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# PROTEIN EXPRESSION CHANGES IN LEAVES OF BALD CYPRESS (*TAXODIUM DISTICHUM* (L.) RICH.) IN RESPONSE TO WATERLOGGING

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*Key words*: *Taxodium distichum*, Waterlogging, Soluble protein, Protein expression

**Abstract**

To gain a better understanding of bald cypress (*Taxodium distichum* (L.) Rich.) response to waterlogging at the protein level, a proteomic approach has been conducted. One-year old bald cypress seedlings were selected for three waterlogging treatments (high, medium and low waterlogging), and one control (75% field capacity). Leave samples were collected at 8, 18, 38 and 60 days under waterlogging conditions. We also carried out a parallel recovery experiment by removing waterlogging at 50 days and sampled the leave ten days later. The results showed that the waterlogging treatments had significant effect on leaf soluble protein content. High and medium waterlogging increased soluble protein contents in the early stage, but decreased soluble protein content in the later stage of treatment. Low waterlogging had the highest soluble protein contents in all sampling days. The leaf protein SDS-PAGE analyses showed that three new protein subunits were found among the 28 - 34 subunits in the image profiles for the waterlogging and 10-day recovery treatments. The 2-DE leaf protein profile analysis revealed that 37 protein spots were differentially expressed, and 41 spots were shown to be up-regulated in bald cypress leaves in comparison with the control. These specific or up-regulated proteins were found to be similar with some enzymes related to aerenchyma formation, anaerobic respiration and other anaerobic polypeptides, suggesting that bald cypress had higher adaptation to waterlogging. The present study presents the evidence to further understanding the molecular response of bald cypress to waterlogging.

## Introduction

Waterlogging is a usual environmental stress in tropical and subtropical regions because of excessive rainfall and poor soil drainage. Most plant species grow slowly or die when oxygen availability is limited due to waterlogging, but some species have an excellent ability to endure waterlogging conditions, and certain species can even grow vigorously in response to less sever waterlogging (Voesenek *et al.* 2006, Bailey-Serres and Voesenek 2008). Traditionally, woody species are classified as wet tolerant, intermediate and intolerant species (Sinclair *et al.* 1987). For example, bald cypress (*Taxodium distichum* (L.) Rich.) and red maple (*Acer rubrum* L.) are wet-tolerant, silver maple (*Acer saccharinum* L.) and speckled alder (*Alnus rugosa* (Duroi.) Clausen) are intermediate, and sugar maple (*Acer saccharum* Marsh) and Norway spruce (*Picea abies* (L.) Karst), which can be injured by very light flooding event, are wet intolerant. Wet-tolerant plants have developed several strategies to cope with the stress, such as going dormant, developing anatomical and morphological traits, changing metabolic pathways and physiological responses (Van der Sman *et al.* 1992, Wang and Cao 2012, Parent *et al.* 2008). Waterlogging reduces gas exchange between plant tissue and the atmosphere because the diffusion of gases in the water is 10,000-fold slower than in the air (Armstrong 1979). Although oxygen deficiency is an important

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reason that affects plant growth, soil chemical properties, such as pH and redox potential, are also altered under waterlogging conditions and may also impact plant growth and survival (Jackson and Colmer 2005, Probert and Keating 2000).

Oxygen deficiency stimulates anaerobic fermentation which adversely affects various morphological and physiological processes, such as photosynthesis, energetic metabolism, redox status, gene expression, as well as protein synthesis and degradation (Jackson 2003). Plant response to abiotic stress includes changes in protein expression and post-translational modification of proteins to activate their defense system against the challenges (Hashiguchi *et al.* 2010). Protein profile is changed under anoxia and specific proteins called anaerobic polypeptides are synthesized, most of which are known as enzymes involved in sugar metabolism, glycolysis, and fermentation pathways (Huang *et al.* 2005). Ahsan *et al.* (2007) found that 35 proteins were differentially expressed in tomato (*Lycopersicon esculentum*) roots in response to waterlogging stress. Among the 29 proteins identified, 16 proteins were up-regulated and 13 proteins were down-regulated. In maize (*Zea mays*), anaerobic treatment drastically altered the pattern of proteins synthesized by primary roots (Sachs *et al.* 1980). Except the classical polypeptides, some other proteins were also differentially regulated under anaerobic conditions (Chang *et al.* 2000).

Bald cypress is a dominant species in bottomlands and coastal regions of the southeastern USA. Its native range extends from Delaware Bay south to Florida and west to East Texas and southeastern Oklahoma, and also inland up the Mississippi and Ohio Rivers north to southern Illinois and Indiana. It is also widely planted as commercial and ornamental tree species in Europe, Asia and elsewhere from temperate to subtropical climates. Bald cypress was introduced to China in 1920s, and now it has been widely planted in swampy ground of Pearl river delta and Yangtze valley. Although the effects of waterlogging is well known on bald cypress's growth, morphology, anatomy, physiology and biochemistry (Pezeshki *et al.* 1999, 1986, 1996; Megonigal *et al.* 1997, Pezeshki and DeLaune 1998; Pezeshki and Santos 1998, Keeland and Sharitz 1995, Pezeshki 1991, 1993, Conner 1992, Kludze *et al.*1994, Donovan *et al.* 1988), protein expression changes under waterlogging is less studied. Determination of novel proteins in response to waterlogging will provide a better understanding of their functions in stress adaptation, and can serve as the basis for effective (gene) engineering strategies to improve stress tolerance (Ahsan *et al.* 2007). The main goal of this study was to identify novel proteins that are differentially regulated upon exposure to waterlogging stress in bald cypress, and provide new insight for the development of this species with enhanced tolerance to waterlogging. We also wanted to know how quick the recovery would be after different levels of waterlogging.

## Materials and Methods

Seven experimental pools, each of which was 3 m (length) × 2.3 m (width) × 1.5 m (depth), were built in the field at Xiashu Forestry Centre of Nanjing Forestry University, Jiangsu province. The pools had holes at different heights on the walls for controlling the water level for different waterlogging treatments. The pools were filled with surface soil (pH 5.10, organic matter 22.5 g $kg^{-1}$, hydrolyze nitrogen 155.6 mg $kg^{-1}$, available phosphor 21.6 mg $kg^{-1}$, potassium 102.3 mg $kg^{-1}$) collected from a local farm. Thirty one-year old seedlings of bald cypress (1.5 cm in diameter and 60 cm in height) were separately transplanted into each pool in April 2009. In total 210 seedlings were planted in the seven pools. Four waterlogging treatments were carried out, including high waterlogging (HWL, water level was 5 cm above soil surface), medium waterlogging (MWL, water level was same as soil surface), low waterlogging (LWL, water level was 5 cm below soil surface), control (CK, soil moisture was about 75% of field capacity). The waterlogging experiment initiated in June 2009 lasted for 60 days. At the same time we used three other pools for a separate after-waterlogging recovery experiment (RHWL, RMWL and RLWL). These three pools were

drained to achieve field capacity from HWL, MWL and LWL at 50 days after waterlogging, and sampled for measurements once at day 60 (i.e. we wanted to examine the 10-day-after-waterlogging recovery effects). Leaf samples were collected from the seedlings in the first group of four pools at 8, 18, 38 and 60 days, respectively under waterlogging condition. The leaf samples were washed six times with distilled water and immediately frozen in liquid nitrogen, and maintained at –70°C for further analyses. The leaves in the 10-day recovery treatments were sampled only at 60 days. All the mature leaf samples were collected from 3 randomly selected seedlings in each treatment.

The soluble protein content in leaf was determined according to the method by Bradford (1976) using bovine serum albumin as the protein standard. TCA-acetone precipitation method was used to extract protein (Wessel and Flugge 1984). A 2 g leaf sample was ground to powder in liquid nitrogen with a pestle. The powder was transferred to a 10 ml centrifuge tube with 10% trichloro acetic acid and 0.07% 2-mercaptoethanol in acetone, and the mixture was vortexed. The suspension was incubated for 20 hrs at –20°C. After the incubation, the suspension was centrifuged at 12000 r $min^{-1}$ for 15 min at 4°C. The supernatant was discarded, and the resulting pellet was vortexed with 0.07% 2-mercaptoethanol in acetone, and incubated for 2 hrs at –20°C, then centrifuged at 12000 r $min^{-1}$ for 15 min at 4 °C. The step was repeated for three times. The resulting pellet (protein sample) was collected, and dried with vacuum drier, and was stored at –20°C for further analyses.

SDS-PAGE was carried out with the PROTEAN II XL Cell (Bio-Rad, Hercules, CA, USA). A 10 g protein sample was suspended with 300 µl sample conditioning solution containing 2% sodium dodecyl sulfonate, 5% β-mercaptoethanol, 10% glycerol, 0.02% bromophenol blue and 0.01M Tris-HCl (pH 6.8) in a centrifuge tube, then the tube was incubated in a water bath for 45 min at 35°C. After incubation, the mixture was centrifuged at 12000 r $min^{-1}$ for 15 min at 4°C. The resulting supernatant was immersed in water bath for 3.5 min at 100°C, then directly used in electrophoretic analysis. 20µl of the supernatant was loaded onto the separating gel and spacer gel with concentrations of 12.9 and 4.4%, respectively. The following conditions were used: 80 V for the first 20 min, then constant 300 V until the end.

For 2-DE analysis, isoelectric focusing was carried out with PROTEAN IEF Cell (Bio-Rad, Hercules, CA, USA). A 20 mg protein sample was suspended 400 µl lysis buffer containing 7 M urea, 2 M thiourea, 4% 3-(N-morpholino) propanesulfonic acid sodium (CHAPS), 1% dithiothreitol, and 2% amphoteric electrolyte (pH 3-10) in centrifuge tube. The tube incubated in a water bath for 1 hrs at 35°C, then centrifuged at 15000 r/min for 15 min at 25°C. 150µl of the supernatant was votexed with hydrated loading buffer containing 6 M urea, 2 M thiourea, 0.001% bromophenol blue, 2 and 0.4% dithiothreitol, and 1% amphoteric electrolyte (pH 3 - 10). 350 µl of samples were loaded into IPG strip focusing tray, and 3 ml mineral oil was added to prevent water evaporation. Hydration and isoelectric focusing were performed according to the following procedures and conditions at 20°C and maximum electric current of 50 µA: 50 V for 12 min, 250 V for 45 min, 1000 V for 105 min, 10000 V for 5 hrs, 10000 V for 60000 V/h, and 500 V for 100 min. After isoelectric focusing, the mineral oil on the strip was absorbed with wet filter paper, the strip were equilibrated for 13 min and 15 min in equilibrium liquid one containing 6 M urea, 2% sodium dodecyl sulfonate, 30% glycerol, 0.002 % bromophenol blue, 1% dithiothreitol, and 0.375 M Tris-HCl (pH 8.8), and equilibrium liquid two containing 6 M urea, 2% sodium dodecyl sulfonate, 30% glycerol, 0.002 % bromophenol blue, 2.5% iodoacetamide, and 0.375 M Tris-HCl (pH 8.8), respectively. SDS-PAGE was performed using a 12.9% polyacrylamide gel with a 4.4% stacking gel. The following conditions were used: 80 V for the first 10 min, then constant 300 V until the end. The 2-DE gels were stained using Coomassie Brilliant Blue (CBB), as described previously (Ahsan *et al.* 2007).

CBB-stained gels were selected for the profile analysis. Gel Imaging System (ChemiDoc XRS, Bio-Rad, Hercules, CA, USA) was used to obtain image from CBB-stained gels. Quantity One 1-D Analysis Software was used for imaging and analyzing one-dimensional electrophoretic gels, and PD Quest 2-DE analysis software (Version 8.0.1; Bio-Rad, Hercules, CA, USA) was used to detect and qualify spots. After automated detection and matching, manual editing was carried out. Each sample generated at least three gels. Only those spots with significant and reproducible changes were considered to be differentially expressed proteins (Ahsan *et al.* 2007).

Protein contents (Table 1) of leaf samples per treatment were analyzed using one way variance analysis (ANOVA). Mean values were tested within each sapling day after waterlogging using Duncan's Multiple Range Test at significant difference $p< 0.05$. SDS-PAGE and 2-DE were repeated at least three times, and the representative images are shown in this study.

## Results and Discussion

There was a significant effect of waterlogging treatment on soluble protein content of bald cypress leaves ($p < 0.001$). No significant differences in soluble protein contents were found between treatments of HWL and MWL, and between LWL and CK in all days (Table 1). Comparing with LWL and CK, HWL and MWL increased soluble protein contents significantly at 8 and 18 d after treatment, but they decreased significantly at 38 and 60 days (Table 1). After 10 days recovery (drain water), the soluble protein contents of RHWL and RMWL treatments increased 22.9 and 25.8% compared with HWL and MWL, while RLWL had no obvious variation compared with LWL. Across all treatment times, the soluble protein content had no significant variation between CK and LWL treatments.

**Table 1. Leaf soluble protein contents (mg/g) of bald cypress (*Taxodium distichum*) under waterlogging treatments for 8, 18, 38 and 60 days.**

| Treatment | 8 d | 18 d | 38 d | 60 d |
|---|---|---|---|---|
| HWL | 50.51 ± 2.05a | 55.01 ± 2.98a | 38.7 ± 4.31b | 31.56 ± 3.93c |
| MWL | 48.86 ± 1.32a | 52.71 ± 2.91a | 34.54 ± 3.91b | 34.03 ± 2.29c |
| LWL | 44.76 ± 1.29b | 46.58 ± 2.38b | 51.92 ± 2.70a | 57.49 ± 2.98a |
| CK | 44.89 ± 3.13b | 45.39 ± 3.66b | 47.76 ± 3.88a | 52.54 ± 5.13a |
| RHWL | - | - | - | 38.81 ± 2.91b |
| RMWL | - | - | - | 42.81 ± 3.13b |
| RLWL | - | - | - | 56.99 ± 4.98a |

Different letters in each column indicate significant differences among the waterlogging treatments and recovery effects, whereas the same letters show no significant difference. HWL, water level was 5 cm above soil surface; MWL, water level was on soil surface; LWL, water level was 5 cm below soil surface; CK, soil moisture was about 75% of field capacity; RHWL, RMWL and RLWL represent the 10-day recovery effects from HWL, MWL and LWL waterlogging for 50 days and then released from waterlogging treatment, respectively.

**SDS-PAGE analysis of leaf proteins**

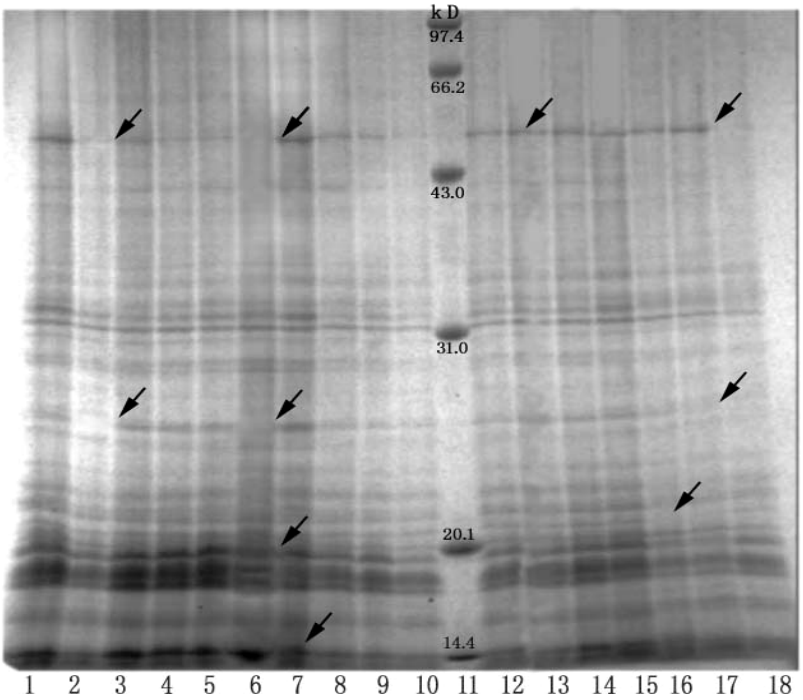


Fig. 1. SDS-PAGE profile of leaf proteins of bald cypress (*Taxodium distichum*) under waterlogging conditions for different days. 1, 8 d (HWL); 2, 8 d (CK); 3, 18 d (HWL); 4, 18 d (MWL); 5, 18 d (LWL); 6, 18 d (CK); 7, 38 d (HWL); 8, 38 d (MWL); 9, 38 d (LWL); 10, 38 d (CK); 11, Molecular weight marker; 12, 60 d (HWL); 13, 60 d (MWL); 14, 60 d (LWL); 15, 60d (RHWL); 16, 60 d (RMWL); 17, 60 d (RLWL); 18, 60 d (CK). HWL, water level was 5 cm above soil surface; MWL, water level was at soil surface; LWL, water level was 5 cm below soil surface; CK, soil moisture was about 75% of field capacity; RHWL, RMWL and RLWL represent the 10-day recovery effects from HWL, MWL and LWL waterlogging treatment for 50 days, respectively. Arrows show differential expression of protein subunits. Samples with 10 μg protein quantity were loaded per lane.

In 1D-SDS-PAGE of proteins, 28 - 34 protein subunits were found in seven treatments at all treatment times, and their molecular weight range from 14 - 94 kD (Fig. 1), and the bands which had higher abundant protein subunits were uniformly distributed in the molecular weights of 14, 17 - 22, 31 - 33 and 51 kD. Under waterlogging and recovery treatments, the protein subunits of 42.3, 52.2 and 26.6 kD were observed clearly on the protein profile at different treatment times, while they could not be observed on the protein profile of CK. Overall, the difference of leaf protein expression between waterlogging treatments and CK mainly showed the changes in the level of expression. For instance, at 18 d after treatment, protein subunits of 14.3, 18.6, 19.3, 20.8 and 21 kD obtained highest protein abundance at all treatment times, and their expression level were higher than that of CK treatment.

The changes in protein expression pattern were further analyzed by 2-DE for better separation and characterization. Statistics showed that isoelectric point and molecular weight of leaf protein spots are mainly distributed between pH 5 - 6.5 and 31.0 - 20.1 kD, and they both fit normal distribution (Fig. 2), suggesting that range of immobilized pH gradient (IPG) gel and concentration of SDS-PAGE separation gel were suitable in the present experiment. The distribution pattern and

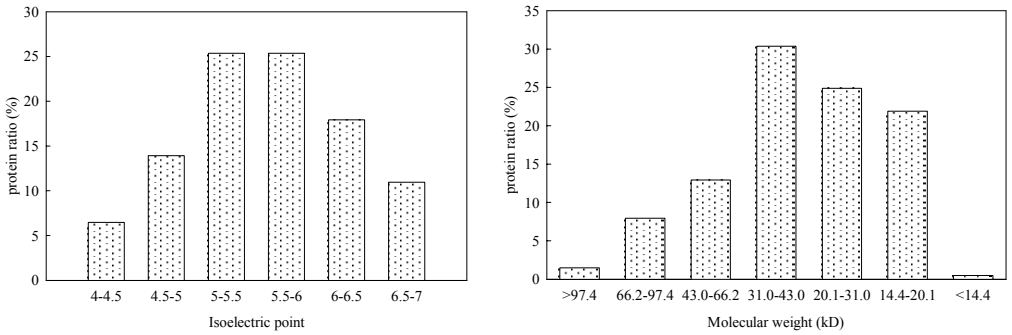


Fig. 2. Isoelectric point and molecular weight distribution of leaf protein spots of bald cypress (*Taxodium distichum*) under waterlogging for 18 days.

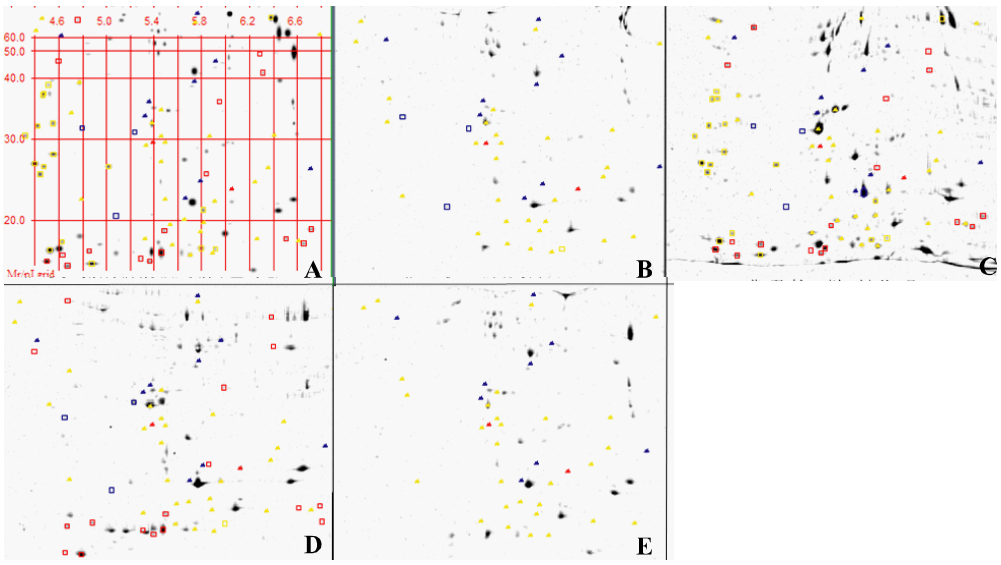


Fig. 3. Distribution of leaf protein spots of bald cypress (*Taxodium distichum*) under waterlogging for 18 days. Abscissa is isoelectric point, and ordinate is molecular weight. A, reference gel; B, LWL (water level was 5 cm below soil surface); C, HWL (water level was 5 cm above soil surface); D, MWL (water level was at soil surface); E, CK (soil moisture was about 75% of field capacity). "□" expressed specific protein spots, and "▲" expressed up-regulated protein spots. Blue indicates that the protein spots were expressed in HWL, MWL and LWL, and red indicates that the protein spots were expressed in HWL and MWL, and yellow represents that the protein spots were only expressed in HWL.

abundance of protein spots in HWL, MWL, LWL and CK treatments was consistent, and the

matching ratio was more than 95% (Fig. 3). This result was in accordance with the result of 1-D-SDS-PAGE. The protein mainly distributed in the molecular weight range of 14 - 97 kD and the isoelectric point range of pH 4.2 - 6.6, and the most protein spots were observed in the isoelectric point of 5.38 and molecular weight of 32.42 kD.

In 2-DE profiles of HWL, MWL, LWL and CK treatments, 162, 177, 195 and 158 protein spots were detected, respectively (Figs. 3, 4 and Table 2). A comparison of 2-DE protein profiles between the three waterlogging and CK samples revealed 37 protein spots were differentially expressed from 201 matched protein spots. Among the differentially expressed proteins, 4 spots were identified from the treatments of HWL, MWL and LWL, and 15 spots from the treatments of HWL and MWL, and 18 spots from the treatment of HWL. The molecular weights of these specific protein spots were mainly distributed around 16, 25 and 31 kD, only three of them had higher molecular weights (76.87, 79.73 and 118 kD). In term of isoelectric point, these specific proteins were mainly distributed around pH 4.5 and 5.4, especially around pH 4.5.

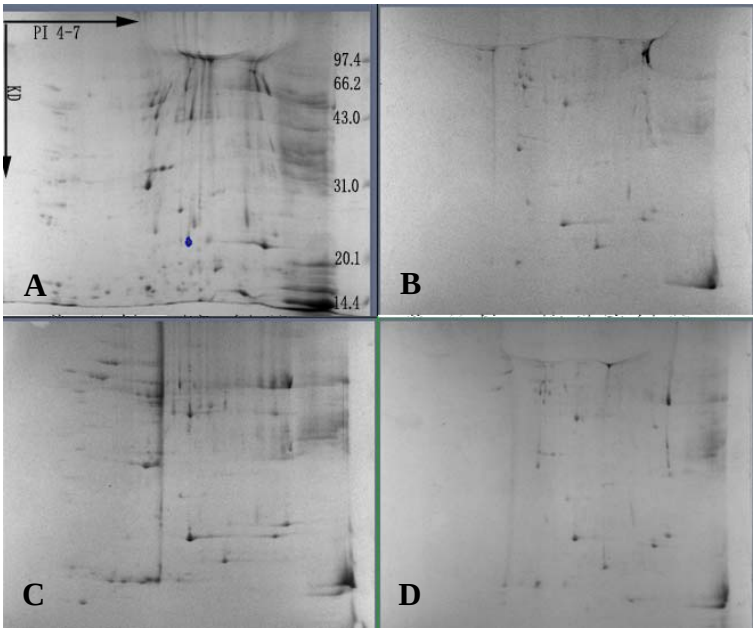


Fig. 4 2. DE maps of leaf proteins of bald cypress (*Taxodium distichum*) under waterlogging condition for 18 days. A, HWL treatment (water level was 5 cm above soil surface). B, LWL treatment (water level was 5 cm below soil surface). C, MWL treatment (water level was at the soil surface). D, CK (soil moisture was about 75% of field capacity). Abscissa is isoelectric point, and ordinate is molecular weight.

Among 201 matched protein spots in the system, 41 spots were shown to be up-regulated in the three waterlogging treatments (Fig. 3 and Table 2). Among 41 up-regulated protein spots, 10 were detected from three waterlogging treatments, and 2 from HWL and MWL, and 29 from HWL. These up-regulated protein spots were mainly distributed around 16 and 31 kD, and their isoelectric points were around pH 4.6 and 5.5 - 6.2.

**Table 2. Waterlogging-induced (after 60 days) differentially expressed proteins in leaves of bald cypress.**

| Expression type | | Index number | Molecular weight (kD) | Isoelectric point | Index number | Molecular (kD) | Isoelectric point |
|---|---|---|---|---|---|---|---|
| Specific expression | Expressed in HWL treatment | 8 | 26.22 | 4.47 | 32 | 17.76 | 4.63 |
| | | 9 | 26.6 | 4.4 | 45 | 32.4 | 4.55 |
| | | 10 | 25.19 | 4.44 | 49 | 38.74 | 4.51 |
| | | 12 | 27.86 | 4.55 | 57 | 15.67 | 4.88 |
| | | 13 | 30.58 | 4.32 | 63 | 26.19 | 5.02 |
| | | 14 | 32.03 | 4.42 | 111 | 17.03 | 5.91 |
| | | 18 | 36.93 | 4.47 | 133 | 17.1 | 5.79 |
| | | 19 | 37.17 | 4.47 | 135 | 21.11 | 5.81 |
| | | 31 | 16.91 | 4.52 | 195 | 79.73 | 6.38 |
| | Expressed in HWL and MWL treatments | 1 | 15.88 | 4.5 | 88 | 18.9 | 5.49 |
| | | 5 | 16.45 | 4.63 | 117 | 25.25 | 5.84 |
| | | 29 | 15.46 | 4.67 | 171 | 48.26 | 6.28 |
| | | 30 | 15.87 | 5.36 | 172 | 40.93 | 6.31 |
| | | 36 | 16.12 | 5.29 | 177 | 18.05 | 6.5 |
| | | 55 | 76.87 | 4.76 | 179 | 19.05 | 6.71 |
| | | 58 | 16.81 | 4.85 | 198 | 17.59 | 6.65 |
| | | 60 | 16.67 | 5.46 | | | |
| | Expressed in HWL, MWL and LWL treatments | 11 | 31.68 | 4.8 | 44 | 31.13 | 5.24 |
| | | 39 | 20.48 | 5.08 | 131 | 118 | 5.83 |
| Up-regulated expression | Up-regulated in HWL treatment | 6 | 22.24 | 4.78 | 94 | 19.54 | 5.54 |
| | | 15 | 34.06 | 4.7 | 96 | 29.54 | 5.49 |
| | | 17 | 39.16 | 4.77 | 108 | 17.4 | 5.55 |
| | | 25 | 78.69 | 4.46 | 109 | 18.78 | 5.78 |
| | | 27 | 66.58 | 4.41 | 110 | 16.61 | 5.88 |
| | | 35 | 18.05 | 5.32 | 113 | 19.8 | 5.81 |
| | | 42 | 29.2 | 5.33 | 114 | 22.02 | 5.9 |
| | | 62 | 26.8 | 5.45 | 118 | 30.51 | 5.86 |
| | | 64 | 32.42 | 5.38 | 130 | 73.83 | 5.74 |
| | | 65 | 30.46 | 5.45 | 149 | 18.02 | 6.26 |
| | | 69 | 34.48 | 5.46 | 156 | 26.02 | 6.35 |
| | | 87 | 16.47 | 5.7 | 162 | 30.58 | 6.4 |
| | | 89 | 20.16 | 5.65 | 178 | 17.88 | 6.6 |
| | | 93 | 22.26 | 5.49 | 193 | 62.37 | 6.78 |
| | Up-regulated in HWL and MWL treatments | 66 | 29.52 | 5.39 | 137 | 23.46 | 6.05 |
| | Up-regulated in HWL, MWL and LWL treatments | 2 | 14.9 | 4 | 115 | 24.34 | 5.79 |
| | | 24 | 61.95 | 4.62 | 120 | 39.38 | 5.73 |
| | | 43 | 33.47 | 5.32 | 181 | 25.87 | 6.7 |
| | | 48 | 35.84 | 5.36 | 123 | 43.83 | 5.92 |
| | | 91 | 22.34 | 5.66 | 132 | 83.66 | 5.76 |

HWL, water level was 5 cm above soil surface; MWL, water level was at soil surface; LWL, water level was 5 cm below soil surface.

It is reported that waterlogging restrains plant growth, development and yield through destroying root systems and resulting in physiological or biochemical alterations due to insufficient oxygen in the soil (Ye *et al.* 2003, Sairam *et al.* 2009a). Nitrogen metabolisms, including nitrogen availability, nitrogen fixation, protein synthesis, and protein degradation have been reported to be involved in waterlogging (Puiatti and Sodek 1999, Jung *et al.* 2008, Webster *et al.* 1986, Schmidt and Stewart 1997). In our study, waterlogging treatments (HWL, MWL and LWL) showed that soluble protein content increased at the early stages of treatments (8 and 18 days), but decreased at later stages (38 and 60 days). The soluble protein content had a significant reduction in treatments of HWL and MWL only at 38 and 60 days, while the treatment of LWL still maintained a higher soluble protein content. Such increase at early stage might be due to an emergency reaction, and had a close relation with abundant expression of anaerobic stress proteins, antioxidant and anaerobic enzymes (Arbona *et al.* 2008, Sairam *et al.* 2009b, Chugh *et al.* 2011, Capelli *et al.* 2008). Such reduction at later stage might be due to the inhibited nitrogen uptake or protein synthesis, and/or enhanced protein degradation under waterlogging condition (Luo *et al.* 2008), and these also are the reasons that the soluble protein content significantly increased in recovery treatments (RHWL and RMWL).

Waterlogging has four main effects that may lead to reduction in plant nitrogen supply, including increasing soil denitrification, reducing decomposition of soil organic matter, decreasing energy supply (low oxygen concentration around root) and reducing soil nitrogen diffusion (Irving *et al.* 2007). This reduction in nitrogen supply is thought to be the actual reason of limited plant growth (Bacanamwo and Purcell 1999), and our findings that LWL treatment maintained high leaf soluble protein content across the total treatment time also explains why bald cypress grow better in LWL conditions than in normal soil moisture (Wang and Cao 2004). Our results are in agreement with other study (Irving *et al.* 2007).

Under waterlogging conditions, the oxygen concentration is low in the plant root zone due to the low diffusion rate of oxygen molecular in water. Low oxygen environments lead to rapid changes in gene transcription, protein synthesis and decomposition, and cellular metabolism (Bailey-Serres and Voesenek 2008). By investigating the induced expression of these proteins in low oxygen levels, we can identify each proteins and their relative genes. These potential genes involved in conferring waterlogging tolerance can be isolated and introduced into transgenic plants in order to identify their possible contribution in stress tolerance (Ashraf 2012). A comparison of 2-DE protein profiles between the waterlogged and control samples revealed 37 protein spots to be differentially expressed in bald cypress leaves, and 41 spots were shown to be up regulated, suggesting that some physiological, biochemical and proteomic processes changed in bald cypress in order to adapt to waterlogging conditions. These processes may include photosynthesis, diseases resistance, energy and metabolism, protein biosynthesis, stress and defense (Irfan *et al.* 2010).

Among specific and up-regulated protein spots, number 44 (molecular weight 31.13 kD, isoelectric point 5.24) expressed in three waterlogging treatments, and number 64 (molecular weight 33.47 kD, isoelectric point 5.32) expressed only in the treatment of HWL, had similar molecular weight and isoelectric points with xyloglucan endotransglycosylase (molecular weight 32.07 kD, isoelectric point 5.16). Xyloglucan endotransglycosylase is correlated with aerenchyma development (Saab and Sachs 1995), implying that the process of aerenchyma development was strengthened in bald cypress under waterlogging conditions. Aerenchyma development is considered a mechanism critical to a plant's ability to cope with anaerobiosis (Wang and Cao 2012), demonstrating that bald cypress had capacity to adapt to waterlogging condotion.

In addition, some specific and up-regulated protein spots were close to ethanol dehydrogenase (molecular weight 41 kD, isoelectric point 5.4 - 5.8), lactic dehydrogenase (molecular weight 21.9, 26.9 and 36.0 kD), and anoxia related-polypeptides (molecular weight 33.0 kD), such as number

123, 62, 48 and 156. These results indicate that anaerobic respiration is enhanced in bald cypress under waterlogging conditions in order to obtain more energy. We did not identify the specific and up-regulated protein spots, and further study will be performed to identify these protein spots by peptide mass fingerprinting (PMF), tandem mass spectrum (MS/MS) and homologous sequence alignment, and to understand water-tolerant mechanisms of bald cypress from proteomics studies.

In the present study, we investigated the response of leaf soluble protein content to waterlogging stress in bald cypress, and compared the leaf protein expression under normal and waterlogging conditions. We have show that there was a significant interaction effect between waterlogging treatment and treatment time on the soluble protein content in bald cypress leaves. Heavy and medium waterlogging treatments increased soluble protein content in the early stage of treatment, but decreased soluble protein content at later stage. SDS-PAGE analysis of leaf proteins showed that there were 28 - 34 protein subunits in the SDS-PAGE profiles, and three protein subunits were found only in waterlogging and recovery treatments. A comparison of 2-DE protein profiles between the waterlogged and control samples revealed 37 protein spots were differentially expressed, and 41 spots were shown to be up regulated in bald cypress leaves. The present study is a good starting point for understanding the molecular response of bald cypress to waterlogging stress, further studies will focus on identification and functional analysis of these specific and up-regulated proteins, and determining their specific role in bald cypress in response to waterlogging.

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