Comparative proteomics analysis of tea leaves exposed to subzero temperature: Molecular mechanism of freeze injury

Hu Yongguang[1], Lu Yongzong[1], Lu Jian[2*]

(1. *Institute of Agricultural Engineering, Jiangsu University, Zhenjiang 212013, Jiangsu, China*;
2. *Institute of Life Sciences, Jiangsu University, Zhenjiang 212013, Jiangsu, China*)

Abstract: Tea freeze injury is one of the most severe agro-meteorological disasters, which leads to sizable losses of tea production in China. The freezing resistant ability of overwintering tea trees becomes weaker and weaker from early-spring to late-spring. If it decreases to critical temperature of -2℃ or lower in the stage with one or two leaves, tea trees suffer from freeze injury and the yield or quality of spring tea production could decrease greatly. Although measurements have been taken to prevent such damage, the physiological and biochemical mechanism of how tea (*Camellia Sinensis*) plant response to freeze injury is still to be elucidated. A comparative proteomics analysis was made on tea leaves at the two-leaf stage. The differential image analysis showed 46 spots with density changes (29 spots increased and 17 spots decreased; $p<0.01$) in the freeze injury group compared with the control group. Thirty eight differential protein spots ($p<0.01$) with good resolution and relatively high abundance in MS were subjected to further protein identification. Among them, all 17 up-regulated spots were collected whereas only six of the down-regulated spots were selected. These differentially expressed proteins including heat shock protein 70, oxygen-evolving enhancer protein, adenosine triphosphate synthase, S-adenosylmethionine synthetase and some enzymes involved in carbohydrate metabolism, were shown responsive to freeze injury. The results would greatly increase the comprehension of the molecular mechanism for freeze injury and provide a better decision making for freeze protection and control.

Keywords: freeze injury, molecular mechanism, freeze protection, comparative proteomics, *Camellia Sinensis*, mass spectrometry

DOI: 10.3965/j.ijabe.20130604.004

Citation: Hu Y G, Lu Y Z, Lu J. Comparative proteomics analysis of tea leaves exposed to subzero temperature: Molecular mechanism of freeze injury. Int J Agric & Biol Eng, 2013; 6(4): 27－34.

1 Introduction

Freeze injury of crops is a kind of agricultural meteorological damage, which mainly induces physiological barriers, inhibition of growth and even death. Low temperature is a key factor of environmental stresses[1]. Following the changes under global climate environmental conditions in recent years, freeze injury has become more and more serious, and many plants, including apple and tea trees, are suffering from it with subsequent great financial losses[2]. For example, freeze injury of crops now has led to the highest financial losses among all kinds of natural damages in USA[3]. Tea production is a very important industry in China, and the tea tree is a subtropical plant, weakly resistant to low-temperature stress. The freezing resistant ability of overwintering tea trees becomes weaker and weaker from early-spring to late-spring. Commonly two weeks before their sprouting, the critical temperature is -5℃, but during their sprouting it decreases to -3℃, and during the one- or two-leaf stage, it decreases to -2℃[4,5].

Received date: 2013-09-02 **Accepted date:** 2013-10-25

Biographies: Hu Yongguang, PhD, Associate Professor, majoring in agro-biological environmental engineering, monitoring and control of frost protection, Email: deerhu@163.com. **Lu Yongzong**, Master student, majoring in monitoring and control of agricultural and biological environment, Email: luyongzong@126.com.

***Corresponding author: Lu Jian**, PhD, Associate Professor, majoring in bioinformatics and comparative proteomics, Tel: +86-511-88797338, Email: lujian@ujs.edu.cn.

Once the tea trees suffer from frost injury, the yield or quality of spring tea production would decrease greatly. In early and middle spring, the weather becomes warmer and warmer and the air temperature is usually above 0 ℃, while sometimes it may decrease sharply to -2 ℃ with the late spring cold. The temporary and sharp drop in temperature induces cold damage and a loss in tea production.

For a long time, the mechanism of how tea trees suffer from freeze injury is a hot and difficult problem. Levitt proposed the sulfhydryl group hypothesis: freeze injury is due to an unfolding and therefore a denaturation of the protoplasmic proteins[6,7]. It results from the formation of intermolecular SS bonds induced by the close approach of the protein molecules due to icing dehydration. Cold resistance is a resistance towards SH oxidation, SH-SS interchange, and subsequently to the formation of these intermolecular SS bonds. Another mechanism of freeze injury is membrane damage theory[8,9]. Much damage to the plants at freezing temperatures is due to cell dehydration, in which water is drawn out as it crystallizes and the organelle or cell membrane shrivels as liquid volume drops. Then the semipermeable cell membrane would be damaged. Additionally, the phase transition of membrane lipids makes parts of enzymes dissociate from the membrane and lose their activation, and then photophosphorylation and oxidative phosphorylation is uncoupled. Hence, the production of Adenosine Triphosphate (ATP) decreases remarkably to disorder the cell metabolism or to even cause the death of the plant. A most recent research gives us a new insight into the mechanism of freeze injury. A kind of lipid can protect plant from freeze injury. Lipids in the membranes of freezing tolerant plants are removed and converted to oil that accumulates in droplets, retaining membrane integrity, keeping membranes from fusing with one another and conserving the energy by storing oil droplets[10].

Above studies focus on the mechanism of freeze injury[6-10], yet it is not clear how cells respond to freeze injury. Comparative proteomics provides us a global method to see how proteins inside the cells change after the plants suffer from freezing. A comparative proteomics analysis of the tea leaves frozen at critical temperature during the two-leaf stage was made. Actually critical temperature is one of the main parameters for freeze protection and control. This study would greatly strengthen the comprehension of the mechanism of freeze injury, and correspondingly provide better practical guidance for freeze protection and control.

2 Materials and methods

2.1 Plant materials

Fuding Dabai is an early-maturing variety of tea trees and tends to suffer from freeze injury. Twenty Fuding Dabai tea trees were well cultured in the experimental tea field, Jiangsu Yinchunbiya Co. Ltd. (Zhenjiang, Jiangsu Province, China), with the temperature controlled above 0℃. In the experiment, ten trees were treated in an incubator for freezing at -2℃, and the other ten trees were cultured above 0℃. The 1st and 2nd leaves were collected from each of the same 10 tea trees. The leaves were briefly washed with sterile water and frozen in liquid nitrogen immediately, and then stored at -80℃ prior to the protein extraction.

2.2 Protein extraction

The extraction of total protein was based on a current method with a slight modification[11]. Five grams leaf samples were weighed and ground in liquid nitrogen, then suspended in Sodium Dodecyl Sulfate (SDS) extraction buffer (0.5% SDS, 10% glycerol, 5% β-mercaptoethanol, 65 mM Tris-HCl [pH 6.8]). After shaking for 1 h, the sample was centrifuged at 12 000 × *g* for 15 min at 4℃. The proteins were precipitated with three to five volumes of acetone containing 10% Trichloroacetic Acid (TCA) and 0.7% β-mercaptoethanol at -20℃ overnight, and then centrifuged at 12 000 × *g* for 15 min at 4℃. The pellet was washed twice with 100% acetone and 80% acetone respectively, then air-dried for 5 min and resuspended in 20 μL rehydration buffer (7 M urea, 2 M thiourea, 4% 3-[(3-Cholamidopropyl) dimethylammonio] Propanesulfonate (CHAPS), 65 mM Dithiothreitol (DTT), 0.2% Bio-Lyte [pH 4-7], and 0.001% bromophenol blue) per 1 mg pellet. Protein content was quantified using the RC DC Protein Assay Kit (Bio-Rad, Hercules, CA, USA).

2.3 Two-dimensional gel electrophoresis (2-DE)

About 1 mg of protein was dissolved in rehydration buffer and applied to Immobilized pH Gradient (IPG) strips (17 cm, pH 4-7, Bio-Rad). The IPG strip was rehydrated for 14 h in 400 μL rehydration buffer containing the protein sample. Isoelectric Focusing (IEF) was performed at 20℃ using a Protean IEF Cell (Bio-Rad) under the following conditions: 250 V for 1 h with a linear increase in voltage, 500 V for 1 h with a linear increase in voltage, 1 000 V for 1 h with a rapid increase in voltage, 10 000 V for 5 h with a linear increase in voltage, and maintained at 10 000 V until a total of 90 000 Volt-hours (Vh) was reached. After IEF, the strips were equilibrated for 15 min in equilibration buffer I (0.375 M Tris-HCl [pH 8.8], 6 M urea, 2% SDS, 20% glycerol, 1% DTT), then re-equilibrated in buffer II containing 2.5% iodoacetamide instead of DTT for 15 min. The strips were transferred onto 12.5% polyacrylamide gels for SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE). Electrophoresis was performed using the PROTEAN II xi Cell system (Bio-Rad) at 25 mA per gel for 20 min, followed by 50 mA until the bromophenol blue marker reached the end of the gel. Gels were run in triplicate for each sample. The gels were stained with modified Colloidal Coomassie Brilliant Blue (CCB) G-250[12]

2.4 Image analysis and statistical analysis

All gels were scanned by Amersham Lab Scan in 300 dpi. Spot detection, quantification, and matching were performed with ImageMaster™ software (Version 5.0, Amersham, Biosciences, General Electrics (GE), CT, USA). A matchset consisting of six images, three for the control group and three for freeze injury group was created, and one image from the control group was selected as the matchset standard for spot matching. The changes in protein levels between the control and treated groups were analyzed by Student's *t* test; only the expression of a protein from the two groups that displayed a difference at the $p<0.01$ level was considered significantly differentially expressed and those proteins that showed a fold change more than the cutoff value were selected for the identification by mass spectrometry.

2.5 Maldi-TOF-MS and database search

Protein spots were cut from gels and proteins in gel pieces were digested and treated as described previously[13]. A mass spectrometry, Bruker UltraFlex III Maldi-TOF/TOF, was employed to analyze the tryptic peptide mixtures. The software FlexAnalysis (Version 2.4) and Biotools (Version 3.0) provided by Bruker Daltonics (Billerica, USA) were used for the analysis of Mass Spectrometry (MS) data. MS data searching was performed by Mascot software (http://www.matrixscience.com) in the databases of NCBI nr, MSDB, and UniProtKB/TrEMBL. The data searching criteria were set as follows: protein masses were unrestricted; peptide mass tolerance ±0.2 Da; max missed cleavages 1. Proteins were subsequently identified using all the data.

3 Results

3.1 The 2-DE and gel image analysis

It is determined from earlier study that the molecular masses of most proteins in the tea leaves ranged between 15 kDa and 120 kDa on SDS-PAGE, and the sample loading of 800 μg of proteins achieved the best sample separation on a 12.5% separation gel for the second dimension of 2-DE. The subsequent 2-DE was performed under these optimal conditions and the migration patterns of proteins from both the control and treated groups are shown in Figure 1. A reference gel marking differentially expressed proteins ($p<0.01$) between the control group and freeze injury group is shown in Figure 2. The initial matching was processed by the ImageMaster™ software. On average, 619 and 578 protein spots were detected in the control and the treated groups respectively. A total of 476 spots were matched on the 2-DE images after visualization by Coomassie brilliant blue G250 staining. The differential image analysis showed that 46 spots with density changes (29 spots increased and 17 spots decreased; $p<0.01$) in the freeze injury group compared with the control group. 38 differential protein spots ($p<0.01$) with good resolution and relatively high abundance in MS were subjected to further protein identification. Among them, all 17 up-regulated spots were collected whereas only six of the down-regulated spots were selected.

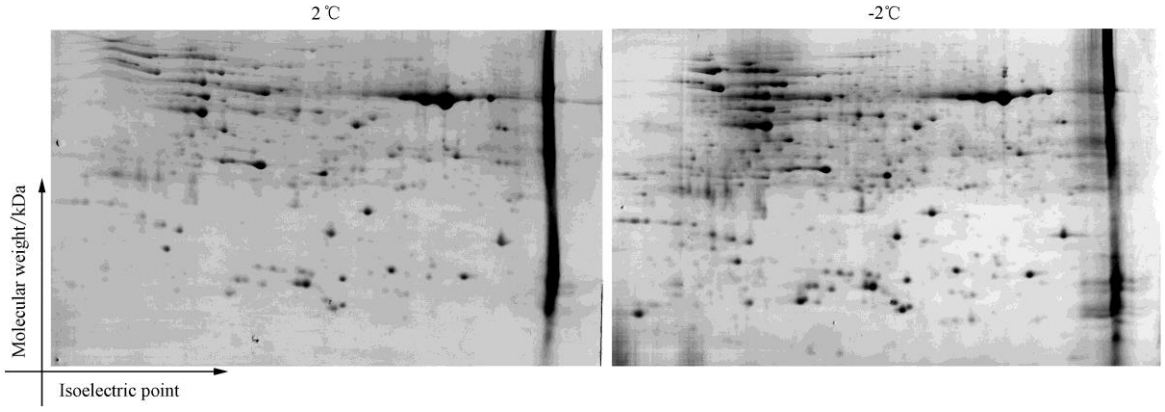

Figure 1 Coomassie-stained 2-D acrylamide gels of the control group (2 ℃), and the freeze injury group (−2 ℃). In the experiment, 800 μg of each protein sample was subject to 2-DE and Coomassie staining

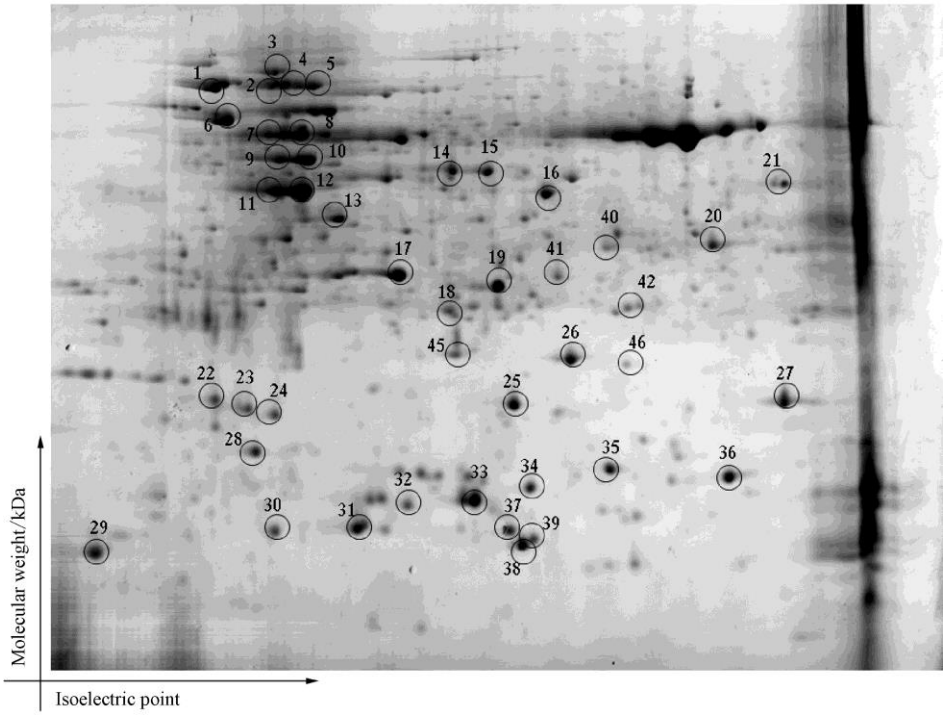

Figure 2 Reference gel map for differential expressed proteins in the freeze injury group (−2℃) compared with the control group (2℃), either up- or down-regulated in the treated group (p<0.01)

3.2 MS analysis and protein identification

Thirty eight differentially expressed proteins were analyzed by Maldi-TOF/TOF-MS, and 23 proteins were identified through Mascot software (http://www.matrixscience.com). Proteins identified from the databases (NCBI nr, MSDB, and UniProtKB/ TrEMBL) are listed in Table 1. Peptide Mass Fingerprintings (PMF) of three new proteins are shown in Figures 3-5 respectively.

4 Discussion

Tea plants are suffered from various stresses, and freezing stress was focused on in this research, which has caused serious problems for tea production. When they suffer freeze-induced damage, the plasma membrane is thought to be the primary site of injury because of its central role in regulation of various cellular processes.

The 70 kDa heat shock proteins (Hsp70s) are a family of conserved ubiquitously expressed heat shock proteins. Proteins with similar structure exist in virtually all living organisms. In the result, four homologous Hsp70 were identified in *Camellia Sinensis*. The Hsp70s are an important part of the cellular machinery for protein folding, and help to protect cells from stress[14,15]. It is

revealed that expressions of the Hsp70 family and other molecular chaperones were increased at high and low temperatures in spinach and tomatoes[16]. Another study shows that accumulation and persistence of heat shock protein 70 in tomato fruit was closely related to cold tolerance[17].

Table 1 List of differentially expressed proteins in the freeze injury group compared with the control group

Spot No.	Protein description	Accession No.	Species	Matched peptides
1	PREDICTED: stromal 70 kDa heat shock-related protein, chloroplastic-like	gi|470129443	Fragaria vesca subsp. Vesca	21/44
2	heat shock 70 protein	gi|2654208	Spinacia oleracea	18/40
3	PREDICTED: heat shock cognate 70 kDa protein 1-like	gi|460411111	Solanum lycopersicum	21/32
5	heat shock protein 70	gi|189380223	Camellia sinensis	18/42
6	unnamed protein product	gi|297734943	Vitis vinifera	16/28
7	hypothetical protein PRUPE_ppa003093mg	gi|462403952	Prunus persica	17/33
8	ATP synthase CF1 alpha subunit (chloroplast)	gi|435856358	Camellia sinensis	27/29
9	ribulose-1,5-bisphosphate carboxylase/oxygenase activase 1	gi|12620881	Gossypium hirsutum	14/28
13	phosphoribulokinase	gi|226935332	Crucihimalaya mollissima	8/24
14	PREDICTED: elongation factor Tu, chloroplastic-like	gi|225456880	Vitis vinifera	8/27
15	PREDICTED: S-adenosylmethionine synthase 2	gi|225432155	Vitis vinifera	10/22
16	glutamine synthetase	gi|67423358	Camellia sinensis	13/32
17	Oxygen-evolving enhancer protein 1, chloroplastic	gi|326487308	Hordeum vulgare subsp. Vulgare	11/43
18	33 kDa precursor protein of oxygen-evolving complex	gi|809113	Solanum tuberosum	10/38
19	oxygen-evolving enhancer protein	gi|344221933	Camellia sinensis	16/44
20	fructose-bisphosphate aldolase 3	gi|432139325	Camellia oleifera	15/60
26	putative reverse transcriptase	gi|4206207	Arabidopsis thaliana	9/18
27	pilus assembly transmembrane protein	gi|470172504	Azoarcus sp. KH32C	7/18
31	hypothetical protein EMIHUDRAFT_468251	gi|485636673	Emiliania huxleyi CCMP1516	12/30
40	PREDICTED: actin-like	gi|356500435	Glycine max	19/45
42	PREDICTED: photosystem II stability/assembly factor HCF136, chloroplastic	gi|225423755	Vitis vinifera	13/55
44	PREDICTED: fructose-1,6-bisphosphatase, cytosolic-like	gi|356559665	Glycine max	11/24
45	ribulose-1,5-bisphosphate carboxylase/oxygenase activase 1	gi|12620881	Gossypium hirsutum	14/16

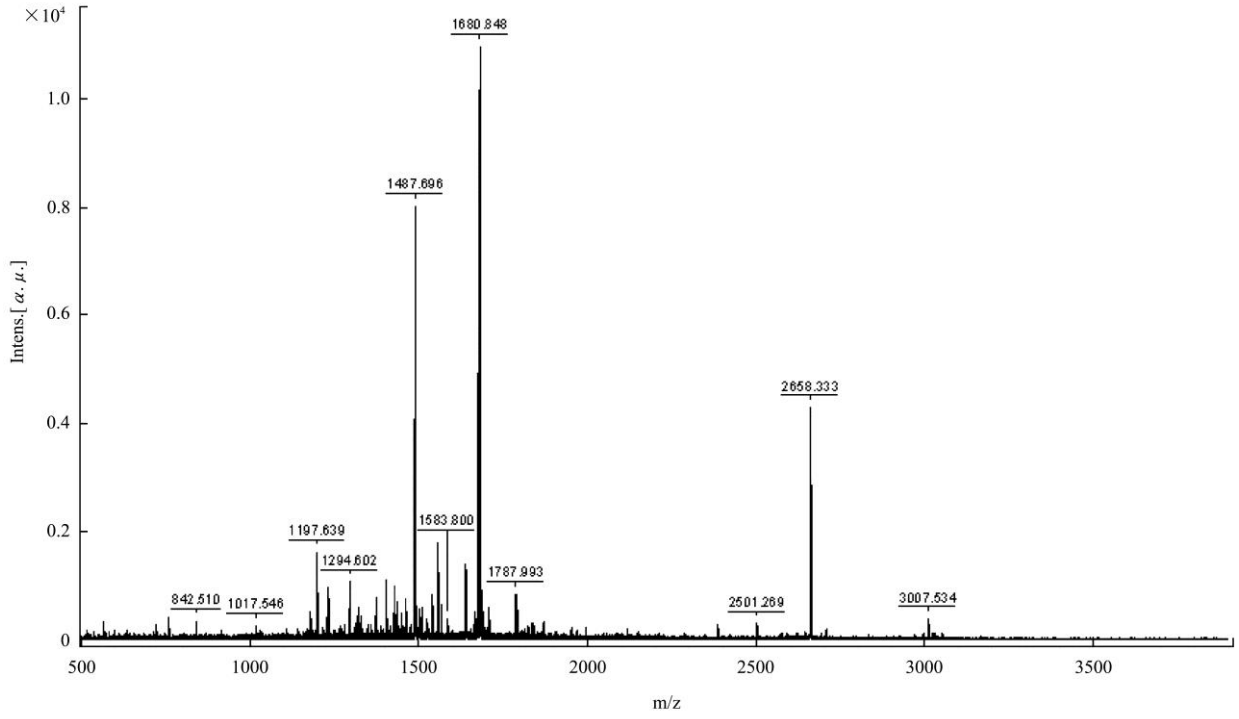

Figure 3 PMF of No. 2 protein spot (heat shock 70 protein, gi|2654208)

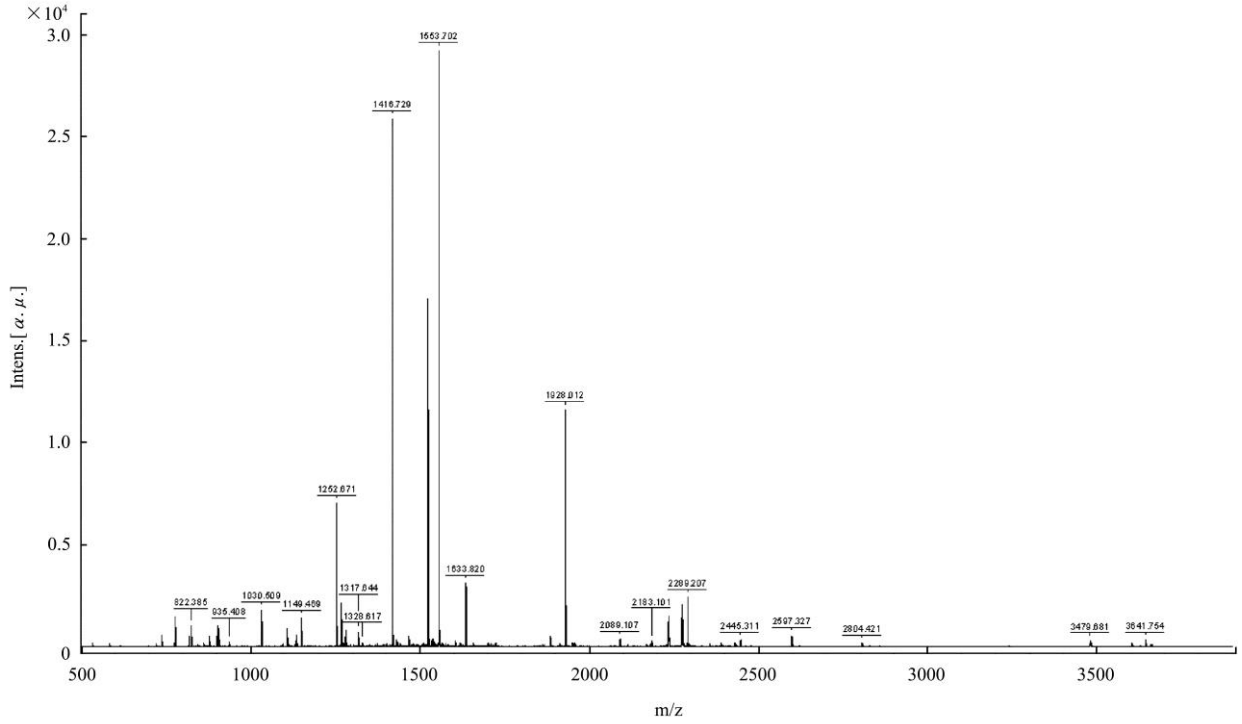

Figure 4 PMF of No. 8 protein spot [ATP synthase CF1 alpha subunit (chloroplast), gi|435856358]

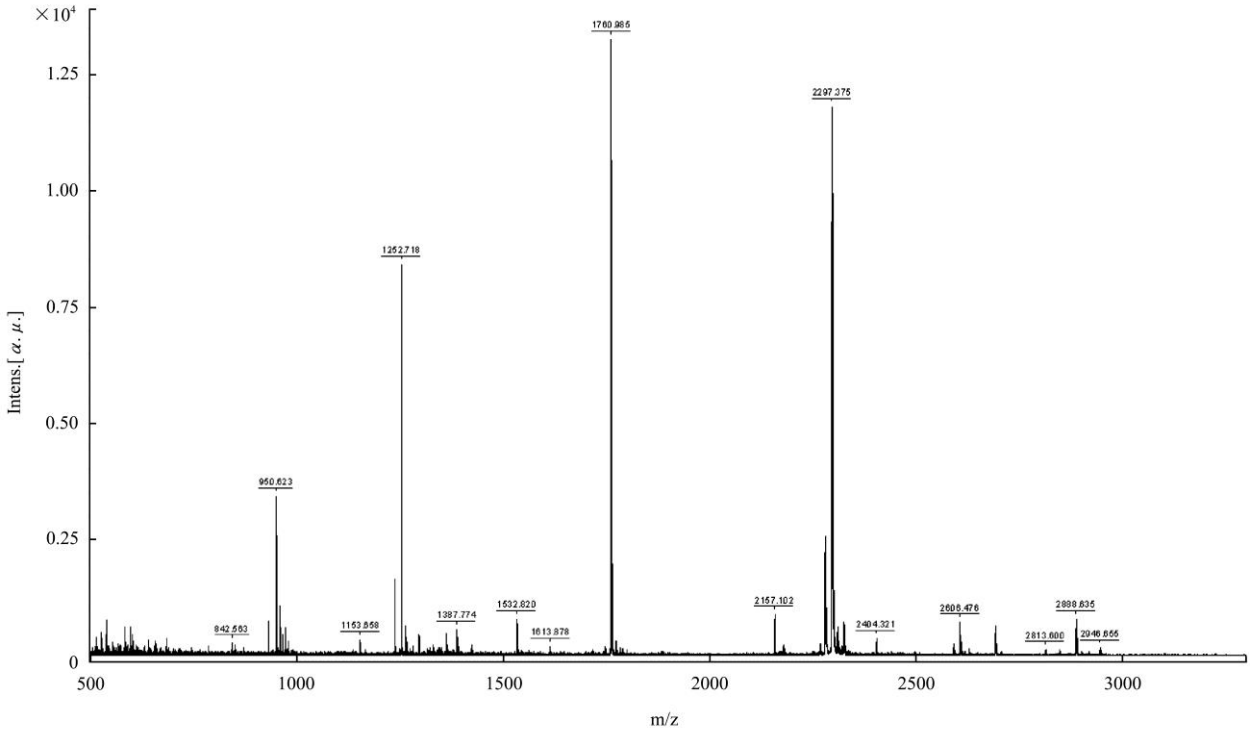

Figure 5 PMF of No. 17 protein spot (Oxygen-evolving enhancer protein 1, chloroplastic, gi|326487308)

Oxygen-evolving enhancer protein seems to be related to water stress, such as early drought-induced changes to the needle proteome of Norway spruce[18], and proteome response of Elymus elongatum to severe water stress and recovery[19]. In the result, the abnormal high expression of Oxygen-evolving enhancer protein seems to be associated with the dehydration induced by freeze injury.

ATP synthase (EC 3.6.3.14) is an important enzyme that provides energy for the cell to use through the synthesis of Adenosine Triphosphate (ATP)[20]. In the experiment, it is likely that the expression of ATP

synthase was increased as a feedback to the inhibition of ATP synthesis.

In molecular biology, the protein S-adenosylmethionine synthetase (EC 2.5.1.6), also known as Methionine Adenosyltransferase (MAT), refers to an enzyme that catalyzes the formation of S-adenosylmethionine (AdoMet) by joining methionine (a non-polar amino acid) and ATP (the basic currency of energy)[21]. The drought response of Theobroma cacao (cacao) and the regulation of S-adenosylmethionine synthetase are involved in polyamine biosynthesis by drought and other stresses[22]. Here the protein S-adenosylmethionine synthetase seems to be related to dehydration induced by low temperature.

Some enzymes involved in carbohydrate metabolism, such as ribulose-1,5-bisphosphate carboxylase oxygenase, phosphoribulokinase and fructose-bisphosphate aldolase showed abnormal expression levels. This means that freeze injury induces the disorder of cell carbohydrate metabolism. Ribulose-1,5-bisphosphate carboxylase oxygenase, commonly known by the abbreviation RuBisCO, is an enzyme involved in the first major step of carbon fixation, a process by which atmospheric carbon dioxide is converted by plants to energy-rich molecules such as glucose. In chemical terms, it catalyzes the carboxylation of Ribulose-1,5-bisphosphate (also known as *RuBP*). It is probably the most abundant protein on the Earth[23,24]. A phosphoribulokinase is an enzyme that catalyzes the chemical reaction: ATP + D-ribulose 5-phosphate $\rightleftharpoons$ ADP + D-ribulose 1,5-bisphosphate.

This enzyme involves in carbon fixation[25,26]. In enzymology, fructose-bisphosphate aldolase, often just aldolase, is an enzyme catalyzing a reversible reaction that splits the aldol, fructose 1,6-bisphosphate, into the triose phosphatesdihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (GAP).

5 Conclusions

With the anomaly and deterioration of the global climate, tea plants are suffering from freeze injury, causing huge damages and losses, but freeze injury of tea (*Camellia Sinensis*) leaves is a complicated biophysical and biochemical process. The comparative proteomics study and analysis were conducted with tea leaves exposed to subzero temperature, to provide some clues that freeze injury is closely related to cellular dehydration induced by freezing temperatures. After the protein extraction and two-dimensional gel electrophoresis, the differential image analysis showed 46 spots with density changes (29 spots increased and 17 spots decreased; $p<0.01$) in the freeze injury group compared with the control group. Thirty eight differential protein spots ($p<0.01$) with good resolution and relatively high abundance were subjected to further protein identification by mass spectrometry.

Hsp70 families, the 70 kDa heat shock proteins, are involved in the process, combined with some proteins that respond to dehydration, such as oxygen-evolving enhancer proteins and S-adenosylmethionine synthetase, inducing the disorder of cellular substance and energy metabolism.

Based on the molecular response to freezing stress, critical temperature of tea plants could be screened, which would provide a better decision making for freeze protection and control.

Acknowledgements

The authors would like to thank Dr. Jun Li for Maldi TOF/TOF MS analysis. We are grateful for the financial supports by the National High Technology Research and Development Program of China (2012AA10A508), the National Natural Science Foundation of China and Jiangsu Province (31101089/BK2010326) and Jiangsu Agriculture Science and Technology Innovation Fund (CX(12)3025).

[References]

[1] Stigter K. Applied agrometeorology. Heidelberg: Springer. 2010.

[2] Gu L, Hanson P J, Post W M, Kaiser D P, Yang B, Nemani R, et al. The 2007 eastern US spring freeze: increased cold damage in a warming world? BioScience, 2009; 58(3): 253-262.

[3] Melo-Abreu J P, Snyder R L. Frost Protection: Fundamentals, Practice and Economics. Food and Agriculture Organization of the United Nations. Rome. 2005.

[4] Yang Y. Cultivation of Chinese Camellia Sinensis.

Shanghai: Shanghai Scientific and Technical Publishers. 2005.

[5] Zhu X, Ma P, Wang J. Climatic analysis on cold injury of Camellia Sinensis in Rizhao region. China Tea, 2008; 30(2): 28-29.

[6] Levitt J. The Hardiness of Plants. New York: Academic Press. 1956.

[7] Levitt J. A sulfhydryl-disulfide hypothesis of frost injury and resistance in plants. Journal of Theoretical Biology, 1962; 3(3): 355-391.

[8] Steponkus P L. Role of the plasma membrane in freezing injury and cold acclimation. Annual Review of Plant Physiology, 1984; 35(1): 543-584.

[9] Steponkus W M, Uemura M, Webb M S. Membrane destabilization during freeze induced dehydration. Current Topics on Plant Physiology, 1993; 10: 37-47.

[10] Moellering E R, Muthan B, Benning C. Freezing tolerance in plants requires lipid remodeling at the outer chloroplast membrane. Science, 2010; 330(6001): 226-228.

[11] Jefferies J R, Brophy P M, Barrett J. Investigation of Fasciola hepatica sample preparation for two-dimensional electrophoresis. Electrophoresis, 2000; 21(17): 3724-3729.

[12] Schroder S, Zhang H, Yeung E S, Jänsch L, Zabel C, Wätzig H. Quantitative gel electrophoresis: sources of variation. Journal of Proteome Research, 2008; 7(3): 1226-1234.

[13] Alam I, Sharmin S, Kim K H, Kim Y G, Lee J, Lee B H. An improved plant leaf protein extraction method for high resolution two-dimensional polyacrylamide gel electrophoresis and comparative proteomics. Biotechnic & Histochemistry, 2013; 88(2): 61-75.

[14] Tavaria M, Gabriele T, Kola I, Anderson R L. A hitchhiker's guide to the human Hsp70 family. Cell Stress Chaperones, 1996; 1(1): 23-28.

[15] Morano K A. New tricks for an old dog: the evolving world of Hsp70. Annals of the New York Academy of Sciences, 2007; 1113: 1-14.

[16] Li Q B, Haskell D W, Guy C L. Coordinate and non-coordinate expression of the stress 70 family and other molecular chaperones at high and low temperature in spinach and tomato. Plant Molecular Biology, 1999; 39(1): 21-34.

[17] Sabehat A, Weiss D, Lurie S. The correlation between heat-shock protein accumulation and persistence and chilling tolerance in tomato fruit. Plant Physiology, 1996; 110(2): 531-537.

[18] Blodner C, Majcherczyk A, Kues U, Polle A. Early drought-induced changes to the needle proteome of Norway spruce. Tree Physiology, 2007; 27(10): 1423-1431.

[19] Gazanchian A, Hajheidari M, Sima N K, Salekdeh G H. Proteome response of Elymus elongatum to severe water stress and recovery. Journal of Experimental Botany, 2007; 58(2): 291-300.

[20] Li C Y, Weiss D, Goldschmidt E E. Effects of carbohydrate starvation on gene expression in citrus root. Planta, 2003; 217(1): 11-20.

[21] Horikawa S, Sasuga J, Shimizu K, Ozasa H, Tsukada K. Molecular cloning and nucleotide sequence of cDNA encoding the rat kidney S-adenosylmethionine synthetase. The Journal of Biological Chemistry, 1990; 265(23): 13683-13686.

[22] Bae H, Kim S H, Kim M S, Sicher R C, Lary D, Strem M D, et al. The drought response of Theobroma cacao (cacao) and the regulation of genes involved in polyamine biosynthesis by drought and other stresses. Plant Physiology and Biochemistry, 2007; 46(2): 174-188.

[23] Dhingra A, Portis A R, Daniell H. Enhanced translation of a chloroplast-expressed RbcS gene restores small subunit levels and photosynthesis in nuclear RbcS antisense plants. Proceedings of the National Academy of Sciences of the United States of America, 2010; 101(16): 6315-6320.

[24] Feller U, Anders I, Mae T. Rubiscolytics: fate of Rubisco after its enzymatic function in a cell is terminated. Journal of Experimental Botany, 2008; 59(7): 1615-1624.

[25] Hurwitz J, Weissbach A, Horecker B L, Smyrniotis P Z. Spinach phosphoribulokinase. The Journal of Biological Chemistry, 1956; 218(2): 769-783.

[26] Jakoby W B, Brummond D O, Ochoa S. Formation of 3-phosphoglyceric acid by carbon dioxide fixation with spinach leaf enzymes. The Journal of Biological Chemistry, 1956; 218(2): 811-822.