iTRAQ Proteomic Analysis Reveals That Metabolic Pathways Involving Energy Metabolism Are Affected by Tea Tree Oil in Botrytis cinerea

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Tea tree oil (TTO) is a volatile essential oil obtained from the leaves of the Australian tree Melaleuca alternifolia by vapor distillation. Previously, we demonstrated that TTO has a strong inhibitory effect on Botrytis cinerea. This study investigates the underlying antifungal mechanisms at the molecular level. A proteomics approach using isobaric tags for relative and absolute quantification (iTRAQ) was adopted to investigate the effects of TTO on B. cinerea. A total of 718 differentially expression proteins (DEPs) were identified in TTO-treated samples, 17 were markedly up-regulated and 701 were significantly down-regulated. Among the 718 DEPs, 562 were annotated and classified into 30 functional groups by GO (gene ontology) analysis. KEGG (Kyoto Encyclopedia of Genes and Genomes) enrichment analysis linked 562 DEPs to 133 different biochemical pathways, involving glycolysis, the tricarboxylic acid cycle (TCA cycle), and purine metabolism. Additional experiments indicated that TTO destroys cell membranes and decreases the activities of three enzymes related to the TCA cycle. Our results suggest that TTO treatment inhibits glycolysis, disrupts the TCA cycle, and induces mitochondrial dysfunction, thereby disrupting energy metabolism. This study provides new insights into the mechanisms underlying the antifungal activity of essential oils.

Keywords: iTRAQ, proteomics, essential oil, Botrytis cinerea, antifungal

INTRODUCTION

Botrytis cinerea, one of the most destructive fungal pathogens, causing gray mold rot in a wide range of fresh fruits and vegetables. The resulting reduction in shelf life is responsible for enormous economic losses in the produce industry. Although chemical fungicides are widely used to control the incidence of the disease, this practice potentially introduces harmful substances into the food chain, and also selects for B. cinerea strains with increased drug resistance (Brul and Coote, 1999; Leroux et al., 2002). These limitations provide a strong stimulus to explore safer and more effective antifungal agents. Essential oils are promising natural substitutes that offer disease control by inhibiting pathogen growth (Prakash et al., 2012). For example, the essential oils of Angelica archangelica L. (Apiaceae) roots and Solidago canadensis L. have been characterized and tested in vitro as antifungal agents against B. cinerea (Fraternale et al., 2014; Liu et al., 2016). Lemongrass essential oil significantly reduces the incidence of B. cinerea and prolongs the shelf-life and sensory properties of frozen mussels and vegetables (Abdulazeez et al., 2016). Essential oils of aromatic plants, which belong to the Lamiaceae family such as origanum (Origanum syriacum L. var. bevanii),
lavender (Lavandula stoechas L. var. stoechas) and rosemary (Rosmarinus officinalis L.), have been reported to cause considerable morphological degenerations of the fungal hyphae of B. cinerea and suppress in vivo disease development on tomato against B. cinerea (Soylu et al., 2010).

Tea tree oil (TTO) is a volatile natural plant essential oil obtained from the leaves of the Australian tree Melaleuca alternifolia by vapor distillation (Homer et al., 2000). The oil exhibits a broad spectrum of antimicrobial activities against a variety of bacteria, fungi, and virus (Carson et al., 2006; Miao et al., 2016). Growth and metabolic activity of Escherichia coli and Candida albicans are inhibited after treatment with TTO (Gustafson et al., 1998; Bona et al., 2016). Our previous studies showed that TTO treatment effectively inhibits spore germination and mycelial growth of B. cinerea, modifies its morphology and cellular ultrastructure, and controls gray mold on strawberry and cherry fruits (Shao et al., 2013a; Li et al., 2017a). TTO’s antifungal mechanism in B. cinerea involves the loss of membrane integrity and the subsequent release of intracellular compounds, probably due in part to changes in membrane fatty acid and ergosterol composition (Shao et al., 2013b; Li et al., 2017a). TTO also causes mitochondrial damage in B. cinerea, disrupting the tricarboxylic acid (TCA) cycle and leading to the accumulation of reactive oxygen species (ROS) (Li et al., 2017b). Metabolomic analysis by quadrupole time-of-flight mass spectrometer was consistent with these results (Xu et al., 2017). However, the molecular mechanisms underlying the effects of TTO against B. cinerea have not yet been associated with specific proteins.

Proteomics can be used to study the changes in protein levels under stress conditions in great detail (Franco et al., 2013), and has been applied to investigate the mode of action of the antimicrobial agent apidaecin IB against membrane proteins in E. coli cells (Zhou and Chen, 2011). Other studies have revealed that proteins related to energy and DNA metabolism, and amino acid biosynthesis are down-regulated in E. coli JK-17 in the presence of rose flower extract (Cho and Oh, 2011). Syzygium aromaticum essential oil perturbs the expression of virulence-related genes involved in the synthesis of serine protease, flagella, and lipopolysaccharide in Campylobacter jejuni (Kovács et al., 2016). In this study, we conducted a proteomics analysis using isobaric tags for relative and absolute quantification (iTRAQ) to study B. cinerea to identify proteins and potential mechanisms underlying the antifungal activity of TTO.

MATERIALS AND METHODS

B. cinerea Growth and Exposure to TTO

Highly virulent B. cinerea (ACCC 36028) was purchased from the Agricultural Culture Collection of China and grown at 25°C on potato dextrose agar (PDA, containing 1 L potato liquid, 20 g/L glucose, and 15 g/L agar) before use. TTO was purchased from Fuzhou Merlot Lotus Biological Technology Company (Fujian Province, China). The primary components of TTO are terpinen-4-ol (37.11%), γ-terpinene (20.65%), α-terpinene (10.05%), l, 8-cineole (4.97%), terpinolene (3.55%), p-cymene (2.14%), and α-terpineol (3.82%), as specified by the supplier. B. cinerea cultures were maintained on PDA at 25°C for 3 days. Spore suspensions were harvested by adding 10 mL sterile 0.9% NaCl solution to each petri dish and then gently scraping the mycelial surface three times with a sterile L-shaped spreader to free the spores. The spore suspension was adjusted using a hemocytometer to 5 x 10⁶ spores/mL. One milliliter suspension was inoculated into 250 mL flasks containing 150 mL sterile potato dextrose broth medium and cultured at 25°C on a rotary shaker at 150 revolutions per minute for 3 days. Before mycelia were harvested, TTO was added to the medium to a final concentration of 5 mL/L, and cultures incubated for another 2 h (Xu et al., 2017). Mycelia were collected and rinsed three times with 0.1 M phosphate buffered saline (PBS) (pH 7.4). Samples were stored at ~80°C. Cultures without TTO were used as a control. Three samples were prepared in parallel for each condition.

Protein Extraction

Approximately 200 mg of frozen mixed mycelium from control or TTO treated cultures was ground into powder in liquid nitrogen and suspended in 25 mL 10% (v/v) trichloroacetic acid in acetone containing 65 mM dithiothreitol (DTT). The suspension was vortexed and incubated at ~20°C for 2 h, centrifuged at 12,000 × g for 45 min at 4°C, and the supernatant discarded. The precipitate was rinsed three times with chilled acetone. The pellet was vacuum dried and dissolved in lysis buffer (4% SDS, 100 mM Tris-HCl, 100 mM DTT, pH 8.0). After incubation for 5 min in boiling water, the suspension was sonicated on ice at 50 W for 5 min. The crude extract was incubated in boiling water again for 5 min, and clarified by centrifugation at 14,000 × g for 40 min at 20°C. To digest protein in the supernatant, 200 µL UA buffer (8 M urea, 150 mM Tris-HCl, pH 8.5) was added and the mixture was centrifuged at 14,000 × g for 30 min at room temperature. This step was repeated three times. Subsequently, 100 µL 50 mM iodoacetamide (IAM) was added, the samples were incubated for 30 min in darkness, and then centrifuged at 14,000 × g for 30 min at room temperature. The precipitate was resuspended in 100 µL UA buffer and samples were centrifuged at 14,000 × g for 30 min at room temperature. 100 µL dissolution buffer was added, followed by centrifugation at 14,000 × g for 30 min at room temperature. This step was repeated three times. The supernatant was removed, the pellet was dissolved in 40 µL trypsin buffer, incubated at 37°C for 18 h, and clarified by centrifugation at 14,000 × g for 30 min at room temperature. Finally, 40 µL 25 mM dissolution buffer was added and samples were centrifuged at 14,000 × g for 30 min at room temperature. The supernatant was transferred to a new tube and quantified with the Bradford assay using BSA as the standard, and SDS-PAGE was performed to verify protein quality.

iTRAQ Labeling and Strong Cation Exchange (SCX) Fractionation

iTRAQ labeling was performed according to the manufacturer’s instructions. Peptides were prepared using the 8-plex iTRAQ labeling kit (AB Sciex, CA, USA). Control replicates were labeled with reagents 113, 114, and 115, and the TTO treatment
replicates were labeled with reagents 116, 117, and 118. The labeled peptide mixtures were pooled and dried by vacuum centrifugation.

The labeled peptide mixtures were dissolved in 3 mL buffer A (10 mM KH$_2$PO$_4$ in 25% acetonitrile, pH 3.0) and loaded onto a polysulfoethyl 4.6 × 100 mm column (5 µm, 200 Å, PolyLC, Inc., Maryland, USA). The peptides were eluted at a flow rate of 1 mL/min with a gradient of buffer A for 30 min, 5–70% buffer B (10 mM KH$_2$PO$_4$, 500 mM KCl in 25% acetonitrile, pH 3.0) for 65 min, and 70–100% buffer B for 80 min. The eluted peptides were pooled into 10 fractions, desalted on C18 cartridges (Sigma), and vacuum-dried.

**LC-MS/MS Analysis**

For nano LC–MS/MS analysis, 10 µL of supernatant from each fraction was injected into an Obitrap-Elite (ThermoFinnigan) equipped with an Easy nLC (Proxeon Biosystems, now Thermo Fisher Scientific). The mobile phase was a mixture of water containing 0.1% formic acid and acetonitrile with 0.1% formic acid isocratically delivered by a pump at a flow rate of 250 nL/min. The elution gradient was: 0–105 min, 0–50% B; 105–110 min, 50–100% B; 110–120 min, 100% B. The MS scanning range was 300–1,800 m/z, MS resolution was 70,000, the number of scans range was 1, and the dynamic exclusion time was 40 s. The MS/MS activation type was HCD, the isolation window was 2 m/z, the MS/MS resolution was 17,500, the normalized collision energy was 30 eV, and the underfill ratio was 0.1%.

**Analysis of Differentially Expression Proteins**

For protein quantitation, one protein was required to contain at least two unique peptides. The quantitative protein ratios were weighted and normalized by the median ratio in Mascot (http://www.matrixscience.com). When differences in protein expression between TTO-treated and control groups were >1.5-fold or <0.67-fold, with p < 0.05, the protein was considered to be differentially expressed.

**Bioinformatic Analysis**

Gene Ontology (GO) is a standardized gene function classification system that describes the properties of proteins.
| Accession | Protein name                                      | Score | Sequence coverage (%) | Fold | p-value |
|-----------|--------------------------------------------------|-------|-----------------------|------|---------|
| gi|154691848 | cytochrome c                                     | 96.3  | 37.9                  | 0.328| 0.007   |
| gi|347441783 | citrate synthase                                 | 133.1 | 8.0                   | 1.819| 0.028   |
| gi|472236008 | malate dehydrogenase protein                     | 957.7 | 55.4                  | 2.120| 0.017   |
| gi|472241505 | oxoglutarate dehydrogenase protein               | 698.3 | 27.2                  | 1.611| 0.037   |
| gi|347827327 | pyruvate carboxylase                              | 2,263.6 | 38.7              | 1.751| 0.027   |
| gi|347833674 | phosphoenolpyruvate carboxykinase                | 548.7 | 30.2                  | 1.625| 0.044   |
| gi|347839725 | succinyl-CoA ligase subunit alpha                 | 420.3 | 24.3                  | 1.612| 0.040   |
| gi|347826865 | fructose-1,6-bisphosphatase                       | 308.1 | 39.1                  | 1.640| 0.031   |
| gi|154323902 | enolase                                          | 2,009.9 | 46.6               | 1.621| 0.008   |
| gi|472238209 | glucose-6-phosphate isomerase protein             | 574.2 | 29.9                  | 1.980| 0.032   |
| gi|472246374 | phosphoglycerate mutase protein                   | 54.3  | 2.6                   | 1.576| 0.021   |
| gi|472240435 | 6-phosphofructokinase protein                     | 539.9 | 28.1                  | 1.775| 0.022   |
| gi|472237248 | bisphosphoglycerate-independent phosphoglycerate protein | 823.0 | 44.1                  | 2.164| 0.018   |
| gi|347841748 | fructose-bisphosphate aldolase                   | 1,045.2 | 42.2               | 1.725| 0.027   |
| gi|536718572 | phosphoglycerate kinase 1                        | 587.5 | 40.2                  | 1.723| 0.040   |
| gi|347833674 | phospho-2-dehydro-3-deoxyhexoionate aldolase     | 548.7 | 30.2                  | 1.870| 0.029   |
| gi|347835540 | phosphoglycerate mutase family protein           | 36.0  | 4.7                   | 1.792| 0.015   |
| gi|472240974 | 6-phosphofructo-2-kinase fructose bisphosphatase protein | 98.8  | 9.4                   | 1.851| 0.037   |
| gi|347441437 | inosine 5-monophosphate dehydrogenase            | 581.8 | 19.9                  | 1.606| 0.020   |
| gi|347841600 | adenine phosphoribosyltransferase                | 182.4 | 37.8                  | 1.777| 0.022   |
| gi|347829189 | adenosine kinase                                 | 465.9 | 31.3                  | 1.956| 0.016   |
| gi|347441679 | adenosylhomocysteinase                           | 1,287.4 | 61.7                | 1.881| 0.027   |
| gi|347837737 | S-adenosylmethionine synthetase                  | 423.1 | 30.1                  | 2.004| 0.008   |
| gi|347831618 | AMP deaminase 3                                  | 111.1 | 4.5                   | 1.673| 0.029   |
| gi|347828730 | adenylosuccinate synthetase                      | 333.0 | 30.9                  | 1.602| 0.036   |
| gi|347837737 | S-adenosylmethionine synthetase                  | 423.1 | 30.1                  | 2.004| 0.008   |
| gi|347837845 | adenylyl cyclase-associated protein              | 417.9 | 20.7                  | 1.810| 0.022   |
| gi|472242224 | guanyl-nucleotide exchange factor protein        | 65.4  | 1.5                   | 1.674| 0.004   |
| gi|154691052 | uracil phosphoribosyltransferase                 | 90.6  | 9.4                   | 1.796| 0.046   |
| gi|154697015 | nucleoside diphosphate kinase                    | 522.4 | 42.8                  | 1.935| 0.010   |
| gi|347840376 | UTP-glucose-1-phosphate uridylyltransferase      | 1,333.6 | 45.7               | 1.623| 0.038   |
| gi|347832865 | ribulose-phosphate 3-epimerase                   | 38.6  | 7.9                   | 2.204| 0.031   |
| gi|154300519 | alcohol dehydrogenase protein                    | 167.7 | 16.5                  | 1.960| 0.026   |
| gi|347836330 | alcohol dehydrogenase (NADP dependent)           | 281.1 | 24.4                  | 2.019| 0.020   |
| gi|347441999 | zinc-containing alcohol dehydrogenase            | 636.5 | 44.8                  | 1.656| 0.032   |
| gi|347440923 | aldehyde dehydrogenase                           | 1,070.9 | 48.0               | 1.865| 0.021   |
| gi|154703069 | ATP synthase D chain, mitochondrial              | 252.1 | 26.4                  | 1.924| 0.050   |
| gi|563295821 | ATP synthase subunit e, mitochondrial            | 60.2  | 9.9                   | 1.757| 0.033   |
| gi|347839842 | ATP citrate lyase subunit                        | 549.0 | 37.5                  | 1.589| 0.023   |
| gi|154703371 | vacuolar ATP synthase subunit E                  | 93.3  | 12.7                  | 2.382| 0.013   |
| gi|154692979 | vacuolar ATP synthase subunit D                  | 74.8  | 19.5                  | 1.715| 0.024   |
| gi|347441643 | vacuolar ATP synthase subunit H                  | 307.6 | 22.3                  | 1.761| 0.028   |
| gi|472245494 | vacuolar ATP synthase catalytic subunit a protein | 577.7 | 27.8                  | 1.580| 0.012   |
| gi|347835157 | v-type proton ATPase subunit B                   | 274.1 | 17.6                  | 2.041| 0.019   |
| gi|507414957 | mitochondrial import protein 1                   | 31.1  | 8.6                   | 1.872| 0.043   |
| gi|472243251 | mitochondrial pyruvate dehydrogenase kinase protein | 61.4  | 3.4                   | 2.632| 0.009   |
| gi|229891130 | amino-acid acetyltransferase, mitochondrial      | 44.2  | 2.1                   | 2.115| 0.022   |
| gi|3282211  | isocitrate lyase 1, partial                      | 27.8  | 2.5                   | 1.874| 0.029   |
| gi|347832197 | malate synthase                                  | 46.4  | 5.7                   | 1.875| 0.048   |
| gi|347840647 | acetyl-CoA carboxylase                            | 2,370.7 | 33.8              | 1.622| 0.039   |
| gi|347842358 | acetyl-CoA acetyltransferase                     | 449.4 | 46.3                  | 1.982| 0.018   |

(Continued)
TABLE 1 | Continued

| Accession | Protein name                                                                 | Score | Sequence coverage (%) | Fold<sup>a</sup> | p-value   |
|-----------|------------------------------------------------------------------------------|-------|-----------------------|------------------|-----------|
| gi|347841050 | fatty acid synthase                                                          | 1,414.5 | 25.2                | 1.693 | 0.042 |
| gi|347845418 | fatty acid synthase beta subunit dehydratase protein                         | 1,668.6 | 24.8                | 1.567 | 0.045 |
| gi|347841364 | NADP-specific glutamate dehydrogenase                                         | 1,138.8 | 46.9                | 1.840 | 0.021 |
| gi|347827914 | homocitrate synthase                                                          | 454.5 | 39.0                | 1.501 | 0.031 |
| gi|347837008 | homoserine kinase                                                             | 190.1 | 28.7                | 1.920 | 0.042 |
| gi|347836521 | GABA transaminase                                                             | 483.9 | 27.7                | 1.544 | 0.018 |
| gi|472224205 | aspartate aminotransferase protein                                            | 385.8 | 26.1                | 1.837 | 0.048 |
| gi|347841990 | tryptophan synthase                                                           | 611.0 | 28.3                | 1.542 | 0.024 |
| gi|347832506 | threonine synthase                                                            | 348.4 | 16.4                | 1.560 | 0.047 |
| gi|154692005 | cysteine synthase                                                             | 292.8 | 25.0                | 1.589 | 0.028 |
| gi|347833148 | glutamine synthetase                                                          | 484.0 | 26.9                | 1.778 | 0.015 |
| gi|347839014 | histidine biosynthesis protein                                                 | 184.6 | 9.3                 | 1.840 | 0.027 |
| gi|347828253 | dihydrodipicolinate synthetase family protein                                 | 518.7 | 28.0                | 1.869 | 0.013 |
| gi|347836881 | D-3-phosphoglycerate dehydrogenase                                           | 666.4 | 25.5                | 1.758 | 0.018 |
| gi|472242394 | saccharopine dehydrogenase protein                                            | 338.7 | 36.2                | 1.743 | 0.039 |
| gi|347441047 | glycine dehydrogenase                                                         | 286.9 | 12.3                | 1.708 | 0.029 |
| gi|507414630 | C-1-tetrahydrofolate synthase                                                 | 905.4 | 31.2                | 1.737 | 0.031 |
| gi|347831191 | glutamate carboxypeptidase protein                                             | 298.0 | 23.2                | 1.977 | 0.020 |
| gi|347841903 | methionine aminopeptidase 1                                                   | 221.2 | 20.3                | 2.040 | 0.021 |
| gi|332313356 | methionine aminopeptidase 2                                                   | 73.1 | 10.3                | 2.044 | 0.027 |
| gi|347829817 | serine/threonine protein kinase                                                | 32.6 | 4.4                 | 1.693 | 0.037 |
| gi|472244536 | glutamate-cysteine ligase protein                                              | 61.6 | 3.6                 | 1.698 | 0.037 |
| gi|347829487 | 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase         | 2,013.2 | 41.1      | 2.505 | 0.004 |
| gi|347836712 | glycine cleavage system H protein                                             | 116.0 | 22.0                | 1.982 | 0.025 |
| gi|472236211 | amino acid permease protein                                                   | 39.7 | 4.0                 | 1.999 | 0.031 |
| gi|347830997 | peptide methionine sulfoxide reductase                                        | 82.8 | 20.5                | 1.919 | 0.029 |
| gi|472243795 | aromatic-l-amino-acid decarboxylase protein                                   | 287.8 | 12.1                | 1.936 | 0.015 |
| gi|347833024 | lysine decarboxylase-like protein                                             | 79.7 | 8.6                 | 1.585 | 0.033 |
| gi|472246546 | glutathione-dependent formaldehyde dehydrogenase                             | 587.5 | 48.7                | 1.666 | 0.043 |
| gi|347840830 | NADH-cytochrome b5 reductase                                                  | 305.5 | 23.0                | 1.545 | 0.029 |
| gi|347827019 | cytochrome P450 monoxygenase                                                  | 31.4 | 2.4                 | 1.722 | 0.042 |
| gi|125949746 | calcineurin                                                                   | 194.3 | 12.4                | 1.777 | 0.023 |
| gi|154289817 | chitin synthase                                                               | 129.2 | 4.7                 | 1.555 | 0.023 |
| gi|347840218 | sorbitol dehydrogenase                                                        | 28.3 | 2.9                 | 1.706 | 0.028 |
| gi|347440923 | aldehyde dehydrogenase                                                        | 1,070.9 | 48.0      | 1.865 | 0.021 |
| gi|347833737 | mitochondrial peroxiredoxin Prx1                                              | 42.8 | 7.6                 | 1.856 | 0.044 |
| gi|347828993 | antioxidant                                                                   | 129.5 | 33.1                | 2.127 | 0.028 |
| gi|347839043 | superoxide dismutase                                                          | 163.1 | 17.0                | 1.717 | 0.012 |
| gi|166409444 | flavohemoglobin                                                               | 294.7 | 35.7                | 1.994 | 0.009 |
| gi|347828340 | oxidoreductase                                                                | 305.1 | 14.93               | 2.119 | 0.045 |
| gi|347841065 | nuclear control of ATPase protein                                             | 84.7 | 4.7                 | 0.219 | 0.001 |
| gi|347836808 | heat shock protein 70                                                         | 3,060.8 | 53.2      | 1.750 | 0.014 |
| gi|472242753 | 30 kda heat shock protein                                                     | 296.7 | 47.5                | 1.959 | 0.019 |
| gi|347827157 | heat shock protein 90                                                         | 1,603.7 | 37.7      | 1.650 | 0.032 |
| gi|347830903 | heat shock protein ST1                                                        | 689.1 | 35.8                | 2.451 | 0.011 |
| gi|347830415 | heat shock protein Hsp68                                                      | 1,199.5 | 34.3      | 1.817 | 0.020 |
| gi|347833633 | heat shock protein                                                            | 748.3 | 34.3                | 1.999 | 0.020 |
| gi|154288804 | short chain dehydrogenase                                                     | 105.9 | 20.7                | 2.142 | 0.005 |
| gi|347840162 | translation initiation factor 3                                               | 284.6 | 46.8                | 1.905 | 0.031 |
| gi|472245156 | eukaryotic translation initiation factor 3 subunit                           | 749.4 | 18.9                | 1.890 | 0.015 |
| gi|229463757 | eukaryotic translation initiation factor 3 subunit H                          | 195.8 | 20.7                | 1.851 | 0.013 |
| Accession | Protein name | Score | Sequence coverage (%) | Fold<sup>3</sup> | p-value |
|-----------|--------------|-------|------------------------|-----------------|---------|
| gi|229501208 | eukaryotic translation initiation factor 3 subunit K | 232.7 | 33.5 | 1.751 | 0.044 |
| gi|347841080 | eukaryotic translation initiation factor 2 subunit alpha | 193.5 | 17.1 | 1.574 | 0.030 |
| gi|347830243 | eukaryotic translation initiation factor 4e | 151.7 | 12.0 | 1.798 | 0.044 |
| gi|347840917 | actin-depolymerizing factor 1 | 519.9 | 53.6 | 1.959 | 0.018 |
| gi|3182891 | actin | 1,055.4 | 52.8 | 1.555 | 0.035 |
| gi|347831507 | actin binding protein | 276.9 | 16.6 | 1.942 | 0.003 |
| gi|347840551 | actin related protein 2/3 complex | 217.4 | 21.9 | 1.835 | 0.013 |
| gi|347838304 | F-actin capping protein beta subunit isoforms 1 and 2 | 156.0 | 27.7 | 1.595 | 0.044 |
| gi|205716451 | actin cytoskeleton-regulatory complex protein end 3 | 109.4 | 10.4 | 1.827 | 0.022 |
| gi|347827028 | actin lateral binding protein | 691.2 | 50.3 | 2.621 | 0.002 |
| gi|347441258 | myosin regulatory light chain cdc4 | 327.6 | 43.9 | 1.775 | 0.049 |
| gi|347838471 | transcription factor HMG | 321.9 | 28.4 | 1.608 | 0.038 |
| gi|347838526 | transcription factor CCAAT | 42.1 | 3.2 | 4.970 | 0.001 |
| gi|374093884 | transcription regulator PAC1, partial | 267.7 | 17.2 | 2.290 | 0.003 |
| gi|472234708 | transcription factor protein | 388.6 | 51.4 | 2.634 | 0.003 |
| gi|472244889 | transcription factor CBF/NF-Y | 328.9 | 25.8 | 1.858 | 0.011 |
| gi|347840266 | transcription factor Zn, C<sub>2</sub>H<sub>2</sub> | 50.5 | 1.7 | 3.407 | 0.003 |
| gi|347837101 | EF-hand calcium-binding domain protein | 1,298.4 | 40.7 | 1.654 | 0.021 |
| gi|347223545 | cell division control protein cdc48 protein | 103.7 | 20.5 | 1.930 | 0.025 |
| gi|206557271 | cell division cycle protein 123 | 38.6 | 3.9 | 1.809 | 0.050 |
| gi|347828695 | apoptosis-inducing factor 3 | 267.7 | 17.2 | 2.290 | 0.003 |
| gi|347224209 | thioredoxin protein | 386.6 | 51.4 | 2.634 | 0.003 |
| gi|347844889 | sulfate adenylyltransferase protein | 328.9 | 25.8 | 1.858 | 0.011 |
| gi|347839319 | protein disulfide-isomerase | 542.3 | 39.1 | 1.862 | 0.031 |
| gi|347442007 | transaldolase | 1,216.6 | 50.2 | 1.984 | 0.022 |
| gi|154703353 | elongation factor 1-alpha | 2,637.4 | 50.0 | 1.831 | 0.034 |
| gi|347830450 | elongation factor 2 | 1,896.6 | 44.6 | 1.688 | 0.020 |
| gi|3472244387 | elongation factor 1-beta protein | 597.2 | 40.0 | 2.006 | 0.024 |
| gi|347841449 | NAD-dependent formate dehydrogenase | 1,663.0 | 50.1 | 1.931 | 0.042 |
| gi|347835785 | 26S protease regulatory subunit 6A | 355.1 | 27.6 | 1.848 | 0.017 |
| gi|3472242788 | proteasome component p35 protein | 101.7 | 23.9 | 1.942 | 0.023 |
| gi|347841691 | arp2/3 complex subunit Arc16 | 249.2 | 41.7 | 1.792 | 0.020 |
| gi|154319207 | 26S protease regulatory subunit 7 | 221.9 | 19.4 | 2.009 | 0.026 |
| gi|347833025 | proteasome subunit alpha type 1 | 133.2 | 16.9 | 1.706 | 0.025 |
| gi|347441407 | protein kinase C substrate | 282.5 | 18.1 | 1.703 | 0.028 |
| gi|347827686 | sec14 cytosolic factor | 240.1 | 41.4 | 1.711 | 0.030 |
| gi|347840528 | peptidyl-prolyl cis-trans isomerase D | 431.3 | 39.9 | 2.070 | 0.019 |
| gi|63236153 | inorganic pyrophosphatase | 317.8 | 29.7 | 1.714 | 0.015 |
| gi|347830035 | aldose 1-epimerase | 338.4 | 29.6 | 2.114 | 0.040 |
| gi|347831189 | carbohydrate-Binding Module family 48 protein | 330.4 | 27.1 | 3.744 | 0.014 |
| gi|347839149 | carbohydrate-Binding Module family 50 protein | 196.5 | 25.3 | 2.276 | 0.047 |
| gi|347841295 | cystathionine beta-synthase | 416.0 | 26.0 | 1.790 | 0.031 |
| gi|347842143 | dihydrolipoyl dehydrogenase | 303.6 | 25.9 | 1.788 | 0.022 |
| gi|347836348 | protein phosphatase Pp2a regulatory subunit A | 414.1 | 21.1 | 1.576 | 0.045 |
| gi|347838932 | class III aminotransferase | 340.3 | 23.9 | 1.844 | 0.015 |
| gi|347831623 | amidophosphoribosyltransferase | 1,467.6 | 20.8 | 1.573 | 0.025 |
| gi|347236449 | enoyl- hydratase isomerase protein | 101.1 | 19.1 | 1.849 | 0.026 |
| gi|347237246 | tubulin-specific chaperone c protein | 222.7 | 20.7 | 1.621 | 0.044 |
| Accession | Protein name | Score | Sequence coverage (%) | Fold<sup>a</sup> | p-value |
|-----------|--------------|-------|------------------------|------------------|---------|
| gi|347826988 | trans-2-enoyl-CoA reductase | 31.9 | 1.9 | 0.031 | 0.001 |
| gi|347837864 | 1,3,8-naphthalenetiol reductase | 89.0 | 19.6 | 2.213 | 0.029 |
| gi|472243905 | casein kinase i protein | 148.3 | 19.8 | 1.591 | 0.043 |
| gi|347831955 | acetate kinase | 193.1 | 18.9 | 1.726 | 0.015 |
| gi|347839614 | aspartyl aminopeptidase | 293.3 | 18.8 | 1.564 | 0.036 |
| gi|472238538 | 3-hydroxybutyryl-dehydrogenase protein | 133.3 | 17.2 | 1.645 | 0.025 |
| gi|347441025 | arf gtpase-activating protein | 249.9 | 17.2 | 2.074 | 0.008 |
| gi|347828551 | phosphatidyl synthase | 72.6 | 9.4 | 1.967 | 0.029 |
| gi|154294387 | mitogen-activated protein kinase | 101.9 | 17.1 | 1.664 | 0.039 |
| gi|472240101 | alpha beta hydrolase fold-3 domain protein | 45.3 | 9.0 | 1.812 | 0.020 |
| gi|347827703 | BAR domain protein | 271.6 | 43.4 | 1.751 | 0.037 |
| gi|347830570 | Thujapil family protein | 645.5 | 37.0 | 1.703 | 0.016 |
| gi|347832713 | DUF1688 domain-containing protein | 437.7 | 27.6 | 1.726 | 0.034 |
| gi|472245392 | DUF718 domain-containing protein | 286.0 | 23.6 | 1.947 | 0.021 |
| gi|472245612 | c6 finger domain protein | 74.8 | 22.4 | 1.840 | 0.045 |
| gi|347836108 | C2 domain-containing protein | 248.4 | 22.4 | 1.782 | 0.029 |
| gi|347833490 | DUF757 domain-containing protein | 101.1 | 16.2 | 2.252 | 0.036 |
| gi|472236354 | yip1 domain-containing protein | 66.0 | 11.1 | 2.052 | 0.033 |
| gi|347838200 | FAD binding domain-containing protein | 117.4 | 10.7 | 2.072 | 0.015 |
| gi|347836441 | DUF89 domain-containing protein | 69.4 | 6.0 | 1.638 | 0.027 |
| gi|472240877 | bar domain-containing protein | 69.4 | 5.9 | 1.784 | 0.040 |
| gi|347832303 | acyl-CoA dehydrogenase domain protein | 202.2 | 19.9 | 2.010 | 0.042 |
| gi|472237107 | saff domain-containing protein | 94.8 | 8.5 | 1.933 | 0.015 |
| gi|347828586 | CUE domain-containing protein | 53.8 | 3.1 | 3.833 | 0.008 |
| gi|472244807 | calponin domain protein | 79.3 | 2.9 | 2.067 | 0.033 |
| gi|563296966 | KH domain protein | 31.2 | 1.7 | 1.900 | 0.011 |
| gi|347829378 | R2H domain-containing protein | 32.3 | 1.6 | 1.938 | 0.001 |
| gi|347836748 | pemilio domain-containing protein | 37.9 | 1.4 | 2.313 | 0.007 |
| gi|347836261 | methyltransferase domain-containing protein | 27.9 | 2.9 | 0.031 | 0.001 |
| gi|154691472 | eukaryotic peptide chain release factor subunit 1 | 426.9 | 30.8 | 1.912 | 0.036 |
| gi|347837479 | glia maturation factor gamma | 102.7 | 30.6 | 1.703 | 0.028 |
| gi|347837628 | CORD and CS domain-containing protein | 134.3 | 29.8 | 1.787 | 0.013 |
| gi|347828828 | ruvB-like helicase 1 | 417.5 | 30.4 | 1.502 | 0.035 |
| gi|347742085 | CND8 | 99.4 | 6.3 | 0.405 | 0.001 |
| gi|156051430 | 40S ribosomal protein S3 | 1,591.3 | 60.8 | 1.638 | 0.040 |
| gi|347827805 | 40S ribosomal protein S5 | 418.3 | 38.5 | 1.531 | 0.044 |
| gi|347835120 | 40S ribosomal protein S6 | 332.8 | 34.3 | 1.763 | 0.046 |
| gi|347836429 | 40S ribosomal protein S7 | 276.1 | 30.4 | 1.857 | 0.007 |
| gi|156043471 | 40S ribosomal protein S8 | 688.8 | 40.2 | 1.584 | 0.026 |
| gi|154291145 | 40S ribosomal protein S10 | 106.2 | 25.4 | 1.891 | 0.016 |
| gi|156061679 | 40S ribosomal protein S13 | 404.4 | 33.8 | 1.867 | 0.035 |
| gi|472237384 | 40S ribosomal protein S18 | 546.8 | 42.3 | 1.902 | 0.018 |
| gi|347837250 | 40S ribosomal protein S19 | 363.8 | 51.0 | 2.715 | 0.025 |
| gi|347841467 | 40S ribosomal protein S21 | 157.1 | 63.6 | 2.762 | 0.018 |
| gi|347829326 | 40S ribosomal protein S23 | 190.6 | 20.0 | 1.898 | 0.048 |
| gi|156065881 | 40S ribosomal protein S24 | 348.1 | 32.6 | 1.861 | 0.040 |
| gi|156065633 | 40S ribosomal protein S25 | 174.3 | 26.8 | 2.073 | 0.037 |
| gi|347832333 | 40S ribosomal protein S27 | 322.4 | 37.8 | 1.823 | 0.028 |
| gi|347828118 | 40S ribosomal protein S29 | 126.9 | 42.9 | 2.508 | 0.013 |
| gi|347827513 | 40S ribosomal protein S30 | 63.1 | 16.1 | 0.199 | 0.002 |

(Continued)
| Accession  | Protein name                                                | Score | Sequence coverage (%) | Fold | p-value |
|------------|-------------------------------------------------------------|-------|-----------------------|------|---------|
| gi|347828771 | 60S ribosomal protein L44 | 97.8 | 13.2 | 2.919 | 0.014 |
| gi|156062084 | 60S ribosomal protein L9 | 1,053.6 | 63.4 | 1.571 | 0.031 |
| gi|2229891536 | 54S ribosomal protein L4, mitochondrial | 54.3 | 6.8 | 0.375 | 0.024 |
| gi|156037530 | 60S ribosomal protein L12 | 608.9 | 40.0 | 1.562 | 0.010 |
| gi|347832401 | 60S ribosomal protein L13 | 444.7 | 33.0 | 1.662 | 0.032 |
| gi|347835805 | 60S ribosomal protein L6 | 611.8 | 33.0 | 1.670 | 0.023 |
| gi|347836248 | 60S ribosomal protein L10 | 126.5 | 11.3 | 2.336 | 0.039 |
| gi|347839766 | 60S ribosomal protein L16 | 271.7 | 29.7 | 2.055 | 0.039 |
| gi|154316257 | 60S ribosomal protein L17 | 563.9 | 30.5 | 2.136 | 0.011 |
| gi|154310248 | 60S ribosomal protein L19 | 409.8 | 29.4 | 2.652 | 0.009 |
| gi|347840178 | 60S ribosomal protein L21 | 247.9 | 35.6 | 1.977 | 0.029 |
| gi|347830985 | 60S ribosomal protein L23 | 425.6 | 48.9 | 1.936 | 0.030 |
| gi|347835534 | 60S ribosomal protein L24 | 274.0 | 29.0 | 2.291 | 0.015 |
| gi|347831348 | 60S ribosomal protein L26 | 236.5 | 36.8 | 2.174 | 0.030 |
| gi|347441549 | 60S ribosomal protein L27a | 708.8 | 30.5 | 1.603 | 0.018 |
| gi|347841474 | 60S ribosomal protein L28 | 271.7 | 29.7 | 2.055 | 0.039 |
| gi|154315039 | 60S ribosomal protein L35 | 140.2 | 18.9 | 2.744 | 0.024 |
| gi|156036474 | 60S ribosomal protein L36 | 166.0 | 35.9 | 1.648 | 0.038 |
| gi|154297848 | 60S acidic ribosomal protein P0 | 1,277.7 | 41.7 | 1.896 | 0.029 |
| gi|347833237 | 60S acidic ribosomal protein P1 | 553.2 | 41.2 | 2.379 | 0.011 |
| gi|347835558 | 60S acidic ribosomal protein P2 | 500.4 | 55.9 | 2.178 | 0.012 |
| gi|347441053 | ribosome associated DnaJ chaperone Zuotin | 635.2 | 25.3 | 1.863 | 0.029 |
| gi|156044830 | ribosome biogenesis protein Nhp2 | 106.9 | 9.8 | 1.594 | 0.024 |
| gi|229485391 | ribosome biogenesis protein P0a | 56.1 | 4.2 | 1.636 | 0.045 |
| gi|347837666 | nuclear transport factor 2 | 236.2 | 28.2 | 2.249 | 0.020 |
| gi|472246396 | nuclear segregation protein | 466.5 | 27.0 | 3.04 | 0.013 |
| gi|347835094 | leucyl-tRNA synthetase | 722.1 | 25.9 | 1.809 | 0.016 |
| gi|347835240 | methionyl-tRNA synthetase | 183.7 | 18.9 | 1.931 | 0.029 |
| gi|347828755 | tryptophanyl-tRNA synthetase | 283.9 | 23.4 | 1.864 | 0.037 |
| gi|563295297 | ribosome associated DnaJ chaperone Zuotin | 635.2 | 25.3 | 1.863 | 0.029 |
| gi|156044830 | ribosome biogenesis protein Nhp2 | 106.9 | 9.8 | 1.594 | 0.024 |
| gi|229485391 | ribosome biogenesis protein P0a | 56.1 | 4.2 | 1.636 | 0.045 |
| gi|347833265 | aspartyl-tRNA synthetase | 271.9 | 15.1 | 1.861 | 0.017 |
| gi|347836347 | phenylalanyl-tRNA synthetase beta chain | 159.9 | 13.5 | 2.148 | 0.003 |
| gi|347842507 | tRNA methyltransferase | 31.7 | 2.9 | 1.735 | 0.006 |
| gi|347837080 | polyadenylate-binding protein | 621.1 | 19.8 | 1.755 | 0.039 |
| gi|563295292 | histone H1-binding protein | 84.1 | 7.0 | 1.894 | 0.025 |
| gi|347223767 | oxysterol-binding protein | 154.5 | 6.5 | 3.378 | 0.014 |
| gi|154692219 | glycogen synthase | 204.9 | 11.1 | 1.884 | 0.038 |
| gi|154308576 | glucose-6-phosphate 1-dehydrogenase | 365.5 | 25.1 | 1.986 | 0.023 |
| gi|347833053 | 1,3-beta-glucan biosynthesis protein | 131.7 | 10.6 | 2.131 | 0.033 |
| gi|347841047 | plasma membrane stress response protein | 34.6 | 2.0 | 3.195 | 0.009 |
| gi|347830640 | methylethyltetrahydrofolate reductase | 196.2 | 13.4 | 1.552 | 0.019 |
| gi|154309515 | Ca/CaM-dependent kinase-1 | 141.7 | 18.4 | 1.566 | 0.036 |
| gi|347829911 | GTP-binding nuclear protein Ran | 301.8 | 38.1 | 1.732 | 0.025 |
| gi|472236275 | tRNA splicing endonuclease subunit protein | 96.8 | 14.5 | 2.013 | 0.007 |
| gi|347831289 | RNA binding effector protein Scp160 | 853.4 | 22.1 | 1.568 | 0.050 |
| gi|347839263 | DNA-directed RNA polymerase I subunit | 49.6 | 14.1 | 2.662 | 0.041 |

(Continued)
TABLE 1 | Continued

| Accession | Protein name                                      | Score | Sequence coverage (%) | Fold | p-value |
|-----------|--------------------------------------------------|-------|-----------------------|------|---------|
| gi|347441996| HAD superfamily hydrolase                        | 203.1 | 32.5                  | 1.599| 0.041   |
| gi|347840552| ubiquitin carboxyl-terminal hydrolase            | 362.9 | 27.1                  | 1.976| 0.026   |
| gi|347837756| ubiquitin-like protein SMT3                      | 34.9  | 18.8                  | 2.301| 0.030   |
| gi|472238757| ubiquitin-activating enzyme e1 protein            | 489.3 | 17.3                  | 1.665| 0.016   |
| gi|154695558| ubiquitin-conjugating enzyme E2                  | 36.3  | 7.5                   | 1.579| 0.042   |
| gi|472241717| ubiquitin thioesterase protein                   | 56.4  | 8.3                   | 1.749| 0.027   |
| gi|47440894 | translocon beta subunit Stb1                    | 225.3 | 44.6                  | 1.753| 0.042   |
| gi|472236180| minor allergen alt a 7 protein                   | 282.3 | 47.8                  | 2.844| 0.005   |
| gi|472235513| anthranilate synthase component 2 protein        | 392.7 | 20.7                  | 1.590| 0.029   |
| gi|347832723| nipsnap family protein                           | 154.3 | 19.9                  | 1.633| 0.026   |
| gi|347832071| phosphoglucomutase                               | 1,936.2| 53.1                | 1.896| 0.017   |
| gi|347829895| phosphomannomutase                               | 182.7 | 21.5                  | 1.854| 0.028   |
| gi|347832016| N-acetylglucosamine-phosphate mutase             | 436.9 | 26.4                  | 1.853| 0.011   |
| gi|472241846| UDP-galactopyranose mutase                       | 549.0 | 33.1                  | 2.149| 0.020   |
| gi|347841593| UDP-N-acetylglucosamine pyrophosphorylase        | 519.9 | 35.0                  | 1.922| 0.008   |
| gi|472237006| UDP-glucose 4-epimerase gal10 protein             | 191.1 | 20.5                  | 1.867| 0.009   |
| gi|472241485| mannose-1-phosphate guanylyltransferase alpha-a  | 584.1 | 36.3                  | 1.631| 0.033   |
| gi|472241485| nad h-dependent d-xylene reductase xyl1 protein  | 247.9 | 28.6                  | 1.541| 0.046   |
| gi|347828612| transketolase                                    | 1,284.8| 41.2                | 2.020| 0.013   |
| gi|154321267| phosphoketolase                                  | 883.5 | 24.4                  | 1.836| 0.042   |
| gi|347842358| acetyl-CoA acetyltransferase                     | 449.4 | 46.3                  | 1.982| 0.018   |
| gi|347830285| phospho-2-dehydro-3-deoxyheptonate aldolase      | 460.2 | 36.1                  | 1.950| 0.027   |
| gi|347840715| 3-isopropylmalate dehydratase                    | 593.0 | 29.8                  | 1.519| 0.019   |
| gi|472240697| cyanide hydratase/nitrilase                      | 353.7 | 17.0                  | 2.551| 0.012   |
| gi|347832595| aldo/keto reductase family oxidoreductase        | 497.6 | 42.5                  | 1.999| 0.018   |
| gi|154322845| aldo/keto reductase                              | 327.8 | 28.9                  | 1.724| 0.044   |
| gi|347836965| nitroreductase family protein                    | 228.3 | 32.7                  | 1.893| 0.018   |
| gi|154293270| glucose 1-dehydrogenase                          | 263.4 | 27.8                  | 1.636| 0.043   |

Fold: the average ratio (control/TTO-treated) of protein levels from three biological replicates as determined by iTRAQ approach. A protein was considered a differential expression protein as it exhibited a >1.5-fold or <0.67-fold change and P < 0.05.

using three attributes: biological process, molecular function, and cellular components (Ashburner et al., 2000). A GO analysis (http://www.geneontology.org) was conducted to assign functional annotations for differentially expression proteins (DEPs), and the Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg) was used to predict the primary metabolic and signal transduction pathways in which the identified DEPs are involved.

Confocal Laser Scanning Microscopy
To assess the effects of TTO on the cytoplasmic membranes of B. cinerea, confocal laser scanning microscopy (LSM 880, Carl Zeiss, Germany) was performed, using the fluorescent indicator propidium iodide (PI) (Sigma-Aldrich, USA) and a modified protocol (Lee and Kim, 2017). B. cinerea cells containing 4 × 10^6 spores/ml were added to each glass tube and incubated with TTO (final concentration 5 mL/L) with shaking at 200 rpm at 25°C for 2 h. The cells were washed and resuspended in 0.5 mL PBS (pH 7.4), stained with PI (10 μM final concentration) for 30 min at room temperature in the dark, and then washed twice with PBS. Images were acquired using confocal laser scanning microscopy. The experiment was repeated three times.

Measurement of Enzyme Activities Related to TCA Cycle
Using the protocol described above (see Protein Extraction), ground mycelium was suspended in PBS (pH 7.4) and centrifuged at 10,000 × g for 10 min at 4°C. Enzyme activities were measured in the supernatant for malate dehydrogenase (MDH), citrate synthase (CS), and oxoglutarate dehydrogenase (OGDH), using kits purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China), following the manufacturer’s instructions. Protein concentration was determined using a method based on the (Bradford, 1976) assay. MDH activity was calculated as μmol of NAD reduced per minute per mg of protein (U/mg protein). One unit of CS activity was defined as the amount of enzyme that produces 1 μmol of citric acid per minute (U/mg protein). OGDH activity was defined as the amount of enzyme that produces 1 nmol of NADH per minute (U/mg protein). Measurements were performed at 595 nm using three replicates for each sample.
RESULTS

Identification of B. cinerea Proteins by iTRAQ

A total of 204,639 spectra were generated by iTRAQ proteomic analysis using control and TTO-treated B. cinerea and were analyzed using the Mascot search engine. As shown in Figure 1A, 17,337 spectra matched known spectra, comprising 10,001 peptides, 9,720 unique peptides, and 2,397 proteins from control and TTO-treated samples. The distribution of the number of peptides, predicted molecular weights, and isoelectric points, and peptide sequence coverage are shown in Figures 1B–D, respectively. Over 87% of the proteins were represented by at least two peptides. Molecular weights ranged from 20 to 200 kDa, and isoelectric points ranged from 5.0 and 7.0. Approximately 51% of identified proteins had more than 10% peptide sequence coverage.

Identification of Differentially Expressed Proteins Using iTRAQ

The threshold for differential expression (TTO-treated vs. control) was a protein level difference >1.5 or <0.67, with a \( p < 0.05 \). 718 differentially expressed proteins were identified in the TTO sample, of which 17 were up-regulated and 701 were down-regulated. Details for each protein are provided in Table 1.

GO Analysis of DEPs

GO analysis was conducted to identify significantly enriched GO functional groups. DEPs were categorized by biological process, cellular component, and molecular function. Of the 718 DEPs, 562 were annotated and classified into 30 functional groups (Figure 2). Biological processes accounted for 12 GO terms (with “metabolic process” accounting for 44.11% of these, and “cellular process” 34.32%). Cellular components accounted for 7 GO terms, dominated by “cell” (31.60%) and “cell part” (31.60%). Molecular functions accounted for 11 GO terms, the most abundant being “catalytic” (44.72%) and “binding” (43.61%).

The agriGO analysis tool was used to detect and visualize significantly enriched GO terms associated with the 562 annotated proteins, with an adjusted \( p \)-value cutoff of 0.05. Significant functional groups included “regulation of biological quality” (GO:0065008, \( p = 0.033 \)) and “primary metabolic process” (GO:0044238, \( p = 0.016 \)). There are 5 DEPs, accounting for about 45.45% of the total protein in regulation of biological quality. And 189 DEPs, accounting for about 73.82% of the total protein in primary metabolic process.

KEGG Analysis of DEPs

Proteins typically do not exercise their functions independently, but coordinate with each other to complete a series of biochemical reactions. Pathway analysis can help reveal cellular processes involved in disease mechanisms or drug action. Using the KEGG database as a reference, 562 DEPs were linked to 133 different pathways. Glycolysis, the TCA cycle, and purine metabolism were among the pathways most significantly altered by exposure to TTO.

DISCUSSION

The antifungal activity of essential oils is probably based on their ability to significantly reduce total lipid and ergosterol content, thereby disrupting membrane permeability and resulting in leakage of cell components such as ATP, DNA, and potassium ions (Tian et al., 2011; Tao et al., 2014; Cui et al., 2015). Our previous study demonstrated that TTO considerably increases membrane permeability, causing extrusion of abundant material (Shao et al., 2013b; Yu et al., 2015) and decreasing intracellular ATP in B. cinerea (Li et al., 2017b). In this study, observations using confocal laser scanning microscopy indicate that TTO damages the B. cinerea cell membrane, potentially causing the release of internal material such as ATP.

Levels for many DEPs related to glycolysis metabolism, such as glucose-6-phosphate isomerase, 6-phosphofructokinase, phosphoenolpyruvate carboxykinase, fructose-1, 6-bisphosphatase, and enolase, are decreased by TTO treatment. Using the KEGG database as a reference, 562 DEPs were linked to 133 different pathways. Glycolysis, the TCA cycle, and purine metabolism were among the pathways most significantly altered by exposure to TTO.
in the second step of glycolysis (Achari et al., 1981). 6-phosphofructokinase is a key enzyme in the control of the glycolytic pathway in nearly all cells (Wang et al., 2016). The activity of this enzyme is controlled by several metabolites, most notably its two substrates, fructose 6-phosphate and ATP. Glycolysis is also an important pathway for energy production in the cytosol of plant cells. Our results suggest that TTO inhibits glycolysis and may affect energy supply in B. cinerea.

Mitochondria are the primary sites of aerobic respiration in eukaryotic cells. They generate energy for cellular functions through oxidative phosphorylation and the TCA cycle, and also play a crucial role in regulating the apoptosis (Shaughnessy et al., 2014). In this study, several proteins associated with the mitochondrial respiratory chain and TCA cycle, such as ATP synthase D chain, ATP synthase subunit e, MDH, CS, and OGDH, were significantly down-regulated in cells treated with TTO (Table 1). ATP synthase D chain and ATP synthase subunit e are involved in the biosynthesis of ATP. Dill oil inhibits mitochondrial ATPase activity and dehydrogenase activities, and affects mitochondrial function in Aspergillus flavus (Tian et al., 2012). Mustard essential oils decrease intracellular ATP and increase extracellular ATP in E. coli O157:H7 and Salmonella typhi (Turgis et al., 2009). Citral decreases intracellular ATP content, increases extracellular ATP content, inhibits the TCA pathway, and decreases the activities of CS and α-ketoglutarate dehydrogenase in Penicillium digitatum (Zheng et al., 2015). Our additional study demonstrates that TTO treatment significantly inhibits the activities of MDH, CS, and OGDH (Figure 4). In our previous study, we found that TTO decreases intracellular ATP and the activities of MDH, succinate dehydrogenase, ATPase, CS, isocitrate dehydrogenase, and α-ketoglutarate dehydrogenase, disrupting the TCA cycle in B. cinerea (Li et al., 2017b). The down-regulation of two MDHs suggests that the Krebs cycle is not completely functional in Paracoccidioides lutzii upon exposure to argentilactone (Prado et al., 2014). Together, these results imply that TTO affects proteins in B. cinerea involved in glycolysis, the TCA cycle, and ATP synthesis, thereby disrupting the TCA cycle, interrupting energy metabolism, and inducing mitochondrial dysfunction.

Cytochrome c (cyt c) is a hemoglobin located in the inner mitochondrial membrane, and is responsible for transferring electrons between mitochondrial electron transport chain complexes III and IV (Reed, 1997; Lo et al., 2017). ATP is produced by the aerobic mitochondrial respiratory chain. Abnormal cyt c disrupts the mitochondrial respiratory chain and impacts ATP production (Zhou et al., 2015). Our study shows that cyt c is up-regulated in B. cinerea after TTO treatment at 5 mL/L (Table 1). The increase in cyt c levels may improve the performance of the oxidative
respiratory chain, perhaps as a protective response to TTO toxicity.

Purines are one of the building blocks for nucleic acids. Their synthesis pathways generate many kinds of energy molecules (Qian et al., 2014). Inosine 5'-monophosphate dehydrogenase (IMPDH) is a rate-controlling enzyme in the de novo synthesis of the guanine nucleotide, and plays crucial roles in cell growth and proliferation (Fotic, 2016). IMPDH inhibition reduces guanine nucleotide pools and interrupts cellular functions such as DNA replication, RNA synthesis, and signal transduction (Weber, 1983; Weber et al., 1996). These effects are associated with cell cycle disruption, cellular differentiation, and apoptosis (Vitale et al., 1997; Yalowitz and Jayaram, 2000). Nucleoside diphosphate kinases (NDPK) are critical enzymes related to the maintenance of intracellular nucleotide levels, and catalyze the conversion of nucleoside triphosphates to nucleoside diphosphates in all living organisms (Véron et al., 1994). Both NDPK and AK can mediate the conversion of adenosine into ATP by ADP and AMP (Senft and Crabtree, 1983). In our study, TTO treatment decreased IMPDH levels (Table 1). Furthermore, levels of adenosine kinase AK and NDPK were also reduced after TTO treatment (Table 1). From these results, we can conclude that TTO may block the accumulation of energy and disrupt the cell cycle, ultimately inducing apoptosis.

CONCLUSION

The effect of TTO treatment on proteins in B. cinerea is summarized in Figure 5. We found that important metabolic pathways, including glycolysis, the TCA cycle, and purine metabolism, were compromised by TTO treatment, while cyt c increased. We conclude that the disruption of energy
metabolism by TTO contributes to its antifungal activity against
*B. cinerea*.

**AUTHOR CONTRIBUTIONS**

JX and XS designed the experiments. JX and YW performed the
experiments. FX and HW analyzed the data. JX, XS, and HW
drafted the manuscript. All authors read and approved the final
manuscript.

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