Proteome analysis of an ectomycorrhizal fungus
Boletus edulis under salt shock

Yu LIANG*, Hui CHEN, Mingjuan TANG, Shihua SHEN*

Key laboratory of Photosynthesis and Environmental Molecular Physiology, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, P. R. China

ABSTRACT

Soil salinization has become a severe global problem and salinity is one of the most severe abiotic stresses inhibiting growth and survival of mycorrhizal fungi and their host plants. Salinity tolerance of ectomycorrhizal fungi and survival of ectomycorrhizal inocula is essential to reforestation and ecosystem restoration in saline areas. Proteomic changes of an ectomycorrhizal fungus, Boletus edulis, when exposed to salt stress conditions (4% NaCl, w/v) were determined using two-dimensional electrophoresis (2DE) and mass spectrometry (MS) techniques. Twenty-two protein spots, 14 upregulated and 8 downregulated, were found changed under salt stress conditions. Sixteen changed protein spots were identified by nanospray ESI Q-TOF MS/MS and liquid chromatography MS/MS. These proteins were involved in biosynthesis of methionine and S-adenosylmethionine, glycolysis, DNA repair, cell cycle control, and general stress tolerance, and their possible functions in salinity adaptation of Boletus edulis were discussed.

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Introduction

Soil salinization has become a severe global problem, with 950 M ha of the earth’s soil being saline soil (Li et al. 2005). In China, for example, there are more than 30 M ha of saline soil and 9 M ha of secondary salinized soil (Wang 1993; Li et al. 2005). The methods to improve the stress resistance and survival of plant seedlings have become more and more important for reforestation and ecosystem reestablishment in saline areas. There are ca 6 K ectomycorrhizal fungal (EMF) species (Molina et al. 1992), and over 3 % of plant species could form ectomycorrhizas with EMF. The important role of EMF in forest establishment and recovery has been well documented, and the formation of ectomycorrhizae is essential for the survival of EM trees, as well as successful reforestation in saline areas (Lamhamedi et al. 1992; Martins et al. 1996; Amaranthus 1998; Duñabeitia et al. 2004; Turjaman et al. 2005). Two intrinsic characteristics may influence the functioning of EMF species in reforestation in saline areas: (1) host specificity of the fungus in the soil; (2) the tolerance of inoculated or native EMF species to salt stress.

King bolete (Boletus edulis) is an EMF widely distributed in temperate and subtropical forests (Mao 1997; Dunstan et al. 1998; Hall et al. 1998; Vance et al. 2001). It belongs to EMF species with a broad host range and could form mycorrhizal relationships with conifers, oak, birch, fir, etc (Molina et al. 1992; Mao 1997). Abiotic stress tolerance varies among different EMF species, and B. edulis was reported as a tolerant species...
and could grow even at a water potential of ~3 MPa (Coleman et al. 1989). The broad host range and high stress tolerance have made B. edulis a suitable EMF species that could be introduced with various host tree species in reforestation and ecosystem reestablishment in saline areas.

High salinity usually causes water deficit, ion toxicity, and nutrient deficiency leading to molecular damage, growth arrest, and even death of organisms. Some fungal species, e.g., yeasts, can tolerate high saline environments and have developed defence systems, including osmolyte synthesis and cation transport systems for sodium exclusion under salt stress (Garcia et al. 1997). Transcriptional data from some recent studies on yeast have indicated that responses to salt stress require the activity of several pathways and many genes are involved (e.g. Gasch & Werner-Washburne 2000; Posas et al. 2000). However, the lack of knowledge regarding genetic mutations and genomic information of ECM fungi, make it difficult to understand the molecular mechanisms of these fungi in response to salinity stress.

Proteomics is a useful tool to study organism responses under abiotic stresses, offering a powerful tool for the identification of proteins associated with a particular environmental signal (e.g. Novotna et al. 2003; Kav et al. 2004; Qi et al. 2004). Unlike methods to study individual proteins, proteomics is used to study a global expression profile of proteins, which better enable us to understand the systematic and integrative responses of organism cells to environmental stresses, as well as interactions and inter-regulations among proteins under these stresses. Proteomics has been widely applied to study cellular responses of plants and microbes under abiotic stress (e.g. Cui et al. 2005; Topanurak et al. 2005; Yan et al. 2005), and great achievements have been made to understand the integrative molecular regulation under these stresses.

In the present study, proteomic changes of the EMF, Boletus edulis, under saline stress (4 % NaCl, w/v) were studied, and our objective was to find which proteins were involved in responses to salinity and to better understand the adaptation mechanisms of B. edulis to salt stress.

## Materials and methods

### Fungal materials and salt treatment

Boletus edulis was provided by professor Liang-dong Guo and the fungal materials were kept in the Institute for Microbial Resources of the institute of Microbiology, Chinese Academy of Sciences. Mycelia of B. edulis were first grown on potato-dextrose agar (PDA) for a week (25 °C). Rounded pieces of mycelia (5.5 mm diam) were taken from the colony margin and grown at 25 °C on potato-dextrose agar media containing 0, 1, 2, 4, 8 % NaCl (w/v), respectively. Radial growth of B. edulis colonies at different NaCl concentrations was measured.

Mycelia of B. edulis were grown in sterilized potato-dextrose liquid culture (25 °C, 160 rev min⁻¹). After one-week of growth, solid NaCl was added to obtain a final concentration of 4 % (w/v). B. edulis treated with NaCl for 6 h and untreated controls (three replications) were centrifuged at 6000 g for 5 min at 4 °C to retrieve the mycelia.

### Protein extraction

Proteins were extracted using a modified protocol according to Shen et al. (2003). Five hundred milligram samples were ground into fine powder in liquid nitrogen with a precooled mortar and pestle, and homogenized in 2 ml homogenization buffer containing 20 mM Tris-HCl (pH 7.5), 250 mM sucrose, 10 mM ethylene glycol-bis (β-aminoethylether)N,N,N',N'-tetraacetic acid (EGTA), 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM dithiothreitol (DTT) and 1 % Triton X-100. The homogenate was transferred into an eppendorf tube and centrifuged at 15,000 g for 15 min at 4 °C. The supernatant was transferred to a new tube and the protein was precipitated using 1/4 volume 50 % cold trichloroacetic acid (TCA) in an ice water-bath for 30 min. The mixture was centrifuged at 15,000 g for 15 min at 4 °C, and the supernatant was discarded. The pellet was washed with acetone three times, centrifuged and vacuum-dried. The dried powder was dissolved in sample buffer containing 9 M urea, 4 % Nonidet P-40 (NP-40), 2 % ampholine pH 3.5-10 (GE Healthcare Bio-Science, Little Chalfont) and 5 % 2-mercaptoethanol. The protein concentrations in the buffer were determined according to Bradford (1976), with bovine serum albumin as the standard.

### Two-dimensional gel electrophoresis (2DE)

2DE was carried out according to Shen et al. (2003). First-dimensional isoelectric focusing (IEF) was performed in a 13 cm-long glass tube, 3 mm diam. The gel mixture consisted of 4 % acrylamide, 8 % urea, 5 % ampholine (1 part pH 3.5–10, 1 part pH 5–8) and 2 % NP-40. IEF was performed at 200, 400, and 800 V for 30 min, 15 h and 1 h, respectively. About 500 μg protein was loaded. After the first-dimensional run, gels were incubated in equilibration buffer [0.05 M Tris–HCl pH 6.8, 2.5 % SDS, 10 % (v/v) glycerol and 5 % 2-mercaptoethanol] for 15 min twice. The second-dimensional electrophoresis was performed on vertical slab gels (135 × 170 × 1 mm), and a Laemmli buffer system (1970) was used to cast 5 % stacking gel and 15 % resolving gel. After electrophoresis, the gels were stained with 0.1 % Coomasie brilliant blue (CBB) R-250.

### Image analysis

The stained gels were scanned at 600 dots per inch (dpi) resolution using a UMAX Power Look 2100XL scanner (Maxium Tech, Taipei). The transparency mode was used to obtain a greyscale image. The image analysis was performed with ImageMaster™ 2D Platinum software (GE Healthcare Bio-Science). The optimized parameters were as follows: saliency 2, partial threshold 4, and minimum area 50. The percentage of spot volumes (%) between control and salt treatment were compared using Student’s t-test, and only significantly changed protein spots (p < 0.05) were subject to protein identification.
Protein spots were excised from the gels manually and cut into small pieces. Protein digestion was performed according to Shen et al. (2003) with slight modification. Each small gel piece with protein was washed with 25 % (v/v) ethanol and 7 % acetic acid for about 12 h or overnight at room temperature, destained with 50 mM NH₄HCO₃ in 50 % (v/v) methanol for 1 h at 40 °C. The protein in the gel piece was reduced with 10 mM DTT in 100 mM NH₄HCO₃ for 1 h at 60 °C and incubated with 40 mM iodoacetamide in 100 mM NH₄HCO₃ for 30 min at room temperature. The gel pieces were minced and lyophilized, then rehydrated in 25 mM NH₄HCO₃ with 10 ng sequencing grade modified trypsin (Promega, Madison, WI) at 37 °C overnight. After digestion, the protein peptides were collected, and the gels were washed with 0.1 % trifluoroacetic acid (TFA) in 50 % acetonitrile three times to collect the remaining peptides. The peptides were desalted by ZipTipC₁₈ (Millipore, Bedford, MA) and co-crystallized with one volume of saturated α-cyano-4-hydroxycinnamic acid in 50 % (v/v) acetonitrile containing 1 % TFA.

The desalted protein samples were subject to nanospray electrospray ionization quadrupole time-of-flight mass spectrometry (ESI Q-TOF MS/MS) or liquid chromatography mass spectrometry (LC-MS/MS).

Q-TOF MS/MS

The applied spray voltage was 800–1000 V, with a sample cone working on 25–40 V. The MDP detector working voltage was 2250 V, and energy adjustable collision cell was filled with pure argon gas. MS/MS data were processed using MassLynx 3.5 and searched against NCBI protein sequence databases with the MS/MS ion searching program MASCOT (http://www.matrixscience.com).

LC MS/MS

Liquid chromatography (LC) was performed on a surveyor LC system (Thermo Finnigan, San Jose, CA). The C18 column was obtained from Column Technology (Fremont, CA). Mobile phase A was 0.1 % formic acid in water, and mobile phase B was 0.1 % formic acid in acetonitrile. The tryptic peptide mixtures were eluted using a gradient of 2–98 % B over 180 min. The MS/MS was performed on a LTQ linear ion trap mass spectrometer (Thermo Finnigan, San Jose, CA) equipped with an electrospray interface and operated in positive ion mode. The capillary temperature was set to 170 °C and the spray voltage was 3.4 kV. The acquired MS/MS spectra were compared against the NCBI Basidiomycota protein database using the TurboSEQUEST program in the BioWorks 3.1 software suite (Thermo Finnigan). An accepted SEQUEST result had to have a Xcorr score of at least 0.1 (regardless of charge state). Peptides with a +1 charge state were accepted if they were fully tryptic-case digested and had a cross-correlation (Xcorr) of at least 1.9. Peptides with a +2 charge state were accepted if they had an Xcorr ≥ 2.2. Peptides with a +3 charge state were accepted if they had an Xcorr ≥ 3.75.

Results and discussions

Mycelial growth of Boletus edulis under different NaCl concentrations

The radial growth of the Boletus edulis mycelia on PDA with different NaCl concentrations is shown in Fig 1. Mycelial growth of B. edulis was inhibited by NaCl except on the PDA medium with 1 % NaCl. After 2 d, the radial growth B. edulis on PDA with NaCl concentration equal or lower than 4 % was found to fit a linear growth model. The growth of B. edulis was greatly inhibited on PDA medium with 8 % NaCl. After one-month of growth, the diameter of mycelial colony reached to 5.8 ± 0.3 cm (mean ± s.e., data not shown). Tresner & Hayes (1971) determined the NaCl tolerance of 975 terrestrial fungal species and found that Basidiomycetes were the least tolerant with about 75 % of species being unable to withstand 4 % NaCl. Our results indicated that B. edulis had a relatively higher NaCl tolerance and could grow well on PDA medium containing 4 % NaCl. The high salinity tolerance enables B. edulis to survive and grow in almost all saline areas. Considered the wide host range of B. edulis, it would be a suitable EMF species to apply to reforestation and ecosystem restoration of saline areas.

2-DE and image analysis

Over 700 reproducible protein spots were detected on the each gel of control and salt stress treatment with the pH range of 3.5–10 and the mass range of 20–120 kDa (Fig 2). Eighteen protein spots, comprising ten upregulated (U1-U10) and eight downregulated proteins (D1-D8), were found significantly changed under salt stress. The folds, which are defined as the ratio of mean percentages of spots volume (vol%) on the treated gels to those on the control gels, ranged from 1.79 to 17.55 for the upregulated proteins, and 0.05 to 0.52 for the downregulated proteins.

Fig 1 – Radial growth of Boletus edulis mycelia on PDA with different NaCl concentrations (bars indicate standard deviations).
Twelve changed protein spots, comprising six upregulated and six downregulated proteins, were identified using ESI Q-TOF MS/MS and LC MS/MS (Table 1). These identified proteins were involved in cellular processes, such as biosynthesis of methionine (Met) and S-adenosylmethionine (AdoMet; Fig 3). MHMT catalyses the synthesis of cystathionine from cysteine and O-phospho-L-homoserine (OPH) in a $\gamma$-replacement reaction (Ravanel et al. 1998). MHMT is involved in L-methionine biosynthesis from 5-methyltetrahydropteroyltriglutamate (5-CH$_3$-H$_4$-pteroyl-(glu)$_3$) and homocysteine (Whitfield et al. 1970; Urbanowski et al. 1987). RMT3 catalyses the transfer of a methyl group from S-adenosylmethionine (AdoMet) to a guanidino nitrogen of arginine, resulting in S-adenosyl-homocysteine (AdoHcy) and methylarginine (Bedford & Richard 2005).

Biosynthesis of Met and AdoMet might play an important role in fungal responses to salt stress. It has been reported that a yeast methionine biosynthetic gene $\text{HAL2 (MET22)}$, is capable of improving growth under salt stress, and methionine supplementation may also improve the tolerance of yeast to NaCl (Gläser et al. 1993). AdoMet is an important metabolite participating in many cellular processes, e.g. methylation of proteins, nucleic acids, polysaccharides and fatty acids, biosynthesis of ethylene and polyamines, cell wall synthesis and modifications (Espartero et al. 1994).

The upregulation of MHMT is expected to enhance the biosynthesis of methionine and the downregulation of RMT3 may reduce the AdoMet consumption in arginine methylation of proteins. The changes of these two enzymes may cause the accumulation of methionine and AdoMet in the fungus, which could meet the increased demand in stress-tolerance-related processes such as hormone synthesis and cell wall modifications. It is interesting to find that CGS was downregulated in our study. One possible reason, as suggested by Kim et al. (2002), may be the negative regulation of methionine accumulation. The role of CGS in the biosynthesis of methionine and S-adenosylmethionine is still unclear, and results of transgenic studies showed that CGS repression did not cause a decrease of cystathionine concentrations in plants (Kreft et al. 2003).

Proteins involved in glycolysis

Enhanced glycolysis and increased expression of related enzymes under abiotic stresses have been well documented (e.g. Umeda & Uchimiya 1994; Suzuki et al. 2005). In the present study, glyceraldehyde-3-phosphate dehydrogenase (U2) involved in glycolysis was upregulated, which may be related to glycolysis enhancement and be responsible for the energy supply under salt stress. Another possible function of increased glycolysis under salt stress is osmoregulation, for the biosynthesis of glycerol, a common osmolyte of fungi, is connected with glycolysis (Blomberg 2000). In a recent study on proteomic changes of yeast to salt stress, enzymes in glycolysis also changed significantly (Gori et al. 2007), which indicates the important role of glycolysis regulation during salinity adaptation of fungi.

Proteins related to DNA repair

Two parallel pathways, one error free and another error prone (mutagenic), were found in the ubiquitin-related DNA repair system. In Saccharomyces cerevisiae the homologue of...
Table 1 – Significantly changed proteins identified in this study

<table>
<thead>
<tr>
<th>Spot no.</th>
<th>Mr/pI</th>
<th>Matched sequences (Q-TOF MS/MS) or no. of matched peptides (LC MS/MS)</th>
<th>Coverage</th>
<th>Name</th>
<th>Organism</th>
<th>Accession no.</th>
<th>Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>U1²</td>
<td>85593/5.89</td>
<td>FPTTTIESPQFK LDADVISEAARK</td>
<td>3 %</td>
<td>5-Methyltetrahydropteroylglutamate-homocysteine S-methyltransferase</td>
<td>Cryptococcus neoformans</td>
<td>AAW46187</td>
<td>2.39**</td>
</tr>
<tr>
<td>D¹³</td>
<td>60243/6.10</td>
<td>VNANAEACIR FNNSGALYYGNSK</td>
<td>6 %</td>
<td>Cystathionine gamma-synthase (EC 2.5.1.48)</td>
<td>Neurospora crassa</td>
<td>P38675</td>
<td>0.23*</td>
</tr>
<tr>
<td>D²³</td>
<td>65727/4.75</td>
<td>NAGLDQDSQSEADDPDK</td>
<td>3 %</td>
<td>Arginine N-methyltransferase</td>
<td>Cryptococcus neoformans</td>
<td>XP_570924</td>
<td>0.38*</td>
</tr>
<tr>
<td>U²³</td>
<td>36033/7.01</td>
<td>SVNNNIPSTGAAK LTQLAPF</td>
<td>10 %</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>Schizopyllum commune</td>
<td>P32638</td>
<td>2.23*</td>
</tr>
<tr>
<td>D⁴³</td>
<td>89457/4.9</td>
<td>13</td>
<td>10 %</td>
<td>MMα2</td>
<td>Cryptococcus neoformans</td>
<td>XP_567564</td>
<td>0.52*</td>
</tr>
<tr>
<td>U³³</td>
<td>98144/5.26</td>
<td>TAVALSFGGVAK</td>
<td>1 %</td>
<td>Probable DNA replication licensing factor [nimQ]</td>
<td>Neurospora crassa</td>
<td>CAD72055</td>
<td>17.55**</td>
</tr>
<tr>
<td>U⁴³</td>
<td>72873/5.57</td>
<td>FNGDGQGSK QVSEMVPNLTDK</td>
<td>3 %</td>
<td>Similar to CaN425/CarDF1; Candida albicans</td>
<td>Debaryomyces hansenii</td>
<td>Q8BBF7</td>
<td>1.79*</td>
</tr>
<tr>
<td>D³³</td>
<td>22802/5.31</td>
<td>LLLGOSVGGQ TTISEYVR</td>
<td>9 %</td>
<td>Ypt1</td>
<td>Schizosaccharomyces pombe</td>
<td>CAA63619</td>
<td>0.05**</td>
</tr>
<tr>
<td>D⁵³</td>
<td>65096/6.05</td>
<td>GGVLPDLSQAPK</td>
<td>2 %</td>
<td>Unnamed protein product similar to ca/CaSNF5.3f</td>
<td>Debaryomyces hansenii</td>
<td>CAG89206</td>
<td>0.41*</td>
</tr>
<tr>
<td>U⁵³</td>
<td>59019/5.30</td>
<td>EADNDAGLATVAR</td>
<td>2 %</td>
<td>calcineurin A</td>
<td>Aspergillus oryzae</td>
<td>BAA69952</td>
<td>2.02**</td>
</tr>
<tr>
<td>U⁶³</td>
<td>70581/5.13</td>
<td>13</td>
<td>12 %</td>
<td>Heat shock protein HS51</td>
<td>Puccinia graminis</td>
<td>Q01877</td>
<td>1.79**</td>
</tr>
<tr>
<td>D⁶³</td>
<td>61191/4.21</td>
<td>GVBVTSQRSGTAHSLNTR</td>
<td>6 %</td>
<td>Hypothetical protein UM02452.1</td>
<td>Ustilago maydis</td>
<td>XP_758599</td>
<td>0.26**</td>
</tr>
</tbody>
</table>

a Functional catalogue of protein: 1 metabolism, 2 energy, 3 DNA repair, 4 cell cycle and development, 5 stress tolerance, 6 protein with unknown function.

b Fold: the ratio of mean spot vol% of the treatment gels to that of the control gels. * and ** indicate significant changes at 0.05 and 0.01 levels.
Salt stress tolerance proteins

Heat shock protein HSS1 (U6) belongs to the heat shock protein family. Proteins in this family are induced by a variety of environmental stresses and have been mentioned as contributing to salt tolerance (Lewis et al. 1995; Lahav et al. 2004). Calcineurin A (U5) is the catalytic subunit of calcineurin, a Ca\(^{2+}\) and calmodulin-dependent serine-threonine protein phosphatase (Rusnak & Mertz 2000). Calcineurin is essential for NaCl tolerance of yeast and could promote yeast survival under environmental stress (Mendoza et al. 1994; Bultynck et al. 2006). The role of calcineurin in salt tolerance is thought to be mediated by transcriptional and post-translational mechanisms (Rusnak & Mertz 2000), and it was reported that calcineurin-deficient yeast exhibited decreased tolerance to Na\(^+\) and Li\(^+\) (Nakamura et al. 1993; Mendoza et al. 1994). The upregulation of two stress tolerance proteins, calcineurin A and heat shock protein HSS1, may enhanced salt tolerance of Boletus edulis and promotes survival of the fungus under salt stress.

In conclusion, proteins related to multiple cellular processes, e.g. metabolism, energy related processes, DNA repair, cell cycle control, and stress tolerance, were found involved in the stress responses of B. edulis to salt stress in the present study. Paying more attention to these cellular processes rather than individual stress-tolerance related proteins will be helpful for better understanding of the global response mechanisms of organisms to abiotic stresses.

Acknowledgements

This work was supported by the Knowledge Innovation Project of the Institute of Botany, the Chinese Academy of Sciences (C-810). We are grateful to Liang-dong Guo for providing fungal isolates. We also thank Kate Partner and two anonymous reviewers for their comments and suggestion on an early version of this paper.

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