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Physiological and Protein Responses to Drought in Four Pine Seedlings

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Abstract

Physiological and protein responses to drought stress in four pine seedlings, *Pinus armandi* Franch. (Pa), *Pinus tabulaeformis* Carr. (Pt), *Pinus bungeana* Zucc. ex Endl. (Pb) and *Pinus sylvestris* L. var. *mongolica* Litv. (Ps), were investigated using differential proteomics and water physiological indices. Firstly, the water physiological data showed that the decline rate of net photosynthesis rate, stomatal conductance, leaf water potential, turgor pressure except for under moderate drought stress was as follows: Pa>Ps>Pt>Pb. Pb and Pa always maintained the highest and lowest swelling pressure, respectively. Secondly, cluster analysis of 343 proteins indicated that the four pine species were classified into three groups with a genetic distance coefficient of 0.065. That is, five-needle-pine group (Pa), three-needle-pine

group (Pb), two-needle-pine group (Pt and Ps), and the genetic distance between Pb and Pa was the farthest. The result was consistent with the declined rate in above physiological indices. Finally, for the differential proteomics analyzed, a total of 13 different proteins (P values < 0.01) changed significantly, the number of differentially expressed proteins was more in Pa (accounting for 46.2%) than the other three species, and 8 proteins were identified using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). These proteins are quite diverse in their functions and involved in photosynthesis, osmotic regulation and functioning as signal transduction. These results suggested that the sensitivities of the four pine species to drought were possibly related to genetic distance.

Key words: drought stress, drought-resistance, proteomics, genetic distance.

Introduction

Trees in their lifetime are usually subjected to extremely harsh environmental stresses. Drought stress is particularly prominent among a variety of natural stresses associated with predicted changes in climate. Thus, understanding the genetic basis and molecular mechanisms of drought stress adaptation is of particular

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importance for forest tree species given the possibility of future rapid climatic changes.

Studies on the drought resistance of forest tree species have mostly focused on changes in physiological adaptation, such as photosynthesis (BASSMAN and ZWIER, 1991; DICKMAN *et al.*, 1992; NI and PALLARDY, 1992), transpiration (SCHLTE and MORSHALL, 1982), respiration (ZHANG, 2000), physiological mechanisms, such as antioxidant defense (HU *et al.*, 1999), stomatal regulation (RHODENBANGH and PALLARDY, 1993), osmotic adjustment (KOSTER, 1991), and hormonal regulation mechanism (AASAMAA *et al.*, 2002; ROBERT and LENOBLE, 2002). Recently, molecular studies on stress responsive gene expression, protein and metabolite profiling in forest tree species have been undertaken. As a large-scale study of proteins, proteomics is now being widely applied to many forest tree species, such as the maritime pine (COSTA *et al.*, 1999; GION *et al.*, 2005), Huashan pine (HE *et al.*, 2007), Monterey pine (VALLEDOR *et al.*, 2010), poplar (RENAUT *et al.*, 2004; PLOMION *et al.*, 2006; BOGEAT-TRIBOULOT *et al.*, 2007; HE *et al.*, 2008), spruce (LIPPERT *et al.*, 2005) and oak (JORGE *et al.*, 2006). Also, there are some reports on the application of proteomics to the study of responses of forest trees to drought (COSTA *et al.*, 1998; JORGE *et al.*, 2006; PLOMION *et al.*, 2006; BOGEAT-TRIBOULOT *et al.*, 2007; HE *et al.*, 2007, 2008). These works established the validity of the technique in screening drought-induced protein variations, and offered important information about the practical application of proteomic studies for understanding the molecular mechanisms of stress response and forest yield.

In our previous research (ZHANG *et al.*, 2000), we have systematically studied the drought tolerance characteristics of 32 different afforestation tree species in Northern China, and divided these trees into two types and 4 subtypes according to water eco-physiological characteristics. We have concluded that *Pinus armandi* Franch. (Pa), *Pinus tabulaeformis* Carr. (Pt), *Pinus bungeana* Zucc. ex Endl. (Pb), *Pinus sylvestris* L. var. *mongolica* Litv. (Ps) belong to the high potential delay dehydration species based on their drought-resistance mechanism. This study aims to investigate the molecular mechanism and sensitivity difference of the four pine species applying proteomics and physiology technology, and we hope to provide some insights into the selection of forest tree species and molecular breeding in the arid and subarid areas of Northern China.

Materials and Methods

Plant material and treatment

Three-year-old seedlings (1–2 cm in diameter, 20–30 cm in height) of *Pinus armandi* Franch. (Pa, five needles in one bundle), *Pinus tabulaeformis* Carr. (Pt, two needles in one bundle), *Pinus bungeana* Zucc. ex Endl. (Pb, three needles in one bundle), *Pinus sylvestris* L. var. *mongolica* Litv. (Ps, two needles in one bundle) were transplanted into 18 cm × 26 cm × 34 cm plastic pots containing 10 kg of a mixture of clay soil/sand/peat moss (5:3:2) and grown for three months in a naturally-lighted greenhouse under a 25/20 °C (day/night) regime, a

relative humidity (RH) of 80–90%, and a CO₂ concentration of 375 ± 10 μmol·mol⁻¹. Shading was utilized when the photosynthetic photon flux density exceeded the light saturation point (about 800 μmol m⁻² s⁻¹). Thereafter, the plants were divided into three groups on June 26, 2005. At the beginning of the experiment, the soil water potential (ψ_{swp}) was brought to about -0.5 MPa.

Three treatments (T0, T1 and T2), each with three biological replicates, were applied as follows. T0 was an unstressed control, in which ψ_{swp} was maintained at about -0.5 MPa by regular watering during the whole experiment (i.e., from day 0 to day 15). Six current-year fresh needle samples were taken from each seedling for protein extraction on day 7 and 15. T1 was the moderate drought stress treatment, in which watering was withheld until the ψ_{swp} was brought to about -1.5 MPa (around day 7), three seedlings were harvested for protein extraction. T2 was the severe drought stress treatment, in which watering was withheld until the ψ_{swp} was brought to about -3.0 MPa (around day 15), three seedlings were harvested for protein extraction.

Measurements of stomatal conductance (Gs), net photosynthetic rates (Pn) and leaf water potential (ψ_{lwp})

Measurements of Gs and Pn were determined three times at around nine o'clock in the morning with a LI-6400 Portable Photosynthesis System (LI-COR, Inc., Lincoln, NE, USA) (CUI *et al.*, 2006), and the changes in Pn and Gs in the four pine species were shown in *Figure 1A* and *B*. Pre-dawn ψ_{swp} , an indicator of plants' water status, was measured using the WP4 Dewpoint Potential Meter (Decagon Devices, Inc., Pullman Washington, USA). The leaf water potential (ψ_{lwp}) was measured using plant pressure chamber (A-3000) (Spectronics Corporation, USA) (*Figure 1D*). Turgor pressure (ψ_p) was estimated by the pressure-volume (pV) curves using pressure chamber (TYREE and HAMMEL, 1972; LI, 1989; ZHANG and ZHENG, 2012), the methods were as follows: pV curve of each tree were made in the mild drought stress (ψ_{lwp} : -0.5 ~ -1.0 MPa), moderate drought stress (ψ_{lwp} : -1.5 ~ -2.0 MPa), severe drought stress (ψ_{lwp} : <-2.0 MPa), and the linear relationship between the ψ_p and ψ_{lwp} was estimated according to pV curve, finally ψ_p was calculated (*Figure 1C*). The linear relationship between ψ_p and ψ_{lwp} was shown in *Table 1* (ZHANG *et al.*, 2000).

Two-dimensional gel electrophoresis (2-DE) and gel analysis

Needle proteins were extracted as described previously (HE *et al.*, 2005). Proteins from 2 g needles were extracted in 20 mL extraction buffer (5% sucrose, 4% sodium dodecyl sulfate (SDS) and 5% 2-mercaptoethanol) with the addition of 0.2 g polyvinylpyrrolidone (PVPP) for 10 min at room temperature with gentle stirring, followed by centrifugation at 10,000 × g for 20 min. The clear supernatant was heated at 100 °C for 3 min and then precipitated with eight volumes of cold acetone containing 0.07% 2-mercaptoethanol. After at least 1 h at -20 °C, the mixture was

Figure 1. – Progression of P_n (A), G_s (B), ψ_p (C) and ψ_{lwp} (D) of *Pinus armandi* Franch. (Pa), *Pinus tabulaeformis* Carr. (Pt), *Pinus bungeana* Zucc. ex Endl. (Pb), and *Pinus sylvestris* L. var. *mongolica* Litv. (Ps) during drought treatment. Statistical analysis (two-way ANOVA) indicated the differences in P_n , G_s , ψ_p and ψ_{lwp} were significant between species and at different soil water potentials (P values < 0.05).

Table 1. – The relationship between the ψ_p and ψ_{lwp} ($\psi_p = a + b\psi_{lwp}$).

Species	Ψ_{swp} (MPa)	Ψ_{lwp} (MPa)	a	b	R^2	Species	Ψ_{swp} (MPa)	Ψ_{lwp} (MPa)	a	b	R^2
Pa	-0.5±0.10	-1.40±0.07	1.1919	0.4053	0.9698	Pt	-0.5±0.07	-1.35±0.03	1.6445	0.5719	0.9913
	-1.5±0.15	-1.91±0.05	1.1919	0.4053	0.9698		-1.5±0.01	-1.78±0.02	1.4440	0.4885	0.9612
	-3.0±0.15	-2.29±0.04	1.1919	0.4053	0.9698		-3.0±0.04	-2.14±0.01	1.6564	0.5372	0.9993
Ps	-0.5±0.05	-1.38±0.04	1.6395	0.7148	0.9996	Pb	-0.5±0.04	-1.31±0.02	1.9594	0.7669	0.9971
	-1.5±0.10	-1.86±0.03	1.6725	0.5886	0.9934		-1.5±0.05	-1.58±0.01	1.6980	0.5412	0.9831
	-3.0±0.11	-2.22±0.02	1.6725	0.5886	0.9934		-3.0±0.14	-1.77±0.01	1.6980	0.5412	0.9831

Pa, *Pinus armandi* Franch.; Pt, *Pinus tabulaeformis* Carr.; Pb, *Pinus bungeana* Zucc. ex Endl.; Ps, *Pinus sylvestris* L. var. *mongolica* Litv.; ψ_p , turgor pressure; ψ_{lwp} , leaf water potential; Ψ_{swp} , pre-dawn soil water potential.

centrifuged at $10,000 \times g$ for 20 min. The pellet was resuspended in 10 mL of extraction buffer and centrifuged at $10,000 \times g$. After washing once or twice with 80% cold acetone, the pellet was precipitated by adding four volumes of cold acetone, and then was lyophilized and solubilized with 12.5 μL lysate buffer per 1 mg of pellet. Concentrations were determined by the Bradford method (BRADFORD, 1976). For isoelectric focusing (IEF), the IPGphor system (Amersham Biosciences, Uppsala, Sweden) and pH 3–10 immobilized pH gradient (IPG) strips (18 cm, nonlinear) were used. A total of 1.2 mg combined proteins was mixed with a strip rehydration solution (8 mol L^{-1} urea, 2% 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulphonate (CHAPS), 20 mmol L^{-1} dithiothreitol (DTT), 0.5% IPG buffer and trace bromophenol blue). The IPGphor system was then programmed as follows: 12 h (active rehydration), 1 h at 500 V, 1 h at 1000 V, and 8–10 h at 8000 V. The focused

strips were equilibrated twice for 15 min in 10 mL equilibration buffer (50 mmol L^{-1} Tris-HCl, pH 8.8, 6 mol L^{-1} urea, 30% (v/v) glycerol and 2% (w/v) SDS) containing 100 mg DTT with gentle shaking. During the second equilibration, 250 mg iodoacetamide was used instead of DTT. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a PROTEAN II xi Cell (Bio-Rad, Hercules, California, USA). A total of 48 2-D gels (15% acrylamide) were stained with Coomassie Brilliant Blue (CBB) R-250, and images of the gels were captured with a scanner (UMAX Powerlook 2100 XL; UMAX, Taiwan, China).

Forty-eight gels were digitized and analyzed using ImageMaster™ 2D Platinum Software (Version 5.0; Amersham Biosciences, Uppsala, Sweden) for spot detection, spot matching, background subtraction, normalization and statistical analyses. The spot detection was initially performed with software automatically.

Since many spots were omitted or were not circled properly by the automatic mode, spot detection was corrected manually. *T*-test was performed to determine the significant differences between two groups. *P* values < 0.01 were considered statistically significant. The spot of interest was selected if significant changes did not occur between different stages of control samples (excluding developmental differences), but did occur between control and treated samples at the same stage.

Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) analysis

CBB-stained protein spots were excised manually from the gels and washed three times with ultra-pure water. Spots were washed subsequently in destaining buffer [50% acetonitrile (ACN), 25 mmol L⁻¹ NH₄HCO₃] and freeze-dried. The gels were swollen for 1 h at 4°C in 10 ng μL⁻¹ trypsin (Sequencing grade modified Trypsin; Promega, Madison, USA) in 20 mmol L⁻¹ NH₄HCO₃ and incubated for 16 h at 37°C.

The spots were incubated for 1 h in 5% trifluoroacetic acid (TFA) at 37°C, and the supernatant was removed and saved in an Eppendorf tube. Then the spots were incubated for 1 h in 2.5% TFA/50% ACN at 30°C and the second supernatant was pooled with the first supernatant. Next 5 μL of 100% ACN was added to the spots for 5 min, the third supernatant was pooled with the two former supernatants and then lyophilized. MS analysis was conducted with a MALDI-TOF MS (Voyager DE-Pro; Applied Biosystems, Framingham, MA, USA) in reflector mode over a mass range of 500–3,500 Da. The 0.2 μL peptide mixture was mixed with 0.2 μL matrix solution (α-cyano-4-hydroxycinnamic acid, 5 mg/ml in 100% methanol) on the target plate. For each sample, spectra from 100 shots at several different positions were combined to generate a peptide mass fingerprint (PMF) for database searches for protein identification. The PMF obtained from each digested protein were compared with PMFs in the nonredundant National Center for Biotechnology Information database (NCBI nr) using Mascot search (Matrix Science, <http://www.matrixscience.com/>). The search was performed within green plants (Viridiplantae). A mass tolerance of ±100 ppm and one incomplete cleavage was allowed. Oxidation (M) as Variable modifications was selected.

Data analysis

The presence or absence of protein spots was scored as 1 or 0 respectively. The data were analyzed using NTSYSpc2.10e (NEI, 1972). Coefficients of genetic distances and similarity based on pairwise comparisons for protein profiles (12 individuals) among the four pine species were calculated. Due to the bad definition of the borders, only the spots located in a central zone of the 2-D gels were retained. Reference gels were created for each patch, and then matched together one after the other, leading to a global synthetic gel. Statistical analysis of the eco-physiological data (Pn, Gs, ψ_{p} , ψ_{swp} , ψ_{p}) was performed using Microsoft Office Excel and SPSS 16.0, *P* values < 0.05 were considered statistically significant.

Results and Discussion

Progression of physiological characteristic, pre-dawn leaf water potential (ψ_{lpw}) and turgor pressure (ψ_{p}) during drought stress

Under drought stress, we analyzed the changes of net photosynthetic rate (Pn), stomatal conductance (Gs), turgor pressure (ψ_{p}) and leaf water potential (ψ_{lpw}) value in the four pine species (Pa, Ps, Pt, Pb). Statistical analysis (two-way ANOVA) indicated the differences in Pn, Gs, ψ_{p} and ψ_{lpw} were significant between species and at different soil water potentials (*P* values < 0.05). With increasing degree of drought stress, Pn and Gs decreased gradually (Figure 1A and B), and the decline rate under T1 and T2 stresses was as follows: Pa > Ps > Pt > Pb (Figure 2). Our previous study (ZHANG *et al.*, 2000) has pointed out that the Pn of seedlings decreased significantly under drought stress, and the tree species

Figure 2. – Declined rate of Pn, Gs, ψ_{p} and ψ_{lpw} of *Pinus armandi* Franch. (Pa), *Pinus tabulaeformis* Carr. (Pt), *Pinus bungeana* Zucc. ex Endl. (Pb), and *Pinus sylvestris* L. var. *mongolica* Litv. (Ps) under different drought stress; Pn', Gs', ψ_{p} and ψ_{lpw} represent the declined rate under moderate drought stress; Pn'', Gs'', ψ_{p} and ψ_{lpw} represent the declined rate under severe drought stress.

Figure 3. – The dendrogram of polymorphism site of *Pinus armandi* Franch. (Pa), *Pinus tabulaeformis* Carr. (Pt), *Pinus bungeana* Zucc. ex Endl. (Pb), and *Pinus sylvestris* L. var. *mongolica* Litv. (Ps) proteins based on genetic distance.

Supplementary Table 1. – Genetic similarity coefficients within the four pine species.

	Pa	Ps	Pt	Pb
Pa	1.0000			
Ps	0.9194	1.0000		
Pt	0.8922	0.9472	1.0000	
Pb	0.8562	0.8997	0.9116	1.0000

Supplementary Figure 1. – Representative CBB-stained 2-D gel of needle proteins in response to drought stress in *P. armandii* Franch (4A). The proteins were separated by IEF/SDS-PAGE. Locations of the 6 drought responsive proteins are indicated by open circles and target plots (framed regions) in D (A, B, C). The framed regions are enlarged in Supplementary Fig. 1B. Needles were sampled on normal water, moderate drought stress ($\psi_{\text{swp}} \approx -1.5$ MPa) and severe drought stress ($\psi_{\text{swp}} \approx -3.0$ MPa) (i.e. Panel CK, Panel D1 and D2), and the 2-D gels were analyzed. $\equiv$ point to spots whose intensity notably increased (total 3 spots), $\equiv$ point to spots whose intensity notably increased at moderate drought stress and decreased at severe drought stress (total 1 spots) and $\equiv$ point to the spots whose intensity decreased at moderate drought stress and increased at severe drought stress (total 2 spot) (B). The detailed changes of ratio were showed in Table 2.

were more sensitive to water deficit when the decline rate of Pn was much larger. It is more likely that drought affects the ability of the plant to conduct water to leaves to support the stomata opening, transpiration, and finally, CO_2 assimilation. The highest ψ_p value was always maintained in the Pb of the four pine species under drought stress, while the lowest p value in the Pa (Figure 1C) and the decline rate under T2 stress was as follows: Pa > Ps > Pt > Pb (Figure 2). Hsiao et al (HSIAO et al., 1973, 1985) pointed out maintenance of a certain degree of swelling pressure was very important for plant growth and survival when their water potential declined. Besides, the lowest ψ_{lwp} value was always maintained in the Pa of four pine species under drought stress, while the highest in the Pb (Figure 1D). The declined rate of ψ_{lwp} was as follows: Pa > Ps > Pt > Pb (Figure 2). These results possibly indicated that in the four pine species, Pa was the most sensitive to drought stress, followed by Ps, Pt and Pb.

The clustering analysis based on protein polymorphism site

Based on the established sucrose extraction method of needle protein (HE et al., 2005), 343 proteins separated with 2-dimensional electrophoresis (2-DE) from the four pine species under T0 control treatment were analyzed by UPGMA cluster system, and the genetic distance varied from 0.05 to 0.16. UPGMA cluster analysis indicated that the four pine species were classified into three groups when genetic distance coefficient was about 0.065 (Figure 3). That is, one was five-needle-pine group (Pa), one was three-needle-pine group (Pb), and the other was two-needle-pine group (Pt and Ps). The genetic similarity coefficient between Pb and Pa, Pt, Ps was 0.86, 0.91, 0.90 (Supplementary Table 1), respectively. It suggested that the genetic relationship between Pb and Pa was farther than that between Pb and another two. The result was consistent with the declined rate in Pn,

Supplementary Figure 2. – Representative CBB-stained 2-D gel of needle proteins in response to drought stress in *P. tabulaeformis* Carr. The proteins were separated by IEF/SDS-PAGE. Locations of the 2 drought responsive proteins are indicated by open circles and target plots (framed regions) in D (A, B). The framed regions are enlarged in Supplementary Fig. 2B. Needles were sampled on normal water, moderate drought stress ($\psi_{\text{swp}} \approx -1.5$ MPa) and severe drought stress ($\psi_{\text{swp}} \approx -3.0$) (i.e. Panel CK, Panel D1 and D2), and the 2-D gels were analyzed. $\equiv$ point to spots whose intensity notably increased (total 1 spots), $\equiv$ point to spots whose intensity notably increased at moderate drought stress and decreased at severe drought stress (total 1 spots) (B). The detailed changes of ratio were showed in Table 2.

Table 2. – Identification of needle proteins responding to drought stress using protein fingerprinting (MALDI-TOF MS).

Index no. ^a	Theoretical		Experimental		Sequences cover (%) ^b	Similar proteins	Species	NCBI accession no.	Stress behaviour of spots ^c		
	Mass (kDa)	pI	Mass (kDa)	pI					CK	D1/T	D2
Pad1	45.6	5.49	15-24	3.3-3.8	32	MAPK ^c	<i>Solanum tuberosum</i>	gi 21165523	1	6.5289	6.6612
Pad2			18-25	3.9-4.5		Unidentified			1	3.7819	3.9582
Pad3			6-15	5.2-5.7		Unidentified			1	9.7756	2.8851
Pad4	51.5	6.18	12-16	5.5-6.2	28	RuBisCO LSU ^d	<i>Fagonia cretica</i>	gi 6687544	1	0.1301	1.2147
Pad5			13-18	5.7-6.2		Unidentified			1	0.1058	1.1621
Pad6	12.6	8.57	12-16	5.7-6.5	29	putative translation factor	<i>Pinus pinaster</i>	gi 20218809	1	1.6322	1.7085
Ptd1	45.6	5.49	15-24	3.8-4.3	32	MAPK ^c	<i>Solanum tuberosum</i>	gi 21165523	1	1.2562	1.8256
Ptd2			16-25	5.8-6.3		Unidentified			1	1.3140	0.1524
Pbd1			6-15	6.5-7.0		Unidentified			1	5.5266	6.3741
Pbd2			18-24	6.1-6.7		Unidentified			1	3.1245	1.2243
Pbd3			30-36	6.3-6.9		Unidentified			1	2.8872	3.5571
Psd1	25.5	6.25	18-27	5.7-6.3	48	dehydrin	<i>Glycine max</i>	gi 37495453	1	0.8406	1.1017
Psd2	4.0	9.39	12-21	6.7-7.5	91	Photosystem I H protein	<i>Camptochaete deflexa</i>	gi 33307527	1	0.0810	0.0745

^a Pads = needle proteins of *P. armandi* Franch from drought experiment (Supplementary Figure 1),

Ptds = needle proteins of *P. tabulaeformis* Carr. from drought experiment (Supplementary Figure 2),

Pbds = needle proteins of *P. bungeana* Zucc. ex Endl. from drought experiment (Supplementary Figure 3),

Psds = needle proteins of *P. sylvestris* L. var. *mongolica* Litv. from drought experiment (Supplementary Figure 4),

^b Percentage of amino acids in reference protein covered by matching peptides from MALDI-TOF analysis.

^c MAPK: mitogen-activated protein kinase.

^d RuBisCO LSU: ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit.

^e The change of fold in different proteins divided by reference gels (controls) during drought stress.

Gs, ψ_{Iwp} and ψ_{p} value except for ψ_{p} under T1 stress, that is, $\text{Pa} > \text{Ps} > \text{Pt}$, suggesting that the sensitivity to drought was possibly related to genetic relationship based on UPGMA dendrogram of polymorphism site of proteins, and it is necessary to deeply discuss the molecular variance in stress tolerance of these pine species using proteomics method.

Variations of drought responsive proteins in the four pine species

More than 500 reproducible leaf proteins were detected by 2-DE. Six differential proteins were notably affected by drought stress in *P. armandi* Franch. Of which,

three (Pad1, Pad2, Pad6) showed an increase in intensity, one (Pad3) showed increased intensity when ψ_{swp} values reached about -1.5 MPa and decreased intensity when ψ_{swp} values reached about -3.0 MPa, and two showed decreased intensity when ψ_{swp} values reached about -1.5 MPa and increased intensity when ψ_{swp} values reached about -3.0 MPa during drought stress (Supplementary Figure 1A and 1B; Table 2 and 3).

Two differential proteins were notably affected by drought stress in *P. tabulaeformis* Carr.. Of which one (Ptd1) showed an increase in intensity, one (Ptd2) showed increased intensity when ψ_{swp} values reached about -1.5 MPa and decreased intensity when ψ_{swp} val-

Table 3. – Needle proteins responding to drought stress in four pine species.

Species	Expression changes under drought stress				Total	Percent (%)
	Increased	Decreased	Increased at first and then decreased	Decreased at first and then increased		
<i>P. armandi</i> Franch (Pa)	3		1	2	6	46.2
<i>P. tabulaeformis</i> Carr. (Pt)	1		1		2	15.4
<i>P. bungeana</i> Zucc. ex Endl. (Pb)	2		1		3	23.0
<i>P. sylvestris</i> L. var. <i>mongolica</i> Litv. (Ps)		1		1	2	15.4
Total	6	1	3	3	13	100

ues reached about -3.0 MPa during drought stress (Supplementary Figure 2A and 2B; Table 2 and 3).

Three differential proteins were notably affected (p -value <0.01) by drought stress in the *P. bungeana* Zucc.ex Endl.. Of which, two (Pbd1, Pbd3) showed an increase in intensity, one (Pbd2) showed increased intensity when ψ_{swp} values reached about -1.5 MPa and decreased intensity when ψ_{swp} values reached about -3.0 MPa during drought stress (Supplementary Figure 3A and 3B; Table 2 and 3).

Two differential proteins were notably affected (p -value <0.01) by drought stress in the *P. sylvestris* L. var. *mongolica* Litv. Of which, one (Psd1) showed decreased intensity when ψ_{swp} values reached about -1.5 MPa and increased intensity when ψ_{swp} values reached about -3.0 MPa, one (Psd2) showed a decline in intensity during drought stress (Supplementary Figure 4A and 4B; Table 2 and 3).

Functional annotation of stress proteins

In our study, thirteen proteins were found to change very significantly (p -value <0.01) in abundance under drought stress. These proteins are mainly involved in functional proteins (dehydrins, Photosystem II H protein, Ribulose-1,5-bisphosphate carboxylase oxygenase)

and regulatory proteins (mitogen-activated protein kinase, putative translation factor).

The activity and abundance of many functional proteins changed through their regulation of signal transduction and gene expression. Plants will establish a new balance according to their interaction under drought stress. In this study we found some osmotic regulation proteins, such as dehydrins and photosynthesis proteins (photosystem II H protein, ribulose 1,5 – bisphosphate carboxylase/oxygenase large subunit).

Dehydrins (DHN), also known as late embryogenesis proteins (LEA), are members of a protein family that are expressed after plants are exposed to stresses with a dehydrative component such as drought, low temperature, salinity and ABA (WELLING *et al.*, 2004; PUHAKAINEN *et al.*, 2004; RORAT *et al.*, 2006; WACHOWIAK *et al.*, 2009). Dehydrins are ubiquitous plant proteins, and have a wide range of molecular masses from 9 to 200 kDa. Dehydrins has high thermal stability and hydrophilic property, and can replace partially some water molecules to maintain cellular dissolved state, thus avoiding the collapse of cellular structure, especially the membrane structure when plants were exposed to drought stress. Dehydrins also have the function of molecular chaperones to maintain the natural structure and

Supplementary Figure 3. – Representative CBB-stained 2-D gel of needle proteins in response to drought stress in *P. bungeana* Zucc.ex Endl. The proteins were separated by IEF/SDS-PAGE. Locations of the 3 drought responsive proteins are indicated by open circles and target plots (framed regions) in D (A,B,C). The framed regions are enlarged in Supplementary Fig. 3B. Needles were sampled on normal water, moderate drought stress ($\psi_{\text{swp}} \approx -1.5$ MPa) and severe drought stress ($\psi_{\text{swp}} \approx -3.0$ MPa) (i.e. Panel CK, Panel D1 and D2), and the 2-D gels were analyzed. $\circ$ point to spots whose intensity notably increased (total 2 spots), $\bullet$ point to spots whose intensity notably increased at moderate drought stress and decreased at severe drought stress (total 1 spots) (B). The detailed changes of ratio were showed in Table 2.

Supplementary Figure 4. – Representative CBB-stained 2-D gel of needle proteins in response to drought stress in *P. sylvestris* L. var. *mongolica* Litv (7A). The proteins were separated by IEF/SDS-PAGE. Locations of the 2 drought responsive proteins are indicated by open circles and target plots (framed regions) in D (A, B). The framed regions are enlarged in Supplementary Fig. 4B. Needles were sampled on normal water, moderate drought stress ($\psi_{\text{swp}} \approx -1.5$ MPa) and severe drought stress ($\psi_{\text{swp}} \approx -3.0$ MPa) (i.e. Panel CK, Panel D1 and D2), and the 2-D gels were analyzed. $\Rightarrow$ point to spots whose intensity notably decreased at moderate drought stress and increased at severe drought stress (total 1 spots). $\Rightarrow$ point to spots whose intensity notably decreased (total 1 spots) (B). The detailed changes of ratio were showed in Table 2.

function of protein under drought stress. Some studies showed the accumulation of dehydrins in plants tissues in response to dehydrative stress have been shown to enhance survival of herbaceous and woody plants in low temperature or under drought conditions (KONTUNEN-SOPPELA *et al.*, 2000; RICHARD *et al.*, 2000; WELLING and PALVA, 2006). In our study, we found that dehydrins (Psd1) expression was notably decreased at moderate drought stress and increased at severe drought stress in Ps, indicating that the drought resistance of Ps was enhanced under drought stress.

Photosystem II (PSII), including at least 12 kinds of protein components (psbA-F, psbH, psbI, psbK-N), originally detected as a 9 kDa phosphoprotein in pea thylakoid membranes (BENNET, 1977), is a pigment-protein complex that functions as a light-dependent water-oxidizing plastoquinone reductase. The PSII complex, located in the thylakoid membrane of higher plants, algae and cyanobacteria, is highly conserved among all these oxygenic photosynthetic organisms. It drives the water oxidation process of photosynthesis, which splits water into reducing equivalents and molecular oxygen by solar energy (SHI and SCHRODER, 2004). PSIIH, which is encoded by psbH, is a small membrane-spanning subunit of the PSII core complex of cyanobacteria and plants, and involved in stabilization, assembly or dimerization of the PSII complex. The small protein may facilitate fast dynamic conformational changes that the PSII complex needs to perform an optimal photosynthetic activity. Photosynthesis of higher plants under drought stress is one of the most sensitive physiological processes. In our study, we found that the expression of

PSIIH protein (Psd2) was decreased and even disappeared in Ps under drought stress. It implied Ps's normal photosynthesis was affected under drought stress.

Since RuBisCO is often rate-limiting for photosynthesis in plants, it may be possible to improve photosynthetic efficiency by modifying RuBisCO genes in plants to increase its catalytic activity and/or decrease the rate of the oxygenation activity (SPREITZER and SALVUCCI, 2002). This could improve biosequestration of CO_2 and be an important climate change strategy, so the degradation of RuBisCO will directly affect photosynthesis and nitrogen balance. Many studies indicated that some proteins, in particular RuBisCO LSU, were degraded easily under stress. For example, 19 protein spots were identified as the degradation fragments of RuBisCO LSU in rice under low temperature stress. The RuBisCO LSU degradation band (50 kDa) was found in wheat leave under the aging process induced by darkness and under the natural aging process (RUI and XU, 2004). In addition, RuBisCO LSU was increased in the marine pine needles (COSTA *et al.*, 1998) and sugar beet leaves (HORTON, 2000; HAJHEIDARI *et al.*, 2005) under drought stress conditions. It is likely that the increase of RuBisCO LSU under stress conditions come from the degradation of RuBisCO which eventually inhibited photosynthesis (MEDRANO *et al.*, 1997). RAKWAL *et al.* (RAKWAL and AGRAWAL, 2003) indicated tissue necrosis was found in the leaf and stem of rice under the treatment of jasmonic acid, and RuBisCO subunit significantly reduced. In our study, we found that in Pa, the RuBisCO LSU (Pad4) was decreased at moderate drought stress and increased with severe drought stress. It may suggest

that drought stress led to the degradation of RuBisCO, which then reduced photosynthesis of Pa (*Figure 1*).

Conifers can sense drought stress through various sensors or receptors, and the signal was transferred to the nucleus through signal transduction, and eventually regulated gene expression. In our study, we identified some protein kinases, such as mitogen-activated protein kinase and putative translation factor.

The mitogen-activated protein kinase (MAPK, 38-55 KDa) cascade plays an important role in signal transduction pathways in eukaryotes. The basic MAPK cascade consists of three interlinked protein kinases. The first component, the MAPK kinase kinase (MAPKKK), activates a MAPK kinase (MAPKK) through double serine (Ser) and threonine (Thr) phosphorylation. In turn, phosphorylated MAPKK activates the third component of the pathway, i.e., MAPK, through double phosphorylation of specific Thr and tyrosine (Tyr) residues in a conserved tripeptide motif (T-X-Y) (HAMEL *et al.*, 2005). Plant MAPK cascades are important amplifying modules that can rapidly transduce stress signals into various appropriate intracellular responses. And several dozens of MAPKs have been identified and isolated from Arabidopsis (MIZOGUCHI *et al.*, 1993), maize (LALLE *et al.*, 2005), tobacco (WILSON *et al.*, 1995), Petunia (DECROOC-FERRANT *et al.*, 1995), oat (HUTTLY and PHILLIPS, 1995), wheat (TAKEZAWA, 1999), barley (KNETSCH *et al.*, 1996), rice (SONG and GOODMAN, 2002; LIEBERHERR *et al.*, 2005) and *Chorispora bungeana* (ZHANG *et al.*, 2006), respectively. Many results clearly revealed that MAPKs played important roles in signal transduction in response to differentiation, development, and a variety of biotic and abiotic stresses (HUTTLY and PHILLIPS, 1995; JONAK *et al.*, 1996; KOVTUN *et al.*, 1998; BOGRE *et al.*, 1999) such as pathogen attack, hormones, wounding, cold, salt, drought, oxidative stress, ozone and sugar starvation, etc. Especially, HAMEL *et al.* (2005) suggest that biotic and abiotic challenges activate MAPKs in poplar, as in herbaceous species, which then function as a convergence point for pathogen defense and oxidant stress signaling cascades. In this study, we found that the expression of MAPK in Pa (Pad1) and Pt (Ptd1) were gradually increased with the intensification of drought stress. It indicated the MAPK was correlated with signal transduction in response to drought in the two pine species. Furthermore, we found that the expression of putative translation factors (Pad6) in Pa were increased under drought stress, it indicated that the translation factors in Pa play an important role in response to drought stress.

Comparative analysis of differential proteins and physiological responses in four pine species under drought stress

Thirteen differential proteins (P values < 0.01) were detected in four pine species under drought stress (*Table 3*), and they are mainly involved in photosynthesis, osmotic regulation and signal transduction and so on. Six, two, three and two differential proteins were tested respectively in Pa, Pt, Pb and Ps under drought stress. In addition, the MAPK was found not only in the Pa but also in the Pt (Pad1/Ptd1) (*Table 2*). It suggested that

the two pine species have some interesting correlations in responding to drought stress. It is worth noting that six of 13 (46.2%) differential proteins in Pa were detected under drought stress, and it was more than other three pine seedlings in the number of differential proteins (*Table 3*). These might indicate that five-needle-pine group (Pa) was more sensitive than two- or three-needle-pine group (Ps, Pt and Pa) in responding to drought stress. According to the changes in amplitude of the net photosynthetic rate, stomatal conductance, leaf water potential (*Figure 2*) and protein polymorphism genetic distances (*Figure 3*) and genetic similarity coefficient, we can conclude that Pa and Pb have the farthest relationship in drought response and genetic relationship than any other two species. These results were consistent with our previous conclusion based on water physiological characteristics and comprehensive evaluation index system of drought tolerance in trees (ZHANG *et al.*, 2000). We have successfully compared the difference of drought sensitivity and tolerance in four pine species not only from eco-physiology but also from biochemistry, especially proteomics, and provide important information about the practical application of proteomic studies in deeply molecular mechanism of stress response. We hope this study will provide some insights into the selection of forest tree species and molecular breeding in the arid and subarid areas of Northern China.

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