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Proteomic analysis of the heat stress response in leaves of two contrasting chrysanthemum varieties

Yang Zhang[1,2,3], Ming Sun[1,2,3,*], Qixiang Zhang[1,2,3]

[1]College of Landscape Architecture, Beijing Forestry University, Beijing, 100083, China
[2]Beijing Key Laboratory of Ornamental Plants Germplasm Innovation and Molecular Breeding, Beijing, 100083, China
[3]National Engineering Research Center for Floriculture, Beijing, 100083, China

[*]Corresponding author: sun.sm@163.com

Abstract

High temperature stress causes damages to chrysanthemum by adversely affecting its growth and development. To identify differentially expressed proteins under heat stress, heat-tolerant variety (*Chrysanthemum nankingense*) and heat-susceptible variety (*Chrysanthemum* 'Wanfen') were selected to contrast the full leaf proteome profiles with or without 6 h of heat stress (40°C), a parameter established in our previous study. Isobaric tags for relative and absolute quantitation (iTRAQ) was used in combination with tandem mass spectrometry and multidimensional liquid chromatography technology. Results of physiological tests showed that the variation of relative ion leakage and malondialdehyde were larger in *Chrysanthemum* 'Wanfen' than those in *Chrysanthemum nankingense*. In proteomics experiments, 250 proteins were identified, of which 43 were differentially expressed. Functional classification indicated that most of proteins were involved in photosynthesis, metabolic processes, oxidation-reduction processes and transport. Furthermore, 12 differentially expressed proteins were analyzed at the mRNA level under different durations (0 h, 3 h and 6 h) of heat treatment by qRT-PCR. The results showed that under heat stress the expression patterns of the rest 11 genes coding the altered proteins were correspondent to their respective protein levels with the exception of actin.

Keywords: Chrysanthemum; iTRAQ; Proteomics; RT-PCR; High temperature.
Abbreviations: ACN_ acetonitrile; HSP_ Heat shock protein; iTRAQ_ isobaric tags for relative and absolute quantitation; MDA_ malondialdehyde; Rubisco_ Ribulose-1,5-bisphosphate carboxylase/oxygenase.

Introduction

Heat stress is usually defined as an increase in temperature beyond a threshold level that is sufficient to damage the growth and development of plants. Generally, 10 to 15°C above normal temperatures is considered as heat stress or heat shock. Currently, the global temperature is continuing to increase due to human activities and the increasing concentration of greenhouse gases (Neilson et al., 2010). Heat stress due to high ambient temperatures is a serious threat to plants, which can lead to protein denaturation and aggregation and the inactivation of enzymes, among other problems (Wahid et al., 2007). Chrysanthemums are the most common cut flower, garden flower and potted flower in the world. The optimum temperature range for its growth and development is between 15 and 25°C. Above 25°C would adversely affect its growth, especially in the summer. It has been reported that high temperatures may cause inhibition of several physiological and biochemical activities, such as decreasing the activity of enzymes, protein synthesis, photosynthesis, and cell membrane stability and lead to reproductive barriers, flowering delay, spindling morphological growth (Nan and Sun 1994; Jia et al. 2009). Therefore, it is essential to understand the mechanism of chrysanthemum adapting to heat stress and explore appropriate for improving chrysanthemum's heat-tolerance. Plants have sophisticated and elaborate mechanisms to adapt to the environmental stresses (Ahuja et al., 2010), which provide opportunities to improve their tolerance to heat stress. Attempts to improve the chrysanthemum's heat tolerance by conventional breeding protocols such as grafting (Fang et al., 2009) and short time heat acclimation (Li et al., 2007) have obtained limited success. Subsequently, molecular breeding and genetic engineering techniques have been employed as new ways combat the problem (Hong et al., 2009; Katiyar-Agarwal et al., 2003; Sanmiya et al., 2004). The identification of specific genes expressed in response to stress and their functions in stress adaptation provides a solid foundation for generating effective strategies to improve stress tolerance. However, genomic information is not adequate to establish the actual structure and regulation networks of the proteins under heat stress. Consequently, proteomics has emerged as a powerful tool for the quantitative analysis of proteomes influenced by different physiological environments including high temperature and other stresses (Shakeel et al., 2013; Wei et al., 2009; Fan et al., 2011; Marsh et al., 2010). In addition, proteomic studies also provides the complete proteome at the organelle, cellular, tissue and organ levels (Holzmeister et al., 2011; Drakakaki et al., 2012; Bandehagh et al., 2011; Lundby et al., 2012) and serves as a bridge between transcriptomics and metabolomics (Cook et al., 2004). Comparative quantitative proteomic analysis becomes increasingly popular because it is high-throughput, highly reproducible with high sensitivity (Chong et al., 2006). Isobaric tags for relative and absolute quantitation (iTRAQ) technology is one of the most popular techniques and allows up to eight samples to be simultaneously quantified. It overcomes the limitations of a two-dimensional

electrophoresis, which measures reporter ion peak intensities with a direct connection of tandem mass spectrometry (MS/MS) spectra (Chen et al., 2002). Within the past few years, this technology has developed quickly, and enabled an insight into the biochemical proteome under different conditions (Jone et al., 2006; Fukao et al., 2011; Lan et al., 2011; Lee et al., 2007). Using this technology several post-harvest-related proteins were identified associated with cassava root post-harvest physiological deterioration (Owiti et al., 2011) and 223 plasma membrane-associated proteins were also discovered from lily pollen grain and tube (Han et al., 2010). However, few proteomic studies regarding heat stress have been reported (Chu et al., 2012). To better understand how chrysanthemums adapt to heat stress, we used iTRAQ technology and qRT-PCR. The heat-tolerant variety *Chrysanthemum nankingense* and heat susceptible one *Chrysanthemum* 'Wanfen' are selected based on our previous study on the evaluation of 12 varieties for heat tolerance (Wang et al., 2012). By comparing the expression of heat-tolerant and heat-susceptible chrysanthemum leaves, our objectives are to identify heat stress related proteins, to illuminate their expression variation and to understand the relationship at transcription and translation level, which would facilitate to reveal the mechanism of heat tolerance and confirming the effective strategies to breed heat-tolerant varieties.

Results and Discussion

Thermostability of cell membranes after heat stress

Increased electrolyte leakage has long been used as an indicator of membrane damage to measure the heat tolerance in various plants (Blum et al., 2001; Lee et al., 2007; Shakeel et al., 2013). In this study, the relative electrolyte leakage of *C.* 'Wanfen' tissue was higher than that of *C. nankingense* after 6 h of heat stress (Fig. 1). Malondialdehyde is one of the products of membrane lipid peroxidation caused by stress, which can reflect the damage of the membrane. An increase in malondialdehyde concentration is recorded here, which is consistent with the measured electrolyte leakage amount. Taken together, both results have shown that heat-susceptible chrysanthemum was more susceptible to high temperature than heat-tolerant chrysanthemum.

ITRAQ analysis of chrysanthemum leaf proteins under heat stress

In this study, a total of 250 unique proteins were identified (unused ProtScore> 1.2) with iTRAQ, which was performed twice on leaves of control and heat-treated plants. A total of 43 proteins were differentially expressed in various comparisons (*C.* 'Wanfen' 6 h / *C.* 'Wanfen' control; *C. nankingense* 6h / *C. nankingense* control; *C. nankingense* 6 h / *C.* 'Wanfen' 6 h; *C. nankingense* control / *C.* 'Wanfen' control). 20 and 6 proteins were significantly changed in the comparison of *C.* 'Wanfen' 6 h / *C.* 'Wanfen' control and *C. nankingense* 6h / *C. nankingense* control respectively, and only two heat shock proteins were observed to increase in the two comparisons. When *C. nankingense* 6 h was compared with *C.* 'Wanfen' 6 h, 25 proteins were found to be differentially expressed (Table 1) and functionally categorized according to biological process by WEGO. More than 7 functional categories were selected to evaluate the data sets. They were mainly involved in photosynthesis, metabolic processes, oxidation-reduction processes, transport and translation. The biological relevance of these differentially expressed proteins is discussed below.

Proteins involved in photosynthesis

Photosynthesis is particularly sensitive to high temperature stress. It can be completely inhibited before other stress symptoms occur. In this study, 5 differentially expressed proteins have been identified related to photosynthesis. The expression levels of all of the 5 proteins from *C. nankingense* were higher than those of *C.* 'Wanfen' after a 6 h heat stress. Among them, the ribulose-1,5-bisphosphate carboxylase/ oxygenase large subunit, chloroplast ribulose 1,5-bisphosphate carboxylase/oxygenaseactivase and photosystem I P700 chlorophyll A apoprotein A1 were significantly down-regulated after 6 h of heat stress compared to the control in *C.* 'Wanfen', whereas these protein levels were not significantly changed in *C. nankingense*. This suggests that these proteins are likely involved in protecting the plant from high temperature damages. Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) is one of the most abundant soluble proteins in plants and is a key enzyme controlling net photosynthesis. It consists of several large and small subunits and is regulated by rubiscoactivase. Rubiscoactivase is responsible for removing inhibitory molecules from the large-subunit active site (Spreitzer, 1999), and elevated temperatures can inhibit the light activation of Rubisco and affect photosynthesis (Feller et al., 1998). A recent study aiming to identify the mechanism of heat tolerance and the role of stromal proteomes in the heat-tolerant plant *Agave americana* also found that Rubisco was down-regulated, which agrees with our result (Shakeel et al., 2013). The expression level of rubiscoactivase in heat-tolerant chrysanthemum was also much higher than in heat-susceptible chrysanthemum after 6 h of heat stress in this study, which suggests that rubiscoactivase might be a biomarker for the evaluation of chrysanthemum heat-tolerance. The cytochrome b_6/f complex transfers electrons from PSII to PSI via quinone and plastocyanin. Rowland et al. (2010) observed cytochrome b_6/f complex was up-regulated, most likely to alleviate the damages from heat stress. Similar result has also been found in this study. In another study, Bukhov et al. (1999) noted that PSI stromal enzymes and chloroplast envelopes were thermostable, and PSI drives the cyclic electron pathway, which contributes to the thylakoid proton gradient and is activated at high temperature. In contrast, PSII was highly heat-susceptible, and its activity was dramatically reduced at high temperatures. This may explain why the fold change for the PSI P700 chlorophyll A apoprotein A1 is larger than that for the PSII 44 kDa protein as shown in Table 1.

Proteins involved in metabolic processes

Four metabolic-related proteins are shown in Table 1. Fructose-1, 6-bisphosphatase is involved in carbohydrate metabolism and is related to the synthesis of sucrose. In order to clarify the contribution of FBPase, the amount of final dry matter and the photosynthetic activity were compared between transgenic and wild-type tobacco and it was found that a >1.7-fold increase in FBPase expression had a positive effect on photosynthesis (Tamoi et al., 2006). The study supports our results here that the quantity of FBPase in heat tolerant plants is higher than that insusceptible ones, while increasing carbon fixation activity is beneficial to the photosynthesis and energy accumulation. Beta-1, 3-glucanase PR2 (Kim et al., 2010) is also involved in carbohydrate metabolism and catalyze the hydrolysis of beta-1,3-glucan. Its activity increased in response to pathogen attack, cold stress and hormonal treatments (Ward et al., 1991; Hincha et al., 1997). However, the level of beta-1,3-glucanase found in this study has been decreased at

Table 1 Differentially expressed proteins of *Chrysanthemum nankingense* 6 h compared to *Chrysanthemum* 'Wanfen' 6 h (p<0.05).

Accession	Protein name	Species	Fold Change	Function
gi|470227716	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	*Artemisia frigida*	9.40	Photosynthesis
gi|441403305	component of cytochrome b_6/f complex (chloroplast)	*Chrysanthemum ×morifolium*	2.75	Photosynthesis
gi|470227708	photosystem I P700 chlorophyll A apoprotein A1 (chloroplast)	*Artemisia frigida*	2.54	Photosynthesis
gi|94502487	photosystem II 44 kDa protein	*Helianthus annuus*	1.67	Photosynthesis
gi|158726716	chloroplast ribulose 1,5-bisphosphate carboxylase/oxygenaseactivase	*Flaveria bidentis*	1.64	Photosynthesis
gi|149207273	fructose-1,6-bisphosphatase	*Flaveria linearis*	3.67	Metabolic process
gi|608712	P-protein of the glycine cleavage system	*Flaveria pringlei*	2.87	Metabolic process
gi|437995	glycine hydroxymethyltransferase	*Flaveria pringlei*	2.49	Metabolic process
gi|296142021	beta-1,3-glucanase PR2	*Chrysanthemum ×morifolium*	0.09	Metabolic process
gi|26324158	elongation factor 1 alpha	*Stevia rebaudiana*	3.22	Transport
gi|51512147	putative dicarboxylate/tricarboxylate carrier	*Helianthus tuberosus*	3.12	Transport
gi|38564733	initiation factor eIF4A-15	*Helianthus annuus*	2.55	Transport
gi|470227702	ATP synthase CF1 alpha chain (chloroplast)	*Artemisia frigida*	4.43	ATP synthesis; transport
gi|452849058	ATP synthase CF1 beta subunit (chloroplast)	*Chrysanthemum indicum*	2.03	ATP synthesis; transport
gi|758362	F1 ATPase	*Helianthus annuus*	1.71	ATP synthesis; transport
gi|269308672	broad substrate reductase/dehydrogenase	*Artemisia annua*	2.73	Oxidation-reduction
gi|189418957	glycolate oxidase	*Mikania micrantha*	2.18	Oxidation-reduction
gi|372863482	ribosomal protein L14 (chloroplast)	*Chrysanthemum indicum*	9.45	Translation
gi|317373797	chloroplast phosphoglycerate kinase 3	*Helianthus annuus*	3.34	Glycolysis
gi|50058096	glutathione reductase	*Zinnia violacea*	0.62	Redox homeostasis
gi|51315699	RecName: Full=Histone H4	*FLATR*	0.33	Nucleosome assembly
gi|499112	nucleoside diphosphate kinase	*Flaveria bidentis*	0.22	GTP biosynthesis
gi|9082317	actin	*Helianthus annuus*	1.64	None
gi|2606077	auxin-induced protein	*Helianthus annuus*	0.34	None
gi|332806715	cyclophilin 2	*Tagetes patula*	0.27	Protein folding

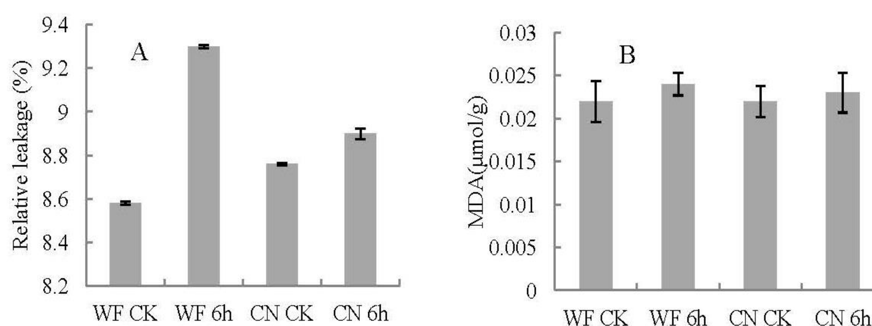

Fig 1. Physiological response of chrysanthemum leaf under heat stress. (A) Relative electrolyte leakage; (B) MDA content.

high temperatures, suggesting that high temperature might inhibit the activity of enzymes.

Proteins involved in transport

We found 6 transport-related proteins with expression levels that were higher in the heat-tolerant chrysanthemum regardless of the heat shock treatment. ATP synthase, a multisubunit complex that spans the inner membrane, is the key enzyme in the ATP synthesis process, and the catalytic sites are primarily found on the beta subunits. ATP is produced from ADP in the presence of a proton gradient across the membrane, and a decrease in the amount of ATP produced might affect energy-dependent processes required for heat stress resistance (Lee et al., 2007; Majoul et al., 2004; Ferreira et al., 2006). Three ATP synthase-related proteins identified in this study were down-regulated after heat stress in the heat-susceptible chrysanthemum, which was consistent with the above studies. However, the higher expression levels in the heat-tolerant chrysanthemum suggest that ATP synthase might be beneficial

Table 2. Primer name and sequence.

Name	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
Rubisco	CACAAACAGAGACTAAAGCAAG	CCATGTACCAGTAGAAGATTCG
Component of cytochrome b_6/f complex	TCACCTTTCCTATTCTTTCCC	TATTGTTACTCTTGCTCCCATC
Photosystem I P700 chlorophyll A apoprotein A1	CTAGCTTGGTTTCAAGATGTAG	CTACTCCAGCGTTTAGAAATTG
Photosystem II 44 kDa	AGCGGCTATATCTGTCTTTG	GATCCTACGTTAGCTCCAAG
Fructose-1,6-bisphosphatase	CTGCATGTATGGTAGCTCTT	CCCAGTTCTTAGCATTTCCT
Initiation factor eIF4A-15	CTACGATATCTTCCAGTTGTTG	GTTTGATACCTTCCAAAGTGAG
ATP synthase CF1 alpha chain	CTGATCTCGATAAAGCTACTCA	CTGTCCAACTTCTAATGAATCG
ATP synthase CF1 beta chain	CTCTAGTTTACGGTCAGATGAA	GTCTTGTTCATTAACATCTCGG
Broad substrate reductase /dehydrogenase	GATATCTTGGTGAATAACGCAG	ATGATCTCAGTTAAGAGGTGTG
Phosphoglycerate kinase 3	TTTGCTGCTATTGTTGGTGG	ATCCGACTGATAAGCCCTGA
ACTIN	ACAACTGCTGAACGGGAAAT	TCATAGACGGCTGGAAAAGG
Heat shock protein 70	GAAGTAGGCTGGGACTGTAAC	TAACTACAAGGGTGAGGAGAA
PP2Acs	ATCAGAACAGGAGGTCAGGG	TAATTTGTATCGGGGCACTT

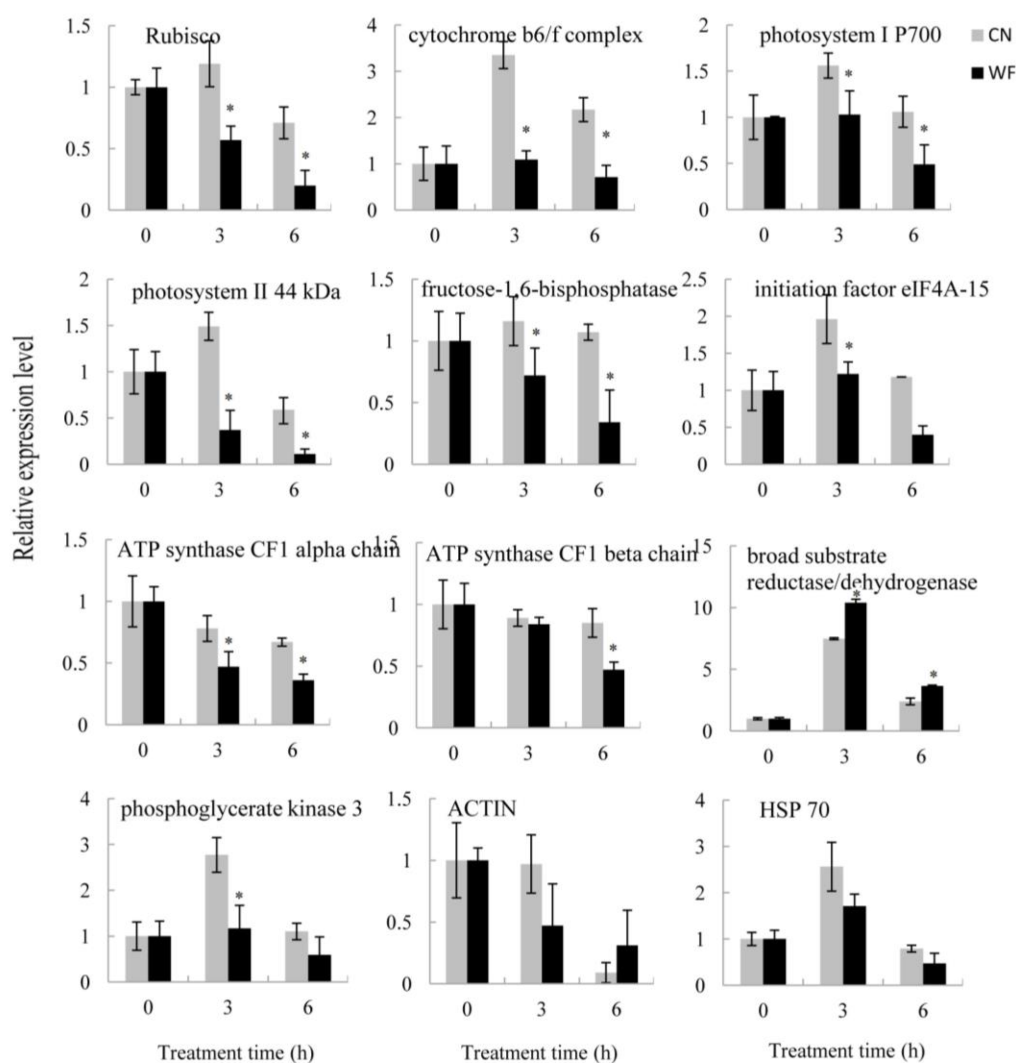

Fig 2. Results of real-time PCR expression analysis of genes for heat responsive proteins in heat-tolerant and heat-susceptible leaves. Significant differences in gene expression are marked with * ($p<0.05$).

in the response to heat stress. The translation process consists of three phases: initiation, elongation and termination. Initiation factor eIF4A-15, as one of the initiation factors, is involved in protein synthesis, and elongation factor 1A takes charge of catalyzing the first step of the elongation cycle (Lamberti et al., 2004). Both proteins were expressed at a higher level in the heat-tolerant chrysanthemum, which revealed that the heat-tolerant chrysanthemum was more actively translating protein. It is possible that the high temperature caused more damage to the heat-susceptiblechrysanthemum's enzymes and inhibited their activity. The dicarboxylate/tricarboxylate carrier, which is responsible for the transport of metabolites between the mitochondria and cytosol, showed a similar expression change as elongation factor 1 alpha protein. The abundant metabolites synthetized by the enzymatic activity of heat-tolerant plants might partially explain their increase heat tolerance.

Proteins involved in oxidation-reduction reactions and other processes

Proteins involved in oxidation-reduction reactions were also identified in this study. Glutathione reductase, Histone H4, nucleoside diphosphate kinase (NDPK), auxin-induced protein and cyclophilin 2 had decreased expression levels in *C. nankingense* compared to *C.* 'Wanfen' regardless of heat shock treatment. This may be associated with specific characteristics of the variety. Although it was shown that over expression of NDPK2 could improve the tolerance of transgenic potatoes to multiple environmental stresses (Tang et al., 2008), the results of this study were different. This finding was most likely caused by the complicated regulation mechanism of NDPKs or desynchronized between the gene and protein level. Histones play a central role in transcriptional regulation, DNA repair, DNA replication and chromosomal stability. Histone H4 is the most conserved histone and was down-regulated after cold stress in rice (Karlie et al., 2011), which was consistent with the results of this study. This might be attributed to the fact that histones are easily and reversibly modified post-translationally, which might protect the plant from the environmental stress (Karlie et al., 2011; Kreps et al., 2002). Except for the above 5 proteins, the expression levels of the other differentially expressed proteins were higher in the heat-tolerant chrysanthemum than on the heat-susceptible chrysanthemum after 6 hours of heat stress. From the expression changes in the expression of glycolate oxidase (a key enzyme in the process of photorespiration), ribosomal proteins (proteins that can bind rRNA and comprise the ribosomal subunits involved in the cellular process of translation) and phosphoglycerate kinase 3 (a key enzyme in glycolysis and gluconeogenesis), we may find some clues to improve chrysanthemum's heat tolerance by enhancing the enzymatic activity of these pathways.

Heat shock protein 70 (HSP70) and heat shock protein 70.58 (HSP70.58), as the only two up-regulated and differentially expressed proteins in both varieties after heat stress in this study, belong to the HSP 70 family. HSP70, an extensively studied protein, is considered to be involved in protein translation and translocation, protein folding, protein chaperoning, proteolysis, suppressing aggregation and reactivating denatured proteins (Wahid et al., 2007; Zhang et al., 2005). It has been verified in many studies that HSP70 is beneficial to improve plant tolerance to high temperature (Zhao et al., 2012; Kim and An, 2013).

Real-time PCR analysis

To realize the expression relationship between transcriptional and translational levels, we further studied the expression patterns of the genes for the twelve differentially expressed proteins by qRT-PCR. As shown in Fig.2, six genes had a similar expression pattern, with a tendency to increase after 3 h and then decrease at 6 h. This may reflect that a short-duration heat stress can stimulate the activity of enzymes, whereas longer periods of stress caused damage to the physiology of the plant. Besides, the majority of the 12 genes had a significant higher expression level in heat-tolerant chrysanthemum. Compared to protein expression after 6 h of heat stress, not all of the genes exhibited parallel expression patterns between protein and mRNA levels. This phenomenon has been reported in other studies (Wei et al., 2009; Lee et al., 2007). The discrepancies could be related to translational regulation, post-translational processing, protein degradation or a combination of these. In general, these results provided an indication that these genes were important to the response to high temperature and would be potential targets to improve the heat tolerance of plants by increasing their expression levels.

Materials and Methods

Plants materials and growth conditions

The heat-susceptible (*C.* 'Wanfen') and heat-tolerant (*C. nankingense*) plants were propagated by placing healthy stem cuttings into a suitable substrate with a turf: perlite (1:1). The plants were grown to a height of 10 to15 cm in a greenhouse for approximately 3 months. Healthy and pest-free plants (both susceptible and resistant to heat) were kept in a growth cabinet for 24 hours with the temperature maintained at 20°C/18°C (day/night) with a 12 h photoperiod. Then, one group was moved to a growth cabinet (40°C) for 6 h for heat shock treatment. Meanwhile, a control experiment was conducted in a 20°C growth cabinet for 6 h. The relative humidity in the growth cabinet was maintained at 75%. All of the collected materials were frozen immediately in liquid nitrogen.

Relative ion leakage and lipid peroxidation measurements

A relative electrolyte analysis was conducted according to Lee et al. (2007) with some modifications. Fresh leaf square pieces (0.2 gram,1 cm length) were soaked in 10 mL centrifugal tubes containing 10 mL Milli-Q water, then pumped in a vacuum drier for 30 min and shaken every 10 min. The samples were incubated at 25°C for 20 min, and the electrical conductivity of the bathing solution (L_t) was measured with a conductivity meter. Then, the tubes were placed into boiling water (100°C) for 15 min. The sample was allowed to cool to 25°C before the electrical conductivity (L_0) was measured again. The relative ion leakage was calculated according to the formula $L_t/L_0 \times 100\%$. Lipid peroxidation was measured as the amount of malondialdehyde (MDA) determined by thiobarbituric acid reactive substance (TBARS) as described by Panda (2003).

Protein extraction

Total protein was extracted from the leaves of 4 different treatments ('Wanfen' control, 'Wanfen' heat shock for 6 h, *C. nankingense* control, *C. nankingense* heat shock for 6 h). Leaves (1 g) were ground to a fine powder in a mortar and pestle in liquid nitrogen. At a proportion of 1:5 (powder: lysis buffer), lysis buffer (2M thiourea, 7M urea, 4%CHAPS) was mixed with the powder thoroughly, supersonicated for 60 s, and the mixture was centrifuged for 1 h at 40,000×g. The supernatant was transferred to a new tube and precipitated overnight in four volumes of pre-cooled acetone (-20°C)

containing 10% trichloroacetic acid at -20°C. The protein was collected by centrifugation for 10 min at 40,000×g. The supernatant was discarded, and the pellet was washed with 100% cold acetone twice or until the extract was no longer green. The protein pellet was dried by lyophilization and stored at -80°C or was immediately resuspended in lysis buffer. The protein concentration was determined by the Bradford method (Sigma-Aldrich).

Trypsin digestion

Protein reduction, denaturation and blocking of cysteine residues were performed according to the manufacturer's instructions (AB Sciex). Briefly, 200 μg were treated with 4 μL of the reducing reagent at 60°C for 1 h and alkylated using 2 μL of cysteine blocking reagent at room temperature for 10 min. Next, the protein solution was transferred into a 10K Millipore, centrifuged for 20 min at 12,000 rpm, and the liquid was discarded. After the addition of 100 μL dissolution buffer, the sample was centrifuged for 20 min at 12,000 rpm to recover the pellet, and this procedure was repeated 3 times. Finally, the proteins were digested overnight at 37°C with 4 μg trypsin in a total volume of 50 μL, centrifuged again for 20 min at 12,000 rpm in a new tube to collect the peptide solution.

ITRAQ labeling

Labeling of the samples (100 μg) with the 8-plex iTRAQ reagents (AB Sciex) was performed according to the manufacturer's recommendations. One unit of iTRAQ reagent label (defined as the amount of reagent required to label 100 μg of proteins) was thawed and reconstituted. Each diluted label (113, 114, 115 and 116) were added to 50 μL of the respective samples (*C.* 'Wanfen' 6 h, 0 h and *C.nankingense* 0 h, 6 h) and incubated at room temperature for 2 h. The reaction was quenched by adding 100 μL ultrapure water to each sample. Before mixing the total samples, 1 μL of each sample was mixed to confirm successful labeling by MALDI-TOF-MS. Then, the total labeled samples were mixed in equal ratios, dried in a vacuum centrifuge and stored at -20°C.

Fractionation by off-line reverse phase HPLC

The dried iTRAQ-labeled peptides were redissolved in isometric solvent A (98% ddH$_2$O, 2% ACN) and centrifuged for 20 min at 14,000×g. The supernatant (50 μL) was injected into a RIGOL L-3000 HPLC system equipped with a BioBasic SCX (4.6 mm×250 mm, 5 μm 100 Å, Thermo) flowing at 0.7 mL/min. The gradient was set as follows: from 5% to 8% solvent B (98% ACN, 2% ddH$_2$O) in 5 min, 8% to 18% B in 30 min, 18% to 32% B in 27 min, 32% to 95% in 2 min, held in 95% B for 4 min, and back to 5% B in 4 min. Fractions were collected every 1 min, and a total of 48 fractions were collected.

Nano-MS/MS analysis

The nano-MS/MS analysis was conducted with a Triplequadrupole TOF 5600 system (AB SCIEX, Concord, ON). When all the fractions were merged together, 30 μL solvent A (2% ACN, 0.1% formic acid) was added to the samples. The samples were vortexed and centrifuged for 10 min at 12,000 rpm. The supernatant (8 μL) was injected and eluted with a loading pump rate of 2 μL/min for 15 min, then separated with a 101 min mobile phase gradient (5% to 10% B in 0.1 min, 10% to 25% B in 60 min, 25% to 48% in 25 min, 48% to 80% B in 1 min, and held in 80% B for 4 min, then back to 5% B in 1 min, held at 5% for 10 min) at a flow rate of 0.3 μL/min. Solvent B was 98% ACN, 0.1% formic acid. Data were acquired using an ion spray voltage of 2.5 kV, curtain gas of 30 PSI, nebulizer gas of 6 PSI, and an interface heater temperature of 150°C. The MS was operated with an RP of 30,000FWHM for TOF-MS scans. For IDA, survey scans were acquired in 250 ms, and up to 20 product ion scans were collected if it exceeded a threshold of 125 counts per second (counts/s) with a +2 to +5 charge state. A rolling collision energy setting was applied to all precursor ions for collision-induced dissociation. Dynamic exclusion was set for ½ of peak width (8 s), and then the precursor is refreshed from the exclusion list.

Data analysis

Protein Pilot 4.0 (AB SCIEX, Foster City, CA), including the Paragon and Pro Group TM algorithms, was used to simultaneously identify and quantify proteins in the iTRAQ experiments. Database searches were performed against the NCBI Asteraceae protein database with the following parameters: iTRAQ 8 plex peptide labeled; trypsin digestion with only 1 maximum miss cleavage; carboxymate for cysteine residue; instrument, oxidation for methionine; qTOF ESI; identification focus, biological modifications. The tolerances were specified as ±0.05 Da for peptides and ±0.05 Da for MS/MS fragments. The FDR was controlled at 1% using the integrated tools in ProteinPilot. An unused score>1.2 was required for all of the identified proteins. A differentially expressed protein had to match the following standards, which were selected in the previous work: being quantified in at least three spectra so that a p-value could be generated; having a p-value of <0.05 and an error factor of <2 (Snelling et al., 2007; Luo et al., 2009); having a 50% fold-change, i.e., an expression ratio of >1.50 or <0.67. The significantly differentially expressed proteins were assigned to a hierarchical biological process using the Web Gene Ontology Annotation Plotting tool (WEGO, http://www.geneontology.org).

Real-time PCR analysis

Total RNA of heat-tolerant and heat-susceptible leaves was extracted from plants treated with 0 h, 3 h or 6 h of heat shock. The primers used for real-time PCR were in Table 2. RT-PCR was performed in a 10 μL volume containing 5 μL SYBR Premix Ex Taq (TaKaRa), 1 μL cDNA template, 0.2 μL each primer and 3.6 μL ddH$_2$O. The cycling conditions were comprised of an initial denaturation of 95°C/60 s, followed by 40 cycles of 95°C/15 s, 55°C/15 s and 72°C/45 s (Gu et al. 2011). After the amplification steps, the melting curve was estimated for each primer pair to verify whether only one specific product had been amplified. Three replications were performed, and the results were averaged. PP2Acs was used as a reference gene according to a pre-experiment. The 2C$_t$ method was applied to calculate the relative quantification (Livak and Schmittgen, 2001).

Conclusions

Proteomics approach offers a more direct and effective way to elucidate a plant's response to stress. To our knowledge, this is the first report on analyzing differentially expressed proteins and their transcripts in chrysanthemum during heat stress. The proteomic data shown here suggest that the heat stress response was accompanied by multiplex processes such as photosynthesis, metabolic processes, and transport. The more highly expressed proteins in heat-tolerant chrysanthemum, such

as cytochrome b_6/f complex, fructose-1,6-bisphosphatase, initiation factor eIF4A-15, glycolate oxidase, ribosomal protein L14 and chloroplast phosphoglycerate kinase 3 will be good candidates for further study to improve resistance to high temperature. HSP70 was the only protein with increased levels in both chrysanthemum varieties and was also noticed to play a pivotal role in protecting chrysanthemum from heat stress damages. Because the chrysanthemum is not a model species and genomics information has not been well developed, there are still limitations to thoroughly unveil the mechanism of the response to heat stress. To better understand heat stress, genomic and proteomic data should be assembled together to identify more genes in chrysanthemum and to provide a solid foundation for the development of heat tolerant varieties. Here we just have studied the proteomic changes in short-duration heat stress of two chrysanthemum varieties. Further investigations are to carried out for the changes under longer term heat stress with a diverse collection of chrysanthemum varieties.

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