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Full Length Article

Effects of Drought Stress and Rehydration on Physiological Parameters and Proline Metabolism in Kiwifruit Seedling

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Abstract

Drought stress is the most crucial abiotic stress, which restricts development of plants. The effect of long-term drought and rehydration on the exchange of physiological parameters and the expression of proline metabolic pathway related genes and proteins were studied in 'Hong Yang' kiwifruit seedlings. Relative water content (soil and leaf) and chlorophyll content were reduced with prolonged drought treatment. However, the contents of Malondialdehyde (MDA) and osmotic adjustment substance (soluble sugar and proline), and protective enzyme superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) showed a stage-dependent increase. Expressions of proline biosynthesis related genes, including *AcP5CS*, *AcP5CR*, *AcOAT*, *AcDREB1* and *AcDREB2* demonstrated that these genes were significantly up-regulated by drought stress, and reached a maximum, then followed by a decrease. Our results indicate that the glutamine pathway is predominant under drought stress in kiwifruit seedling and a reduction of catabolic gene (*AcProDH*) was adopted as a defense strategy in adverse conditions. These results suggested that drought stress adaption in kiwifruit was governed by proline biosynthesis, antioxidative enzymes, and osmoregulation.

Keywords: Drought stress; Gene expression; Kiwifruit; Osmoregulation

Introduction

Kiwifruit (*Actinidia chinensis*) is an important fruit crop in the world which is very sensitive to drought. Drought has significant impacts on the growth of kiwifruit, even cause plant death. In kiwifruit breeding programs, how to improve drought tolerance of kiwifruit is the main focused area. Previous studies show the responses of plant under drought stress cause a series of physiological and multigenic alterations, such as gene expression, the osmotic adjustment and the antioxidation pathway (Shinozaki and Yamaguchi-Shinozaki, 2007; Waraich *et al.*, 2017). During drought stress, plant accumulate reactive oxygen species (ROS) in mitochondria and chloroplasts (Mittler, 2002). There are many enzymatic or non-enzymatic mechanisms limit the accumulation of ROS. ROS-scavenging system such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX), monodehydroascorbate reductase (NADH) plays an important role in plant response during drought stress (Wang *et al.*, 2011).

Under drought stress, another characteristic of plant response is by reducing water loss, and increase the subcellular structures such as the osmotic adjustment of Malondialdehyde (MDA), soluble protein, soluble sugars and proline, to protect and stabilize the internal metabolic processes (Szabados and Savouré, 2010). Plant osmotic adjustment system reduces the drought stress by maintaining cell metabolic activities (Morgan, 1984; Singha *et al.*, 2017; Zahoor *et al.*, 2017). Proline is the most widely distributed osmotic regulator in plant cells (Jung *et al.*, 2010), which is also known to protect membranes and proteins from physical damage (Kishor *et al.*, 2005; Szabados and Savouré, 2010). Drought stress can lead to increase content of proline in plants, a large number of accumulated proline can maintain the osmotic balance of the cell fluid to reduce the stress on cell damage (Trovato *et al.*, 2008). Plant proline synthesis has two pathways, including glutamate and ornithine pathway (Yang *et al.*, 2009; Iqbal *et al.*, 2014). Δ1-pyrroline-5-carboxylate synthetase (P5CS) and ornithine δ-aminotransferase (δ-OAT) were the key

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enzymes of two synthetic pathways (Szabados and Savouré, 2010). *Arabidopsis thaliana* δ-OAT gene-transformed tobacco can significantly increase the proline content of tobacco and enhance the early tolerance of tobacco (Roosens *et al.*, 1998), while this gene transformation of rice, improve the salt tolerance of rice (Wu *et al.*, 2003). Under drought stress, the expression of P5CS gene and its protein activity was positively correlated with proline content (Dobrá *et al.*, 2011; Wang *et al.*, 2011). Proline dehydrogenase (ProDH) plays an important role in the proline degradation pathway (Kishor *et al.*, 2005). Δ1-pyrroline-5-carboxylate reductase (*P5CR*) is another key enzyme, which can be regulated by osmotic stress at the transcriptional level for the accumulation of proline (Ronde *et al.*, 2004). The expression of DREB transcriptional factors during drought stress have been confirmed to play an important role to protect cell stability (Morran *et al.*, 2011; Zhao *et al.*, 2012; Duan *et al.*, 2017).

However, the effects of drought stress and rehydration on the proline metabolism and related genes were not well identified. To better characterise kiwifruit adaptation, we measured the physiological parameters and gene expression in proline metabolism during drought stress and rehydration. The drought resistance mechanism of kiwifruit seedlings was discussed.

Materials and Methods

Plant Material and Treatment

The experimental material was 'Hong Yang' (*Actinidia chinensis*) kiwifruit, which was seedling with six ages, 10.0 + 1.15cm in average plant height and 0.5 + 0.07 cm in stem. The explants were derived from Pujiang kiwifruit production base (30°2'N; 103°29'E), and were organized in the tissue culture laboratory of Sichuan Agricultural University, Chengdu(30°67'N,104°06'E), Sichuan Province. Kiwifruit 'Hong Yang' seedlings were grown in the plastic pots in the chamber with turfy soil and vermiculite (3:1) at Sichuan Agricultural University. The seedlings were conducted a full irrigation before the drought treatment, then stopped for a duration of irrigation. The soil relative water content (SRWC) was determined at the same time every day with WET Sensor (WET-2). Samples were measured at 0, 3, 6, 9, 12, and 15 days after stopping the water supply and on the seventh day after rehydration points after treatment and used for following assay. Leaf samples were collected from at least 5 plants each time, frozen with the liquid nitrogen and stored at -80°C.

Determination of Physiological Parameters

The leaf relative water content (LRWC) (%) was determined by drying method (Li *et al.*, 2011). The photosynthesis pigment contents (mg g^{-1} FW) were determined according to the Lichtenthaler method (Morran *et al.*, 2011). The activities of SOD ($U \cdot g^{-1}$ FW) were determined by nitrogen blue tetrazolium (NBT) reduction method (Rao *et al.*, 1996). POD activity (U g^{-1} FW) was measured by guaiacol method (Zhang *et al.*, 2017); CAT activity ($U \cdot g^{-1}$ FW) was measured by UV absorption monitoring the disappearance of H_2O_2 (Zhang *et al.*, 2017). MDA content (μmol g^{-1} FW) was measured by thiobarbituric acid method (TAB); soluble sugar content (mg g^{-1} FW) determination using anthrone colorimetry as reported by Wang *et al.* (2011). Free proline (μg g^{-1} FW) was according to the method of Kishor *et al.* (2005). The above indicators are repeated three times.

Gene Expression Analysis by qRT-PCR

The total RNA was extracted from the leaves of kiwi by modified CTAB method. RNA integrity was detected by 1% agarose gel electrophoresis, and cDNA was reverse transcribed using TURE-script 1st Strand cDNA Synthesis Kit with gDNA Eraser kit. QRT-PCR was performed using an i-Cycler iQ5 (Bio-Rad, Hercules, CA, USA). The gene relative expression level was calculated through the comparative CT (2-△△CT) method. Gene specific primers (Table 1) were designed using Primer5 software.

Statistical Analysis

All data were expressed as the means ± standard deviations of three replicates. One-way ANOVA and Tukey text were analyzed with *SPSS* package v. 17.0 (IBM Corporation, USA). Differences were considered as statistically significant at the $P < 0.05$ level.

Results

Relative Water and Photosynthetic Pigment Contents

As showed in Fig. 1, during the drought stress, the soil relative water content (SRWC) decreased continuously. SRWC have fallen by 76% at day-15 compared with day-0. The leaf relative water content (LRWC) in the seedlings of 'Hong Yang' kiwifruit decreased moderately in the late post-drought period, while after 7 days of rehydration, the LRWC had some recovery, but significantly lower than that of control.

Chlorophyll and *β*-carotene (*β*-Car) which was the important photosynthesis pigment for assisting in light absorption. Total chlorophyll content was significantly decreased during the drought period, but showed a slightly increase when rehydrated at day-22 (Fig. 2). This variation was associated with both Chl *a* and Chl *b* under drought stress. The ratio of Chl *a/b* was decreased at day-3, and then increased to the peak, and then decreased from day-9 to day-15. The content of *β*-Car was decreased slightly during drought stress, and then showed an increase after rehydration (Fig. 2).

Table 1: Information of primers used in this study

Genes	Accession number	Primer sequences (5'-3')
AcP5CS	Achn109361	Forward: CTTAGTGACCGATAACGATT
		Reverse: GCTCTCCTAGTGCTTATTG
AcP5CR	Achn136451	Forward: GTCATCATCTTCTCTGTCAA
		Reverse: GCTTCTCCGAAAGTAGTG
AcOAT	Achn090001	Forward: TCGTAGAATCGTGGATAATC
		Reverse: CTAATGATGAAGGCAGGAT
AcProDH	Achn293881	Forward: ATGCTGAATTACGCTCTC
		Reverse: ACTTACAGAAGATTGTGGAA
AcDREB1	Achn049811	Forward: CGGAGGAATTGGGCGAGAT
		Reverse: ATCAACCGAGTCAATCAGCACTA
AcDREB2	Achn131671	Forward: CTACAGAGGGATAAGGATGAG
		Reverse: ATTCGAGACCGCTTGTTG
Actin	AY680701.1	Forward: GTGCTCAGTGGTGGTTCAA
		Reverse: GACGCTGTATTTCCTCTCAG

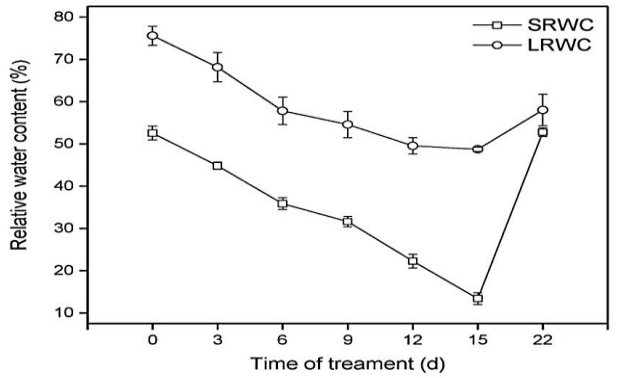

Fig. 1: Soil relative water content and relative water content in kiwifruit seedling leaves after withholding water and rehydration. Values represent the mean ± SD of 6 replicate samples tested in replicate

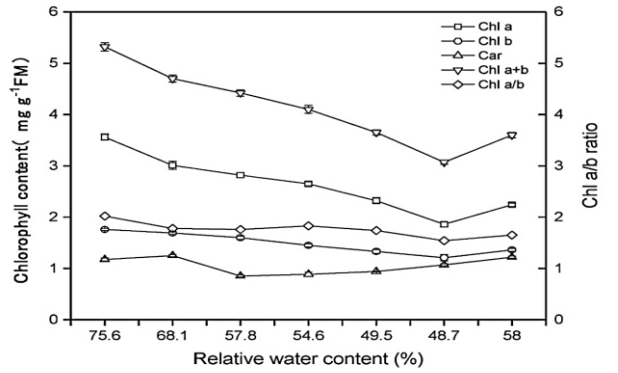

Fig. 2: Contents of chlorophylls and *β*-carotene after withholding water and rehydration. Values represent the mean ± SD of 6 replicate samples tested in replicate

Membrane Indices and Enzyme Activities

MAD is a product of membrane lipid peroxidation, and its content can reflect the degree of damage to plants. As shown in Fig. 3A, the MDA contents increased persistently during drought stress, and then decreased after rehydration. However, proline content increased slightly and reached the max value at day-6, and sharply decreased at day-9 (Fig. 3B). The soluble sugar content increased slightly at day-6, then declined by more than 50% at day-15, but raised again at day-22 after rehydration (Fig. 3C).

During the drought stress, the activity of SOD, POD and CAT showed same variation. SOD activities reached the peak at day-6 and decreased at day-15 to a minimum, which was 42.8% lower than that of the control (Fig. 3D). POD activities reached a peak at day-12, which was 307.6% higher than that of the control (Fig. 3E). CAT activities increased significantly from day-3 to day-9 and suddenly decreased from day-12 to day-15 (Fig. 3F). Subsequently, the activities of CAT cannot be restored after 7 days of rehydration.

Gene Expression in Proline Metabolism under Drought Stress

As shown in Fig. 4, drought stress induced the expression of proline accumulation genes *AcP5CS*, *AcP5CR* and *AcOAT*. *AcP5CS* and *AcP5CR* expression were up-regulated under drought stress with a 23.8- and 9.2-fold increase at day-9, respectively. *AcP5CS* showed instant response at day-3, and maintained a high level until day-12 then attenuate its gene expression at day-15. While the expression of *AcOAT* showed slight upregulation at day-3, and reached a peak value at day-6 then decreased from day-9 to day-15. On the contrary, the expression of proline dehydrogenase (ProDH) was down-regulated. *AcProDH* transcripts were decreased significantly from day-3 to day-15, but after 7 days of rehydration it increased significantly higher than the untreated level.

The expression levels of *AcDREB1* and *AcDREB2* were also analyzed during drought stress. The expression of *AcDREB1* in kiwifruit leaves was up-regulated 8.5-, 26.7-, 13.3- and 9.1-fold after 3, 6, 9 and 12 days drought stress, respectively, which was significantly higher than the untreated level. The expression level of *AcDREB2* in leaves of kiwi showed nearly the same pattern with *AcDREB1*, which was also much higher than the untreated level (Fig. 4).

Discussion

In the kiwifruit plantation in China, drought restrains kiwifruit production (Montanaro *et al.*, 2007; Morandi *et al.*, 2011). Kiwifruit responded the drought stress by different physiological metabolism (Wang *et al.*, 2011). Our studies found that the RWC was observably reduced and photosynthesis was restrained under drought stress (Fig. 1 and 2), which were similar with physiological responses in *Cotinus coggygria* seedlings (Li *et al.*, 2011). The reduction of chlorophyll contents also indicated that photosynthesis was influenced during drought treatment.

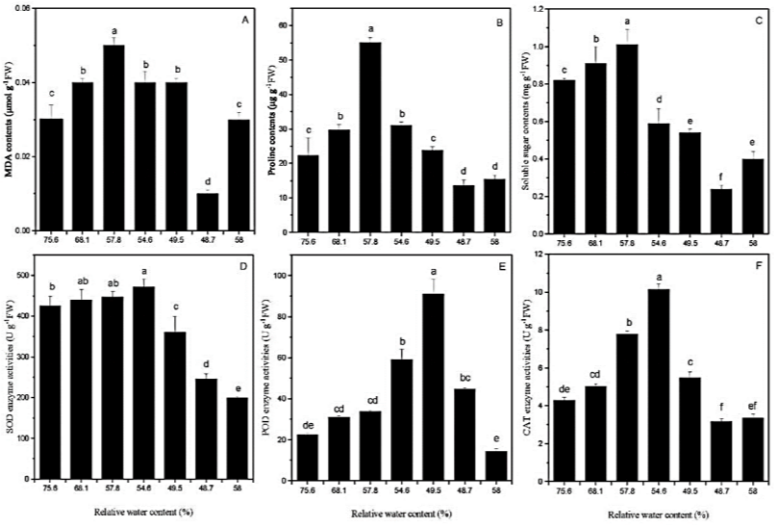

Fig. 3: Changes of physiological indices in kiwifruit after withholding water and rehydration. Values represent the mean ± SD of 6 replicate samples tested in replicate. Bars with different uppercase letters show significant differences at the $P < 0.05$ level

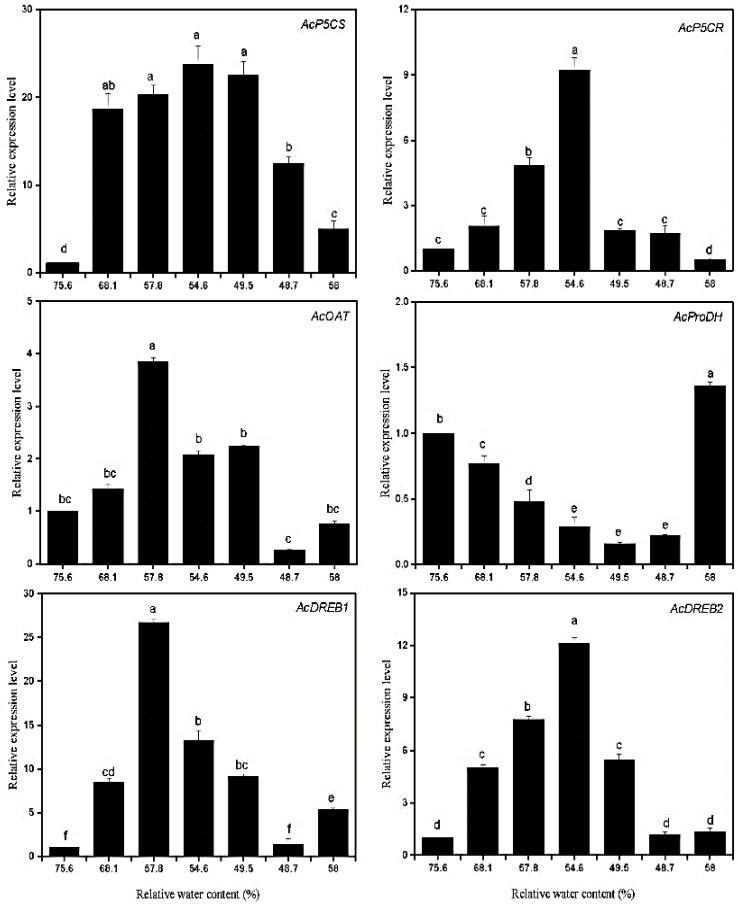

Fig. 4: Expressions of proline metabolism related genes *AcP5CS*, *AcP5CR*, *AcOAT*, *AcProDH*, *AcDREB1* and *AcDREB2* after withholding water Values represent the mean ± SD of three biological replicates tested in triplicate. Bars with different uppercase letters show significant differences at the $P < 0.05$ level

MDA is the product of membrane lipid peroxidation, which indicates the degree of oxidative stress of plants (Wang *et al.*, 2011). We confirmed that drought stress has led to a significant increase in MDA content. Under drought stress, reactive oxygen species (ROS) accumulates as a product of oxidative stress, leading to the damage of macromolecules in cells. The antioxidant enzymes such as SOD, POD, and CAT are considered to eliminate ROS under stress (Zang *et al.*, 2014). SOD, POD and CAT activities in leaves of kiwifruit firstly increased, and then decreased during the drought stress. This indicates that there is an upper limit to the drought response of the kiwifruit seedlings, and severe drought has caused damage to the leaves of kiwifruit.

Proline and soluble sugar are several important components involved in plant osmotic protection. When the external environment changes, the plant can be through the accumulation of proline and soluble sugar levels to change its osmotic adjustment (Wan *et al.*, 2014). The increase of proline content in kiwifruit leaves at earlier stage under drought stress showed that kiwifruit seedlings can adjust the content of osmotic substances to adapt to drought stress. Undulations of soluble sugar content in leaves of kiwifruit suggested that soluble sugar play an important role under drought stress in kiwifruit seedlings as well as proline.

Stress regulates the expression of proline metabolic pathways and causes the increase of proline content in plants to adapt to poor environment (Jaarsma *et al.*, 2013). In our study, the changes of *AcP5CS*, *AcP5CR* and *AcOAT* expression were found to be up-regulated under drought stress. On the contrary, the expression of *AcProDH* was down-regulated (Fig. 4). The results suggested that proline degradation in kiwifruit may be needed to support plant growth under drought stress, and proline biosynthesis may help to maintain the balance of redox in plant cell. The expression levels of *AcP5CS* was highly induced than *AcOAT* indicated that the accumulation of proline was dominated by the glutamate pathway in the first 6 days under drought stress, followed by the dominance of the ornithine pathway which was similar with Zhao *et al.* (2012). Previous studies have shown that DREB is an important transcription factor in the downstream gene expression during drought stress (Zegaouia *et al.*, 2017). The expression levels of *AcDREB1*and *AcDREB2* were identified as being strongly induced, which demonstrated the positive function of DREB gene under drought stress.

Conclusion

Taken together, these results suggested that kiwifruit seedling was susceptible to drought stress, and the protection role of physiological and molecular responses only lasted for day-3 to day-9 after withholding water. After rehydration, some physiological and molecular parameters of kiwifruit seedling can be restored, indicating that it has a certain drought resistance. Moreover, adaptation to drought stress was a complex process involved in osmoregulation, antioxidative enzymes and proline biosynthesis related genes in kiwifruit seedling.

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