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Research Paper

Proteomic analysis of *Camellia sinensis* (L.) reveals a synergistic network in the response to drought stress and recovery

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ABSTRACT

Drought is a crucial limiting factor for tea yield and quality. To systematically characterize the molecular response of tea plants to drought stress and its capacity to recover, we used iTRAQ-based comparative proteomic approach to investigate the effects of drought on protein expression profiles in tea seedlings subjected to different drought treatments. A total of 3274 proteins were identified, of which 2169 and 2300 showed differential expressions during drought and recovery, respectively. Functional annotation showed that multiple biological processes were regulated, suggesting that tea plants probably employed multiple and synergistic resistance mechanisms in dealing with drought stress. Hierarchical clustering showed that chlorophyll *a/b*-binding proteins were up-regulated in DB and RE, suggesting that tea plants might regulate expression of chlorophyll *a/b*-binding proteins to maintain the photosystem II function during drought stress. Abundant proteins involved in sulfur-containing metabolite pathways, such as glutathione, taurine, hypotaurine, methionine, and cysteine, changed significantly during drought stress. Among them, TL29 interacted with LHCb6 to connect S-containing metabolites with chlorophyll *a/b*-binding proteins. This suggests that sulfur-containing compounds play important roles in the response to drought stress in tea plants. In addition, the expression of PAL was up-regulated in DA and down-regulated in DB. Cinnamyl alcohol dehydrogenase, caffeic acid O-methyltransferase, and 4-coumarate-CoA ligase also showed significant changes in expression levels, which regulated the biosynthesis of polyphenols. The results indicate that slight drought stress might promote polyphenol biosynthesis, while serious drought stress leads to inhibition. The expression of lipoxygenase and short-chain dehydrogenase increased during slight drought stress and some volatile metabolite pathways were enriched, indicating that drought stress might affect the tea aroma. The study provides valuable information that will lay the foundation for studies investigating the functions of drought response genes in tea leaves.

1. Introduction

Tea (*Camellia sinensis* L.) is the most widely consumed non-alcoholic beverage in the world, which can be grown in tropical to subtropical climates. (Zheng et al., 2016). It is always subjected to environmental stress, especially drought, which reduces yield by as much as 40% (Gupta et al., 2013). Tea leaves wilt under drought stress. This is accompanied by a decrease in chlorophyll and carotenoid levels, impaired membrane integrity, and damage to cell structure (Das et al., 2015). Moreover, tea quality-related compounds, such as catechins, caffeine, theanine, and some free amino acids, are also diminished in response to drought stress (Wang et al., 2016a,b). Consequently, it is vital to study the drought-resistance mechanism for exploring relative genetic resources, improving water use efficiency/drought resistance,

Abbreviations: SSH, suppression subtractive hybridization; iTRAQ, isobaric tags for relative and absolute quantitation; SRWC, soil relative water content; LRWC, leaf relative water content; SCX, strong cation exchange; KEGG, Kyoto encyclopedia of genes and genomes; GO, gene ontology; COG, cluster of orthologous group; GSH, glutathione; AsA, ascorbic acid; TL29, thylakoid lumen 29; LHCb6, light harvesting complex photosystem II subunit 6

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and developing water-saving cultivation for reducing losses (Egert and Tevini, 2002).

Over the past decades, thousands of genes and dozens of metabolic and signaling pathways have been identified in plants suffering from drought stress (Molina et al., 2008; Zhou et al., 2007). For example, 108 transcript-derived fragments were identified as differentially expressed in a drought-tolerant genotype (Gupta et al., 2013). A total of 123 putative drought-responsive genes were identified in young roots subjected to drought stress using SSH (Gupta et al., 2012). The development of RNA-seq has made it possible to construct global transcriptomic profiles of drought-stressed tea plants. Liu et al. (2016) detected 5955 differentially expressed genes associated with exposure to a range of drought stress intensities. Most of these studies focused on physiological or transcriptomic approaches. To date, little research has focused on uncovering proteomic changes under drought stress. However, some studies have shown that transcript levels were not well correlated with associated proteins due to post-transcriptional and post-translational regulation (Peng et al., 2015). Thus, it is important to increase our knowledge of how the expression of protein levels responds to drought. The elucidation of the fundamentals of this process complements the transcriptome studies, and also provides direct insight into the translational level that is under investigation.

To develop a better understanding of the underlying metabolic processes and the molecular mechanisms of drought stress tolerance, an iTRAQ-based shotgun quantitative approach was adopted in this study to obtain a global view of the proteome response to the drought and recovery. These results provide insights into the complex molecular mechanisms associated with drought stress in tea plants, from which we were able to infer the actual mechanisms of drought tolerance in tea plants.

2. Materials and methods

2.1. Plant materials and treatment

Uniformly developed seedlings of a drought-tolerant variety (cv. Yingshuang), from Institute of Tea Research, Qingdao Agricultural University, Qingdao, P. R. of China, were established in pots (22-cm diameter, height 23 cm) each containing the same volume of soil. The pots were weighed before starting the drought condition. There were two watering regimes: control plants were watered to field capacity daily while plants under drought treatment were not watered until the cultivar displayed wilting symptoms. Four time points were selected for the experiments: 0, 5, and 15 d after the beginning of the drought stress. Plants were then re-watered to field capacity and the final sample was collected 3 d later. They are identified as CK, DA, DB, and RE, in this order. The third mature leaves of the plants were fixed in liquid nitrogen and stored at −80 °C in a refrigerator before processing.

2.2. Soil relative water content and leaf relative water content measurements

In order to measure the drought stress intensity, the soil relative water contents and leaf relative water contents were measured at different stages of the well-watered and water deficit treatments. Before the physiological characteristics were assessed, the soil relative water content (SRWC, %) was monitored at four different times. The soil water content (v/v) was measured according to the method of Maritim (Maritim et al., 2015). Leaf relative water content (LRWC, %) was recorded during control, drought, and re-watered conditions (Barrs and Weatherley, 1962). Each biological replicate included three plants, and three replicates were used for both control and drought-stressed plus re-watered plants.

2.3. Protein extraction, digestion, and iTRAQ labeling

Proteins were isolated from the third mature leaves. The leaves from two biological replicate samples were well ground in liquid nitrogen, then extracted with chilled acetone containing 10% (v/v) TCA, 10% PVPP, 10 mM DTT (final concentration), and incubated at −20 °C overnight. The supernatant was discarded after centrifugation at 4 °C, 12,000*g*. The precipitate was air-dried after washing twice with chilled acetone. It was then dissolved in Lysis Buffer 3 (2 M Thiourea, 7 M Urea, 4% CHAPS, 40 mM Tris-HCl, pH 8.5). 10 mM DTT (final concentration) was added for disulfide reduction. After incubating for 1 h at 56 °C, 5 mM IAM was added, followed by incubation for a further 45 min in the darkroom. Chilled acetone was added (5 × volume), and the sample was held overnight at −20 °C to precipitate proteins. The pellet was air-dried for 5 min after centrifugation at 4 °C, 12,000*g*, and was dissolved in Lysis Buffer 3 and sonicated for 3 min. Finally, after centrifugation at 4 °C, 12,000*g*, for 15 min, the supernatant was transferred to a new tube and the protein concentrations were estimated using the Bradford assay, with BSA as a standard (Bradford, 1976).

The total protein (100 μg) was digested with Trypsin Gold (Promega, Madison, WI, USA) with the ratio of protein: trypsin = 30:1 at 37 °C for 16 h. The iTRAQ peptide samples were labeled in eight batches with the 8-plex iTRAQ reagent (Applied Biosystems) according to the manufacturer's protocol. The CK samples were labeled with iTRAQ reagents 113 and 115, DA samples were labeled with iTRAQ reagents 114 and 116, DB samples were labeled with iTRAQ reagents 117 and 119, and RE samples were labeled with iTRAQ reagents 118 and 121, respectively.

2.4. SCX fractionation and LC–MS/MS analysis

SCX chromatography was performed with a LC-20AB HPLC Pump system (Shimadzu, Kyoto, Japan). The iTRAQ-labeled peptide mixtures were reconstituted with 4 mL buffer A (25 mM NaH_2PO_4 in 25% ACN, pH 2.7) and loaded onto a 4.6 × 250 mm Ultremex SCX column containing 5-μm particles (Phenomenex). The peptides were eluted at a flow rate of 1 mL/min with a gradient of buffer A for 10 min, 5–60% buffer B (25 mM NaH_2PO_4, 1 M KCl in 25% ACN, pH 2.7) for 27 min, and 60–100% buffer B for 1 min. The system was then maintained at 100% buffer B for 1 min before equilibrating with buffer A for 10 min prior to the next injection. Elution was monitored by measuring the absorbance at 214 nm, and fractions were collected every 1 min. The eluted peptides were pooled into 20 fractions, desalted with a Strata X C18 column (Phenomenex). The collected fractions were resuspended and concentrated by vacuum centrifugation and reconstituted in buffer A (2%ACN, 0.1%FA). The mass spectroscopy analysis was performed with a Q EXACTIVE (QE, Thermo Fisher Scientific, San Jose, CA) coupled online to the HPLC. The MS survey scan followed the method of Li et al. (2015).

2.5. Data analysis

Raw data were converted with Proteome Discoverer 1.2 (PD 1.2, Thermo), and the Mascot search engine (Matrix Science, London, UK; version 2.3.02) was employed for identification and quantification with a database containing 29,726 sequences (Charbonneau et al., 2007). Specifically, an automatic decoy database generated by choosing the decoy checkbox, was performed in Mascot. To reduce the probability of false peptide identification, only peptides shown by a Mascot probability analysis to be greater than "identity" at the 95% confidence level were counted as identified. Proteins with a more than 1.2-fold change and P-values < 0.05 were determined as differentially expressed proteins. Functional annotations of the proteins were conducted using Blast2GO program against the non-redundant protein database (NR;