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Proteome analysis of an ectomycorrhizal fungus *Boletus edulis* under salt shock

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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.

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

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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 osmotic adjustment by 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 Center 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 pre-cooled 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 M 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 % Coomassie 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.