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Physiological and proteome analysis suggest critical roles for the photosynthetic system for high water-use efficiency under drought stress in *Malus*

Shasha Zhou[a,1], Mingjun Li[a,1], Qingmei Guan[a], Fengli Liu[a], Sheng Zhang[b], Wei Chen[b], Lihua Yin[a], Yuan Qin[a], Fengwang Ma[a,*]

[a] State Key Laboratory of Crop Stress Biology for Arid Areas, College of Horticulture, Northwest A&F University, Yangling, Shaanxi 712100, China
[b] Institute of Biotechnology, Cornell University, Ithaca, NY 14853, USA

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ABSTRACT

Water use efficiency is an important indicator for plant adaptation and resistance to drought conditions. We previously found that under moderate drought stress, the water use efficiency of cv. 'Qinguan' apple (*Malus domestica* Borkh.) (tolerant to drought) was enhanced, while that of cv. 'Naganofuji No. 2' was not enhanced. In this research, we also found that instantaneous water-use efficiency of cv. 'Qinguan' was higher than that of cv. 'Naganofuji No. 2', mainly because of its higher net photosynthesis rate. To dissect the potential mechanisms underlying this phenomenon, we performed a comparative iTRAQ-based proteomics analysis with leaves of drought-treated cv. 'Qinguan' and 'Naganofuji No. 2'. We identified 4078 proteins, of which 594 were differentially abundant between drought and well-watered leaves. The majority of increased proteins were predicted to be involved in photosynthetic pathway in drought treated cv. 'Qinguan' leaves, indicating that regulation of photosynthesis plays an important role for higher water use efficiency under drought stress. Enzyme activity assays were performed to validate the proteomics data. Our results suggested that the main regulatory mechanisms for high water use efficiency of cv. 'Qinguan' under moderate drought stress included the maintaining of Calvin cycle function by increasing key enzymes, stabilization of photosynthetic electron transfer and keeping reactive oxygen species at normal level by regulation of photosynthetic electron transfer chain, photorespiration and reactive oxygen species scavenging capability, thus prevented photoinhibition, reduced reactive oxygen species production and enhanced net photosynthesis rate. In addition, the response of signal regulatory proteins and abiotic stress-responsive proteins to drought also helped plants to cope with such stress.

1. Introduction

Water deficit, one of the most common challenges to field-grown plants, affects growth and productivity [1]. The resistance of plants results from several physiological and biochemical mechanisms [2]. Photosynthetic components are some of the most vulnerable to stresses, with plant responses manifested by changes in pigment complexes, reduced photosynthetic rates, destroyed chloroplast structures, or restrictions to electron transport and enzyme activities [3]. Within a genetic/physiological context, drought resistance refers to the ability of one genotype to yield 'better' under such stress [4], that is to say greater water use efficiency (WUE). WUE, is defined as the ratio of biomass yield to water consumption, and is also defined as the ratio of net photosynthesis rate (Pn) to transpiration rate (Tr) (WUEi, instantaneous water use efficiency) [5]. In fact, WUE is an important index of plant adaptability and resistance to drought.

Abbreviations: APX, ascorbate peroxidase; AsA, ascorbate; CAT, catalase; Ci, intercellular CO_2 concentration; DHAR, dehydroascorbate reductase; Gs, stomatal conductance; H_2O_2, hydrogen peroxide; iTRAQ, Isobaric Tags for Relative and Absolute Quantitation; MDHAR, monodehydroascorbate reductase; NPQ, non-photochemical quenching; Pn, net photosynthesis rate; Pr, photorespiration rate; ROS, reactive oxygen species; SOD, superoxide dismutase; Tr, transpiration rate; WUE, water-use efficiency; WUEi, instantaneous water-use efficiency.

* Corresponding author. Tel.: +86 29 87082648; fax: +86 29 87082648.
E-mail addresses: zhoushasha198609@126.com (S. Zhou), limingjun@nwsuaf.edu.cn (M. Li), qguan@nwsuaf.edu.cn (Q. Guan), 782283217@qq.com (F. Liu), sz14@cornell.edu (S. Zhang), wc456@cornell.edu (W. Chen), yinlhua@163.com (L. Yin), gougouqy@163.com (Y. Qin), fwm64@nwsuaf.edu.cn, fwm64@sina.com (F. Ma).
[1] These authors contributed equally to this work.

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Through research efforts to improve crop WUE, the mechanisms and regulatory genes underlying high efficiency of water use have been studied in a range of plants, including Arabidopsis (*Arabidopsis thaliana*) [6], soybean (*Glycine max*) [7], tobacco (*Nicotiana tabacum*) [8]. However, WUE is a complex and comprehensive trait, involving many physiological pathways.

High-throughput methods have facilitated the steps in identifying key regulatory processes and genes that provide a theoretical basis for breeding high WUE plant varieties, especially under drought. Although several transcriptome analyses have been performed with samples under drought, those quantitative mRNA data have not necessarily been useful for inferring the effects of this stress on protein expression [9]. This is because post-transcriptional regulation [10] can result in poor correlations between transcripts and their cognate proteins. By contrast, proteomics studies can complement transcriptome investigations, and also lead more direct insight into the metabolic processes associated with such experimental conditions [11]. Therefore, proteomics have proven to be a powerful tool in exploring biochemical pathways and the complex response mechanisms of plants to various abiotic stresses [12].

Apple (*Malus domestica* Borkh.) is one of the most economically important fruit worldwide. It is often cultivated in arid and semi-arid areas. One example, the Loess Plateau in China, is the largest and most ideal region for apple production in the country because of abundant light and the wide range in temperatures between day and night. However, because water is short there, per-acre yield is very low. Thus, understanding the molecular mechanisms for high WUE in apple is critically needed for breeders to develop cultivars with improved efficiency and higher productivity in dry climates.

In a previous study with 31 genotypes of apple cultivars reported that cv. 'Qinguan' was more tolerant to drought and had the highest WUE under long periods of moderate drought, and that deficit treatment improved its WUE. By contrast, cv. 'Naganofuji No. 2' was more sensitive to drought, and under the same moderate drought condition, its WUE was not obviously altered from that of well-watered control plants [5]. In the current study, we have used both of those cultivars to understand molecular mechanisms for drought adaptability and WUE. An integrated physiological and iTRAQ (Isobaric Tags for Relative and Absolute Quantitation)-based [13] comparative proteomics analysis was conducted with leaf samples collected after long-term moderate drought. Our ultimate objective is to investigate the key regulatory processes and genes contributing to higher WUE behavior during long-term moderate drought for cv. 'Qinguan' apple and obtain data that could be applied toward breeding high-WUE perennial fruit tree varieties which can acclimate to moderate drought.

2. Materials and methods

2.1. Plant material and stress treatment

The experiments were conducted at Northwest A&F University, Yangling, China (34°20′ N, 108°24′ E). Buds of cv. 'Qinguan' or cv. 'Naganofuji No. 2' apple (*M. domestica* Borkh.) grafted onto the uniform apomixic rootstock of *M. hupehensis* Rehd. var. Pingyiensis at the beginning of March in 2012. The plants were grown in plastic pots (38 cm high, the diameter was 23 cm) filled with a local 5:1 loess soil (0.95% organic matter, 50.34 mg kg^{-1} alkali hydrolyzable nitrogen, 30.97 mg kg^{-1} available phosphorus, and 50.65 mg kg^{-1} available potassium): sand medium (v:v). The plants were located in a greenhouse under ambient light, at 20–35°C and 50–75% relative humidity. At the end of May, uniform trees of each cultivar (100 trees each) were divided into two groups to render the following treatments (50 trees for every treatment): (1) well-watered, daily irrigated to maintain 75–85% field capacity (FC); and (2) moderate drought, daily irrigated to achieve 45–55% FC. The groups were designated as follows: QG-CK, well-watered cv. 'Qinguan'; QG-D, moderately drought-stressed cv. 'Qinguan'; CF-CK, well-watered cv. 'Naganofuji No. 2'; CF-D, moderately drought-stressed cv. 'Naganofuji No. 2'. These treatments continued for three months. At the end of treatment, for each treatment, 30 plants were selected as samples. For each plant, two mature leaves (newly generated during drought and had the same developed days) were used to measure the gas exchange parameters, photorespiration and non-photochemical quenching, and then were removed and quickly frozen in liquid nitrogen, stored at −80°C for chlorophyll, superoxide anion, hydrogen peroxide (H_2O_2), ascorbate contents, antioxidant enzymes activity analysis and protein extraction for iTRAQ-based comparative proteomics analysis. Leaves from every five trees was one biological replicate, every treatment had five biological replicates. The similar leaves (newly generated during drought and had the same developed days) were selected for chlorophyll fluorescence measurement. Until then, QG-CK plants were 1.46 m high in average, QG-D plants were 0.97 m high in average, CF-CK plants were 1.26 m high in average, CF-D plants were 0.88 m high in average.

2.2. Evaluation of gas exchange parameters

A LI-Cor 6400 portable photosynthesis system (LI-COR, Huntington Beach, CA, USA) was used to monitor gas exchange parameters, including net photosynthesis rate (Pn), transpiration rate (Tr), stomatal conductance (Gs), and intercellular CO_2 concentration (Ci). On sunny days between 0900 h and 1100 h, 10 mature leaves per treatment were tested from different trees for every treatment.

2.3. Measurement of chlorophyll content

The method of Chen et al. [14] was used for measurement of chlorophyll content. Chlorophylls and carotenoid in leaves were extracted with 80% acetone, and then analyzed using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan).

2.4. Calculations of chlorophyll fluorescence

A chlorophyll (Chl) fluorescence transient was detected with an M-PEA-2 Multifunction Plant Efficiency Analyzer (Hansatech, UK). Dark-adapted leaves from each treatment were analyzed via JIP-tests [15] according to the following parameters: F_o, the minimum fluorescence intensity; F_m, the maximum fluorescence intensity; F_k, F_j, and F_i are the fluorescence intensities at approximately 0.3 ms, 2 ms (J-step), and 30 ms (I-step), respectively; $F_v/F_m = (F_m - F_o)/F_m$ reflects the maximum trapping efficiency of P680; $W_t = (F_t - F_o)/(F_j - F_o)$, represents the curve formed by the ratios of variable fluorescence F_t (t represents any time) to amplitude $F_j - F_o$; and $W_k = (F_k - F_o)/(F_j - F_o)$ represents the ratio of variable fluorescence F_k to amplitude $F_j - F_o$.

$ET_o/TR_o = 1 - V_J$, with $V_J = (F_j - F_o)/(F_m - F_o)$ represents relative variable fluorescence at J-step; $RE_o/TR_o = 1 - V_I$, with $V_I = (F_i - F_o)/(F_m - F_o)$ represents relative variable fluorescence at I-step; $RE_o/ET_o = (1 - V_I)/(1 - V_J)$; $M_o = 4(F_k - F_o)/(F_m - F_o)$; and $Sm = (Area)/(F_m - F_o)$ (Area is normalized total complementary area). ET_o/TR_o is the probability of an electron moving from Q_A^- to the intersystem electron carriers; RE_o/TR_o is the probability of an electron moving to reduce the final electron acceptors at the P700 acceptor side; RE_o/ET_o is the probability of an electron from the intersystem electron carriers moving to reduce the final electron acceptors at the P700 acceptor side; Mo is the approximate initial slope of the fluorescence transient, reflecting the maximum