Biochemical and Biophysical Research Communications xxx (2015) 1–8

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

iTRAQ-based quantitative proteomic analysis reveals proteomic changes in leaves of cultivated tobacco (*Nicotiana tabacum*) in response to drought stress

He Xie [1], Da-Hai Yang [*, 1], Heng Yao, Ge Bai, Yi-Han Zhang, Bing-Guang Xiao

Tobacco Breeding and Biotechnology Research Center, Yunnan Academy of Tobacco Agricultural Sciences, Key Laboratory of Tobacco Biotechnological Breeding, National Tobacco Genetic Engineering Research Center, Kunming 650021, China

ARTICLE INFO

Article history:
Received 24 November 2015
Accepted 30 November 2015
Available online xxx

Keywords:
Tobacco
Proteome
iTRAQ
Drought
Redox
Heat shock protein

ABSTRACT

Drought is one of the most severe forms of abiotic stresses that threaten the survival of plants, including crops. In turn, plants dramatically change their physiology to increase drought tolerance, including reconfiguration of proteomes. Here, we studied drought-induced proteomic changes in leaves of cultivated tobacco (*Nicotiana tabacum*), a solanaceous plant, using the isobaric tags for relative and absolute quantitation (iTRAQ)-based protein labeling technology. Of identified 5570 proteins totally, drought treatment increased and decreased abundance of 260 and 206 proteins, respectively, compared with control condition. Most of these differentially regulated proteins are involved in photosynthesis, metabolism, and stress and defense. Although abscisic acid (ABA) levels greatly increased in drought-treated tobacco leaves, abundance of detected ABA biosynthetic enzymes showed no obvious changes. In contrast, heat shock proteins (HSPs), thioredoxins, ascorbate-, glutathione-, and hydrogen peroxide (H_2O_2)-related proteins were up- or down-regulated in drought-treated tobacco leaves, suggesting that chaperones and redox signaling are important for tobacco tolerance to drought, and it is likely that redox-induced posttranslational modifications play an important role in modulating protein activity. This study not only provides a comprehensive dataset on overall protein changes in drought-treated tobacco leaves, but also shed light on the mechanism by which solanaceous plants adapt to drought stress.

1. Introduction

Drought severely affects plant growth and development. In agriculture, drought causes severe yield reduction all over the world and leads to remarkable economic losses. It has been predicted that due to climate change, especially the increasing temperature and shortage of fresh water, the occurrence of drought will be more frequent in many areas, and drought will increase its impact on food security and ecological stability.

Drought stress changes almost every aspect of plant physiology, including ion and osmotic homeostasis, photosynthesis activity, and growth and development [1]. In response to drought, higher plants rapidly activate the production of abscisic acid (ABA), the major phytohormone that regulates plant tolerance to drought. ABA signaling not only controls the stomatal closure response, which is an effective and necessary way of preserving water, but also regulates the transcript levels of a large number of genes [2], thus playing a critical role in drought tolerance. Using genetic screening, many elements in regulating plant tolerance to drought have been identified [2], including genes involved in ABA biosynthesis and perception [3,4] and various transcription factors [5,6].

The rapid development of next-generation sequencing technologies and bioinformatic tools has made transcriptome analysis a cost-effective and high-throughput approach of gaining insight into plant responses to drought stress. However, increasing lines of evidence have indicated that transcriptome are often inconsistent with proteome, due to the complexity of the regulation of protein abundances [7–9]. Given the critical role of proteins in almost all cellular functions, proteomic analyses, which provide comprehensive qualitative and quantitative information on hundreds to thousands of proteins, have become an important tool for studying various biological processes.

* Corresponding author.
E-mail addresses: xiehe126@126.com (H. Xie), gresea_young@hotmail.com (D.-H. Yang), 334758770@qq.com (H. Yao), 30431@163.com (G. Bai), duckzyhsm@163.com (Y.-H. Zhang), xiaobg@263.net (B.-G. Xiao).
[1] These authors equally contributed to the research.

http://dx.doi.org/10.1016/j.bbrc.2015.11.133

Please cite this article in press as: H. Xie, et al., iTRAQ-based quantitative proteomic analysis reveals proteomic changes in leaves of cultivated tobacco (*Nicotiana tabacum*) in response to drought stress, Biochemical and Biophysical Research Communications (2015), http://dx.doi.org/10.1016/j.bbrc.2015.11.133

Mass spectrometry-based proteomic analyses have been used in studying plant responses to drought. Hajheidari et al. [10] studied the proteomic response of wheat grain to drought, and found that about 60% of the identified proteins whose abundance were affected by drought treatment were related with redox, suggesting the importance of redox in drought tolerance in wheat grain. As probably the first organ to sense drought stress, drought-treated root has been studied in soybean, and it was found that proteins involved in carbohydrate and nitrogen metabolism, cell wall modification, signal transduction, cell defense, and programmed cell death were differentially regulated [11]. Leaves of wheat and barley were also studied for their responses to drought on a proteome level [12,13]. Furthermore, proteomic analysis on rice split-root system and on maize phloem sap shed light on the root-shoot signaling during drought adaptation [14,15]. A phosphoproteomic approach was used to study changes in protein phosphorylation status in maize, providing information of drought-induced protein post-translation modifications [16].

Tobacco (*Nicotiana tabacum*; Solanaceae) has been widely used as a model in plant biology. Here we treated tobacco plants with drought for two days, and when plants were mildly dehydrated, leaves were harvested for isobaric tags for relative and absolute quantitation (iTRAQ)-based proteomic analysis. In total, 5570 proteins were identified, among which 260 and 206 proteins showed increased and decreased abundance, respectively. Most of them are involved in photosynthesis, metabolism, and stress and defense. On the proteomic level, this study provides insight into the physiological reconfiguration in leaves of tobacco, a model of solanaceous plant, in response to drought stress.

2. Materials and methods

2.1. Plant materials, growth conditions, and drought stress treatments

Tobacco (*N. tabacum*) cultivar Honghuadajingyuan seedlings were germinated on 1/2 MS medium and kept in a growth chamber maintained at 25 °C with a 16/8 h light and dark cycles for seed germination. Twelve days old seedlings were then transferred to soil in small plastic pots for 7 days until they were transferred to 1 L plastic pots. When the plants were about 40 days old, watering was stopped, and leaves were harvested after 2 days when they showed a mild dehydration phenotype (drought treatment, T); the leaves from well-watered control group (unstressed control, CK) was harvested at the same time. Leaf samples were immediately frozen in liquid nitrogen and stored at −80 °C.

2.2. Extraction and quantification of ABA

Around 100 mg of frozen leaf tissue were ground in liquid nitrogen and 1 ml of ethyl acetate spiked with 20 ng of the internal standard ($^2H_6$)-ABA was added to each sample. Each sample was vortexed for 10 min, followed by centrifugation at 13,000 *g* for 15 min at 4 °C. The supernatants were evaporated to dryness in a vacuum concentrator (Eppendorf, Hamburg, Germany) at 45 °C, and then samples were resuspended in 600 μl of a methanol: water (70: 30, volume/volume) solution and centrifuged again to remove particles. The supernatants were analyzed on a high performance liquid chromatography-tandem mass spectroscopy (Shimadzu 8040 HPLC-MS/MS system).

2.3. Protein extraction

Samples were initially frozen dried. The powder was resuspended in 200 μl of dissolution buffer (0.5 M triethyl ammonium bicarbonate at pH 8.5). The protein disulfide bonds of the solution sample were reduced for 30 min with 5 mM dithiothreitol at room temperature and alkylated for 30 min with 15 mM iodoacetamide in the dark. Then, 800 μl of cold acetone was used to precipitate proteins at −20 °C for about 2 h. After centrifugation at 13,000 *g* for 20 min at 4 °C, the precipitates were collected and washed with 800 μl of cold acetone for two times. After centrifugation at 13,000 *g* for 20 min at 4 °C, the supernatants were removed and the precipitates were dried and stored at −80 °C for later use.

2.4. Protein quantification and digestion

Total protein concentrations were measured using the Bradford method [17]. For each sample, 100 μg of protein were dissolved in 500 μl of dissolution buffer, and then diluted with 500 μl of 50 mM NH_4HCO_3. After being reduced and alkylated, 2 μg of trypsin were added to the proteins and were incubated overnight at 37 °C for protein digestion. After protein digestion, equal volumes of 0.1% formic acid (FA) were added. Peptides were purified on a Strata-X C18 column for 3 times, washed with 5% acetonitrile (ACN) and 0.1% FA twice, and eluted with 1 ml 80% ACN and 0.1% FA. Eluted peptides were dried in a vacuum concentrator and redissolved in 20 μl of 0.5 M TEAB (tetraethylammonium bromide) for peptide labeling.

2.5. iTRAQ labeling and fractionation

Samples were labeled with iTRAQ Reagent-8 plex Multiplex Kit (AB Sciex U.K.) according to the manufacturer's instructions. All of the labeled samples were mixed with equal amount. Next, the labeled samples were fractionated using a high-performance liquid chromatography (HPLC) system (Thermo Dinoex Ultimate 3000 BioRS) equipped with a Durashell C18 (5 μm, 100 Å, 4.6 × 250 mm) column. A total of 12 fractions were collected.

2.6. LC-MS/MS analysis

LC-MS/MS analysis was performed on an AB SCIEX nanoLC-MS/MS (Triple TOF 5600 plus) system. Samples were chromatographed using a 120-min gradient from 2 to 35% (mobile phase A: 0.1% (v/v) FA, 2% (v/v) ACN; mobile phase B: 0.1% (v/v) FA, 90% (v/v) ACN after direct injection onto a 20 cm PicoFrit emitter (New Objective) packed to 20 cm with Magic C18 AQ 3-μm 200 Å stationary phase. MS1 spectra were collected in the range 360–1460 *m/z* for 250 ms. The 20 most intense precursors with charge state 2–5 were selected for fragmentation, and MS2 spectra were collected in the range 50–2000 *m/z* for 100 ms; precursor ions were excluded from reselection for 15 s.

2.7. Data analysis

The original MS/MS file data were submitted to ProteinPilot Software v4.5 for data analysis. For protein identification, the Paragon algorithm [18] which was integrated into ProteinPilot was employed against Uniprot Bos taurus database (31,872 items, update in April 2015) for database searching. The parameters were set as follows: the instrument was TripleTOF 5600, iTRAQ quantification, cysteine modified with iodoacetamide; biological modifications were selected as ID focus, trypsin digestion, the Quantitate, Bias Correction and Background Correction was checked for protein quantification and normalization. For false discovery rate (FDR) calculation, an automatic decoy database search strategy [19] was employed to estimate FDR using the PSPEP (Proteomics System Performance Evaluation Pipeline Software, integrated in the ProteinPilot Software). Only unique peptides whose confidence was

Please cite this article in press as: H. Xie, et al., iTRAQ-based quantitative proteomic analysis reveals proteomic changes in leaves of cultivated tobacco (*Nicotiana tabacum*) in response to drought stress, Biochemical and Biophysical Research Communications (2015), http://dx.doi.org/10.1016/j.bbrc.2015.11.133

more than 95% were contained in iTRAQ labeling quantification, and protein with the unused value more than 1.3 were considered for further analysis. For biological replicates or technology replicates experiment, the fold change was the ratio of protein abundance between samples to be compared which are determined by the median abundance of its replicate samples, and the p-values provided by student's *t*-test, to reduce the overall false positive; the Benjamini-Hochberg Multiple Hypothesis Testing was used to adjust our statistical confidence measures based on the number of tests performed, that was the corrected p-value (q-value). Proteins with a fold change larger than 1.2 (T:CK ratio≥1.2 or T:CK ratio≤0.83) and q-value less than 0.01 were considered to be significantly differentially expressed. The Hierarchical clustering was performed with Genesis program [20].

2.8. Bioinformatics and annotations

To determine the biological and functional properties of all the identified proteins, the identified protein sequences were mapped with Gene Ontology Terms (http://geneontology.org/). For this, homology search was first performed for all the identified sequences with a localized NCBI blastp program against NCBI nr database. The e-value threshold was set to less than 1e-5, and the best hit for each query sequence was taken account for GO term matching. The GO term matching was performed with blast2go v4.5 pipeline [21]. Clusters of Orthologous Groups of Proteins System (COG, http://www.ncbi.nlm.nih.gov/COG/) were employed for the functional annotation of genes from new genomes and for research into genome evolution. To identify candidate biomarkers, we employed hypergeometric test to perform GO enrichment and Pathway enrichment.

3. Results

3.1. Drought treatment increases ABA levels, but not abundance of ABA biosynthesis enzymes/putative receptors in tobacco leaves

In order to minimize the side-effects induced by severe drought stress, such as program cell death, Tobacco (*N. tabacum*) were regularly watered to avoided dehydration until being treated with drought stress. Then we stopped watering for 2 days, until plants showed a mild drought phenotype (T), and plants well watered were used for comparison (controls, CK) (Supplementary Fig. 1).

Given the important role of plant hormone ABA in mediating drought stress responses, ABA contents were determined in control and drought-treated tobacco leaves (Fig. 1). Consistent with the turgor loss, drought stress increased tobacco leaf ABA concentration ca. 2.7 fold (from 66.0 to 242.7 ng/g fresh weight (FW)). ABA receptors and biosynthesis enzymes were searched in the detected proteins, but neither putative receptor proteins nor biosynthesis enzymes (abscisic-aldehyde oxidase, zeaxanthin epoxidase, and short-chain dehydrogenase) showed significant changes of abundance after drought stress (Supplementary Table 1).

3.2. Identification of differentially expressed proteins in leaves of tobacco response to drought

Total proteins were extracted from the above-mentioned tobacco leaf samples (drought treatment (T) and untreated control (CK); three biological replicates were used for each condition), and were subjected to iTRAQ labeling and 2D LC-MS/MS analysis. A total of 5570 proteins were identified in these samples (Supplementary Table 2), and these proteins covered a wide range of biological processes, including metabolic process (18.61%), cellular process

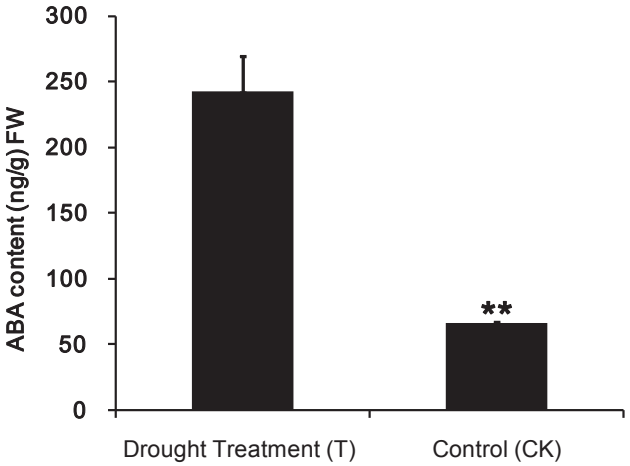


Fig. 1. ABA levels in drought-treated and control tobacco leaves. Tobacco plants were drought-treated for 2 days by stopping watering, and well-watered plants served as controls, and the ABA levels in the leaves were determined (average ± SE, n = 5). Asterisks indicate significant differences between drought-treated plants and control plants determined by Student's *t*-test (**, P < 0.01). FW = fresh weight.

(18.8%), response to stimulus (10.05%), localization (5.69%), and signaling (1.87%) (Fig. 2).

We found that 260 and 206 proteins exhibited increased (T:CK ratio≥1.2, q-value<0.01) and decreased (T:CK ratio≤0.83, q-value<0.01) levels, respectively, between untreated control and drought-stressed samples (Supplementary Table 3). Hierarchical clustering of all differentially regulated proteins indicated that drought treatment induced large changes in protein levels (Fig. 3). Furthermore, gene ontology analysis showed that a large number of proteins involved in “stress and defense” were up- or down-regulated, and a number of proteins in “photosynthesis” and “metabolism” were also differentially regulated (Fig. 4). Thus, drought stress strongly reshapes tobacco proteome by influencing many aspects of plant physiology, including photosynthesis, metabolism, and stress responses.

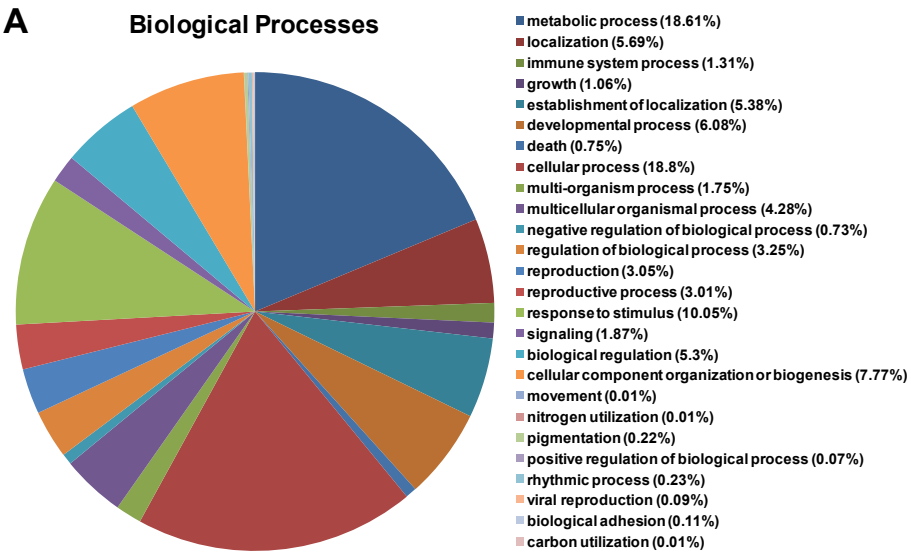
The top 30 up-regulated proteins (Supplementary Table 4) included aquaporins and heat shock proteins (HSPs), and these proteins are known to be important for water transport and stress adaptation [22,23]. Notably, histone H4 isoform X1 (gi|698497211) was the most up-regulated protein (~9-fold increase), indicating that drought stress may influence gene transcription through modification of chromosomal structure. Many chloroplastic proteins, such as chloroplast ribosomal protein L4 (gi|3298439), ABC transporter D family member 2 (chloroplastic isoform X1) (gi|698462547), and cytochrome b6 (gi|80750957), elevated their levels in drought-treated samples.

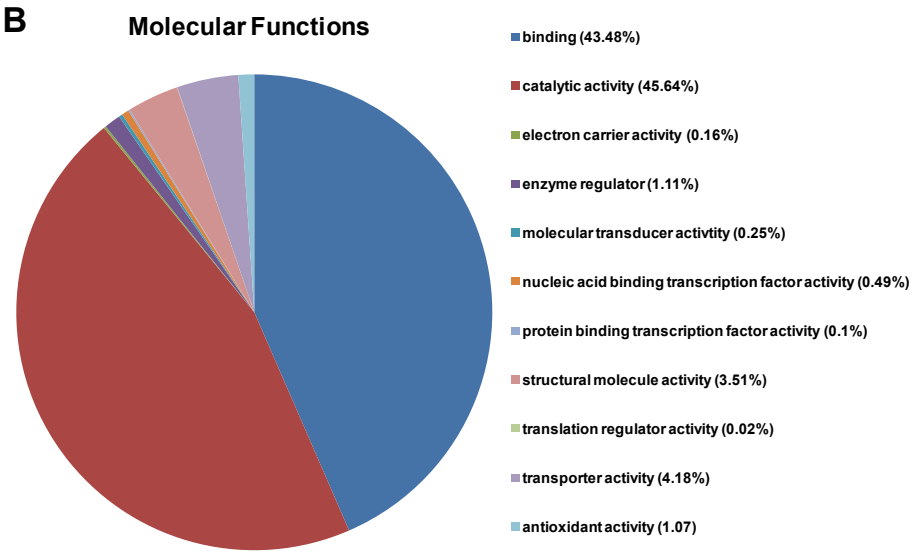
Strikingly, several chloroplast-located proteins were also highly decreased (Supplementary Table 5), such as oxygen-evolving enhancer protein 3-2 (gi|697160242). The parts of the photosynthesis machinery, ribulose bisphosphate carboxylase/oxygenase (RuBisCO) activase (gi|697114648) and the small subunit of RuBisCO (gi|20024) were both highly decreased (58 and 53%, respectively). We noticed that some isoforms of chlorophyll a/b-binding (CAB) protein showed increased levels, but some isoforms of CAB had reduced abundance. These data clearly point to the importance of rearranging chloroplastic proteins in response to drought stress in tobacco.

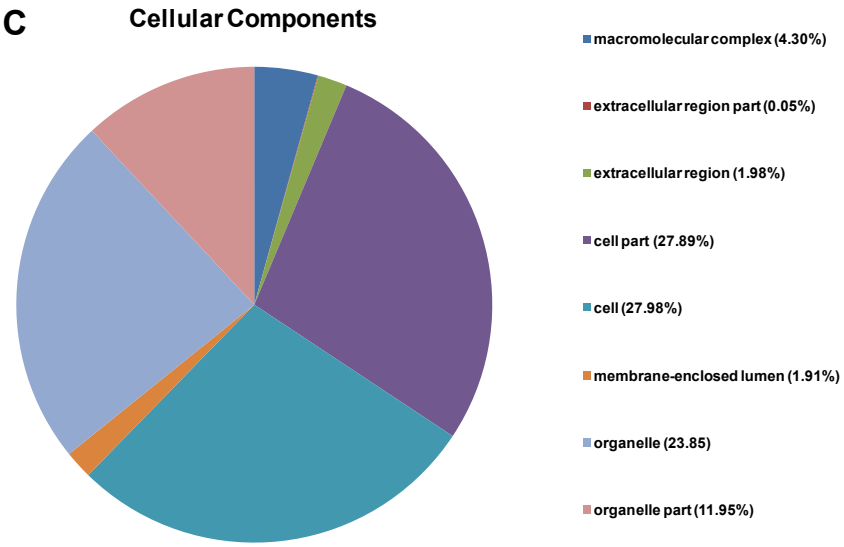
3.3. Analysis of stress-related members of heat shock protein family

Heat shock and redox-related proteins are involved in plant

Please cite this article in press as: H. Xie, et al., iTRAQ-based quantitative proteomic analysis reveals proteomic changes in leaves of cultivated tobacco (*Nicotiana tabacum*) in response to drought stress, Biochemical and Biophysical Research Communications (2015), http://dx.doi.org/10.1016/j.bbrc.2015.11.133







Please cite this article in press as: H. Xie, et al., iTRAQ-based quantitative proteomic analysis reveals proteomic changes in leaves of cultivated tobacco (*Nicotiana tabacum*) in response to drought stress, Biochemical and Biophysical Research Communications (2015), http://dx.doi.org/10.1016/j.bbrc.2015.11.133

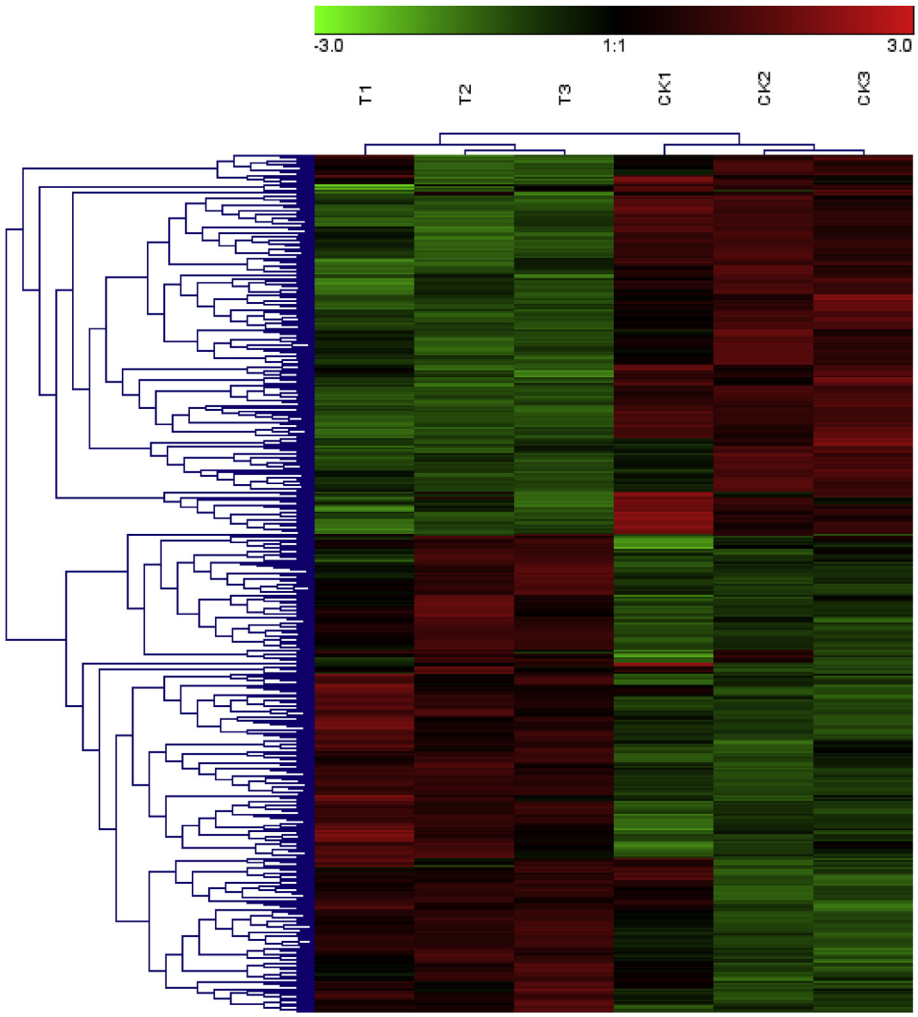


Fig. 3. Hierarchical clustering of differentially expressed proteins under drought stress. Hierarchical clustering of the 466 differentially expressed proteins. Top, sample tree (T1, T2, T3, 3 replicates treated with drought stress; CK1, CK2, CK3, 3 replicates of controls). The color scale bar on the top, green and red indicate decreased and increased levels, respectively, and black indicates no significant changes of proteins in response to drought stress. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

adaptation to various stresses [22,24,25]. Thus, we searched all the drought-induced differentially regulated proteins that belong to these protein families.

Heat shock protein 82 (gi|697107663), which is a HSP90-like protein, showed the highest level of induction (1.38-fold increased) by drought treatment, and increased abundance was found for the heat shock 70 kDa protein 17 (gi|698521745) and heat shock cognate 70 kDa protein 2-like protein (gi|697104555) (Table 1). However, conversely, the levels of three HSPs (gi|697165190, gi|698451062, gi|698477814) decreased (Table 1), suggesting that in adaptation to drought stress, some types of HSPs are required to be reduced.

3.4. Analysis of stress-related members of redox-related protein family

Redox balance plays an important role in protecting plants from drought-induced oxidative stress, and redox signaling also controls a large number of transcripts, that are essential for stress adaptation [26]. Thus, the proteins involved in redox were searched in the differentially expressed proteins (Table 2).

Thioredoxins (Trxs) are well conserved disulfide reductases that control the redox status of target proteins, and in plants, Trxs are shown to be critical for plant tolerance of oxidative stress [27]. Strikingly, five Trxs and one Trx precursor were found, and all these six Trxs exhibited 21–54% decreased levels (Table 2).

Given the importance of ascorbate and glutathione in redox, proteins important for ascorbate and glutathione homeostasis were screened in the identified proteins [28]. The biosynthesis of ascorbic acid (vitamin C; VC; also ascorbate) requires nine steps, starting from hexose phosphate isomerase and ending with L-galactono-1,4-lactone dehydrogenase [29]. Most VC-biosynthetic enzymes did not show significant differences after drought treatment, except a mitochondrial L-galactono-1,4-lactone dehydrogenase showed a minor decrease; in contrast, some ascorbate peroxidases and ascorbate oxidases, two enzymes that consume VC, had somewhat decreased levels (Table 2), suggesting that after drought treatment, tobacco plants may elevate VC content by decreasing the activity of VC catabolism enzymes.

Regarding glutathione-related proteins, we found that two glutathione S-transferases (GSTs) increased 58 and 21%

Fig. 2. Subcellular localization and functional classification of the 5570 tobacco proteins identified in the iTRAQ proteomics analysis. All the identified proteins (5570 in total) were categorized according to (A) Biological processes; (B) Molecular functions; (C) Cellular components.

Please cite this article in press as: H. Xie, et al., iTRAQ-based quantitative proteomic analysis reveals proteomic changes in leaves of cultivated tobacco (*Nicotiana tabacum*) in response to drought stress, Biochemical and Biophysical Research Communications (2015), http://dx.doi.org/10.1016/j.bbrc.2015.11.133

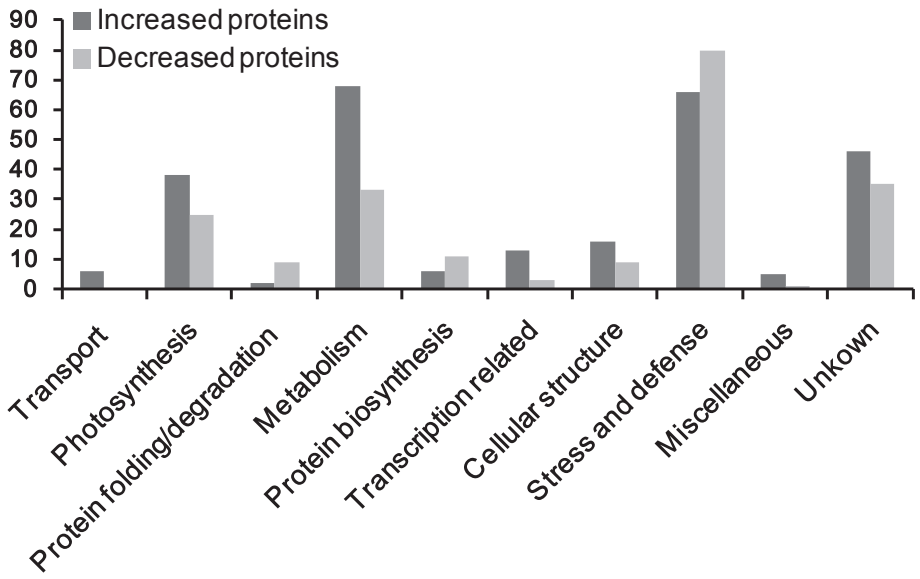


Fig. 4. Functional classification of the differentially expressed proteins in response to drought stress. Differentially expressed proteins were categorized according to their gene ontology terms, and in each category, the numbers of proteins up- and down-regulated are shown (Y-axis indicates the number of proteins with increased or decreased abundance).

Table 1
Heat shock proteins differentially expressed in leaves of drought-stress tobacco plants compared to controls.

Accession[a]	Protein Name[b]	T:CK[c]	p-value[d]
gi|697107663	heat shock protein 82	2.38	0.0000
gi|697104555	heat shock cognate 70 kDa protein 2-like	1.59	0.0013
gi|698521745	heat shock 70 kDa protein 17	1.35	0.0013
gi|697165190	heat shock cognate protein 80	0.78	0.0001
gi|698451062	heat shock protein 90-1-like	0.78	0.0001
gi|698477814	heat shock cognate 70 kDa protein 2	0.78	0.0047

[a] Protein gi number from NCBI.
[b] Name of the protein identified by MS/MS.
[c] The ratio between protein levels in drought stress-treated (T) and control (CK) plants.
[d] Student's *t*-test p-value.

respectively, while another GST showed 33% reduced levels; a glutathione reductases and a glutathione peroxidase-like protein were down- and up-regulated, respectively (Table 2).

Given that H_2O_2 is an important form of redox signaling compound and a product of superoxide, proteins involved in H_2O_2 metabolism were searched in the regulated proteins. We found that several superoxide dismutases (SODs) were reduced (~10%) in drought-treated leaves, including one mitochondrial SOD and one chloroplastic SOD, and several catalases were strongly down-regulated (22–45%) (Table 2). Thus, redox-related physiological changes are an important part of tobacco response to drought stress.

4. Discussion

In this study, taking advantage of iTRAQ-based quantitative proteomics technology, we investigated the response of tobacco to drought stress on a proteome-scale. More than 460 proteins, which were almost 8.4% of all detected 5570 proteins, were up- or down-regulated in drought-treated tobacco leaves, indicating that drought strongly influences plant physiology.

ABA signaling plays a critical role in plant tolerance to drought [30]. Drought-induced increase in ABA content usually results in stomatal closure, by which plants decrease transpiration and conserve water. We detected a 2.7-fold increase of ABA content in tobacco leaves 2 days after stopping watering. However, no protein abundance changes of ABA biosynthesis-related enzymes were detected at that time point. This might be due to the fact that we slowly dehydrated the plants.

Among the top 30 up- and 30 down-regulated proteins, we found that a large portion of these proteins was chloroplastic or mitochondrial (Supplementary Tables 4 and 5). Therefore, it is likely that drought stress strongly affects the function of chloroplasts and mitochondria. Retrograde signaling has been found to be involved in ABA-induced responses [31,32], influencing the communication between nuclear and plastid/mitochondrion. ABA is synthesized from precursors in plastids, and subsequently synthesized and catabolized in cytoplasm [30]. Clearly, retrograde signaling plays an important role in modulating the crosstalk between nuclear and chloroplasts and mitochondria, controlling not only ABA metabolism but also various drought-induced responses.

Increasing lines of evidence have revealed that HSPs are not only important for maintaining protein stability under heat stress, but are involved in various stress responses, including drought. In Arabidopsis, a calcium-dependent protein kinase (CPK), CPK10, is important for ABA-mediated stomatal closure, and it was found that HSP1 interacts with CPK10, and thus *cpk10* and *hsp1* mutants exhibited a similar drought-sensitive phenotype [33]. Transcriptional profiling in Arabidopsis also suggested that HSPs are involved in all stress conditions tested, including drought [22]. Our proteomic data further indicate that modulation of HSPs is an essential part of physiological changes in plant drought tolerance. Importantly, not all HSPs elevate their levels, but some HSPs even reduce their levels during drought stress. Similarly, a HSP70 was found to be reduced in drought-treated rapeseed (*Brassica napus* L.) roots [34], and HSP17 and three HSP70 showed decreased levels in a drought-susceptible cultivar of wheat [10]. How HSPs function in plant drought resistance deserves further in-depth study.

Trxs regulate the redox status of target proteins, by reducing protein disulfide bonds [27]. In the proteomic data, we found that several Trxs had decreased levels in drought-treated tobacco, and no Trxs were increased (Table 2). However, in the seeds of drought-treated wheat, different Trxs had tolerant and susceptible cultivar-specific responses [10].

Please cite this article in press as: H. Xie, et al., iTRAQ-based quantitative proteomic analysis reveals proteomic changes in leaves of cultivated tobacco (*Nicotiana tabacum*) in response to drought stress, Biochemical and Biophysical Research Communications (2015), http://dx.doi.org/10.1016/j.bbrc.2015.11.133

Table 2
ROS (reactive oxygen species)-related proteins differentially expressed in drought-stressed and control plants.

Accession[a]	Protein Name[b]	T:CK[c]	p-value[d]
gi|697148344	thioredoxin-like	0.79	0.0000
gi|698466597	thioredoxin-like 3-2, chloroplastic isoform X1	0.79	0.0059
gi|698568566	thioredoxin M3, chloroplastic	0.75	0.0022
gi|697142568	thioredoxin-like	0.71	0.0000
gi|698428246	thioredoxin	0.46	0.0000
gi|312231980	plastid thioredoxin F precursor	0.57	0.0000
gi|698538296	nucleobase-ascorbate transporter 12	1.35	0.0086
gi|697133021	putative L-ascorbate peroxidase 6	0.89	0.0453
gi|698473253	L-ascorbate peroxidase 1	0.89	0.0000
gi|698558368	L-ascorbate peroxidase 2	0.92	0.0048
gi|698532420	L-ascorbate oxidase isoform X2	0.70	0.0043
gi|697153314	L-ascorbate oxidase homolog isoform X3	0.85	0.0030
gi|697128386	L-galactono-1,4-lactone dehydrogenase, mitochondrial	0.92	0.0022
gi|697140024	glutathione S-transferase-like	1.58	0.0001
gi|698440256	probable glutathione S-transferase parC	1.22	0.0000
gi|697124131	probable glutathione S-transferase	0.67	0.0000
gi|697188031	glutathione reductase	0.88	0.0177
gi|698579037	probable glutathione peroxidase 2	1.24	0.0180
gi|697098099	superoxide dismutase [Cu–Zn]	0.91	0.0025
gi|697171181	superoxide dismutase [Mn], mitochondrial	0.87	0.0000
gi|698489923	superoxide dismutase [Fe], chloroplastic	0.86	0.0000
gi|697100359	catalase isozyme 1	0.55	0.0333
gi|697176653	catalase isozyme 1	0.78	0.0180
gi|698491984	catalase isozyme 1	0.58	0.0408
gi|697155765	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 9, mitochondrial	1.25	0.0024
gi|698549852	NADH dehydrogenase [ubiquinone] iron-sulfur protein 6, mitochondrial	0.53	0.0006
gi|698453009	NADP-dependent malic enzyme	0.80	0.0027

[a] Protein gi number from NCBI.
[b] Name of the protein identified by MS/MS.
[c] The ratio between protein levels in drought stress-treated (T) and control (CK) plants.
[d] Student's *t*-test p-value ($p < 0.01$, and six proteins with $0.01 < p < 0.05$ are also listed).

In addition to Trxs, our proteomic data also revealed a number of proteins related to ascorbate, glutathione, and reactive oxygen species (ROS) were up- or down-regulated in drought-treated tobacco, indicating that regulation of redox pathways is essential for plants tolerance to drought. The involvement of ROS has been intensively studied genetically. In Arabidopsis, drought-induced ABA activates rbohD- and rbohF-mediated H_2O_2 production in guard cells, and H_2O_2 triggers increase of cytosolic [Ca^{2+}], and subsequent closure of stomata [35].

Much research, including studies applying more sensitive proteomic approaches and focusing on more detailed time series, is still needed to understand which proteins are needed for redox homeostasis, and which proteins are important for redox signaling transduction.

Although increase in sensitivity in proteomic analyses is still a long-term goal that is needed to gain further insight into the changes of low abundant proteins, such as transcription factors. Proteomic work focusing on protein posttranslational modifications will also provide important information on the mechanism of drought-induced protein activity regulation.

Conflict of interest

The authors declared that they have no conflicts of interest to this work.

Acknowledgments

This work was supported by Projects (2013YN06 and 2015YN09) from the Yunnan Academy of Tobacco Agricultural Sciences, YNTC, Kunming, China, and Project (110201401011 (JY-11)) from CNTC, China.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.11.133.

References

[1] J.K. Zhu, Salt and drought stress signal transduction in plants, Annu. Rev. Plant Biol. 53 (2002) 247–273.
[2] S.R. Cutler, P.L. Rodriguez, R.R. Finkelstein, et al., Abscisic acid: emergence of a core signaling network, Annu. Rev. Plant Biol. 61 (2010) 651–679.
[3] S.Y. Park, P. Fung, N. Nishimura, et al., Abscisic acid inhibits type 2C protein phosphatases via the PYR/PYL family of START proteins, Science 324 (2009) 1068–1071.
[4] J. Santiago, F. Dupeux, A. Round, et al., The abscisic acid receptor PYR1 in complex with abscisic acid, Nature 462 (2009) 665–668.
[5] T. Yoshida, Y. Fujita, H. Sayama, et al., AREB1, AREB2, and ABF3 are master transcription factors that cooperatively regulate ABRE-dependent ABA signaling involved in drought stress tolerance and require ABA for full activation, Plant J. 61 (2010) 672–685.
[6] P.J. Seo, F. Xiang, M. Qiao, et al., The MYB96 transcription factor mediates abscisic acid signaling during drought stress response in Arabidopsis, Plant Physiol. 151 (2009) 275–289.
[7] Y. Taniguchi, P.J. Choi, G.W. Li, et al., Quantifying E-coli proteome and transcriptome with single-molecule sensitivity in single cells, Science 329 (2010) 533–538.
[8] C. Vogel, E.M. Marcotte, Insights into the regulation of protein abundance from proteomic and transcriptomic analyses, Nat. Rev. Genet. 13 (2012) 227–232.
[9] J.W. Walley, Z.X. Shen, R. Sartor, et al., Reconstruction of protein networks from an atlas of maize seed proteotypes, Proc. Natl. Acad. Sci. U. S. A. 110 (2013) E4808–E4817.
[10] M. Hajheidari, A. Eivazi, B.B. Buchanan, et al., Proteomics uncovers a role for redox in drought tolerance in wheat, J. Proteome Res. 6 (2007) 1451–1460.
[11] I. Alam, S.A. Sharmin, K.H. Kim, et al., Proteome analysis of soybean roots subjected to short-term drought stress, Plant Soil 333 (2010) 491–505.
[12] G. Caruso, C. Cavaliere, P. Foglia, et al., Analysis of drought responsive proteins in wheat (*Triticum durum*) by 2D-PAGE and MALDI-TOF mass spectrometry, Plant Sci. 177 (2009) 570–576.
[13] H.M. Zhang, L.S. Zhang, H. Lv, et al., Identification of changes in *Triticum aestivum* L. leaf proteome in response to drought stress by 2D-PAGE and MALDI-TOF/TOF mass spectrometry, Acta Physiol. Plant 36 (2014) 1385–1398.

Please cite this article in press as: H. Xie, et al., iTRAQ-based quantitative proteomic analysis reveals proteomic changes in leaves of cultivated tobacco (*Nicotiana tabacum*) in response to drought stress, Biochemical and Biophysical Research Communications (2015), http://dx.doi.org/10.1016/j.bbrc.2015.11.133

[14] M. Mirzaei, N. Soltani, E. Sarhadi, et al., Shotgun proteomic analysis of long-distance drought signaling in rice roots, J. Proteome Res. 11 (2012) 348–358.
[15] S. Alvarez, E.L. Marsh, S.G. Schroeder, et al., Metabolomic and proteomic changes in the xylem sap of maize under drought, Plant Cell Environ. 31 (2008) 325–340.
[16] X.L. Hu, L.J. Wu, F.Y. Zhao, et al., Phosphoproteomic analysis of the response of maize leaves to drought, heat and their combination stress, Front. Plant Sci. 6 (2015) 298.
[17] M.M. Bradford, Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[18] I.V. Shilov, S.L. Seymour, A.A. Patel, et al., The paragon algorithm, a next generation search engine that uses sequence temperature values and feature probabilities to identify peptides from tandem mass spectra, Mol. Cell Proteomics 6 (2007) 1638–1655.
[19] H. Choi, A.I. Nesvizhskii, False discovery rates and related statistical concepts in mass spectrometry-based proteomics, J. Proteome Res. 7 (2008) 47–50.
[20] A. Sturn, J. Quackenbush, Z. Trajanoski, Genesis: cluster analysis of microarray data, Bioinformatics 18 (2002) 207–208.
[21] A. Conesa, S. Götz, Blast2GO: a comprehensive suite for functional analysis in plant genomics, Intl. J. Plant Genomics 2008 (2008) 619832.
[22] W.R. Swindell, M. Huebner, A.P. Weber, Transcriptional profiling of Arabidopsis heat shock proteins and transcription factors reveals extensive overlap between heat and non-heat stress response pathways, BMC Genomics 8 (2007) 125.
[23] R. Kaldenhoff, M. Ribas-Carbo, J.F. Sans, et al., Aquaporins and plant water balance, Plant Cell Environ. 31 (2008) 658–666.
[24] V. Banti, E. Loreti, G. Novi, et al., Heat acclimation and cross-tolerance against anoxia in Arabidopsis, Plant Cell Environ. 31 (2008) 1029–1037.
[25] C.H. Foyer, G. Noctor, Redox homeostasis and antioxidant signaling: a metabolic interface between stress perception and physiological responses, Plant Cell 17 (2005) 1866–1875.
[26] M.M. Chaves, M.M. Oliveira, Mechanisms underlying plant resilience to water deficits: prospects for water-saving agriculture, J. Exp. Bot. 55 (2004) 2365–2384.
[27] C. Vieira Dos Santos, P. Rey, Plant thioredoxins are key actors in the oxidative stress response, Trends Plant Sci. 11 (2006) 329–334.
[28] C.H. Foyer, G. Noctor, Ascorbate and glutathione: the heart of the redox hub, Plant Physiol. 155 (2011) 2–18.
[29] G.L. Wheeler, M.A. Jones, N. Smirnoff, The biosynthetic pathway of vitamin C in higher plants, Nature 393 (1998) 365–369.
[30] A. Wasilewska, F. Vlad, C. Sirichandra, et al., An update on abscisic acid signaling in plants and more, Mol. Plant 1 (2008) 198–217.
[31] H.S. Jung, J. Chory, Signaling between chloroplasts and the nucleus: can a systems biology approach bring clarity to a complex and highly regulated pathway? Plant Physiol. 152 (2010) 453–459.
[32] T. Hirayama, K. Shinozaki, Perception and transduction of abscisic acid signals: keys to the function of the versatile plant hormone ABA, Trends Plant Sci. 12 (2007) 343–351.
[33] J.J. Zou, F.J. Wei, C. Wang, et al., Arabidopsis calcium-dependent protein kinase CPK10 functions in abscisic acid- and Ca^{2+}-mediated stomatal regulation in response to drought stress, Plant Physiol. 154 (2010) 1232–1243.
[34] P.P. Mohammadi, A. Moieni, S. Komatsu, Comparative proteome analysis of drought-sensitive and drought-tolerant rapeseed roots and their hybrid F1 line under drought stress, Amino Acids 43 (2012) 2137–2152.
[35] J.M. Kwak, I.C. Mori, Z.M. Pei, et al., NADPH oxidase *AtrbohD* and *AtrbohF* genes function in ROS-dependent ABA signaling in Arabidopsis, EMBO J. 22 (2003) 2623–2633.

Please cite this article in press as: H. Xie, et al., iTRAQ-based quantitative proteomic analysis reveals proteomic changes in leaves of cultivated tobacco (*Nicotiana tabacum*) in response to drought stress, Biochemical and Biophysical Research Communications (2015), http://dx.doi.org/10.1016/j.bbrc.2015.11.133