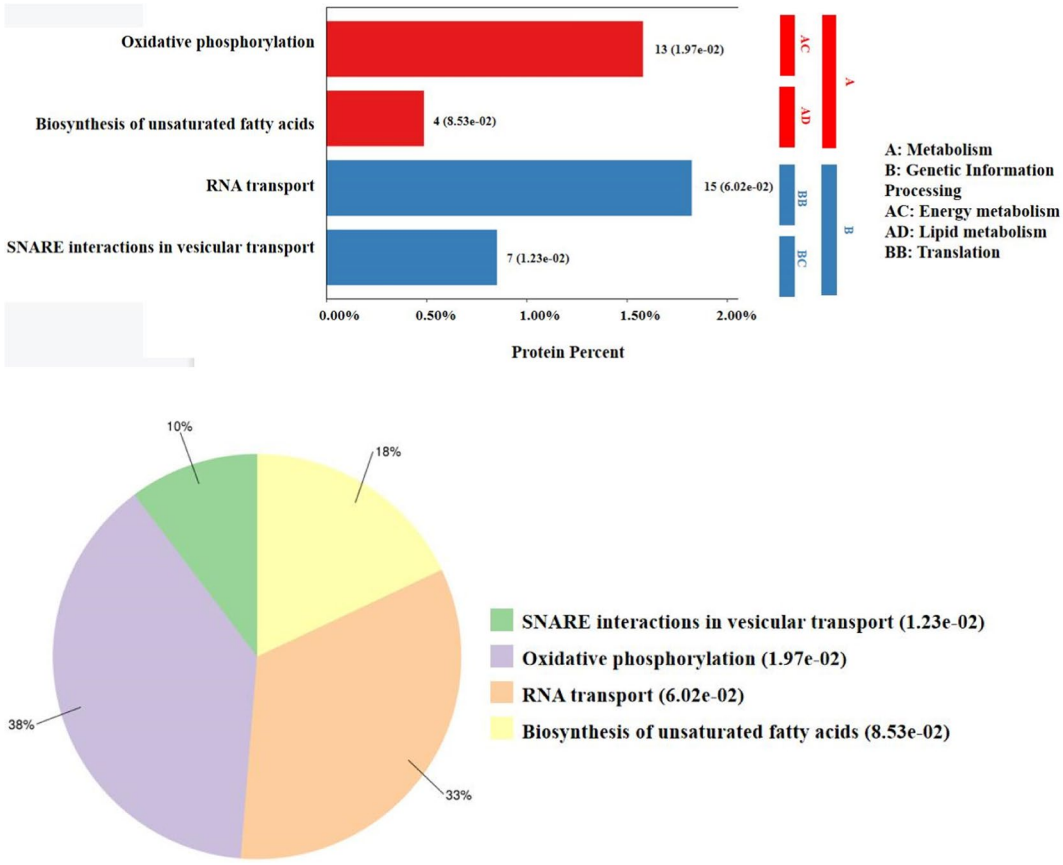

Fig. 5 **A** The significantly enriched KEGG pathways of DEPs, $*p < 0.05$. The ordinate on the left shows the specific metabolic pathway enriched. The ordinate on the right shows the abbreviation of the first and second classification of the metabolic pathway enriched. The number in brackets shows the p-value enriched. **B** The pie chart of DEPs

production performance (Zhu et al., 2020). In our previous studies, at a CHE concentration of 0.017 mg/mL, the inhibition rates of *Septoria microspora* Speg. Spores and *Curvularia lunata* were 96.67% and 84.94%, respectively (Wei et al., 2017). Compared with chelidonidine, sanguinarine and validamycin (7.5×10^{-3} mg/mL), CHE achieved more potent inhibition of the mycelia of *U. virens* in the present study. Thus, CHE possesses good antimicrobial activity and has great potential for controlling plant diseases.

The prevention and treatment of false smut of rice is mainly reliant on prevention, and this is mainly carried out using chemical agents. Hu et al. (2018) used 5% phenylecloazole/dioxime mycosamine for field control of rice false smut, with a prevention efficiency up to 79.89%. However, long-term, repeated use of chemicals can lead to drug resistance and high levels of residues in the environment. Biological control is an effective alternative for modern agriculture. Fu et al. (2019) conducted field efficacy tests of fushi and found that 2.5% stritrin and 15.0% polyantimycin wetting powder had a control effect of > 80.0%, similar to that of chemical controls. CHE, the active agent of a plant fungicide raw material from a pure natural plant extract, is an example of an environmentally friendly modern pesticide that can be applied in liquid dosage form. In the present study, 20% chelerythrine had a clear field control effect on rice false smut when spraying as a 500× liquid. If the spraying concentration is increased, the control effect is improved better, and this will be further investigated in the future. Further field trials will be carried out to compare common pesticides with *Chelidonium majus* active agents.

A decrease in MMP is an indicator of apoptosis. Flow cytometry results showed that the proportion of

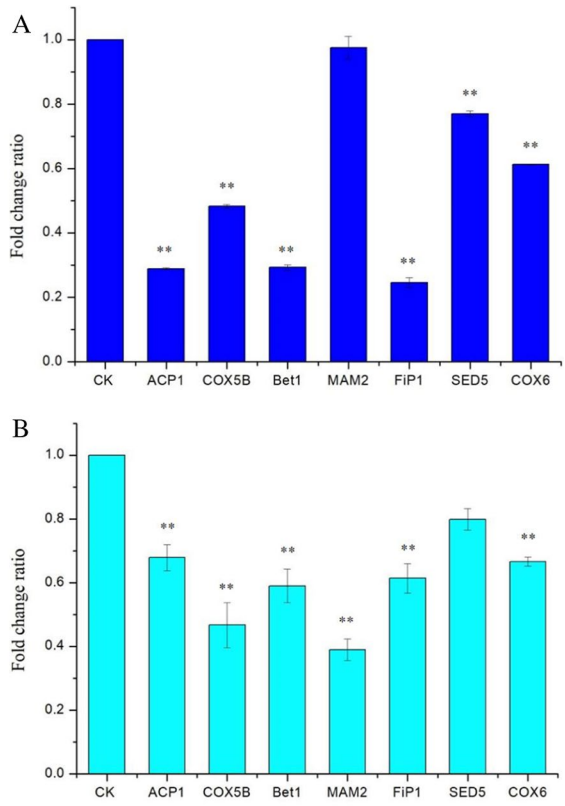

Fig. 6 Graphical representation of the average fold change ratio in expression of the proteins in *Ustilaginoidea virens* cells after CHE treatment. **A** The protein expression in CHE-treated *Ustilaginoidea virens* cells by TMT assay. **B** The protein expression in CHE-treated *Ustilaginoidea virens* cells by PRM assay. Values are means ± SD (n = 3), $^{**}p < 0.01$

damaged cells increased by 31% with increasing CHE concentration. Wei et al. (2020) reported that CHE induced ROS accumulation in of *U. virens*, which is a marker of apoptosis. The results of SEM and TEM demonstrated that CHE destroyed the morphology and structure of hyphae and spores of *U. virens* (Wei et al., 2020). These results imply that CHE induced cell apoptosis.

CHE targets for plant disease control

Expression levels of FIP1 was decreased following CHE treatment. FIP1 affects cell division and elongation, and cell responses to various abiotic stresses (Téllez-Robledo et al., 2019). Downregulation of FIP1 leads to impaired mRNA synthesis and apoptosis. Chen et al. (2017) reported that treatment with ACP1 inhibited the release of ROS in human liver HL-7702 cells, suggesting that ACP1 exhibits antioxidant activity. Downregulation of ACP1 observed in the present study suggests that ROS production was promoted rather than inhibited, leading to apoptosis. These findings suggest that FIP1 and ACP1 are potential targets for plant disease control.

COX plays an important role in mitochondria-mediated endogenous cell apoptosis pathway. The interaction between cytochrome c and cardiolipin in mitochondrial membrane space is the key to initiate apoptosis. Grant et al. (1997) found that COX6 is required for the function of the respiratory ETC, and strains lacking nuclear COX6 are sensitive to H_2O_2. In the present work, expression of COX6 was downregulated. Verena et al. (2019) found that quercetin, one of the main flavonols in pollen, led to changes in the expression of transcripts encoding enzymes related to oxidative phosphorylation, including downregulation of COX5B. Similarly, expression of COX5B was downregulated in the present study, and CHE exposure also altered the abundance of various relevant proteins, as demonstrated by consistent TMT and PRM results.

SNAREs are involved in vesicle docking and fusion in ER-Golgi transport. Vesicle-associated membrane proteins (VAMPs) were the first cargo proteins to be identified that are activity-dependent on bulk endocytosis, which is critical for the progression of this mode of endocytosis (Nicholson-Fish et al., 2015). Maintenance of the Golgi ribbon structure requires normal reverse transport from early endosomes to the trans-Golgi network (TGN), which may be mediated by the formation of VAMP-containing SNARE complexes (Shitara et al., 2013). MAM2 is linked to VAMP2, and Li et al (2019) found that VAMP2 silencing inhibited osteosarcoma cell proliferation, migration and invasion. In the present study, MAM2 was downregulated, hence it may be a potential target for disease control. BET1 is involved in membrane vesicle transport between the ER and Golgi body (Parlati et al., 2000). In our experiment, expression of MAM2 and BET1 was downregulated. CHE altered the expression of these genes in *U. virens* (supplement Table 1), was related to the SNARE vesicle transport pathway, and induced cell apoptosis. SED5 forms a SNARE complex with SEC22, BET1 and BOS1 to mediate the

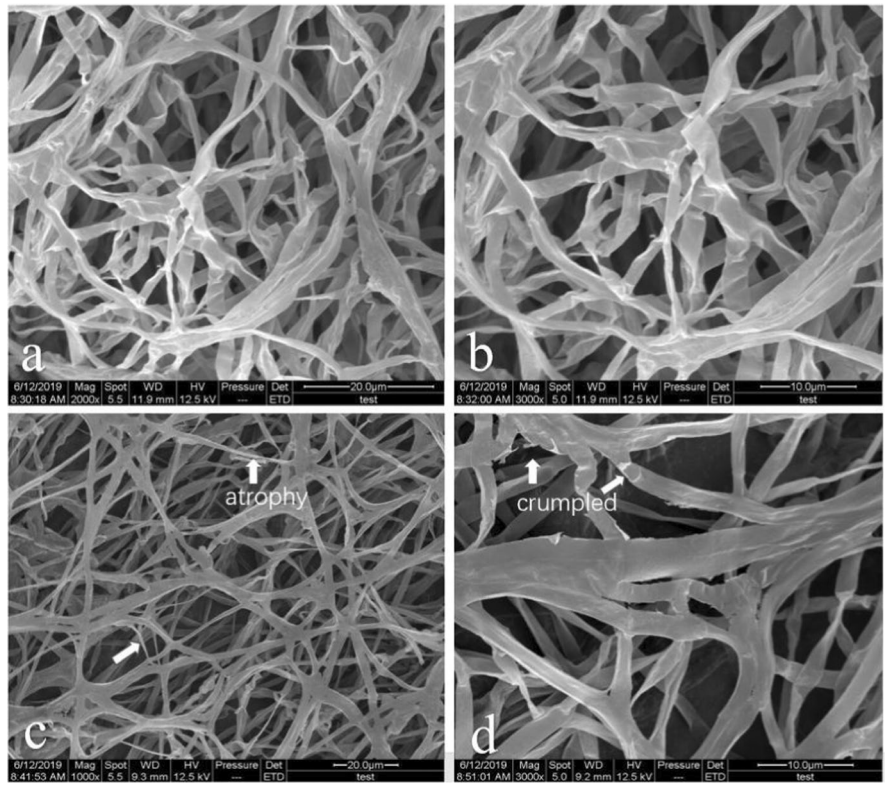

Fig. 7 Scanning electron micrographs of *Ustilaginoidea virens* mycelia. **a** and **b**, Control group. **c** and **d**, Group treated with 15.0 mg/L chelerythrine

fusion of vesicles with the Golgi apparatus (Zou et al., 2017). SED5 deficiency led to defects in the asymmetrical distribution of protein aggregates in yeast undergoing cytokinesis. TMT and PRM results in the present work revealed consistent changes in abundance following CHE treatment.

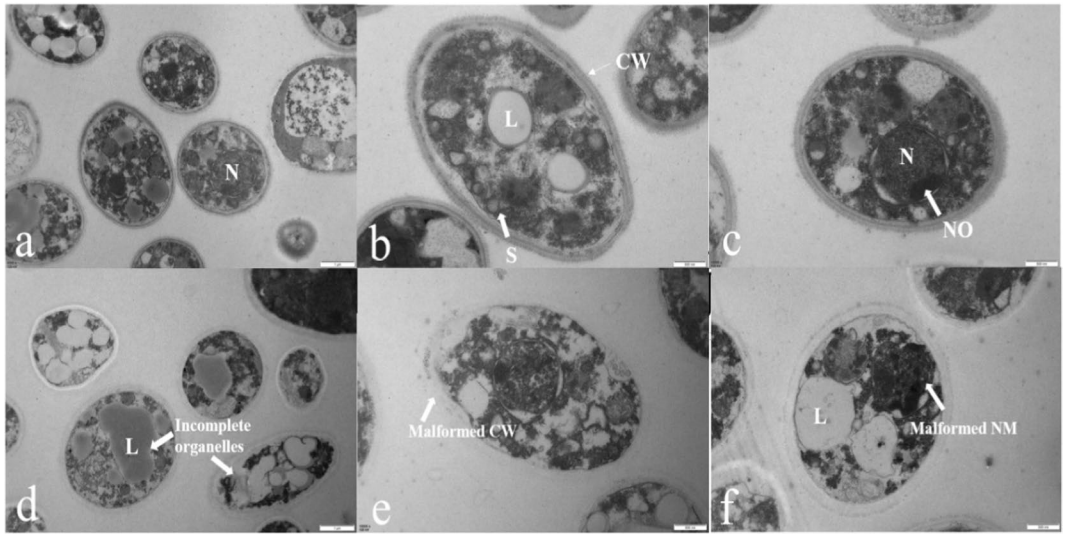

Fig. 8 Transmission electron micrographs of *Ustilaginoidea virens* spores. **a**, **b** and **c**, Control group. **d**, **e** and **f**, Group treated with 15.0 mg/L chelerythrine. CW, Cell wall; L, Lipid globule; N, Nucleus; NO, Nucleolus; IO, Incomplete organelle; MNM, Malformed nuclear membrane. Scale bars are 1 μm in a and d, and 500 nm in b, c, e and f

Conclusions

CHE has potent antimicrobial activity against *U. virens*. The proportion of damaged cells was increased by 31% with increasing CHE concentration. CHE altered the expression of numerous genes, was related to the oxidative phosphorylation pathway, and modified the SNARE vesicle transport pathway. Seven proteins identified by TMT analysis were subjected to PRM, and the observed changes in protein abundance were consistent. The findings will help to elucidate the mechanisms associated with the antimicrobial and apoptosis-inducing effects of CHE in *U. virens.* Although the results suggest that CHE might lead to apoptosis, further studies are necessary, and could assist the development of novel pesticides.

Acknowledgements This project was financially supported by the National Natural Science Foundation of China (No. 51678120). *Magnaporthe oryzae* and *Cochliobolus miyabeanus* strains were kindly provided by the Northeast Agricultural University (Harbin, China) and the Xinyang College of Agriculture and Forestry (Xinyang, China), respectively. *Nigrospora oryzae* and *Aspergillus ruber* strains used in this study were preserved strains from the Northeast Forestry University Microbial Preservation Center (Harbin, China). *U. virens* strains were kindly provided by the Liaoning Academy of Agricultural Sciences (Shenyang, China).

Author contributions Investigation, formal analysis, data curation and writing-original draft preparation were performed by Qing-Hui Wei, Xue-Feng Liu and Yang-Yang Chai. Investigation and data curation were performed by Ming Liang. Min Zhao and Bao-Jiang Zheng proposed the conceptualization, methodology, writing-review & editing and resources. Min Zhao gained acquisition of the financial support for the project leading to this publication.

Data availability Data are available on request to the authors.

Declarations

Competing interests The authors declare no competing financial interests.

References

Adina, W., Faustin, K., Rachel, K., et al. (2005). Control of Golgi morphology and function by Sed5 t-SNARE phosphorylation. *Molecular Biology of the Cell, 16*(10), 4918.

Baghdady, Y. Z., & Schug, K. A. (2019). Qualitative evaluation of high pH mass spectrometry-compatible reversed phase liquid chromatography for altered selectivity in separations of intact proteins. *Journal of Chromatography A, 1599*, 108–114.

Belevich, I., & Verkhovsky, M. I. (2008). Molecular mechanism of protontranslocation by cytochrome c oxidase. *Antioxidants & Redox Signaling, 10*(1), 1–29.

Chen, Q., Tang, H., Zha, Z., Yin, H., Wang, Y., & Wang, Y. (2017). β-D-glucan from Antrodia Camphorata ameliorates LPS-induced inflammation and ROS production in human hepatocytes. *International Journal of Biological Macromolecules., 3*, 45–65.

Cheng, W.P., Zhuang, L.W. (2018). Advances in differential expression and clinical significance about COX6c in diseases. 22 (02), 221–224.程伟鹏, 庄丽维. COX6c在疾病中差异表达及临床意义的研究进展[J]. 生物医学工程与临床, 2018, 22(2):4.

Conesa, A., Götz, S., García-Gómez, J. M., Terol, J., Talón, M., & Robles, M. (2005). Blast2GO: A universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics, 21*(18), 3674–3676.

Egan, J. M., Kaur, A., Raja, H. A., Kellogg, J. J., Oberlies, N. H., & Cech, N. B. (2016). Antimicrobial fungal endophytes from the botanical medicine goldenseal (Hydrastis canadensis). *Phytochemistry Letters., 17*, 219–225.

Fan, H.Y., Xue, G.H., Liu, J.Y., Li, N. (2010). Isolation and Purification of Chelerythrine and Its Anti-fungal Activity. Hubei Agricultural Sciences. 49, 679–682. 范海延, 薛广厚,刘晶瑜等. 白屈菜红碱的分离纯化及其抑菌活性[J].湖北农业科学, 2010, 49(03): 679–682.

FAO. (2018). Rice Market Monitor. 21,1,1–38.

Fu, R.X., Liu, B., Chen, C., et al. (2019). Indoor virulence and field efficacy test of 8 biological agents against Fusarium graminae. Acta Agriculturae Southwest, 32(01): 99–103. 伏荣桃, 刘斌, 陈诚,等. 8种生物药剂对水稻稻曲病菌的室内毒力测定与田间药效试验[J]. 西南农业学报, 2019, 32(1):5

Grant, C. M., Maciver, F. H., & Dawes, I. W. (1997). Mitochondrial function is required for resistance to oxidative stress in the yeast saccharomyces cerevisiae. *FEBS Letters, 410*(2–3), 219–222.

Hu, M.L., Huang, X.D., Luo, L.X. (2018). Compound treatment of rice false smuff with phenylemetronidazole and enoxime mycosine. Plant Protection, 44(3):230–234. 胡茂林,黄晓冬,罗来鑫等.苯醚甲环唑与烯肟菌胺复配防治稻曲病[J].植物保护,2018, 44(03):230–234.

Huang, S., Liu, L., Wang, L., et al. (2019). Research on Advance of Rice False Smut Ustilaginoidea virens (Cooke) Takah Worldwide: Part I. Research Status of Rice False Smut. *Journal of Agricultural Science, 11*(15), 240.

Jia, J.F., Shi, J.Y., Z, B.B., et al. (2022). Effects of polyacetylene alkaloids in stems and leaves of Chrysanthemum chrysanthemi on apoptosis of A549 cells in non-small cell lung cancer. Chinese Journal of New Medicine, 31(09):885–892.贾菊芳,石静亚,张贝贝等.怀菊花茎叶中聚炔类生物碱对非小细胞肺癌A549细胞凋亡的影响[J].中国新药杂志,2022,31(09):885–892.

Jiang, L.L., Zhang, M.J., Meng, M.Z., (2018). And honokiol kill Candida albicans by accumulating ROS and destroying cell membranes. Chinese Journal of Microbiology, 58(03):511–519. 姜路路,张铭嘉,孟美竹等.和厚朴酚通过ROS的积累和破坏细胞膜杀死白色念珠菌 (英文)[J]. 微生物学报, 2018, 58(03): 511–519.

Klopfenstein, D. V., Zhang, L. S., Pedersen, B. S., Ramírez, F., Vesztrocy, A. W., & Aurélien Naldi, A. (2018).

GOATOOLS: A Python library for Gene Ontology analyses. *Scientific Reports., 8*(1), 1–17.

Kumar, A. (2014). Chelerythrine induces reactive oxygen species-dependent mitochondrial apoptotic pathway in a murine T cell lymphoma. *Tumor Biology: The Journal of the International Society for Oncodevelopmental Biology and Medicine., 35*, 129–140.

Li, W. L., Li, L. Y., Feng, A. Q., Zhu, X. Y., & Li, J. X. (2013). Rice False Smut Fungus, *Ustilaginoidea virens*, Inhibits Pollen Germination and Degrades the Integuments of Rice Ovule. *American Journal of Plant Sciences., 4*(12), 2295–2304.

Li, L. J., Wang, X. X., Liu, D. D., et al. (2019). MicroRNA-185 inhibits proliferation, migration and invasion in human osteosarcoma MG63 cells by targeting vesicle-associated membrane protein 2. *Gene, 696*, 80–87.

Lin, W., Huang, J., Yuan, Z., Feng, S., Xie, Y., & Ma, W. (2017). Protein kinase C inhibitor chelerythrine selectively inhibits proliferation of triple-negative breast cancer cells. *Scientific Reports., 7*(1), 2022.

Liu, X.F. (2018). Purification and biological activity of carboxymethyl tuckahoe polysaccharide [D]. South China university of technology, 80–90. 刘晓菲. 羧甲基茯苓多糖的纯化及生物活性研究[D]. 华南理工大学, 2018.

Marei, G. I. K., Rasoul, M. A. A., & Abdelgalei, S. A. M. (2012). Comparative antifungal activities and biochemical effects of monoterpenes on plant pathogenic fungi. *Pesticide Biochemistry and Physiology, 103*(1), 56–61.

Nicholson-Fish, J. C., Kokotos, A. C., Gillingwater, T. H., Smillie, K. J., & Cousin, M. A. (2015). VAMP4 Is an Essential Cargo Molecule for Activity-Dependent Bulk Endocytosis. *Neuron, 88*(5), 973–984.

Parlati, F., McNew, J. A., Fukuda, R., Miller, R., Sllner, T. H., & Rothman, J. H. (2000). Topological restriction of SNARE-dependent membrane fusion. *Nature, 407*(6801), 194–198.

Pencikova, K., Kollar, P., Muller Zavalova, V., Taborska, E., Urbanova, J., & Hosek, J. (2012). Investigation of sanguinarine and chelerythrine effects on LPS-induced inflammatory gene expression in THP-1 cell line. *Phytomedicine: International Journal of Phytotherapy and Phytopharmacology, 19*(10), 890–895.

Shitara, A., Shibui, T., Okayama, M., Arakawa, T., Mizoguchi, I., Sakakura, Y., & Takuma, T. (2013). VAMP4 is required to maintain the ribbon structure of the Golgi apparatus. *Molecular and Cellular Biochemistry., 380*(1), 11–21.

Tang, X., Zhang, J., Liu, L., Yang, D., Wang, H., & He, F. (2017). Synthesis of 13-substituted derivatives of berberine: Aggregation-induced emission enhancement and pH sensitive property. *Journal of Photochemistry and Photobiology a: Chemistry., 337*, 71–81.

Tao, R.T., Ran, L.T. (2018). Antitumor mechanism of chelidonine on KB cell lines. Modern Oncology Medicine, 26(10):1498–1502. 陶然婷,冉令涛.白屈菜碱对KB细胞株的抗肿瘤作用机制探讨[J]. 现代肿瘤医学, 2018, 26(10): 1498–1502.

Téllez-Robledo, B., Manzano, C., Saez, A., et al. (2019). The polyadenylation factor FIP1 is important for plant development and root responses to abiotic stresses. *The Plant Journal*., *99*(6). https://doi.org/10.1111/tpj.14416.

Verena, C., Jana, K., & Karl, F. (2019). Fungicides chlorothanolin, azoxystrobin and folpet induce transcriptional alterations in genes encoding enzymes involved in oxidative phosphorylation and metabolism in honey bees (Apis mellifera) at sublethal concentrations. *Journal of Hazardous Materials., 377*(5), 215–226.

Wang C., Wang Q., Huang Y.J., et al. (2023). Evaluation of antibacterial activity of snake seed and its synergistic effect of inhibitor. Pesticides, 62 (3): 223–226. 王春,王芊,黄元炬等.蛇床子素对稻曲病菌的抑菌活性及诱抗剂与其协同增效作用评价[J].农药,2023,62(03):223–226.

Wei, Q. H., Zhao, M., & Li, X. Y. (2017). Extraction of Chelerythrine and its Effects on Pathogenic Fungus Spore Germination. *Pharmacognosy Magazine, 13*(52), 600–606.

Wei, Q. H., Cui, D. Z., Liu, X. F., Chai, Y. Y., Zhao, N., Wang, J. Y., & Zhao, M. (2020). *In vitro* antifungal activity and possible mechanisms of action of chelerythrine. *Pesticide Biochemistry and Physiology., 164*(2020), 140–148.

Xiao, Z.F., Zhu, C.W., Dai, L.J., Song, H., Zhong, J. (2019). Differential proteomics of tobacco leaves at seedling stage under different potassium concentrations based on label-free technology. Henan agricultural sciences , 48(05): 37–42+77. 肖泽凡,朱崇文,戴林建等.基于Label-free技术的不同钾浓度下烟草苗期叶片差异蛋白质组学研究[J].河南农业科学,2019,48(05):37–42+77.

Yakovlev, Y., Borovskii, G. B., Voinikov, V. K., Grabelnych, O. I., Pobezhimova, T. P., & Antipin, A. I. (2002). An effect of water-soluble proteins of fungi differed in frost-hardy on the energetic activity of isolated plant mitochondria. *Journal of Thermal Biology., 27*(3), 239–244.

Yang, R., Gao, Z. F., Zhao, J. Y., Li, W. B., Zhou, L., & Miao, F. (2015). New Class of 2-Aryl-6-chloro-3,4-dihydroisoquinolinium Salts as Potential Antifungal Agents for Plant Protection: Synthesis, Bioactivity and Structure-Activity Relationships. *Journal of Agricultural and Food Chemistry., 63*(7), 1906–1914.

Zhang, L. L., et al. (2022). Quantitative phosphoproteomic analysis of mice with liver fibrosis by DIA mass spectrometry analysis with PRM verification. *Journal of Proteomics, 271*, 104768–104768.

Zhao, Zh. M., Shang, X. F., Lawoe, R. K., Liu, Y. Q., Zhou, R., & Sun, Y. (2019). Anti-phytopathogenic activity and the possible mechanisms of action of isoquinoline alkaloid sanguinarine. *Pesticide Biochemistry and Physiology, 159*(C), 51–58.

Zhu, L.F., Wang, X.L., Qu, S.H. (2020). Advances in pharmacological effects of sanguinarine. Guangdong Chemical Industry, 20, 47(22): 69–70. 朱丽飞,王小莉,曲书昊. 血根碱的药理作用研究进展[J]. 广东化工, 2020, 47(22): 69–70.

Zou, S. S., Sun, D., & Liang, Y. H. (2017). The Roles of the SNARE Protein Sed5 in Autophagy in Saccharomyces cerevisiae. *Moleculer Cells, 40*(9), 643–654.