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Exogenous abscisic acid significantly affects proteome in tea plant (*Camellia sinensis*) exposed to drought stressLin Zhou¹, Hui Xu¹, Sue Mischke², Lyndel W Meinhardt², Dapeng Zhang², Xujun Zhu¹, Xinghui Li¹ and Wanping Fang^{1,2}

Tea [*Camellia sinensis* (L.) O. Kuntze] is an important economic crop, and drought is the most important abiotic stress affecting yield and quality. Abscisic acid (ABA) is an important phytohormone responsible for activating drought resistance. Increased understanding of ABA effects on tea plant under drought stress is essential to develop drought-tolerant tea genotypes, along with crop management practices that can mitigate drought stress. The objective of the present investigation is evaluation of effects of exogenous ABA on the leaf proteome in tea plant exposed to drought stress. Leaf protein patterns of tea plants under simulated drought stress [(polyethylene glycol (PEG)-treated)] and exogenous ABA treatment were analyzed in a time-course experiment using two-dimensional electrophoresis (2-DE), followed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS). Among the 72 protein spots identified by MALDI-TOF MS, 16 proteins were downregulated and two were upregulated by exogenous ABA. The upregulated proteins have roles in glycolysis and photosystem II stabilization. Twenty-one protein spots were responsive to drought stress and most participate in carbohydrate and nitrogen metabolism, control of reactive oxygen species (ROS), defense, signaling or nucleic acid metabolism. The combined treatments of exogenous ABA and drought showed upregulation of 10 protein spots at 12 h and upregulation of 11 proteins at 72 h after initiation of drought stress. The results support the importance of the role that ABA plays in the tea plant during drought stress, by improving protein transport, carbon metabolism and expression of resistance proteins.

Horticulture Research (2014) 1, 14029; doi:10.1038/hortres.2014.29; published online: 25 June 2014

INTRODUCTION

Tea [*Camellia sinensis* (L.) Kuntze] is a shrub native to China, but cultivated as an important economic crop in more than 50 countries around the world, with an annual production of 4.8 million tons (FAOSTAT, 2012). The top five tea producers are China, India, Sri Lanka, Kenya and Turkey, which collectively share 75% of world production (FAOSTAT, 2012). The crop is typically cultivated in a rain-fed agricultural system, and tea plants are severely affected by various abiotic stresses such as drought, salinity, temperature, heavy metals and soil nutrient deficiency.¹ Among these, drought is a major stress that causes severe damage on the yield and quality of tea products.¹ In recent years, drought-caused damage has become increasingly more frequent and unpredictable due to global climate changes and growing water scarcity.^{2,3}

Understanding drought-induced responses of tea plants is an essential first step for breeding tea varieties with increased drought resistance and for developing crop management practices that can mitigate drought stress. Drought-induced responses have been observed at the levels of morphological, physiological or molecular changes at different stages of tea plant development.¹ Physiological effects of drought stress in tea were increases in proline content, H₂O₂ and superoxide anion, lipid peroxidation, activities of catalase and superoxide dismutase and in water loss rate; and decreases in relative water content, dry mass, chlorophyll, carotenoid, total phenolic contents of leaf and antioxidants such as ascorbate and glutathione.^{1,4} Damayanthi⁵ showed that drought-tolerant tea cultivars maintained a high water status, with a substantial rate of photosynthesis, as a result of osmotic adjustments, elevated total soluble sugars and deep rooting. Drought-induced responses at the gene level have also been investigated in

tea plants. Using suppression subtractive hybridization, Gupta⁶ identified a set of drought-responsive genes under controlled conditions, and validated the identified genes and their patterns of expression under drought conditions in the field. Das⁷ identified differentially expressed genes in roots of tea under drought stress by analyzing a suppression subtractive hybridization cDNA library.

Abscisic acid (ABA) is a plant hormone that functions as an endogenous messenger in response to biotic and abiotic stresses.^{8–11} A well-known response to drought stress is the biosynthesis of ABA, which is the signal that triggers a number of molecular and cellular responses, ultimately resulting in stomatal closure. The mechanism involves ABA's stimulation of second messengers such as reactive oxygen species (ROS), nitric oxide and Ca²⁺, followed by activation and inactivation of protein kinases/phosphatases that target the ion channels to prevent water loss.¹² Previous studies indicated that exogenous ABA treatment can induce drought resistance in tomato,¹³ spring wheat¹⁴ and *Populus*.¹⁵ Increase in ABA levels after exposure to drought has been reported in *Arabidopsis thaliana*,^{16,17} rice¹⁸ and tomato,¹⁹ and an ABA-mimicking ligand that reduces water loss and promotes drought resistance was recently reported in plants.²⁰ Therefore, ABA production has attracted much research attention as a potentially useful trait in selecting for drought tolerance in crops. However, direct application of ABA in actual agriculture was considered impractical, due to the concern of cost-effectiveness.

The analysis of the plant proteome is a powerful approach to evaluate drought-responsive proteins.²¹ Proteomic investigation has been conducted in several tree species such as holm oak, peach, peanut, poplar, rubber tree, white spruce and maritime pine.²² Two-dimensional electrophoresis (2-DE)²³ has been one of the most

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Received: 3 April 2014; Revised: 21 April 2014; Accepted: 30 April 2014

prevalent techniques for analyzing complex protein mixtures and assessing drought-induced responses in different plant species.^{24–29} Using 2-DE technique, Guo *et al.*³⁰ conducted a proteomic analysis to elucidate alterations of protein expression in response to drought in leaves of tea plants. Significant protein changes were found in tea leaves under polyethylene glycol (PEG)-induced stress. The identified proteins have important roles in various physiological processes that are directly or indirectly associated with tea plant's response to stresses.

Despite various reports on the induced physiological and biochemical responses of tea plant, recently reviewed by Upadhyaya and Panda,¹ there is still a large gap in knowledge about underlying molecular mechanisms of drought adaptation in tea. Thus far, there have been no proteomic studies addressing the effect of exogenous ABA on tea plant exposed to drought stress. Moreover, it is known that, in other plant species, different drought tolerance and avoidance strategies require different metabolic adaptations.²¹

In the present study, we analyzed drought and ABA-responsive proteins in tea plant by using 2-DE and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS). Our objective was to evaluate the effects of exogenous ABA on leaf proteome when the plant was exposed to drought stress. Leaf protein patterns of tea plants under simulated drought stress (PEG-treated) and exogenous ABA treatment were analyzed using 2-DE followed by MALDI-TOF MS. This study will contribute to an improved understanding of the molecular basis of drought tolerance in tea plant.

MATERIALS AND METHODS

Plant material and stress treatments

Tea plant cultivar, 'Ying Shuang' (*C. sinensis* cv. Yingshuang) is an improved cultivar bred by the Tea Research Institute, Chinese Academy of Agricultural Sciences. The two-year-old plants were grown in greenhouse (3000 lx, 16 h light/8 h dark) at temperature ranging from 15 °C to 20 °C. The humidity index was set for 60%–70% (College of Horticulture, Nanjing Agricultural University, Nanjing, China). Tea plants were cultured, in groups of 20, in 20 L polyethylene basins with 15 L pH 5.5 nutrient solution (formula provided by Institute of Soil Science, Chinese Academy of Sciences, Nanjing, China).³¹ The nutrient solution was aerated daily at 8:00–10:00 a.m., 12:00–14:00 p.m. and 16:00–18:00 p.m.

Tea plants were sprayed with 250 mL of ABA solution, concentration 50 mg L⁻¹, while controls were sprayed with distilled water. After 3 days, 10% PEG-6000 was used to simulate drought stress. Each treatment had three replicates. During the period of drought stress, treated leaves were sampled at 0 h, 12 h and 72 h after the application of PEG-6000, frozen immediately in liquid N₂ and stored at -70 °C.

Determination of chlorophyll, free proline and malondialdehyde content

Leaf chlorophyll was extracted with ethanol according to Knudson *et al.*³² The concentration of free proline in tea leaves was determined by using the acid-ninhydrin method developed by Bates *et al.*³³ The content of malondialdehyde (MDA) in the leaves was measured by the thiobarbituric acid method, according to Dhindsa *et al.*³⁴ All experiments were carried out with at least three independent repetitions. All statistical analyses were performed with SPSS 20.0 (Windows; SPSS Inc., Chicago, IL, USA) and significance tests were determined by Duncan's test and ANOVA.

Preparation of total protein extracts and 2-DE

Soluble protein was extracted from 1.0 g of each sample by homogenization in liquid N₂ in a mortar, followed by extraction with TCA/acetone precipitation as described by Jacobs *et al.*³⁵ The air-dried pellet (700 mg) was resuspended in 700 µL lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.2% (w/v) Bio-Lyte, 65 mM dithiothreitol, or DTT) and vortexed for 1 h at 25 °C. After centrifugation (14 000 r.p.m., 30 min at 4 °C), the supernatant was isolated. The supernatant was cleared by centrifugation again (14 000 r.p.m., 30 min at 4 °C), and further analysis was performed on the total soluble protein extract in the final supernatant. Protein concentrations were measured with

bovine serum albumin as a standard, according to the Coomassie Brilliant Blue G-250 method.³⁶

Isoelectric focusing (IEF) to separate proteins based on their isoelectric point (pI) was performed in a PROTEAN IEF System (Bio-Rad, Hercules, CA, USA) using 17 cm pH 4–7 immobilized protein gradient strips (Bio-Rad). Strips were rehydrated by loading samples of 2.2 mg protein dissolved in 500 µL rehydration buffer into the channels of the IEF tray and actively rehydrating at 20 °C for 12 h. Focusing was performed at 20 °C, 60 kVh with a maximum current setting of 50 µA strip⁻¹. After IEF, the immobilized protein gradient strips were equilibrated for 15 min in 6 M urea, 20% glycerol, 2% sodium dodecyl sulfate in 0.375 M Tris-HCl pH 8.8, containing 2% (w/v) DTT for the first equilibration step and 2.5% (w/v) iodoacetamide for the second. For the second dimension sodium dodecyl sulfate–polyacrylamide gel electrophoresis, a Protean II Multi Cell (Bio-Rad) was used to separate the proteins by their size. The strips were placed on top of 13.5% (w/v) polyacrylamide gels and electrophoresis was performed at 1 mA gel⁻¹ for 40 min, followed by 15 mA gel⁻¹ for 4 h. After electrophoresis, gels were stained with a modified Coomassie Brilliant Blue staining method.³⁷ Stained gels were scanned by the Versadoc 3000 Imaging System (Bio-Rad). Images were analyzed by using the PDQuest 2-D software (version 8.0.1; Bio-Rad), and several key parameters, such as smooth and saliency, were fixed as constants in the image analysis to obtain comparable data for the quantitative analysis. Spots that showed consistent positions on different gels were considered to be the same proteins. Only those spots whose quantity was changed more than twofold in each of three replicated experiments were collected and sent to Shanghai Boyuan Biological Technology Co. (Shanghai, China) for MS analysis.

MS and database searching

Differentially expressed proteins were destained and digested by using the improved in-gel digestion method developed by Katayama *et al.*³⁸ The excised spots were analyzed using an ABI 4800 MALDI-TOF/TOF Plus mass spectrometer (Applied Biosystems, Foster City, CA, USA). Mass spectra (*m/z* range: 800–3500 Da) were processed and the parameters of peak detection algorithm were used. Our MS data were searched against the National Center Biotechnology Information (NCBI) protein sequence database by an in-house MASCOT (version 2.1; Matrix Science, London, UK) search engine. The search parameters were as follows: one missed cleavage allowed, alkylation of cysteine by carbamidomethylation as a fixed modification and oxidation of methionine as a variable modification. Other search parameters were ±0.15 Da for peptide mass tolerance, ±0.25 Da for fragment mass tolerance and number of queries set as 95. To get more information regarding these proteins, the conserved domains were predicted with BLAST against the NCBI non-redundant database (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>). The similarity of the proteins was also analyzed by BLAST (<http://www.expasy.org/cgi-bin/blast.pl>). The NCBI Reference Sequence (RefSeq) database (<http://www.ncbi.nlm.nih.gov/RefSeq/>) provides a non-redundant collection of sequences representing genomic data, transcripts and proteins. However there is little proteomic information available on *Camellia sinensis*. In order to get the functional information about these proteins, their homologs were searched with BLASTP (<http://www.expasy.org/tools/blast/>) by using their protein sequences as queries.

RESULTS

Responsive change of chlorophyll, proline and MDA under drought stress and ABA treatment

The chlorophyll, proline and MDA content remained unchanged in response to exogenous ABA pre-treatment when measured at 0 h of drought treatment (Figure 1). The drought stress significantly decreased the leaf chlorophyll content (Figure 1a), but treatment with exogenous ABA significantly ameliorated the effects of drought stress on chlorophyll. Proline and MDA content both showed significant increases under drought stress (Figure 1b and 1c). The drought-induced increase in MDA content was significantly repressed by exogenous ABA treatment, similarly to chlorophyll. However, a dramatic further increase in proline content was observed with exogenous ABA treatment of tea plants.

Identification of protein spots related to exogenous ABA treatment
In order to identify the differential expression of proteins under treatment with exogenous ABA, proteins extracted from leaves

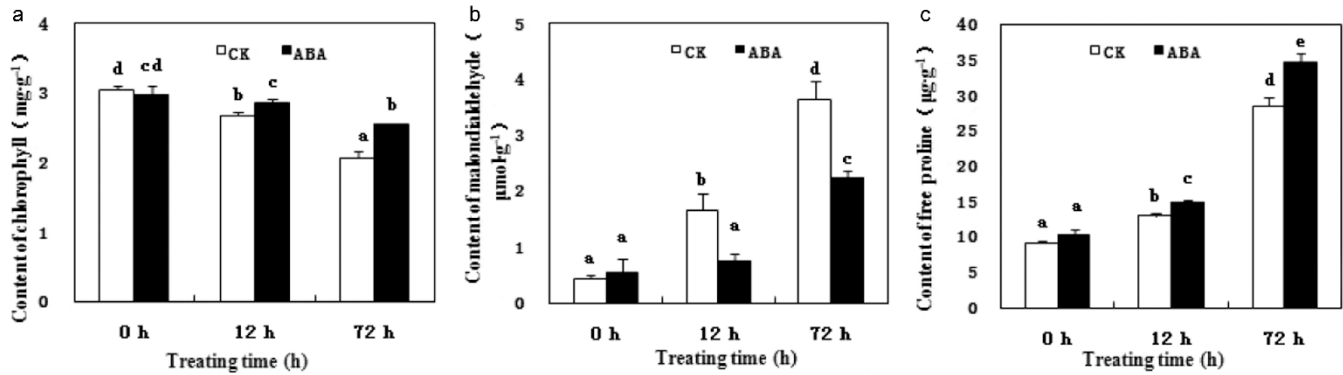


Figure 1. Effects of drought on chlorophyll, proline and MDA content of tea plant. ABA: pre-treated with abscisic acid; CK: control, pre-treated with distilled water.

were separated by 2-DE. More than 700 protein spots were reproducibly detected in each Coomassie Brilliant Blue-stained gel, which included those with molecular weight ranging from 11.0 to 100.0 kDa and pI ranging from 4.55 to 6.85. Eighteen specific proteins (detected in three independent biological replicates on each 2-DE gel) were expressed under ABA treatment (Figure 2). The 18 protein spots were identified by MALDI-TOF analysis and NCBI database search, and 16 of the spots were matched to known proteins, while 2 spots were unnamed proteins without specific functions defined in the NCBI database (Table 1). The identified proteins were involved in photosynthesis (spots 4, 9, 11, 12, 14, 15 and 16); carbon metabolism (spot 1); nitrogen metabolism (spots 6 and 7); lipid metabolism (spot 10); heat-shock (spot 8); energy metabolism (spots 2 and 3); and DNA repair (spot 13). Among the 18 proteins related to ABA treatment, spot 1 (2,3-bisphosphoglycerate-independent phosphoglycerate mutase, iPGAM) and spot 12 (oxygen-evolving enhancer protein 1, chloroplastic, i.e., OEE1) were both significantly increased, while the remaining spots were all significantly decreased. Spots 11 and 12 were OEEs. We found that exogenous ABA upregulated OEE (spot 12) 16-fold, even though spot 11 was decreased.

Identification of drought-responsive protein spots

The proteome of tea plant leaves in response to drought stress was resolved by 2-DE, using triplicate gels. Representative gels are

illustrated in Figure 3, and the global pattern of proteins remained largely unaltered. However, the expression levels of 21 proteins were changed at least 2.0-fold in one or both of the drought treatment stages (Figure 4). Besides the unknown and predicted proteins (spots 14, 18, 19, 20 and 21), other identified proteins were classified into several functional categories including regulatory proteins, energy-related proteins, redox homeostasis-related proteins, etc. (Table 2). The largest functional protein was involved in carbohydrate metabolic processes and nitrogen metabolic processes, which were greatly affected by drought stress. Comparison of the identified proteins between drought-affected and control samples showed all spots except 12, 20 and 21 were significantly downregulated in tea plant for the 12 h samples (Figure 4). Compared with the controls, five protein spots (spots 9, 12, 17, 18 and 19) were upregulated, and 16 spots (spots 1–8, 10, 11, 13–16, 20 and 21) were downregulated (Figure 4). Spots 2 and 20 were present in abundance in the control leaves, but undetected in PEG-treated leaves at 72 h, suggesting that PEG-induced alterations of membrane proteins in tea plant leaves included both quantitative (increased or decreased) and qualitative changes in protein spots. One of inevitable consequences of water stress is that ROS accumulation is enhanced and distributed in different cellular compartments, e.g., chloroplasts, the peroxisomes and the mitochondria. Treatment with 10% PEG-6000 for 12 h and 72 h, damaged the photosynthetic and antioxidant enzyme systems of tea plants.

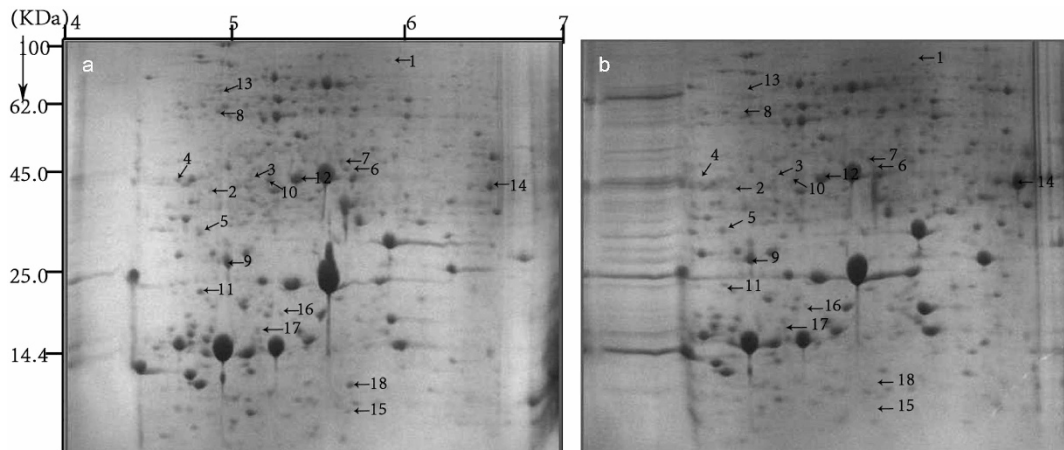


Figure 2. A 2-DE analysis of tea leaf proteins. (a) 2-DE gels of tea leaf subjected to distilled water (control); (b) 2-DE gels of tea leaf subjected to 50 mg L⁻¹ ABA. This is a representative figure from three biological replicas.

Table 1. The identification of differentially expressed proteins in tea leaves subjected to exogenous ABA pre-treatment

Spot no.	Protein name	Accession no. ^a	Species	Theoretical		Experimental		Score ^b	%cov (matching peptides) ^c	
				M _r (kDa)	pI	M _r (kDa)	pI			
1	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	Q42908	<i>Mesembryanthemum crystallinum</i>	61.32	5.39	85.45	6.19	142	8% (3)	+3.28
2	ATP synthase CF1 alpha subunit	YP_538920	<i>Gossypium hirsutum</i>	55.44	5.25	44.32	5.41	544	17% (6)	-4.34
3	ATP synthase CF1 alpha subunit	YP_053140	<i>Nymphaea alba</i>	55.54	5.58	59.38	5.59	351	13% (5)	-3.57
4	Chloroplast ribulose 1,5-bisphosphate carboxylase/oxygenase activase	ABW80752	<i>Flaveria bidentis</i>	48.81	6.10	40.90	5.20	465	15% (5)	-4.01
5	FE861219.1 EST0078 Tender roots cDNA library of tea plant <i>Camellia sinensis</i> cDNA clone 07102311-CHAG_17_03.M13+_C01.ab1, mRNA sequence	FE861219	<i>Camellia sinensis</i>	31.02	5.54	36.35	5.25	143	14% (3)	-8.19
6	Glutamine synthetase	BAD99525	<i>Camellia sinensis</i>	39.40	5.79	44.52	6.00	367	26% (4)	-2.79
7	Glutamine synthetase, chloroplastic	O22506	<i>Daucus carota</i>	48.08	5.63	45.56	5.97	142	14% (3)	-4.01
8	High molecular weight heat shock protein	AAF34134	<i>Malus × domestica</i>	71.57	5.17	60.23	5.42	454	11% (3)	-2.14
9	Light-harvesting complex I protein (Lhca1)	XP_002315298	<i>Populus trichocarpa</i>	26.53	6.10	28.12	5.40	323	13% (2)	-9.07
10	Lipoxygenase	ACJ54281	<i>Camellia sinensis</i>	102.59	6.39	49.36	5.67	91	4% (3)	-4.37
11	Oxygen-evolving enhancer protein	AEN02470	<i>Camellia sinensis</i>	35.54	8.13	21.16	5.30	225	20% (2)	-2.02
12	Oxygen-evolving enhancer protein 1, chloroplastic	P26320	<i>Solanum tuberosum</i>	35.60	5.84	37.16	5.83	340	21% (4)	+16.07
13	Predicted: putative DNA repair protein RAD23-3	XP_002282352	<i>Vitis vinifera</i>	41.98	4.73	69.52	5.39	62	10% (3)	-5.08
14	Ribulose 1,5-bisphosphate carboxylase I large subunit	AAM33278	<i>Stachys affinis</i>	50.19	6.78	37.53	6.72	399	18% (5)	-50.08
15	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	AAB88833	<i>Hypocoum imberbe</i>	50.92	6.13	11.91	6.04	73	2% (1)	-2.82
16	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	AAA21212	<i>Aruncus dioicus</i>	52.13	6.04	19.39	5.70	234	15% (2)	-4.45
17	Unnamed protein product	CBI23038	<i>Vitis vinifera</i>	25.28	6.82	22.75	5.59	117	21% (3)	-4.35
18	Unnamed protein product	CAA32182	<i>Spinacia oleracea</i>	23.20	9.76	13.81	6.05	166	19% (2)	-3.98

^a Accession number in NCBI database.

^b Score is a measure of the statistical significance of a match.

^c Percentage of predicted protein sequence covered by matched peptides.

Comparison of protein profiles under ABA and drought treatments

In order to show the effects of exogenous ABA treatment on these proteins induced by drought stress, the leaf protein pattern in tea plants pre-treated with exogenous ABA was also analyzed using 2-DE. Thirty-three differentially expressed proteins were selected for categorizing (Table 3). Seven of these proteins (spots 6, 7, 28 and 30–33) were predicted proteins, or unknown or unnamed proteins; 19 were in the Rubisco family; and another seven were proteins responding to PEG stress. The latter proteins included apolipoprotein, chloroplast phosphoribulokinase, cytochrome b6-f complex iron-sulfur subunit 2, fructose-bisphosphate aldolase, high molecular weight heat shock protein (Hsp), S-adenosylmethionine synthase 3 and short-chain dehydrogenase/reductase. These proteins play important roles in many life activities of plant, such as transporter activity, photosynthesis, glycolysis, redox reactions and amino-acid biosynthesis. The expression pattern of these proteins in leaves of tea plant pre-treated with exogenous ABA and single drought treatment (Figure 5) suggests that 23 proteins (spots 1, 2, 4, 5, 7, 9–15, 17, 19–21, 23, 25–29 and 31) were upregulated and 10 spots (spot 3, 6, 8, 16, 18, 22, 24, 30, 32 and 33) were downregulated at 12 h; 11 proteins (spots 1, 4, 5, 11, 19, 20, 22, 24, 27, 29 and 30) were upregulated at 72 h; and some proteins were downregulated compared with 0 h (Figures 5 and 6). Apolipoprotein D, chloroplast phosphoribulokinase, fructose-bisphosphate aldolase, high molecular weight Hsp, protein IN2-1 homolog B-like, short-chain dehydrogenase/reductase and most of the Rubisco proteins were upregulated at 12 h in tea plants that had been pre-treated with ABA.

DISCUSSION

Because drought stress is one of the major factors limiting the productivity of tea plant in tea-growing countries, knowledge of the mechanism underlying enhanced tolerance to drought stress in tea plant is of importance. To the best of our knowledge, several studies have explored the transcripts in the leaves and roots of tea plants,^{7,39} but few studies examined proteomics of plant leaves in response to the combination of drought stress and exogenous ABA. In this study, we focused on identifying stress proteins induced in tea plant leaf under drought stress combined with exogenous ABA treatment, and we examined changes in several physiological and biochemical characteristics.

ABA and drought-responsive changes of physiological and biochemical characteristics

Chlorophyll plays an important role in plant photosynthesis, which allows plants to absorb energy from light. Drought stress inhibits the photosynthetic activity in plants by changing chlorophyll content, and a decrease of total chlorophyll content implies lowered light harvesting.⁴⁰ In the present study, the total chlorophyll content was significantly decreased in tea plant under drought stress. Plants can partially protect themselves against biotic and abiotic stresses by accumulating osmolytes. Proline accumulation is one of the most common osmolyte responses to environmental stresses, particularly in plants under drought stress.⁴¹ Previous studies implied that an increase in proline content (251.64%) was observed in 1-year-old clonal seedlings of tea after withholding watering for 5 days.^{4,42} In our study, the increase of proline content under drought

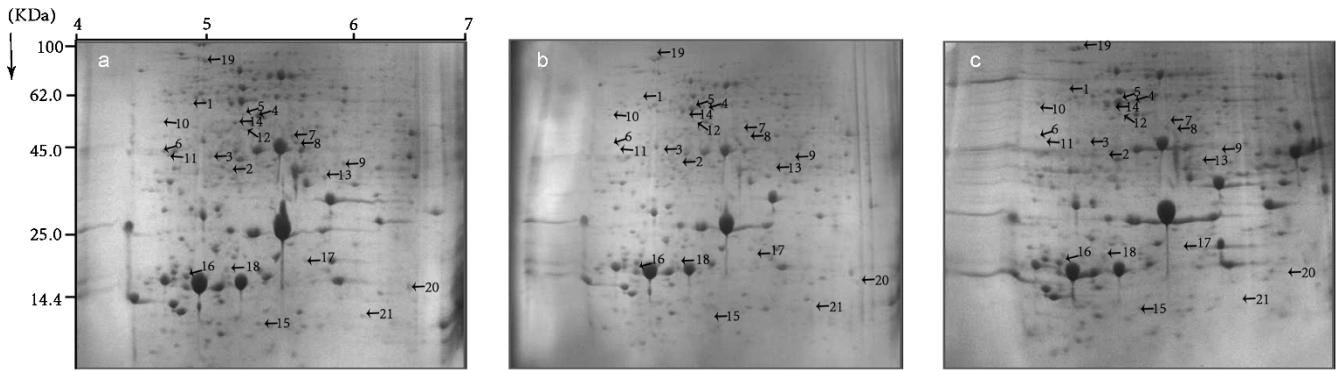


Figure 3. A 2-DE analysis of tea leaf proteins. (a) 2-DE gels of tea leaf subjected to 50 mg L⁻¹ ABA; (b, c) 2-DE gels of tea leaf subjected to drought treatment for 12 h and 72 h after pre-treated with 50 mg L⁻¹ ABA. This is a representative figure from three biological replicas.

stress was greater at 72 h than at 12 h. In many investigations, MDA has been utilized as a suitable biomarker for lipid peroxidation and as an indicator of the extent of ROS damage, thus, the content of MDA is an important indicator of oxidative stress damage.⁴² In this study, drought stress significantly increased the level of MDA, indicating oxidative stress. We found that the contents of chlorophyll and proline in exogenous ABA-pre-treated tea plants were significantly higher than control in response to drought stress. In contrast, higher MDA was observed in control than in ABA treated plants. The variation in chlorophyll, proline and MDA content in this study indicates that exogenous ABA pre-treatment increases the chlorophyll and proline content of the leaf, and reduces membrane lipid peroxidation, thus improves drought tolerance in tea plants pre-treated with exogenous ABA.

Differentially expressed proteins under ABA and drought treatment
Both ABA- and drought-induced changes in protein profile were observed in the present experiment. In plants treated with exogenous ABA, 18 differentially expressed proteins were successfully identified. The present results showed significant ABA-induced increase of iPGAM and OEE proteins, which are respectively involved in glycolysis and maintenance of photosystem II stability.^{43,44} ABA pre-treatment did not increase expression of the remaining proteins. Thus, exogenous ABA may specifically improve glucose metabolism and advance the stability of the photosystem II stabilization.

The responses of plants to drought stress depend on both the species and the severity of water deficiency.⁴⁵ Moreover, differential

expression of drought-responsive protein changes over the length of time plants are subjected to drought stress. The present proteomic study identified numerous drought stress-responsive proteins, such as ascorbate peroxidase, glutamine synthetase, glutathione transferase lambda 2, Hsp 90, the CF1 alpha subunit of ATP synthase and lipoxygenase. Under drought stress, lipoxygenase (Table 2, spot 12) was significantly increased at 12 h, whereas 85.7% (18/21) proteins were downregulated. The downregulated proteins were involved in the ROS pathway (ascorbate peroxidase), photosynthetic carbon metabolism (chloroplast phosphoribulokinase), nitrogen metabolism (glutamine synthetase), energy metabolism (CF1 alpha subunit of ATP synthase) and photosynthesis (light-induced protein). Treatment with 10% PEG 6000 might severely affect the carbon and nitrogen metabolism and growth of tea plant, undermining the antioxidant enzyme system. Drought-dependent changes relating to the antioxidant defense system could contribute to decreased chlorophyll and increased MDA. Lipoxygenase (LOX) reactions may initiate synthesis of a signaling molecule, or be involved in inducing structural or metabolic changes in plant cells.^{46,47} Earlier studies reported that drought stress enhanced LOX gene expression and LOX enzyme activity in leaves.^{48,49} In addition, products of LOXs are involved in diverse cell functions.

Comparison of protein profiles under ABA and drought treatments
The experimental design of the present study enabled the identification of ABA-responsive proteins in drought-stressed tea plants, through the comparison of protein profiles under exogenous ABA

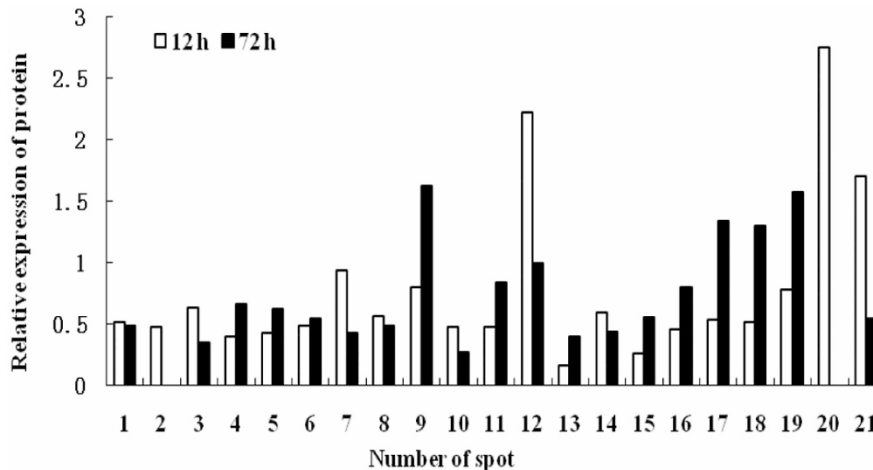


Figure 4. Histograms show the abundance ratio of the identified proteins in tea leaves subjected to drought at 12 h and 72 h

Table 2. The identification of differentially responsive proteins in tea leaves subjected to drought

Spot no.	Protein name	Accession no. ^a	Species	Theoretical		Experimental		Score ^b	%cov (matching peptides) ^c
				<i>M_r</i> (kDa)	pI	<i>M_r</i> (kDa)	pI		
1	39 kDa EF-Hand containing protein	CAA04670	<i>Solanum tuberosum</i>	39.01	4.65	60.13	5.34	50	2% (1)
2	Ascorbate peroxidase	ACB45429	<i>Camellia sinensis</i>	27.75	5.50	38.58	5.58	235	37% (4)
3	ATP synthase CF1 alpha subunit	NP_862739	<i>Calycanthus floridus</i> var. <i>glaucus</i>	55.21	5.20	41.46	5.50	227	10% (3)
4	Chloroplast phosphoribulokinase	BAL03266	<i>Closterium peracerosum</i> - <i>strigosum-littorale</i> complex	45.77	6.28	55.02	5.76	195	12% (3)
5	Chloroplast phosphoribulokinase	BAL03266	<i>Closterium peracerosum</i> - <i>strigosum-littorale</i> complex	45.77	6.28	55.65	5.67	140	12% (3)
6	Chloroplast ribulose 1,5-bisphosphate carboxylase/oxygenase activase	ABW80752	<i>Flaveria bidentis</i>	48.81	6.10	42.69	5.20	354	17% (6)
7	Glutamine synthetase, chloroplastic	O22506	<i>Daucus carota</i>	48.08	5.63	47.88	5.97	195	14% (3)
8	Glutamine synthetase	BAD99525	<i>Camellia sinensis</i>	39.40	5.79	45.99	5.99	361	35% (5)
9	Glutathione transferase lambda 2	NP_191064	<i>Arabidopsis thaliana</i>	33.15	6.78	39.26	6.29	61	4% (1)
10	Heat shock protein 90	ACX42226	<i>Ipomoea nil</i>	90.65	4.92	52.91	5.19	133	5% (4)
11	Light-induced protein, chloroplastic	P80471	<i>Solanum tuberosum</i>	35.67	5.26	41.21	5.22	68	3% (1)
12	Lipoxygenase	ACJ54281	<i>Camellia sinensis</i>	102.59	6.39	49.36	5.67	91	4% (3)
13	Os05g0187000	NP_001054834	<i>Oryza sativa Japonica Group</i>	29.26	6.45	37.22	6.18	76	5% (1)
14	Predicted: sedoheptulose-1,7-bisphosphatase, chloroplastic	XP_002263049	<i>Vitis vinifera</i>	42.90	5.95	52.37	5.62	427	18% (5)
15	Thioredoxin X	ACG44176	<i>Zea mays</i>	19.25	9.20	11.65	5.75	64	6% (1)
16	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	ACY64810	<i>Richea scoparia</i>	52.11	6.08	16.66	5.29	185	12% (3)
17	Ribulose 1,5-bisphosphate carboxylase	CAQ58398	<i>Prismatomeris sp. 1 Davis 4057</i>	52.12	6.34	18.53	6.06	275	12% (5)
18	Predicted protein	XP_002318440	<i>Populus trichocarpa</i>	26.38	8.12	17.76	5.54	277	19% (4)
19	Hypothetical protein VITISV_037064	CAN69818	<i>Vitis vinifera</i>	61.39	5.20	83.44	5.43	421	11% (5)
20	Unknown	ABK92500	<i>Populus trichocarpa</i>	80.87	6.29	15.00	6.84	45	1% (1)
21	Unknown	ABK94111	<i>Populus trichocarpa</i>	16.59	5.14	12.60	6.45	48	12% (2)

^a Accession number in NCBI database.

^b Score is a measure of the statistical significance of a match.

^c Percentage of predicted protein sequence covered by matched peptides.

and drought treatments. The results demonstrated upregulation of apolipoprotein D, fructose-bisphosphate aldolase, high molecular weight Hsp, short-chain dehydrogenase/reductase and Rubisco expression. Apolipoprotein D plays an important role in lipid transportation. Charron *et al.*⁵⁰ demonstrated that apolipoprotein D ortholog, AtTIL lipocalin, is involved in modulating tolerance to oxidative stress and provided the first evidence for a functional role of lipocalins in plants. Fructose-bisphosphate aldolase and short-chain dehydrogenase/reductase respectively participate in gluconeogenesis and the Calvin cycle⁵¹ and in oxidation-reduction processes.⁵² The high molecular weight (*M_r* or relative molecular mass) Hsp may be Hsp 70 (theoretical *M_r* 71.57 kDa, experimental *M_r* 60.23 kDa). Induction of Hsps is important in allowing cells to cope with acute stress, especially stresses affecting the protein machinery of plant cell.⁵³ Previous results showed that ABA-induced H₂O₂ production enhances the synthesis of Hsp70 and upregulates the activities of antioxidant enzymes, resulting in the suppression of cellular ROS levels.⁵⁴ In this study, Hsp70 expression was enhanced by drought in tea plant treated with exogenous ABA, which implied that ABA pre-treatment may improve plant tolerance to drought stress by increasing Hsp70 expression.

Rubisco is the major enzyme assimilating CO₂ from the atmosphere.^{55,56} Drought stress leads to a rapid decrease in the abundance of Rubisco small-subunit transcripts in tomato, *Arabidopsis* and rice.⁵⁷ But the short-term responses of Rubisco to drought stress are not clear, as different studies have produced conflicting results. In our study, drought stress leads to increase in the Rubisco large subunit. This information suggests that ABA-pre-treated tea

plants may accelerate the rate of photosynthesis through Rubisco, and the elevated photosynthesis increases the level of sugars, whose promoting influence on proline accumulation has been demonstrated in several studies.⁴¹ Cytochrome b6-f complex iron-sulfur subunit 2, aldehyde dehydrogenase family 2 member B4 and S-adenosylmethionine synthase 3, which participate in photosynthetic reactions, energy metabolism and S-adenosylmethionine biosynthesis, were downregulated under conditions of drought. Taken together, these results suggest that energy production and metabolism were highly disrupted by drought stress. However, the ABA pre-treatment of tea plants improved transportation of the proteins, carbon metabolism and expression of resistance proteins in the absence of drought. Result of the present study is compatible with those found in plants cells for other species.^{58,59} This study also provides a better understanding of molecular mechanism in drought tolerance of tea plant which would be a valuable resource for the further function study of exogenous ABA.

Poor detection of low-abundance proteins is a common problem when using 2-DE for protein separation in a proteome analysis. Most of the 72 identified protein spots were attributed to Rubisco, because Rubisco is the most abundant protein and makes up more than half of the total leaf proteins in some species.^{60,61} In tea leaves, Rubisco accounts for 50% of soluble leaf protein (20%–30% of total leaf nitrogen). Specific proteins that represent a large proportion of the whole-cell proteins are easily detected after separation, whereas low-abundance proteins may be taken up by the immobilized protein gradient strip in only small quantities and hence, are hardly visualized by 2-DE.^{61,62} It is apparent that

Table 3. The identification of differentially responsive proteins in exogenous ABA pre-treated tea leaves subjected to drought

Spot no.	Protein name	Accession no. ^a	Species	Theoretical		Experimental		Score ^b	%cov (matching peptides) ^c
				M _r (kDa)	pI	M _r (kDa)	pI		
1	Apolipoprotein d, putative	XP_002523505	<i>Ricinus communis</i>	21.63	6.33	16.78	6.63	55	11% (2)
2	Chloroplast phosphoribulokinase	BAL03266	<i>Closterium peracerosum-strigosum-littorale</i> complex	45.77	6.28	55.07	5.67	129	12% (3)
3	Cytochrome B6-F complex iron sulfur subunit 2	ABQ41994	<i>Sonneratia caseolaris</i>	16.83	5.75	18.84	5.61	220	28% (3)
4	Fructose-bisphosphate aldolase	ACG68894	<i>Sesuvium portulacastrum</i>	38.69	6.49	37.27	6.50	45	6% (1)
5	High molecular weight heat shock protein	AAF34134	<i>Malus × domestica</i>	71.57	5.17	60.23	5.42	454	11% (3)
6	Predicted: low-quality protein: aldehyde dehydrogenase family 2 member B4, mitochondrial-like	XP_003528154	<i>Glycine max</i>	59.02	6.30	68.63	6.28	65	4% (2)
7	Predicted: protein IN2-1 homolog B-like	XP_003554349	<i>Glycine max</i>	27.05	5.49	41.20	5.54	73	5% (1)
8	S-adenosylmethionine synthase 3	P50303	<i>Actinidia chinensis</i>	39.83	6.20	64.00	6.16	342	23% (4)
9	Short chain dehydrogenase/reductase	ACN87274	<i>Chelidonium majus</i>	32.98	4.86	41.04	6.35	66	5% (2)
10	Rubisco activase	ABU49419	<i>Camellia sinensis</i>	15.72	5.85	61.25	5.75	96	20% (2)
11	Ribulose bisphosphate carboxylase activase B	AAF71272	<i>Triticum aestivum</i>	48.01	6.92	73.51	5.48	466	18% (6)
12	Ribulose bisphosphate carboxylase large chain	P28426	<i>Ilex crenata</i>	51.97	6.10	20.80	6.56	343	12% (6)
13	Ribulose bisphosphate carboxylase large chain	P28426	<i>Ilex crenata</i>	51.97	6.10	35.11	6.65	346	10% (5)
14	Ribulose bisphosphate carboxylase large chain	P25830	<i>Cerastium glomeratum</i>	53.20	6.22	33.32	6.72	352	14% (4)
15	Ribulose 1,5-bisphosphate carboxylase	CA058398	<i>Prismatomeris</i> sp. 1 Davis 4057	52.12	6.34	15.08	5.52	225	10% (4)
16	Ribulose 1,5-bisphosphate carboxylase	CA058398	<i>Prismatomeris</i> sp. 1 Davis 4057	52.12	6.34	19.87	6.70	260	12% (5)
17	Ribulose 1,5-bisphosphate carboxylase large subunit	AAM33278	<i>Stachys affinis</i>	50.19	6.78	41.26	6.45	399	18% (5)
18	Ribulose 1,5-bisphosphate carboxylase large subunit	CAB02249	<i>Maesa myrsinoides</i>	51.65	6.34	68.80	6.70	422	25% (8)
19	Ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit	AAA21212	<i>Aruncus dioicus</i>	52.13	6.04	16.02	5.17	103	4% (1)
20	Ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit	CAB02254	<i>Stewartia pseudocamellia</i>	49.72	6.46	18.49	6.28	426	11% (4)
21	Ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit	AAK61184	<i>Nyssa yunnanensis</i>	49.87	5.95	12.60	5.25	48	5% (2)
22	Ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit	CAB00004	<i>Margaritaria tetraococca</i>	52.03	6.04	15.01	5.53	262	10% (4)
23	Ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit	AAK61184	<i>Nyssa yunnanensis</i>	49.87	5.95	12.62	5.21	48	5% (2)
24	Ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit	AAD29404	<i>Dactylopetalum ellipticifolium</i>	49.56	6.46	11.03	5.28	114	6% (2)
25	Ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit, partial (chloroplast)	AAV65404	<i>Apodiscus chevalieri</i>	51.64	6.04	38.92	6.53	465	18% (5)
26	Ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit, partial (chloroplast)	AAAB67920	<i>Securigera varia</i>	52.12	6.04	13.68	5.21	232	9% (3)
27	Ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit, partial (chloroplast)	AFI23110	<i>Psychotria graciliflora</i>	20.58	7.63	17.94	5.27	143	31% (1)
28	Predicted: ribulose bisphosphate carboxylase/oxygenase activase, chloroplastic	XP_002282979	<i>Vitis vinifera</i>	48.21	5.50	53.47	5.68	398	16% (4)
29	Chloroplast ribulose 1,5-bisphosphate carboxylase/oxygenase activase	ABW80752	<i>Flaveria bidentis</i>	48.81	6.10	41.32	5.18	285	17% (6)
30	Predicted: uncharacterized protein LOC100251175 isoform 1	XP_002271111	<i>Vitis vinifera</i>	19.01	4.53	21.71	5.15	179	20% (2)
31	Predicted protein	XP_002306773	<i>Populus trichocarpa</i>	41.20	6.67	53.28	6.71	203	9% (3)
32	Unknown	ACU23262	<i>Glycine max</i>	33.46	6.84	11.65	5.75	58	4% (1)
33	Unnamed protein product	CB123038	<i>Vitis vinifera</i>	25.28	6.82	22.75	5.59	117	21% (3)

^a Accession number in NCBI database.

^b Score is a measure of the statistical significance of a match.

^c Percentage of predicted protein sequence covered by matched peptides.

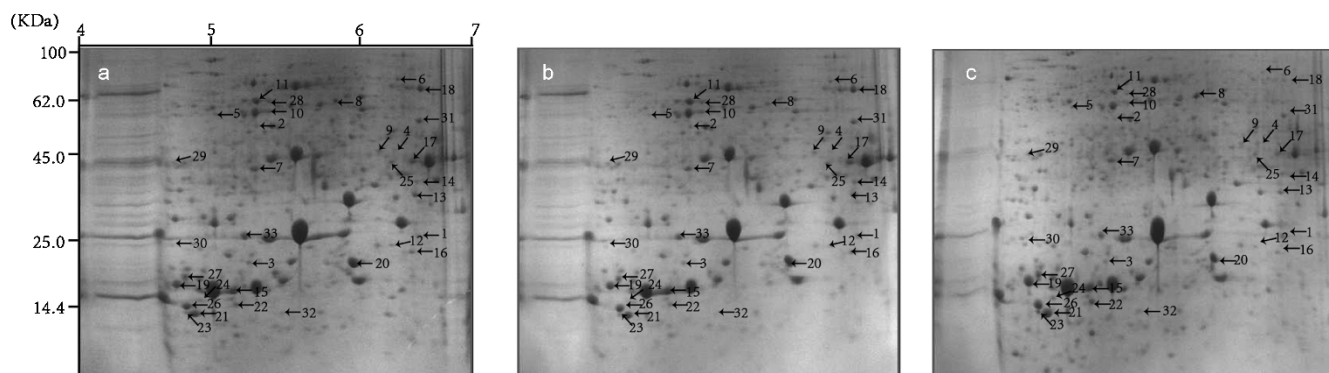


Figure 5. A 2-DE analysis of tea leaf proteins. (a) 2-DE gels of tea leaf subjected to distilled water (control); (b, c) 2-DE gels of tea leaf subjected to drought treatment for 12 h and 72 h. This is a representative figure from three biological replicas.

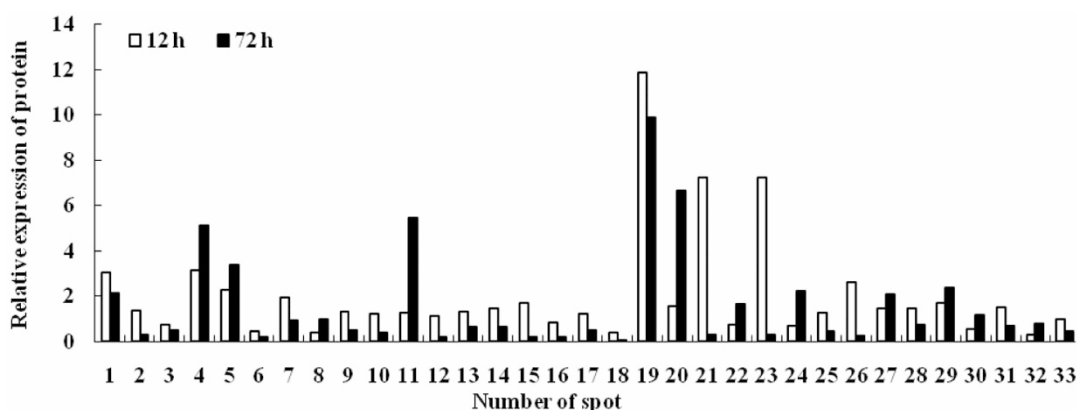


Figure 6. Histograms show the abundance ratio of the identified proteins in exogenous ABA pre-treated tea leaves subjected to drought at 12 h and 72 h

developing efficient methods to minimize the high-abundance proteins has become an important goal in applications of 2-DE for proteome analysis. One solution is to use root tissue harvested from treated and nontreated plants for proteomic identification, because root tissue does not exhibit an over-representation of highly abundant proteins such as Rubisco found in green tissue.⁶³ Roots are also the first sensors of water stress in soil thus would likely provide complementary information in terms of proteins responsive to ABA and drought treatment.

In conclusion, tea has a long legacy in China and its popularity is increasing dramatically throughout the world, making it an important economic crop. Currently, droughts are occurring much more frequently, and in more seasons, and spreading to more regions across the country. Tea production has suffered in both quantity and quality, especially spring tea. In the present study, using comparative proteomics, we identified proteins associated with the drought-stress response and demonstrated ABA regulation of these. This study also demonstrated that ABA pre-treatment was a valuable approach at mitigating drought stress in tea plants under laboratory conditions. This information contributes to the knowledge of the mechanism of resilience to abiotic stress in tea plant, and will be helpful for the further study of the function of exogenous ABA in this species. In future, we hope to extend the findings of this paper to field conditions.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (30800884, 31370688) and the Jiangsu Science and Technology Program of China (BE2011319).

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