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ORIGINAL ARTICLE

Proteomic analysis of cold stress-responsive proteins in *Thellungiella* rosette leaves

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Abstract Low temperature is one of the most severe environmental factors that impair plant growth and agricultural production. To investigate how *Thellungiella halophila*, an *Arabidopsis*-like extremophile, adapts to cold stress, a comparative proteomic approach based on two-dimensional electrophoresis was adopted to identify proteins that changed in abundance in *Thellungiella* rosette leaves during short term (6 h, 2 and 5 days) and long term (24 days) exposure to cold stress. Sixty-six protein spots exhibited significant change at least at one time point and maximal cold stress induced-proteome change was found in long-term cold stress group while the minimal change was found in 6-h cold treatment group. Fifty protein spots were identified by mass spectrometry analysis. The identified proteins mainly participate in photosynthesis, RNA metabolism, defense response, energy pathway, protein synthesis, folding and degradation, cell wall and cytoskeleton and signal transduction. These proteins might work cooperatively to establish a new homeostasis under cold stress. Nearly half of the identified cold-responsive proteins were associated with various aspects of chloroplast physiology suggesting that the cold stress tolerance of *T. halophila* is achieved, at least partly, by regulation of chloroplast function. All protein spots involved in RNA metabolism, defense response, protein synthesis, folding and degradation were found to be upregulated markedly by cold treatment, indicating enhanced RNA metabolism, defense and protein metabolism may play crucial roles in cold tolerance mechanism in *T. halophila*.

Keywords Cold stress · Proteomics · *Thellungiella* · Two-dimensional electrophoresis

Abbreviations

CBB	Coomassie brilliant blue
ESI–MS/MS	Tandem electrospray ionization-mass spectrometry
IEF	Isoelectric focusing
LC–MS/MS	Liquid chromatography–tandem mass spectrometry
MALDI-TOF MS	Matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry
RT-PCR	Reverse transcription-polymerase chain reaction

Introduction

Plants are frequently facing different environmental variations. Among these constraints, low temperature is one of the most crucial factors that impair the distribution, growth

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and productivity of crops or wild-plant species. The mechanisms underlying cold acclimation and tolerance have been intensively studied over the past years and numerous studies on plant response to cold stress have been reported especially in the model plant such as *Arabidopsis thaliana* and rice (Ruelland et al. 2009). Microarray analysis was frequently carried out to examine the global gene expression profile, revealing that many genes are induced or repressed in response to cold (Chen et al. 2002; Fowler and Thomashow 2002). These results provided useful information for understanding the mechanism of cold tolerance and acclimation of plants.

In general, the level of mRNA does not always correlate well with the level of protein mainly due to post-transcriptional regulation (Yan et al. 2006). As a global study of the proteins comprising the proteome, proteomics can be playing an increasingly important role in addressing plant response to environmental changes. The proteomes of various plants in response to different environmental factors including drought, salt, heat and heavy metal have been investigated (Ouerghi et al. 2000; Hajduch et al. 2001; Salekdeh et al. 2002; Majoul et al. 2003). Regarding cold stress, some studies on plant proteomes have been performed, including the analysis of proteins extracted from *Arabidopsis* leaves (Amme et al. 2006), nuclear (Bae et al. 2003), plasma membrane (Kawamura and Uemura 2003), chloroplast lumen and stroma (Goulas et al. 2006) and rice leaves (Cui et al. 2005; Yan et al. 2006; Hashimoto and Komatsu 2007). The differentially expressed proteins identified in above reports include both well-documented stress-responsive proteins and some novel cold-responsive proteins. These results demonstrated the power of the proteomic approach in plant stress tolerance studies.

Plants differ in their cold response and cold-tolerant species may develop efficient strategies to adapt to chilling environment. *Thellungiella halophila* (also called *Thellungiella salsuginea*) is a newly emerging model species for the molecular elucidation of abiotic stress tolerance (Bressan et al. 2001). It is reported that *T. halophila* is more chilling tolerant than *Arabidopsis* and able to complete its entire life cycle at 5/4°C (Griffith et al. 2007). Although a microarray analysis of 3,628 cDNAs revealed 76 differentially expressed transcripts in *T. halophila* in responsive to cold treatment (Wong et al. 2006), large-scale transcriptional studies was hindered by the absence of genome sequence information. Global expression profile of genes at the protein and post-translational level provides a better scenario on how plants respond and adapt to environmental stresses. Hitherto, there has been no large-scale proteomic evaluation of the effects of cold stress done in *T. halophila*. In the present study, a comparative proteomic approach based on two-dimensional electrophoresis (2-DE) was used to investigate responses to short-term- and long-term cold stress in *T. halophila* rosette leaves.

Materials and methods

Plant materials and cold treatment

Shandong ecotype of *Thellungiella halophila* (C. A. Meyer) O. E. Schulz (kindly provided by Prof. Ziyi Cao, Shandong Normal University, China) was germinated and grown in soil under fluorescent light (330 μmol m^{-2} s^{-1}, 16 h light/8 h dark) at 25°C and 35% relative humidity in a growth chamber. Four-week-old seedlings were exposed to a growth chamber maintained at 5/4°C day/night for cold treatment. Four-week-old plants were used as starting material for this study. One set of plants was shifted to low temperature conditions while the control rosette leaves were sampled just before cold treatment (0 h). Fully developed rosette leaves from cold-treated plants were sampled at 6 h, 2, 5 and 24 days after the time the treated plants were shifted to 4°C. The all above samples were frozen in liquid nitrogen, and then stored at −70°C for protein extraction.

Growth parameters and relative electrolyte leakage measurements

Root length, rosette leaf number, root and shoot dry weights were measured for treatment and control groups, respectively. The relative electrolyte leakage (REL) assay was performed according to the method previously described (Yan et al. 2006) and eight replicates were performed for each sample.

Protein extraction and 2-DE analysis

The rosette leaf proteins were extracted using a trichloroacetic acid/acetone method as described previously (Yan et al. 2005). Protein concentration was determined using the Bradford assay (Bioteke, Beijing, China). For 2-DE, 1,100 μg of extracted proteins were loaded onto semi-preparative gels. For isoelectric focusing, the Ettan IPGphor3 system (Amersham Biosciences, Uppsala, Sweden) and pH 4–7 IPG strips (24 cm, linear) were used according to the manufacturer's recommendations. The IPG strips were rehydrated for 13 h in 450 μl rehydration buffer containing protein samples. Focusing was performed in three steps: 500 V for 1 h, 1,000 V for 1 h and 8,000 V for 10 h. The gel strips were equilibrated for 15 min in 10 ml equilibration buffer (50 mM Tris–HCl buffer, pH 8.8, 6 M urea, 30% v/v glycerol, 2% w/v SDS, 1% w/v DTT and 0.002% w/v bromophenol blue). SDS-PAGE was performed with 12.5% gels using the Ettan Six system (Amersham Biosciences).

The gels were run at 5 w per gel for the first 30 min and followed by 17 w per gel. The gels were visualized with Coomassie brilliant blue (CBB) R-250. The gels were scanned using an optical scanner in transmissive mode (Umax, Willich, Germany) at 400 dpi and analyzed with ImageMaster 2-D Elite software (Amersham Biosciences). Each sample was performed by 2-DE at least three repetition for further analysis. The abundance of each protein spot was estimated by the percentage volume (% vol). Only those protein spots with significant and reproducible changes were determined to be differentially expressed proteins.

In-gel digestion and MS analysis

In-gel digestion was performed according to the previously described method (Yan et al. 2006). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis was conducted with a MALDI-TOF/TOF mass spectrometer, a Bruker Autoflex mass spectrometer (Bruker-Franzen, Bremen, Germany). Database searches, including PMF and MS/MS, were performed using MASCOT program (http://www.matrixscience.com). The database was set to NCBInr (updated on 27 October 2008). The taxonomic category selected was green plants. The other parameters for searching were enzyme of trypsin, one missed cleavage, fixed modifications of carbamidomethyl (C) and variable modifications of oxidation (M), peptide tolerance of 100 ppm, MS/MS tolerance of 0.4 Da, peptide charge of 1^+ and monoisotopic. According to the MASCOT probability analysis ($P < 0.05$), only significant hits were accepted for the identification of the protein samples.

For proteins that could not be identified by MALDI-TOF MS, ESI–MS/MS was performed on a Qstar Pulser I Quadrupole TOF-MS (Applied Biosystems/MDS Sciex, Toronto, Canada). The protein identification was determined by MS/MS fragment ion using MASCOT software (Matrix Science Ltd., London, UK) through searching the Swiss-Prot database. The parameters used were monoisotopic peptide masses, ±0.2 Da peptide mass tolerance; one missed cleavage, modifications allowed for oxidation of methionine and carboxyamidomethylation of cysteine.

LC MS/MS was performed for part of proteins that could not be identified by MALDI-TOF MS. The tryptic peptides resulted from in-gel digestion were analyzed with a home-made reversed phase capillary column (50 μm × 10 cm) packed with YMC 5 μm spherical C18 reversed phase particles (YMC, Kyoto, Japan). To elute peptides from the column, an Agilent 1100 series binary pumps system (Agilent Technologies, Santa Clara, CA, USA) was used to generate the following HPLC gradient: 0–40% B in 10 min, 40–100% B in 5 min (A = 0.1 M acetic acid in water, B = 0.1 M acetic acid/70% acetonitrile). The eluted peptides were sprayed directly into an LTQ mass spectrometer (Thermo Electron Corporation, San Jose, CA, USA) equipped with a nano-ESI ion source. The spectra were acquired in data-dependent mode (MS scan 350–2,000 Da, top 3 most abundant MS/MS scans with 30 s dynamic exclusion time). Database searches were performed on an in-house Mascot server (version 2.1, Matrix Science Ltd.).

Quantitative real time PCR

Total RNA in leaves was extracted using RNeasy Plant Mini Kit (Qiagen, Hilde, Germany). The potential contaminating genomic DNA was treated with RQ1 RNase-free DNase (Promega, Madison, WI, USA). The cDNA was synthesized using BcaBEST RNA PCR Kit (Takara, Shiga, Japan) according to the manufacturer's protocol. The primers were designed according to the corresponding *T. halophila* EST sequences in GenBank (Table 1). Quantitative real-time PCR (qPCR) was performed on an ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using SYBR Green Realtime Master Mix (Applied Biosystems). The expression pattern of ubiquitin 10 (UB10, NCBI accession number BQ060353) was used as a reference. The relative gene expression was evaluated using the comparative cycle threshold method (Livak and Schmittgen 2001).

Statistics

Unless stated otherwise, all experiments were repeated at least three times, and within each experiment, treatments were replicated three times, each replication comprising

Table 1 Primer sequences used in qPCR

Spot number	Forward primer	Reverse primer
10	ACGTTAACTGGAAACTTGAGGAC	ATTGTATTGATCAGGTTCGGATT
11	AATGACACTGTTGGTGAGGAAGT	AATTCAGGGTCGTTCTTCTCTTC
18	CCCTGAAATCATGGTTGAGC	AAGAAAAGGCCTCCACATCTC
20	CAAAATTCAACATGCTTCTCAAG	AGATGAAGAACACCTTCATGGTT
Ubiquitin 10	ACAACGTGTAAGGCTAAGATCCA	GGATGTTGTAGTCCGCAAGAGTA

five to eight pooled plants. All data were analyzed using Fisher's protected least significant difference test using SAS statistical software (SAS Institute, Cary, NC, USA).

Results

The physiological responses induced by cold stress in *T. halophila*

The exposure of *T. halophila* seedlings to short-term cold stress (6 h, 2 and 5 days) do not results in obvious changes in morphology and growth parameters (data not shown), while long-term cold treatment (24 days) decreased the growth of *T. halophila* significantly (Table 2). REL is an indicator of membrane damage caused by low temperature. To estimate the effect of cold-induced membrane damage in *T. halophila*, REL was measured in plants after exposure to cold stress for 6 h, 1, 2, 5 and 24 days. As shown in Fig. 1, the REL of seedlings treated for 6 h increased slightly, reached vertex after 1 day and then decreased gradually. The REL value at control condition is relatively higher, which requires further investigation. However, similar REL value was reported in an earlier study (M'rah et al. 2007).

Two-dimensional electrophoresis analysis of total proteins in *T. halophila* rosette leaves

To investigate the dynamic protein expression patterns responsive to temporal and long-term cold stress, the proteome alterations of *T. halophila* rosette leaves after cold treatment for 6 h, 2, 5 and 24 days were examined. Triplicate gels were obtained from three independent experiments and gels from the different experiments showed high reproducibility. The representative gels from the control (0 h) and long-term cold treatment (24 days) plants are illustrated in Fig. 2. More than 1,500 protein spots were detected reproducibly by ImageMaster 2-D Elite software

Table 2 Effect of long-term cold treatment on growth parameters of *T. halophila*

Growth parameters	Control	24 days of cold treatment
Root length (cm)	12.90 ± 1.81	7.45 ± 1.85[a]
Root dry weight (g)	0.028 ± 0.0094	0.0051 ± 0.0014[a]
Shoot dry weight (g)	0.038 ± 0.0041	0.0072 ± 0.0017[a]
Leaf number	30 ± 1.56	12 ± 1.05[a]

Four-week-old seedlings were treated at 5/4°C for 24 days and then root length, leaf numbers, dry weights of root and shoot were measured; the seedlings grown in normal conditions for 24 days were used as control

[a] Treatment effect is significant ($P < 0.01$)

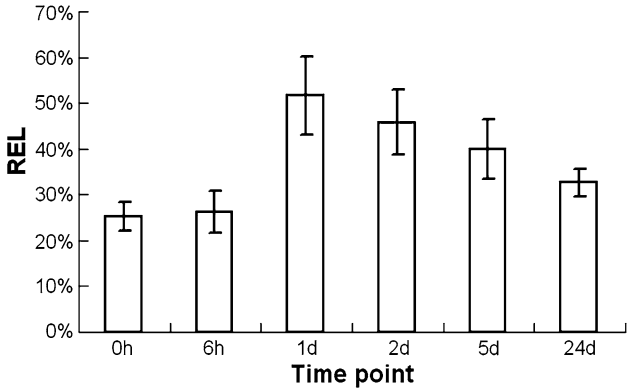


Fig. 1 REL from rosette leaves of *T. halophila*. Four-week-old seedlings were treated at 5/4°C for 6 h, 1, 2, 5 and 24 days, then, REL was measured. Each value represents the mean value ± SD from eight measurements

on CBB-stained gels and all gels showed very similar protein distribution patterns in 2-D image. Quantitative image analysis revealed that a total of 66 protein spots changed their intensities significantly ($P < 0.05$) by more than 1.5-fold when compared with the control and emerged at least at one time point (Fig. 2, also see Suppl. Fig. S1). Three typical regions are enlarged in Fig. 3. Although most spots showed quantitative changes, some spots showed qualitative changes. Some spots [e.g. 1, 2, 3, 4, 32, 33, 49 and 56 identified as SNF2 domain-containing protein/helicase domain-containing protein/zinc finger (C3HC4 type RING finger) family proteins and 2-cysperoxiredoxin, chloroplast, respectively] were absent in the controls, but appeared after cold treatment.

Figure 4 shows the proportion of differentially expressed protein spots during cold treatment. It was clear that smallest changes (1.6%) occurred in the leaf proteome within the first 6 h, with eight more abundant and seven less abundant spots. However, with time, the leaf proteome changed more, and up to 2.3 and 3.4% of the total protein spots changed in abundance at days 2 and 5, respectively. There were 15 more abundant spots, two less abundant spots and five new spots at day 2, and 25 more abundant spots, one less abundant spots and six new spots at day 5. After long-term cold treatment (24 days), largest change (5.6%) occurred in the leaf proteome with 34 more abundant spots, ten less abundant and nine new spots. It seems that long-term cold stress produced more effect on rosette leaf proteome of *T. halophila*. Similar result was reported in a study on chloroplast lumen and stromal proteomes of *Arabidopsis*, in that the longer cold treatment, the more differentially expressed protein spots detected (Goulas et al. 2006). Figure 5 shows the Venn diagram analysis of the quantity of differentially expressed proteins identified from rosette leaves at different time points of cold treatment. There were no large overlaps between cold shock and long-term cold treatment.

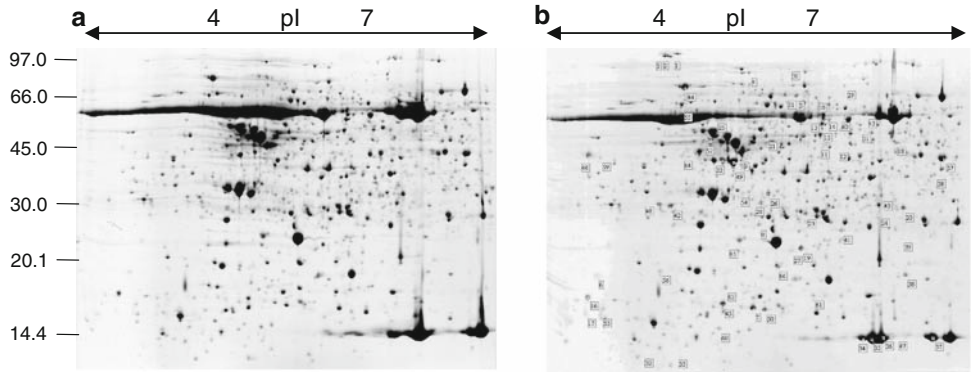


Fig. 2 Representative 2-DE gels of *T. halophila* rosette leaf proteins. 2-DE gels of *T. halophila* rosette leaves in control group (**a**) and 24-day cold treatment group (**b**). 2-DE was performed using 1,100 μg of total protein and 24 cm immobiline dry strips with linear pH gradients from 4 to 7. Gels were stained with CBB R-250. *Labeled spots* indicate differentially expressed proteins showing at least a 1.5-fold change under cold treatments at least at one time point with $P < 0.05$. Positions and sizes of SDS-PAGE molecular weight markers (MW) are shown in kilo daltons

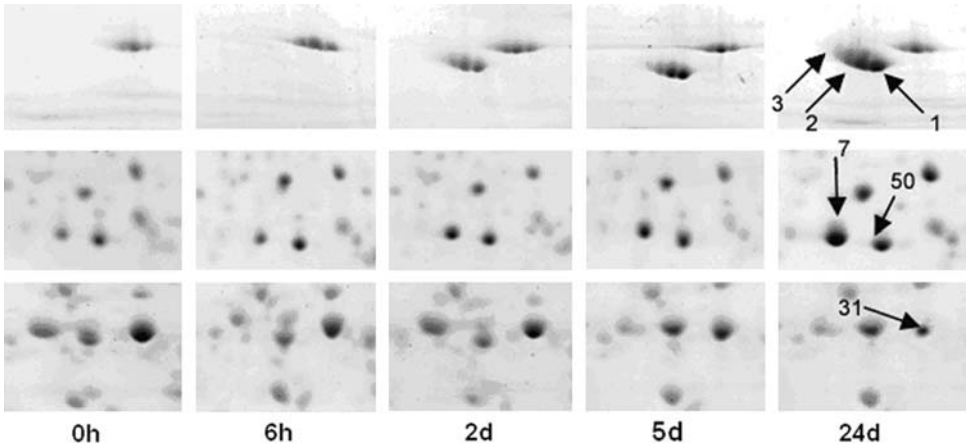


Fig. 3 Expression changes of some cold-responsive protein spots. Four-week-old seedlings were treated at 5/4°C for 6 h, 2, 5, and 24 days, and then total rosette leaf proteins were extracted and separated by 2-DE. *Arrows* indicate proteins showing changes in abundance during cold exposure. Spots *1*, *2* and *3* identified as SNF2 domain-containing protein/helicase domain-containing protein/zinc finger (C3HC4-type RING finger) family proteins were newly induced after the exposure to cold stress for 2, 5 and 24 days. Spots *7* (GRP1A) and *50* (GRP7) were upregulated by cold treatment while protein spot *31* (ATP sulfurylase) was downregulated by cold exposure

Identification of the differentially expressed proteins

The spots of differentially expressed proteins were excised from the gels, in-gel digested by trypsin, and analyzed by mass spectrometer. In total, 50 protein spots were successfully identified either by PMF or MS/MS analysis as shown in Table 3 (also see Suppl. Tables S1, S2, S3 and S4 for more information) representing 40 individual proteins. Many cold stress-responsive proteins identified in our experiments have already been discussed in the context of cold-stress responses. These proteins include, putative 2, 3-bisphosphoglycerate-independent phosphoglycerate mutase, glycine-rich RNA-binding protein 7 (GRP7), ferritin-1, EF-G, phosphoglycerate kinase, ATP synthase CF1 beta chain, UDP-glucose pyrophosphorylase, cysteine proteinase inhibitor, RuBisCO small chain, carbonic anhydrase, and SAL1 phosphatase. Some proteins not yet previously reported to be involved in plant response to cold stress were identified in our study, such as isopropylmalate synthase, cytochrome b6–f complex iron–sulfur subunit, SNF2 domain-containing protein/helicase domain-containing protein/zinc finger (C3HC4 type RING finger) family protein, and so on. These novel cold-responsive proteins may play important roles in cold tolerance of *T. halophila* and their functions are still to be investigated.

Some proteins were identified in more than one spot (Table 3), although they were excised from the same gel. This phenomenon may result from the presence of different protein isoforms, post-translational modification or degradation. For example, RuBisCO small subunit was identified from five spots (spots 34, 35, 36, 37 and 46). Western blots have been performed to confirm the identity of the multiple

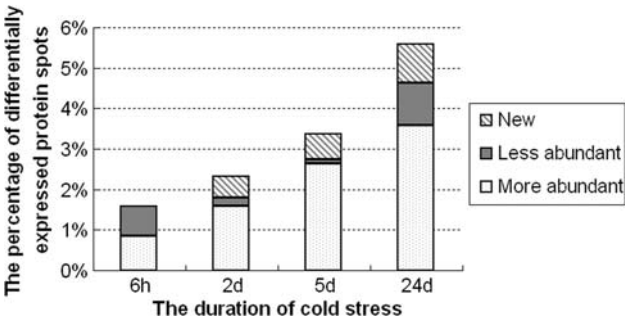


Fig. 4 Summary of the proportion of resolved protein spots that significantly ($P < 0.05$) changed in abundance in the *T. halophila* rosette leaf proteome under cold treatment. The percentages are calculated by dividing the number of resolved protein spots that significantly ($P < 0.05$) changed in abundance by the total number of spots in reference 2-DE gel

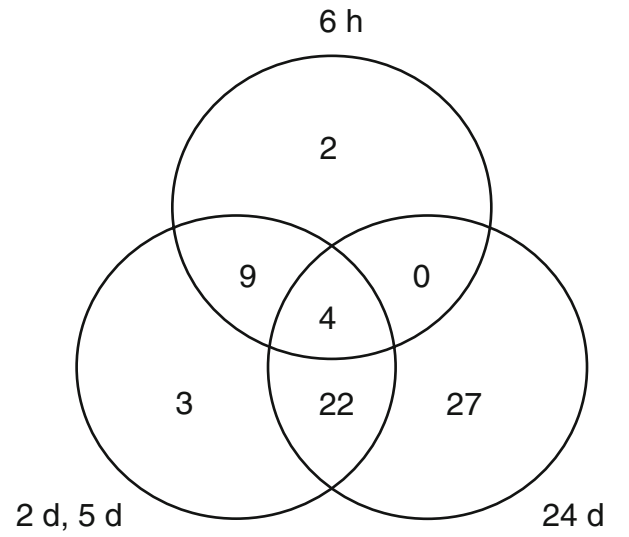


Fig. 5 Venn diagram analysis of the differentially expressed proteins during cold treatment. The *numbers* of differentially expressed spots at different time point are shown in *different segments*

spots of RuBisCO small subunit (data not shown), but the difference in these polypeptides has yet to be determined. SNF2 domain-containing protein/helicase domain-containing protein/zinc finger (C3HC4-type RING finger) family protein was identified in three spots (spots 1, 2 and 3), while putative 2, 3-bisphosphoglycerate-independent phosphoglycerate mutase was identified in three spots (spots 5, 10 and 51).

Fifty identified proteins spots were divided into several groups according to their functions predicted by the gene ontology tool at www.geneontology.org (Fig. 6). The largest group is related to photosynthesis, including 14 cold-responsive protein spots (representing 8 unique proteins) involved in Calvin cycle (spots 18, 34, 35, 36, 37, 40, 43, 47 and 49, identified as glyceraldehyde-3-phosphate dehydrogenase B, chloroplast precursor, RuBisCO small subunits, chloroplast precursor, chloroplast carbonic anhydrase precursor and RuBisCO large subunit), electron transport (spots 16, 17, 26, 61, identified as plastocyanin, oxygen-evolving enhancer 33 and cytochrome b6–f complex iron–sulfur subunit, chloroplast precursor) and photorespiration (spot 15, identified as alanine-2-oxoglutarate aminotransferase 1). The second largest group contains defense and stress-related proteins. The two-third largest groups include proteins involved in RNA metabolism and energy pathway. The two-fourth groups include proteins involved in protein synthesis, folding and degradation, as well as cell wall and cytoskeleton. The remaining groups include metabolisms of nitrogen and sulfur, signal transduction, as well as unclear and unknown function. The subcellular localizations of all identified proteins were analyzed by TargetP program (www.cbs.dtu.dk/services/TargetP) (Emanuelsson et al. 2000) and 46% proteins (23/50) were predicted to be localized in chloroplast. This suggests that the chloroplast is one of the organelles most prone to harm by cold stress in *T. halophila*.

Fifty cold stress-responsive protein spots showed different changes patterns during cold stress, as shown in Table 3, and specific expression pattern may reflect the specific physiological function of the corresponding protein in cold tolerance of plant. It was found that the identified protein spots related to RNA metabolism, defense response, protein synthesis, folding and degradation were all upregulated markedly during cold treatment. It suggests that these cellular processes were enhanced by exposure to cold stress. The identified protein spots involved in photosynthesis and energy pathway displayed diverse change patterns. This may indicate that these processes were rearranged under cold stress. All above adjustments in cellular processes might contribute to cold tolerance in *T. halophila* coordinately.

Comparison of mRNA and protein levels of cold-responsive proteins

To investigate the changes in gene expression at the mRNA level, qPCR analysis were performed for four randomly selected identified proteins at 5 days of cold exposure. All quantitative RT-PCR amplified DNA fragments were sequenced to confirm their identity. For the upregulated protein spots 11 (putative phosphoglycerate kinase) and 20 (cysteine proteinase inhibitor, putative 1), their transcripts were also upregulated and the mRNA of spot 20 showed a higher fold. But for the upregulated protein spots 10 (putative 2,3-bisphosphoglycerate-independent phosphoglycerate mutase) and 18 (glyceraldehyde-3-phosphate dehydrogenase B, chloroplast precursor), the mRNAs were downregulated (Fig. 7), which need further exploration. We also compared the changes in mRNA levels after 3-week

Table 3 Identification of cold stress-responsive proteins in *T. halophila* rosette leaves

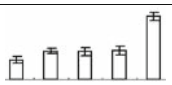
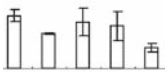
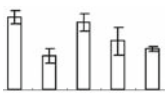
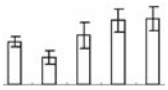
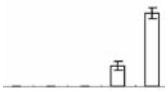
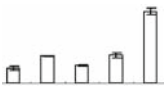
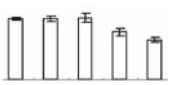
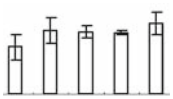
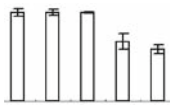
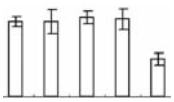
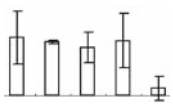
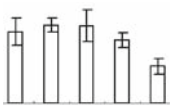
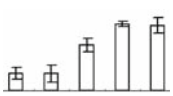
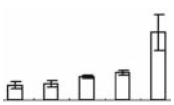
Metabolic group	Spot no.	Expression pattern 0 h, 6 h, 2 d, 5 d, 24 d	Description	Accession no.	Species	Theor./exp. mass (kDa)	Theor./exp. pI	NP	SC (%)
Photosynthesis (28%)	15		Alanine-2-oxoglutarate aminotransferase 1 (GGT1)	gil30688330	*Arabidopsis thaliana*	54/51	6.5/5.8	15	34
	16		Plastocyanin	P00294	*Capsella bursa-pastoris*	10/17	4.1/4.1	2	47
	17		Plastocyanin	P00294	*Capsella bursa-pastoris*	10/15	4.1/4.1	2	47
	18		Glyceraldehyde-3-phosphate dehydrogenase B, chloroplast precursor	P09044	*Arabidopsis thaliana*	48/42	6.8/6.5	3	7
	49		Glyceraldehyde-3-phosphate dehydrogenase B, chloroplast precursor	P25857	*Arabidopsis thaliana*	48/38	6.8/5.3	1	3
	26		PSBO-1 (oxygen-evolving enhancer 33)	gil15240013	*Arabidopsis thaliana*	35/33	5.3/5.4	6	22
	34		RuBisCO small subunit, chloroplast precursor	gil132154	*Raphanus sativus*	21/14	8.2/6.1	7	36
	35		RuBisCO small subunit, chloroplast precursor	gil132154	*Raphanus sativus*	21/14	8.2/6.2	7	36
	36		RuBisCO small subunit, chloroplast precursor	gil132154	*Raphanus sativus*	21/14	8.2/6.3	7	36
	37		RuBisCO small subunit, chloroplast precursor	gil132154	*Raphanus sativus*	21/14	8.2/6.7	7	36
	40		Chloroplast carbonic anhydrase precursor	gil45451864	*Thlaspi caerulescens*	37/32	5.4/6.3	11	40
	43		RuBisCO large subunit	gil4185573	*Gyrostemon sp. Cranfield 02068672*	53/53	6.1/6.1	21	37
	47		RuBisCO small subunit, chloroplast precursor	gil132154	*Raphanus sativus*	21/14	8.2/6.3	12	34
	61		Cytochrome b6-f complex iron-sulfur subunit, chloroplast precursor (Rieske iron-sulfur protein)	Q9ZR03	*Arabidopsis thaliana*	25/18	8.6/5.8	2	12
Defense (16%)	8		Ferritin-1, chloroplast precursor	Q96540	*Brassica napus*	28/26	5.1/5.3	2	9
	20		Cysteine proteinase inhibitor, putative 1	gil24899735	*Arabidopsis thaliana*	22/28	6.0/6.4	6	20

Table 3 continued

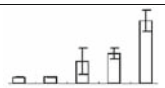
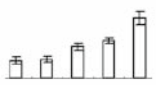
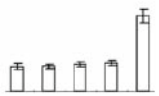
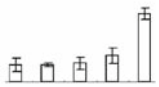
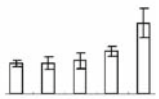
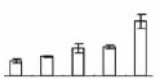
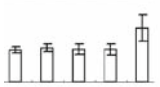
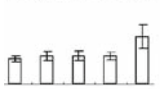
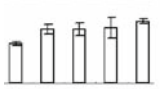
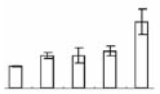
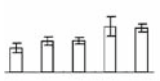
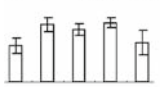
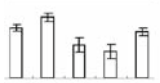
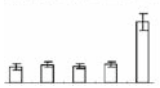
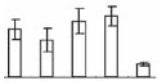
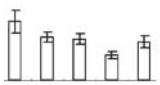
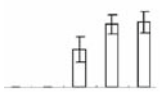
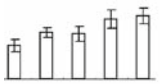
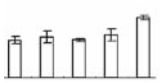
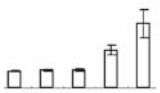
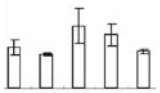
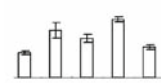
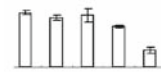
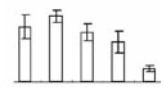
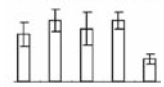
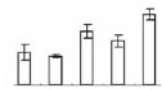
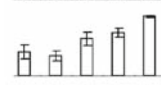
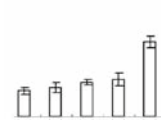
Metabolic group	Spot no.	Expression pattern 0 h, 6 h, 2 d, 5 d, 24 d	Description	Accession no.	Species	Theor./exp. mass (kDa)	Theor./exp. pI	NP	SC (%)
	22		Dehydrin	gil1326161	*Phaseolus vulgaris*	23/39	5.3/5.3	1	4
	38		Lectin	gil126147	*Onobrychis viciifolia*	27/22	5.2/6.5	5	33
	45		Peroxiredoxin-2E, chloroplast precursor	Q949U7	*Arabidopsis thaliana*	25/16	9.7/5.2	2	8
	56		2-cys peroxiredoxin, chloroplast (BAS1)	gil15229806	*Arabidopsis thaliana*	29/23	7.7/4.8	9	36
	58		Aldo-keto reductase	Q84TF0	*Arabidopsis thaliana*	35/36	6.6/6.6	1	3
	65		Peptide methionine sulfoxide reductase	Q9SEC2	*Lactuca sativa*	29/26	8.5/5.3	2	8
RNA metabolism (14%)	1		SNF2 domain-containing protein / helicase domain-containing protein / zinc finger (C3HC4 type RING finger) family protein	gil15226742	*Arabidopsis thaliana*	188/122	6.2/4.9	23	21
	2		SNF2 domain-containing protein / helicase domain-containing protein / zinc finger (C3HC4 type RING finger) family protein	gil15226742	*Arabidopsis thaliana*	188/122	6.2/4.9	23	21
	3		SNF2 domain-containing protein / helicase domain-containing protein / zinc finger (C3HC4 type RING finger) family protein	gil15226742	*Arabidopsis thaliana*	188/122	6.2/4.8	23	21
	7		Glycine-rich RNA-binding protein GRP1A	gil1346180	*Sinapis alba*	16/16	5.2/5.3	7	45
	42		Putative RNA-binding protein cp29	gil15228102	*Arabidopsis thaliana*	31/30	5.1/4.9	6	12
	48		Putative RNA-binding protein cp29	Q9ZUU4	*Arabidopsis thaliana*	31/30	4.9/4.8	2	7
	50		Glycine-rich RNA-binding protein 7	Q03250	*Arabidopsis thaliana*	16/16	6.0/5.5	2	12
Energy pathway (14%)	5		Putative 2,3-bisphosphoglycerate-independent phosphoglycerate mutase	gil23397105	*Arabidopsis thaliana*	61/61	5.3/5.7	2	4
	10		Putative 2,3-bisphosphoglycerate-independent phosphoglycerate mutase	gil21537260	*Arabidopsis thaliana*	61/60	5.5/5.7	15	25
	11		Putative phosphoglycerate kinase	gil21536853	*Arabidopsis thaliana*	42/42	5.5/5.8	10	35
	13		ATPase beta subunit	gil139388101	*Barbarea verna*	54/53	5.5/5.7	28	67
	51		2,3-bisphosphoglycerate-independent phosphoglycerate mutase 1	O04499	*Arabidopsis thaliana*	61/61	5.3/5.4	1	2

Table 3 continued

Metabolic group	Spot no.	Expression pattern 0 h, 6 h, 2 d, 5 d, 24 d	Description	Accession no.	Species	Theor./exp. mass (kDa)	Theor./exp. pI	NP	SC (%)
	57		Fructose-bisphosphate aldolase, cytoplasmic isozyme	O65735	*Cicer arietinum*	39/39	6.6/6.6	2	7
	66		Putative delta subunit of ATP synthase	gil1480014	*Brassica rapa*	15/23	9.2/5.5	16	54
Protein synthesis, folding and degradation (8%)	4		EF-G	gil14532624	*Arabidopsis thaliana*	86/83	5.9/5.4	2	4
	23		PAB1 (20S proteasome alpha subunit B1); peptidase	gil15219257	*Arabidopsis thaliana*	26/27	5.5/5.7	11	44
	44		Heat shock protein 70	gil6746592	*Arabidopsis thaliana*	77/76	5.1/5.0	16	19
	55		Probable protein disulfide-isomerase 1 precursor (PDI 1)	Q9XI01	*Arabidopsis thaliana*	57/56	4.6/4.9	2	5
Cell wall and cytoskeleton (8%)	12		Reversibly glycosylated polypeptide-3	Q8LB19	*Arabidopsis thaliana*	41/41	6.5/5.9	5	20
	14		UGP (UDP-glucose pyrophosphorylase); UTP:glucose-1-phosphate uridylyltransferase	gil15228498	*Arabidopsis thaliana*	52/52	5.8/5.8	9	27
	21		Actin isoform B	gil6683504	*Mimosa pudica*	42/48	5.3/5.5	2	8
	24		Germin-like protein	gil1755184	*Arabidopsis thaliana*	22/26	6.8/6.2	1	8
Metabolisms of nitrogen and sulfur (4%)	31		ATP sulfurylase	gil6606509	*Arabidopsis thaliana*	51/50	6.3/6.2	17	32
	63		Isopropylmalate synthase	Q71QG1	*Brassica oleracea*	55/53	7.3/5.9	1	3
Signal transduction (4%)	30		Double-stranded RNA-binding domain (ISS)	gil116056440	*Ostreococcus tauri*	88/87	5.0/5.5	12	21
	52		SAL1 phosphatase	Q42546	*Arabidopsis thaliana*	38/42	4.9/5.0	1	3
Unclear and unknown function (4%)	19		Unknown protein	gil18391006	*Arabidopsis thaliana*	20/25	5.4/5.2	12	31
	41		SOUL-like protein	gil21592576	*Arabidopsis thaliana*	26/25	4.5/5.8	1	11

NP number of matched peptides, *SC* sequence coverage

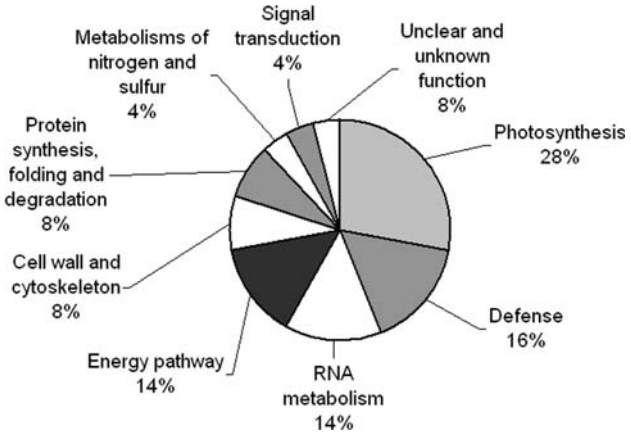


Fig. 6 Functional classification of the 50 identified proteins spots. Four-week-old seedlings were treated at 5/4°C for 6 h, 2, 5 and 24 days, and then total rosette leaf proteins were subjected to 2-DE analysis. Fifty identified differentially expressed protein spots were searched against gene ontology tool (www.geneontology.org) for functional classification

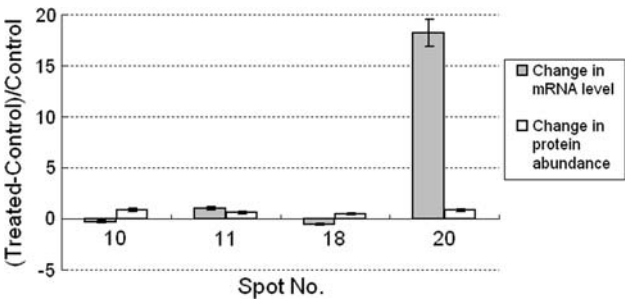


Fig. 7 Comparison of expression patterns in mRNA level and protein abundance of cold-responsive proteins. Spot *10*, putative 2,3-bisphosphoglycerate-independent phosphoglycerate mutase; spot *11*, putative phosphoglycerate kinase; spot *18*, glyceraldehyde-3-phosphate dehydrogenase B, chloroplast precursor; spot *20*, cysteine proteinase inhibitor, putative 1

5/4°C cold treatment reported in an earlier cDNA microarray study (Wong et al. 2006) with the alteration in protein level after 24 days of cold exposure in the present proteomics analysis. As shown in Table 4, although most of the protein spots displayed similar change patterns, two spots (spots 22 and 40 identified as dehydrin and chloroplast carbonic anhydrase precursor, respectively) showed different expression patterns between mRNA and protein level. Our results confirmed the previous evidence that the mRNA level is not necessarily correlated well with the protein level (Yan et al. 2006).

Discussion

Cold stress affects almost all aspects of cellular function in plants and it is, therefore, not surprising that the present comparative proteomics analysis reveals a complex cellular network induced by the low temperature stress. Owing to its high degree of cold tolerance, *T. halophila* is a valuable model for investigating the mechanisms involved in plant cold stress tolerance. Proteomic analysis of cold stress response in *T. halophila* can help to identify key regulators of cold tolerance in plants.

Regulation of chloroplast function under cold stress

The expression regulation of chloroplast proteins is of central importance in cold adaptation (Foyer et al. 1997) and a substantial portion (46%, 23/50) of the identified proteins were predicted to be localized in chloroplast. Eighteen of these have been identified to reside in the chloroplast by previous proteomic studies according to the Plant Proteome Database (PPDB, http://ppdb.tc.cornell.edu/) (Sun et al. 2009). These chloroplast located cold-stress-responsive proteins are associated with various aspects of chloroplast, including Calvin cycle and electron transport (discussed below), chloroplast RNA processing (putative RNA-binding protein cp29), chloroplast protein synthesis and folding (EF-G and heat-shock protein 70), as well as chloroplast redox homostasis (BAS1, Peroxiredoxin-2E and Ferritin-1). This result suggests that the regulation of chloroplast function is central part of cold tolerance mechanism in *T. halophila*.

Plants may adjust photosynthesis via gene regulation to adapt to cold environment and some proteins involved in Calvin cycle and electron transport were found to be altered in abundance in the present study. A RuBisCO large subunit protein (spot 43) decreased in abundance after 24 days of cold treatment, while some RuBisCO small subunit proteins (spots 34, 35, 36, 37 and 47) displayed a heterogeneous change patterns during cold treatment. RuBisCO small subunits were proved to affect RuBisCO catalytic efficiency and specificity (Spreitzer 2003), therefore, the cold-induced changes of RuBisCO small subunits may indicate the adjustment of activity of the RuBisCO complexes under cold stress. At the same time, a chloroplast-localized carbonic anhydrase, which facilitates CO_2 move across the chloroplast envelope, was found to decrease in abundance after 5 and 24 days of cold treatment. The changes in Calvin cycle enzymes observed in this study might be associated with decrease in photosynthetic CO_2 assimilation, possibly resulting in decreased growth rate in the long term. Consistent with changes in Calvin cycle enzymes, three proteins spots representing two electron transport proteins: plastocyanin and cytochrome b6–f complex iron–sulfur subunit were downregulated under long-term cold treatment. It is, perhaps, part of the strategy of *T. halophila* for adapting photosynthesis to low temperatures by adjustment of activity of Calvin cycle enzymes and decreasing electron transfer efficiency.

Table 4 Comparison of mRNA and protein expression profiles of cold-responsive proteins

Spot number	Gene name in *T. halophila* cDNA microarray study	Fold change in mRNA	Fold change in protein abundance
16, 17	Plastocyanin, At1g20340	0.25	0.39, 0.56[a]
41	SOUL heme-binding family protein, At1g17100	2.65	2.94
12	Reversibly glycosylated polypeptide-3, At5g15650	4.81	3.14
20	Cys protease inhibitor, At3g12490	2.85	4.59
22	Dehydrin, At1g76180	No significant change[b]	10.26
40	Carbonic anhydrase 1, chloroplast, At3g01500	No significant change[c]	0.58

[a] Spots 16 and 17 were both identified as plastocyanin

[b] No significant change after 3-week cold exposure, but shows 2.48-fold change under drought stress

[c] No significant change after 3 weeks cold exposure, but shows 0.33-fold change under drought stress

Enhanced RNA metabolism

RNA metabolism, including RNA processing, transporting from nucleus to cytoplasm and mRNA secondary structure stability may be impaired under cold stress, especially for the cold-stress-induced defense-related transcripts (Zhu et al. 2007). Several RNA-binding proteins and helicase-like proteins were all found to be upregulated by cold stress in the present study, including GRP7, glycine-rich RNA-binding protein GRP1A (GRP1A), putative RNA-binding protein cp29 and SNF2 domain-containing protein/helicase domain-containing protein/zinc finger (C3HC4-type RING finger) family proteins indicating enhanced RNA metabolism may play a important role in cold tolerance of *T. halophila*.

Among these cold-responsive nucleic acid-binding proteins, GRPs are suggested to play an important role in post-transcriptional regulation of gene expression in plants under various stress conditions (Mousavi and Hotta 2005). GRP7 has been demonstrated to play a role in the export of mRNAs from the nucleus to the cytoplasm under cold stress conditions in *Arabidopsis* (Kim et al. 2008). Two GRPs, GRP7 and GRP1A were upregulated by cold exposure in *T. halophila*; thus, promoting cold adaptation process by the export of mRNA from the nucleus. RNA-binding protein cp29 is a subunit of the photosystem II and its phosphorylation was reported to relate with the cold tolerance in maize (Mauro et al. 1997). A RNA-binding protein cp29 was proved to be induced by cold stress in *Arabidopsis* in a proteomics study (Amme et al. 2006). The increase in two chloroplast RNA-binding proteins may help to enhance or maintain the chloroplast RNA synthesis under cold conditions. In addition, GRPs and chloroplast RNA-binding proteins are suggested to be part of the plant innate immunity system and increase in their abundance may promote the expression of immunity-related mRNA (Fu et al. 2007).

Three SNF2 domain-containing protein/helicase domain-containing protein/zinc finger (C3HC4 type RING finger) family protein spots were induced by cold treatment and their abundance increased gradually with the duration of the treatment. There are no previous reports showing the involvement of this protein in cold response. This protein contains a DEAD-box helicase domain, which is suggested to be involved in several steps in plant stress tolerance mechanism (Chinnusamy et al. 2008), including the removal of secondary structure in the 5′ UTR of stressed plant mRNA for efficient translation (Vashisht and Tuteja 2006). Thus, GRPs, chloroplast RNA-binding protein and DEAD-box helicase-like protein might function cooperatively to reduce the negative effects of cold stress on RNA metabolism in *T. halophila*.

ROS scavenging mechanisms

Cold stress may disturb cellular redox homeostasis and promotes the production of reactive oxygen species (ROS) and reactive aldehydes (as 4-hydroxy-nonenal and methylglyoxal). Plants develop ROS scavenging mechanisms to cope with the oxidative stress. Because chloroplast is one of major source of ROS, four proteins involved in chloroplast redox homeostasis, including 2-cys peroxiredoxin, chloroplast (BAS1), peroxiredoxin-2E, ferritin-1 and alanine-2-oxoglutarate aminotransferase 1 (GGT1) were found to be upregulated in the present study. BAS1 is involved in the detoxification of alkyl hydroperoxides and its gene expression is regulated under the control of the cellular redox state (Baier and Dietz 1997). Peroxiredoxin-2E reduces hydrogen peroxide and alkyl hydroperoxides with reducing equivalents provided through the thioredoxin or glutaredoxin system and was a component of chloroplast thioredoxin system (Bréhélin et al. 2003). Ferritin-1, an iron-binding protein, is proposed to protect plants from oxidative damage induced by manifold stresses (Deák et al. 1999).

GGT1, an enzyme involved in photorespiration and photorespiration, has been suggested to be important for maintaining electron flow to prevent photoinhibition under stress conditions (Wingler et al. 2000). ggt1 mutation damages the photorespiration, resulting in the accumulation of the toxic metabolites, especially the H_2O_2 (Verslues et al. 2007). It is presumed that the increase in the GGT1 will help to reduce the accumulation toxic metabolites under cold stress. It appears that the control of chloroplast redox homeostasis is important part of cold adaptation mechanism in *T. halophila*.

In addition to the proteins involved in chloroplast redox homeostasis discussed above, two other non-chloroplastic ROS scavenging and defense-related proteins were upregulated by cold exposure in our study, including peptide methionine sulfoxide reductase and aldo-keto reductase. Methionine residues of proteins are a major target for oxidation by ROS and peptide methionine sulfoxide reductase plays protective roles in the cellular response to oxidative stress reducing oxidized MetO back to Met. A peptide methionine sulfoxide reductase *Arabidopsis* mutant, msrb3 has been reported to lose the ability to become tolerant to freezing temperatures following cold pretreatment (Kwon et al. 2007). Aldo-keto reductases can detoxify lipid peroxidation products and glycolysis-derived reactive aldehydes that contribute significantly to cellular damages caused by environmental stresses and improve scavenging capacity of the plant. The increase in aldo-keto reductase abundance is related with its involvement in detoxification process. Indeed, the aldo-keto reductase has been reported to be upregulated by copper stress in root of *Cannabis sativa* (Bona et al. 2007).

Maintaining protein synthesis and folding under cold stress

Cold stress may impose a great influence on the protein synthesis apparatus in plant (Singh et al. 2004). A chloroplast EF-G protein appeared in gel after 2, 5 and 24 days of cold treatment, but there was no spot at the corresponding position in gel of control group. Because chloroplast EF-G is a housekeeping gene, there must be an EF-G protein spot elsewhere in gel of control group. It is postulated that post-translational modification or alternative splicing may occur on EF-G in *T. halophila* under cold stress to maintain the normal operation of protein synthesis. Although EF-G was previously reported to play a role in chloroplast biogenesis and development in *Arabidopsis* (Albrecht et al. 2006), this is the first report showing that chloroplast EF-G is involved in cold response in plant.

Misfolded proteins may accumulate in cell under cold conditions and plants may employ two strategies to cope up with this situation one to refold them and the other to remove them. A chloroplast heat-shock protein 70, hsc70-7, was observed to increase in this study possibly to maintain the normal structure of proteins in chloroplast under cold stress. A probable protein disulfide-isomerase 1 precursor, which plays important roles in the folding of nascent polypeptides and in the proper formation of disulfide bonds in protein folding (Houston et al. 2005) was observed to be upregulated after 5 and 24 days of cold treatment. 26 S proteasome is presumed to play an essential role in the removal of short-lived regulatory proteins and abnormal polypeptides in plants during stress, and PAB1 was reported to be involved in ubiquitin-dependent protein catabolic process (Yang et al. 2004). In our study, a 20 S proteasome alpha subunit B1 (PAB1) was found to increase after 5 and 24 days of cold treatment. This may indicate that a high protein turnover rate is needed for plants to eliminate the misfolded polypeptides under cold environment.

Comparison of cold-induced rosette leaf proteome changes in *Thellungiella* and other plants

The cold stress-induced changes in multiple cellular physiological processes revealed by this study were also reported in previous proteomics researches in *Arabidopsis* (Amme et al. 2006; Goulas et al. 2006) and rice (Cui et al. 2005; Yan et al. 2006; Hashimoto and Komatsu 2007). This may suggest that the cold stress responses in *Thellungiella* are similar, in general, to other plant species.

Because *Thellungiella* is a cold-tolerant relative of *Arabidopsis*, comparison of the cold-induced rosette leaf proteome changes in *Thellungiella* and *Arabidopsis* was performed to advance the understanding of the higher cold tolerance of *Thellungiella*. It was found that some cold-responsive proteins identified in this study were also reported in previous *Arabidopsis* proteomic studies, including putative 2,3-bisphosphoglycerate-independent phosphoglycerate mutase (Amme et al. 2006), heat-shock protein 70, germin-like protein (Bae et al. 2003), ferritin-1 (Kawamura and Uemura 2003), RuBisCO large and small subunits, chloroplast carbonic anhydrase precursor, glyceraldehyde-3-phosphate dehydrogenase B, chloroplast 2-cys peroxiredoxin and putative RNA-binding protein cp29 (Goulas et al. 2006). A few cold-responsive proteins in *Thellungiella* showed similar expression patterns under cold stress in *Arabidopsis*, especially the multiple spots of RuBisCO small subunit, which exhibited diversified expression patterns in both *Thellungiella* and *Arabidopsis*. However, some cold-responsive proteins displayed different expression patterns under cold stress in *Arabidopsis* and *Thellungiella*. For example, glyceraldehyde-3-phosphate dehydrogenase B, chloroplast 2-cys peroxiredoxin and chloroplast carbonic anhydrase precursor were downregulated under cold treatment for 1, 10 and 40 days in *Arabidopsis*, while they were upregulated under cold treatment for

2, 5 and 24 days in *Thellungiella*. The results indicted that the difference in protein expression pattern under cold stress may be part of cold tolerance mechanisms in *Thellungiella*. A similar hypothesis was proposed by Zhu (2000) and supported by a subsequent study (Kant et al. 2006).

The identification of cold-responsive proteins in *Thellungiella* provides not only new insights into cold stress responses but also a good starting point for further investigation of their functions. However, considering the inability of 2-DE-based proteomic study to resolve membrane proteins and low abundant proteins, integrated transcriptomics, proteomics, and metabolomics approaches should be adopted to gain further insight into the complicated but fine-tuned network of plant response to cold stress.

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