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RESEARCH PAPERS

Phosphofructokinase and Glucose-6-Phosphate Dehydrogenase in Response to Drought and Bicarbonate Stress at Transcriptional and Functional Levels in Mulberry[1]

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Abstract—Drought and bicarbonate stress are the most common abiotic stresses limiting plant growth and crop production all over the world. Glycolytic and pentose phosphate pathways have important effects on the resistance response of plants to abiotic stress. Phosphofructokinase (PFK) and glucose-6-phosphate dehydrogenase (G6PDH) are the rate limiting enzymes of the two pathways which control carbon flow through the two pathways, respectively. In *Morus alba* L. leaves, the activities of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) and the proline content increased under drought and bicarbonate stress at first, and then decreased. At the functional level, drought and bicarbonate stress exert an activating effect on the G6PDH activity and an inhibitory effect on the PFK activity in *M. alba* leaves. At the transcriptional level *ATP-PFK, PPi-PFK* and *G6PDH* genes showed no significant change under 80 g/L PEG 6000 treatment. The changes were observed at the *ATP-PFK, PPi-PFK* and *G6PDH* mRNA levels under 30 mM $NaHCO_3$ treatment. Although *M. alba* stopped growing under two different treatments and the changes of G6PDH and PFK are different suggesting that may be additional factors playing important roles in glucose and energy metabolism in plant responses to stresses.

Keywords: Morus alba, bicarbonate stress, glucose-6-phosphate dehydrogenase, phosphofructokinase, drought stress

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INTRODUCTION

Plants exposure to various stresses induces adverse effects on their growth, development, productivity, and yield. The drought and bicarbonate stress are the most frequently occurring abiotic stresses that limit plant growth and crop production all over the world [1, 2]. Both of these stresses cause plants to suffer osmotic stress and produce ROS such as superoxide, hydroxyl radicals, and hydrogen peroxide [3, 4]. Large amounts of ROS results in oxidative damage of DNA, proteins, and lipids [5]; however, bicarbonate stress can also induce an increase in Na^+ and cause a decrease in Zn^{2+}, Fe^{2+}, Ca^{2+}, K^+, Cu^{2+}, Mn^{2+}, and Mg^{2+} contents. On the other hand, unlike NaCl, which is a neutral salt, bicarbonate stress, as an alkali salt, can increase soil pH [6]. In response to a range of stress, plants initiate various adaptations to acquire stress tolerance which comprise morphological and physiological changes accompanied with alterations in molecular, metabolic, and biochemical processes. Under stress conditions the plants alter their metabolism in various ways, including production of compatible solutes (e.g., proline, raffinose, and glycine betaine) that are able to stabilize proteins and cellular structures and/or to maintain cell turgidity by osmotic adjustment, and redox metabolism to remove ROS excess levels and re-establish the cellular redox balance [7]. These changes are closely related to the regulation of the pentose phosphate, and glycolytic pathways, including changes in the pentose phosphate and glycolytic pathways [4, 8–10]. Previous studies have shown that in the presence of oxidants, glucose is channeled towards the pentose phosphate pathway with simultaneous suppression of glycolysis and downstream pathways including the tricarboxylic acid cycle. Stimulation of the pentose phosphate pathway

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Abbreviations: ATP-PFK—ATP-dependent phosphofructokinases; CAT—catalase; D_b—basal diameter; G6PDH—glucose-6-phosphate dehydrogenase; H—plant height; N—number of leaves; N_{80}—blade more than 80 mm wide; NBT—nitro blue tetrazolium; PFK—phosphofructokinases; POD—peroxidase; PPi-PFK—pyrophosphate-dependent phosphofructokinases; SOD—superoxide dismutase.

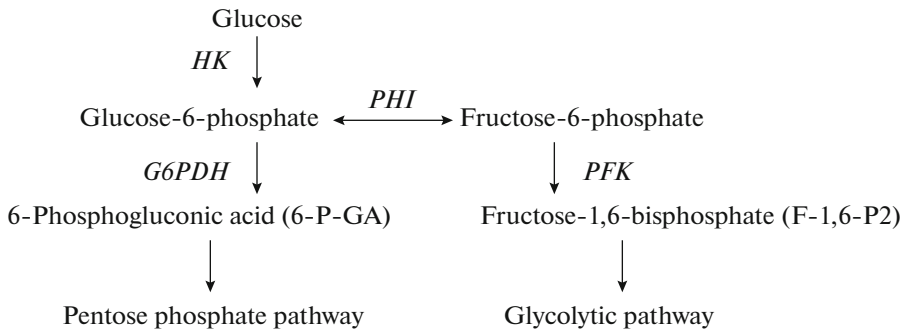

Fig. 1. Glucose-6-phosphate can be irreversibly catalyzed by G6PDH to 6-phosphogluconic acid to enter the pentose phosphate pathway, or alternatively can be catalyzed by phosphohexose isomerase to fructose-6-phosphate and then irreversibly catalyzed by PFK to fructose-1,6-bisphosphate to enter the glycolytic pathway.

enhances the production of NADPH to support antioxidant enzyme activity [11].

The pentose phosphate and the glycolytic pathways are central to the metabolic system of organisms. The pentose phosphate pathway is an important source of reducing equivalents in the form of NADPH for biosynthetic processes such as fatty-acid synthesis and the assimilation of inorganic nitrogen, and modulates the redox state of cells to protect against oxidative stress. This pathway is also the source of biosynthetic precursors for the synthesis of nucleotides, aromatic amino acids, phenylpropanoids and their derivatives. Glycolysis is a fermentative pathway of respiration which has the primary role to provide ATP, NADH and other precursor metabolites for biomass production to meet the energy demands of metabolic activities. The glucose-6-phosphate dehydrogenase (G6PDH) and phosphofructokinases (PFK) are the rate limiting enzymes of two pathways that control the carbon flow through the two respective pathways. Hexoses are partitioned into the glycolytic cycle and pentose phosphate pathway [12]. Glucose-6-phosphate is at the carbon interface between the pentose phosphate and the glycolytic pathway in vivo and can be irreversibly catalyzed by G6PDH to 6-phosphogluconic acid to enter the pentose phosphate pathway or alternatively can be catalyzed by phosphohexose isomerase to fructose-6-phosphate and then irreversibly catalyzed by PFK to fructose-1,6-bisphosphate to enter the glycolytic pathway (Fig. 1). This experiment aimed to determine the regulatory mechanisms of G6PDH and PFK under different stress conditions focussing on the different characteristics of two enzymes in response to drought stress and bicarbonate stress at the transcriptional and functional levels. By studying the response of these two enzymes under different stresses, it is possible to gain insight into how plants use changes in metabolism to adapt to environmental changes and why plants possess different morphological and physiological changes under different stresses.

Morus alba is a fast growing deciduous plant that grows under different climatic conditions [13]. Reports indicate that mulberry leaves contain proteins, carbohydrates, calcium, iron, ascorbic acid, β-carotene, vitamin B-1, folic acid and vitamin D [14]. In addition to their use in animal and insect feeds, they have been shown to possess medicinal properties as a diuretic, in hypoglycemic and hypotensive activities [15]. Mulberry has received wide attention in recent years for its strong environmental adaptability and the outstanding ecological functions of water conservation, clean air and other ecological functions [16]. In this experiment, the key metabolic enzyme activity and gene expression were measured in order to understand the metabolic adaptation mechanism under drought and bicarbonate stress, and to provide a theoretical basis for use mulberry in Karst area remediation.

MATERIALS AND METHODS

Plant material and seedling growth. Seeds of *Morus alba* L. were obtained from the Guizhou Academy of Agricultural Sciences, China. Seedlings were sown directly in perlite and germinated at 25°C in a greenhouse. After germination, all seedlings were transferred to 1/2 Hoagland nutrient solution. The environmental conditions for germination were as follows: 12-h photoperiod with average day/night temperatures of 25/20°C and PPFD of 300 μmol/(m^2 s) with 65% relative humidity. Plant height (H), basal diameter (D_b), number of leaves (N) and blades more than 80 mm wide (N_{80}) were selected as growth indices. Following stress treatment, plants were measured once every two days until all indices remained constant which were considered as the endpoint when the seedlings had stopped growing.

Eight-week-old plants were used for the following treatments: (i) drought stress was performed with two levels of PEG 6000 (80 or 160 g/L) in 1/2 Hoagland nutrient solutions; (ii) bicarbonate stress was performed with two levels of bicarbonate (15 or 30 mM, as $NaHCO_3$) in 1/2 Hoagland nutrient solution. The solution pH was adjusted to 7.8 in all treatments by

addition of NaOH and solutions replaced every day after the imposition of stress treatments.

Enzyme extraction. Enzymes were extracted according to the method as described earlier [17] with some modifications. 0.1 g of leaves were ground in liquid nitrogen and 1 mL of extraction buffers added containing 50 mM Hepes-Tris (pH 7.8), 3 mM $MgCl_2$, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride and 1 mM dithiothreitol. The homogenate was centrifuged at 12000 *g* for 20 min at 4°C.

Proline content assay. Proline content was assayed as described earlier [18]. Proline was extracted from 0.1 g of fresh leaves with 3 mL of methanol : chloroform : water (12 : 5 : 1, v/v). After centrifugation at 10000 *g* for 5 min, the supernatant was used for proline estimation. 1 mL of supernatant was transferred to a test tube and heated in water bath until methanol evaporation, after which 0.33 mL ninhydrin solution, 0.33 mL of glacial acetic acid and 0.33 mL of water were added to the sample. The tubes were then cooled to room temperature and 2 mL of toluene were added. After 30 s of shaking, two phases were separated, and the absorbance of the upper phase was measured at 520 nm.

SOD, POD, and CAT activity assay. Superoxide dismutase (SOD) and peroxidase (POD) activities were assayed as described earlier [19]. SOD activity was determined by measuring the ability to inhibit photochemical reduction of nitro blue tetrazolium (NBT). The assay buffer containing 0.3 mL of 20 μM riboflavin, 150 mM L-methionine, 600 μM NBT, and enzyme extract containing 100 μg protein in the final volume of 3 mL. The reaction started after riboflavin was added. The reaction lasted for 20 min under a PPFD of 170 μmol/(m^2 s) provided by a white fluorescent lamp. The system devoid of enzymes served as a negative control. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the reduction of NBT when monitored at 560 nm.

POD activity was assayed in 2 mL of 100 mM potassium phosphate buffer (pH 6.5) containing 40 mM guaiacol, 10 mM H_2O_2, and enzyme extract containing 100 μg protein at 436 nm [20]. The activity was based on the rate of tetraguaiacol production using an extinction coefficient of 25.5/(mM cm).

CAT activity was assayed according to the method described by Aebi [21], by measuring the decrease at 240 nm for 1 min, due to H_2O_2 consumption.

Glucose-6-phosphate dehydrogenase (G6PDH) activity assay. G6PDH activity was assayed as described above [22] with some modifications. 200 μL aliquot of extract was added to either 1.8 mL of the total dehydrogenase (G6PDH+6PGDH) assay buffer containing 50 mM Hepes-Tris (pH 7.8), 3.3 mM $MgCl_2$, 0.5 mM D-glucose-6-phosphate disodium salt, 0.5 mM 6-phosphogluconate, and 0.5 mM NADP-Na_2 or 1.8 mL the 6-phosphogluconate dehydrogenase (6PGDH) assay buffer, containing 50 mM Hepes-Tris (pH 7.8), 3.3 mM $MgCl_2$, 0.5 mM 6-phosphogluconate, and 0.5 mM NADP-Na_2. The reduction of $NADP^+$ to NADPH was measured as the rate of change in the absorbance at 340 nm during the initial 5 min. The G6PDH activity was calculated as the total dehydrogenase activity with the 6PGDH activity subtracted.

Phosphofructokinase (PFK) activity assay. ATP-PFK and PPi-PFK were assayed as described earlier [23] with some modifications. A 200 μL aliquot of the extract was added to 1.8 mL of assay buffer containing 50 mM Hepes-Tris (pH 7.8), 2.5 mM $MgCl_2$, 0.1 mM NADH, 5 mM F-6-P, 2 units/mL aldolase, 1 unit/mL triosephosphate isomerase, 2 units/mL α-glycerol-3-phosphate dehydrogenase and either 1 mM ATP or 1 mM PPi. The oxidation of NADH to NAD^+ was measured as the changing rate of the absorbance at 340 nm during the initial 5 min. The PFK activity is equal to ATP-PFK activity plus PPi-PFK activity.

Total RNA extraction and gene expression analysis. Total RNA was extracted using Trizol (Takara, Japan) according to the supplier's instruction. The extracted RNA was treated with DNase to eliminate genomic DNA contamination. Total RNA was used to synthesize oligo(dT)-primed cDNA with reverse transcription kit (Takara) according to the manufacturer's instructions. Semi-quantitative Real-Time PCR was performed using a StepOnePlus™ (Life Tech, United States) using SYBR RT-PCR Kit (Takara) according to the manufacturer's instructions. PCR conditions consisted of denaturation at 95°C for 30 s followed by 40 cycles of denaturation at 95°C for 5 s followed by annealing at 60°C for 34 s. The *M. alba* actin gene was used as an internal control. Relative gene expression was calculated as described earlier [24], data analysis was carried out using the comparative Ct ($2^{-\triangle\triangle Ct}$) method. The gene-specific primers that were used for the amplification on the basis of the gene are listed in Table 1.

Statistical analysis. Data are presented as the mean ± SD. The experiments were performed using at least three independent measurements. Statistical analysis was performed using ORIGIN software (v. 9.0). Differences were considered significant when the *p* value was less than 0.05.

RESULTS

Plant Growth

On the basis of previous studies, treatments were made with two levels of PEG 6000 (80 or 160 g/L) and two levels of bicarbonate (15 or 30 mM, as $NaHCO_3$). It was observed that *M. alba* under 80 g/L PEG 6000 treatment, 160 g/L PEG 6000 treatment, 15 mM $NaHCO_3$ treatment and 30 mM $NaHCO_3$ treatment stopped growing on the 8th, 4th,10th, and 8 th day,

Table 1. List of primers used for semi-quantitative real-time PCR

Primer	Forward primer(5'–3')	Reverse primer(5'–3')
ATP-PFK	CTGTTGCCTGATTCCAGAGTC	TACACCTTCAGCCACCACTATA
PPi-PFK	CAAGCCATCCAAGAGCAGTT	GCTTCCTCTTCTCCAATTCAGT
G6PDH	GGCGTGTTCCTGGCAACTTAT	AGGTTACTGCGGTCCAATCTCA
Actin	CGGTGTGATGGTTGGTATGG	TGTAGAAGGTGTGATGCCAGAT

Table 2. Growth parameters of plants under 80 g/L PEG 6000 treatment and 30 mM $NaHCO_3$ treatment

Growth time, days	Control				80 g/L PEG 6000				30 mM $NaHCO_3$			
	H, cm	D_b, mm	N	N_{80}	H, cm	D_b, mm	N	N_{80}	H, cm	D_b, mm	N	N_{80}
0	17.6	2.24	11	1	17.3	2.32	10	1	17.7	2.92	11	3
2	18.9	2.44	11	1	17.9	2.42	11	1	18.7	2.93	11	4
4	20.9	2.53	12	2	18.4	2.44	11	1	19.4	3.00	11	4
6	22.5	2.77	13	3	18.3	2.47	11	1	19.3	3.09	11	4
8	23.8	2.79	13	3	18.5	2.47	11	1	19.3	3.12	11	4
10	25.1	2.92	14	4	18.5	2.39	11	1	19.3	3.08	11	4

respectively. Plants treated with 80 g/L PEG 6000 and 30 mM $NaHCO_3$ stopped growing simultaneously. Two groups of plants were selected for further study. The growth parameters of plants under 80 g/L PEG 6000 treatment and 30 mM $NaHCO_3$ treatment are shown in Table 2.

SOD, POD, CAT Activities and Proline Content

To determine the nature of the antioxidant responses to drought and bicarbonate stresses, we measured the enzymatic activity of SOD, POD, CAT and proline contents in the seedling leaves.

The Figs. 2a, 2b, and 2c show the course of the activities of SOD, POD and CAT enzymes, and proline content in mulberry leaves. Compared with controls, the activities of SOD, POD, and CAT all increased under drought and bicarbonate stresses. However, the activity of these three kinds of antioxidant enzymes all slightly decreased on the 8th day.

The Fig. 2d shows that proline content increased under both drought and bicarbonate stresses. The proline content under drought stress sharply decreased on the 6th day. However, the decrease in proline content under bicarbonate stress appeared on the 8th day. The proline contents under drought and bicarbonate stresses were both lower than in the control on the 8th day.

Phosphofructokinase (PFK) Activity

PFK activity is equal to ATP-PFK activity plus PPi-PFK activity. The enzyme activity under 80 g/L PEG 6000 treatment changed rapidly (Fig. 3). Before the 2d day, the modulating of the PFK activity had been completed. From the 2d to the 8th day, it was around 65% percent of PFK activity under control treatment which was shown to be stable.

On the 2d day, under 30 mM $NaHCO_3$ treatment PFK activity was only about 22% of its activity under control treatment (Fig. 3). On the 8th day , enzyme activity was almost 4-fold higher than on the 2d day, and PFK activity under this treatment was nearly equal to its activity under control condition.

Glucose-6-Phosphate Dehydrogenase (G6PDH) Activity

A large increase in enzyme activity was shown on the 2d day, reaching maximum at 4 d under 80 g/L PEG 6000 treatment (Fig. 4), which was six times higher than its activity under control treatment. After this, the enzyme activity decreased sharply; on the 8th day, its activity was around 30% of that on the 4th day. However, G6PDH activity remained twice as high as its activity under control treatment. The enzyme activity was low and almost equal to that of the control on the 2d day under 30 mM $NaHCO_3$ treatment (Fig. 4). Further, G6PDH activity increased slightly and on the 8th day, its activity was only 1.6 times higher than under control.

mRNA of Genes Encoding PPi-PFK and ATP-PFK

To analyze the mRNA expression of genes encoding PPi-PFK and ATP-PFK under drought stress and bicarbonate stress, we performed semi-quantitative RT-PCR analysis. The Figs. 5a, and 5b show time course of mRNA of PPi-PFK and ATP-PFK genes in leaves. These figures show that under 80 g/L PEG 6000 treatment, mRNA expression of genes encoding PPi-PFK and ATP-PFK both had no marked changes compared to control and were relatively stable. The

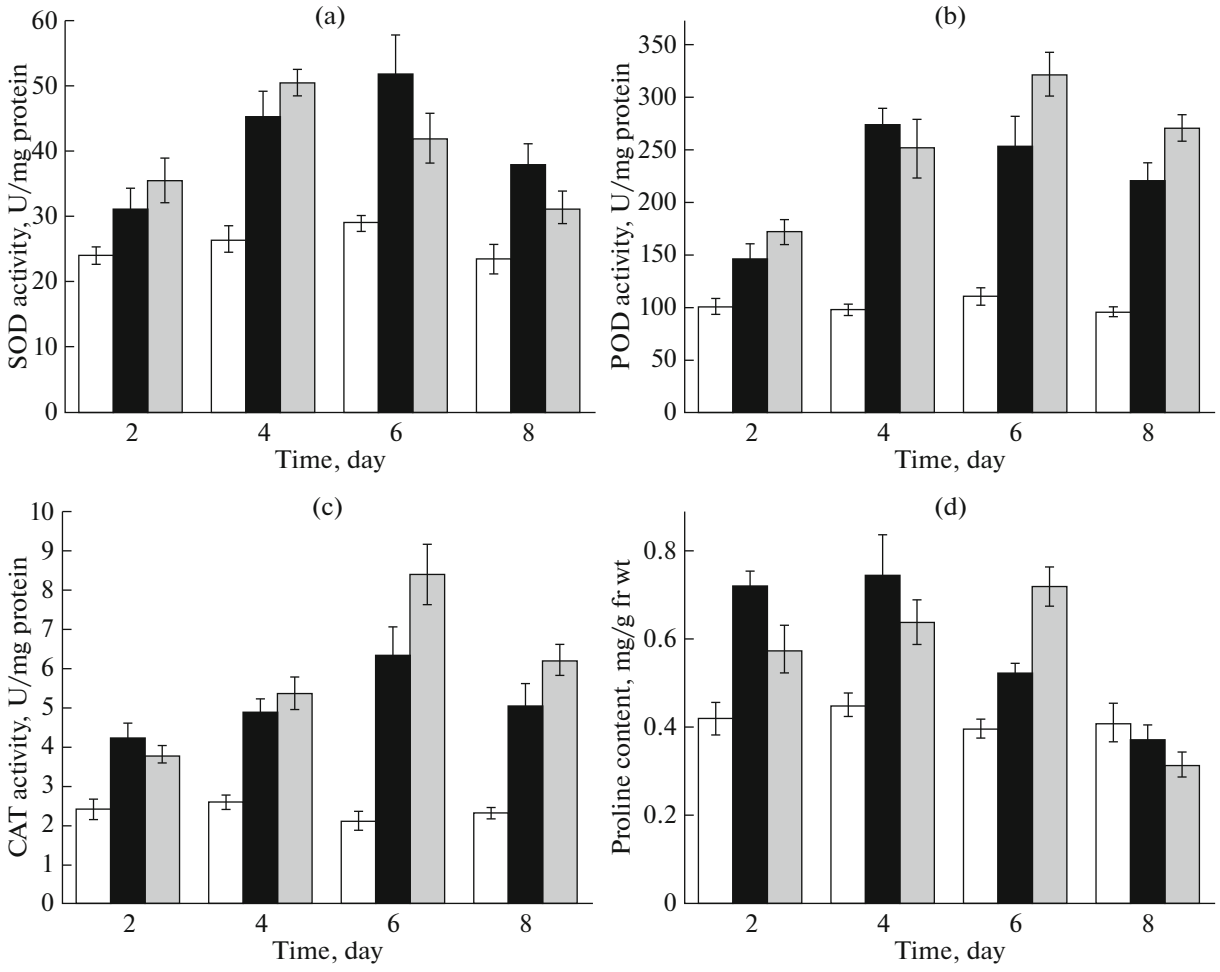

Fig. 2. The activities of SOD, POD and CAT enzymes and proline content under 80 g/L PEG 6000 treatment (black column), 30 mM $NaHCO_3$ treatment (gray column) and control treatment (white column) for 2, 4, 6, and 8 d in mulberry leaves. The values are shown as mean ± SD, $n = 3$. Both stress treatments significantly different as compared to control ($p < 0.05$).

mRNA of ATP-PFK genes under 30 mM $NaHCO_3$ treatment, gradually increased over time reaching a peak on the 6th day after which it fell to a level slightly higher than that under control treatments (Fig. 5a). The mRNA of PPi-PFK genes was much higher than that of control level on the 2d day which then gradually decreased (Fig. 5b). On the 8th day, mRNA level of PPi-PFK genes was even slightly lower than that under control treatment.

mRNA of Genes Encoding G6PDH

Figure 5c shows the time course of mRNA *G6PDH* genes in leaves. This figure shows that under 80 g/L PEG 6000 treatment, the mRNA level of *G6PDH* was relatively stable and had no marked changes compare to control. Under 30 mM $NaHCO_3$ treatment, the mRNA of *G6PDH* genes gradually increased after the 2d day reaching maximum on the 4th day. Later, the level of mRNA *G6PDH* decreased sharply and was shown to be lower than that under control treatment on the 8th day.

DISCUSSION

Under drought and bicarbonate stresses, the enzymatic activity of SOD, POD, CAT and proline content increased in leaves. This suggested that the plant "tried" to reduce the damage from ROS through enhanced antioxidant enzyme activity and reduced osmotic stress; however, in the later stage of stress treatment, the enzymatic activity of SOD, POD, CAT and proline content all decreased. This reflected the overall reduction in metabolic activity of the plant under long-term stress conditions.

Both drought stress and bicarbonate stress exerted activating effects on G6PDH and have an inhibition effect on the PFK activity in leaves. Similar results were observed with different plant species in previous studies [9–11, 17]. These reflect that carbon flux can be diverted from the catabolic glycolytic pathway to

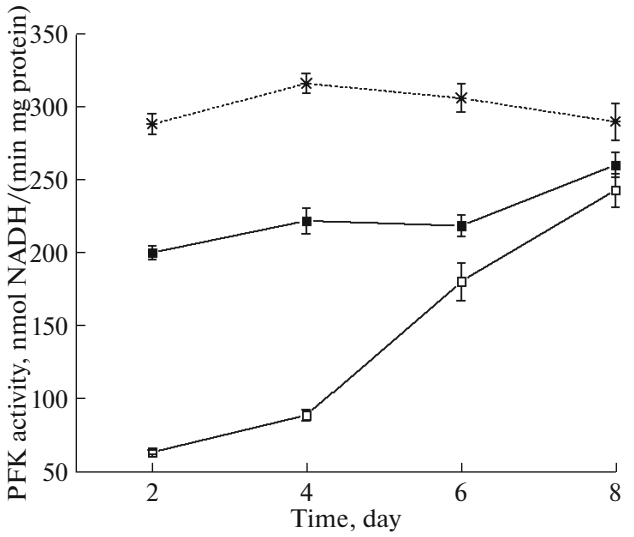

Fig. 3. The activities of PFK enzyme under 80 g/L PEG 6000 treatment (■), 30 mM $NaHCO_3$ treatment (□) and control treatment (*) on the 2d, 4th, 6th, and 8th day in mulberry leaves. The values are shown as mean ± SD, $n = 3$. Both stress treatments significantly different as compared to control ($p < 0.05$).

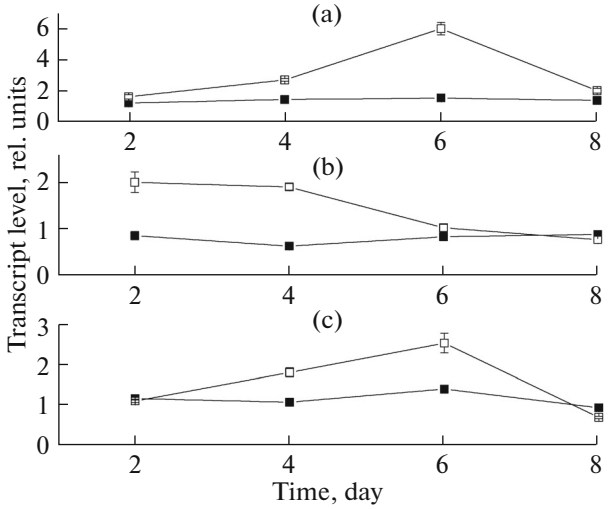

Fig. 5. The transcript level of genes encoding ATP-PFK (a), PPi-PFK (b) and G6PDH (c) under 80 g/L PEG 6000 treatment (■) and 30 mM $NaHCO_3$ treatment (□) on the 2d, 4th, 6th, and the 8th day in mulberry leaves. The values are shown as mean ± SD, $n = 3$. For the data analysis we used the comparative Ct ($2^{-\triangle\triangle Ct}$) method; transcript level of control is 1.

the anabolic pentose phosphate pathway in the leaves of this plant under these two abiotic stresses. The maintenance of PFK activity under stress in this plant suggests that it tends to maintain glycolytic metabolism as a way to sustain the ATP supply under stress conditions [9]. The inhibition of glycolysis has been suggested as a mechanism for accumulating sugars as an energy source for recovery and fast growth when water has become available [25]. The increase in G6PDH activity indicates that plant needs more biosynthetic precursors and NADPH under stress conditions.

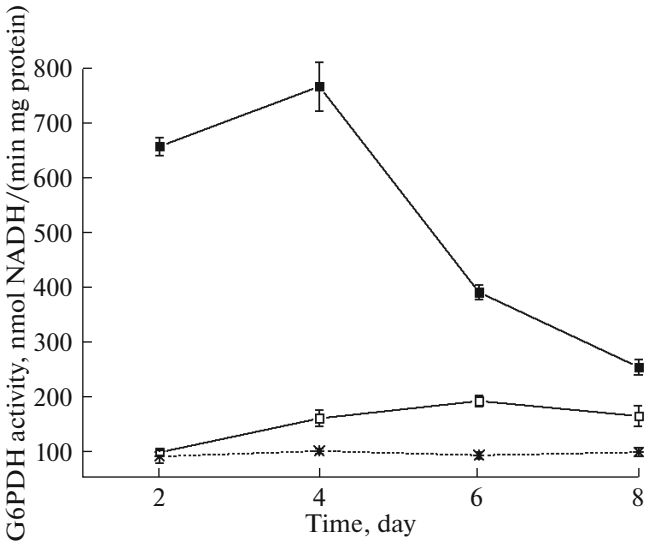

Fig. 4. The activities of G6PDH enzyme under 80 g/L PEG 6000 treatment (■), 30 mM $NaHCO_3$ treatment (□) and control treatment (*) on the 2d, 4th, 6th, and the 8th day in mulberry leaves. The values are shown as mean ± SD, $n = 3$. Both stress treatment significantly different as compared to control ($p < 0.05$).

But our results show that there are some differences between drought and bicarbonate stresses. At functional level, under 80 g/L PEG 6000 treatment, the PFK activity has a rapid change and then was stable (Fig. 3). It suggesting that PFK activity is sensitive to drought stress and reflects its weaker regulating ability under this stress condition. The PFK activity decreased under 30 mM $NaHCO_3$ treatment at first but then was shown to increase. This suggests that HCO_3^- could play a unique role under HCO_3^- treatment; that it induces abiotic stress in the glycolytic pathway but that it can also be used as an alternative source of inorganic carbon for this kind of plant. Moreover, some studies indicate that the bicarbonate addition may increase photosynthesis. The utilization of bicarbonate by terrestrial plants involves the action of carbonic anhydrase which is continuously activated under stress [26]. The PFK activity decreased at first but was then shown to increase under HCO_3^- treatment due to the activity of carbonic anhydrase induced by bicarbonate stress. The specific effect of HCO_3^- may also change PFK and G6PDH activity and mRNA level encoding these genes were more complex than those observed under drought stress (Figs. 3, 4, 5). It has been reported that the plant

growth can be inhibited under very low concentrations (1 mM) of bicarbonate [27]. But in our pre-experiments, it was found that some plants grew more vigorously under 10 mM $NaHCO_3$ treatment. This may have been related to the sensitivity of different plants to some metal ions, and these metal ions are closely related to the activity of the enzyme. For example, in some studies, the plants were divided into Zn-efficient and Zn-inefficient genotypes [28]. The activity of many important enzymes in the process of photosynthesis depends on the presence of metal ions: e.g., the activity of carbonic anhydrase relies on the presence of Zn^{2+}, the activity of phosphoenolpyruvate carboxylase and ribulose bisphosphate carboxylase relies on the presence of Mg^{2+}. As bicarbonate stress may induce a decrease in Zn^{2+}, Ca^{2+}, K^+, Cu^{2+}, Mn^{2+}, and Mg^{2+} levels in plants, it is possible to inhibit the activity of photosynthetic enzymes. But bicarbonate may also provide HCO_3^- as a substrate for photosynthesis, especially when plants closed their stomata under stress conditions. The combined effect may be related to the concentration of bicarbonate and the plant species, which need to be further examined to understand their regulating ability.

The G6PDH is a key enzyme of the pentose phosphate pathway which is the most important NADPH generating pathway. As Fig. 4 shows, under 80 g/L PEG 6000 treatment, G6PDH activity was sensitive and had large increase initially under drought stress. The enzyme activity had diminished quickly which may be due to the fact that all eukaryotic G6PDHs are feedback inhibited by NADPH [29] and as a consequence of overall reduction of glucose supply occurring simultaneously. The results showed that the stress conditions had little effect on the enzyme activity under 30 mM $NaHCO_3$ treatment at the beginning. However, the cumulative effect of continued stress was shown to cause a slight rise in enzyme activity as time went on.

In our study, the mRNA level of *ATP-PFK, PPi-PFK,* and *G6PDH* had not be significant changed under 80 g/L PEG 6000 treatment. It means that the changes of these enzyme activities were only related to metabolic profile regardless of gene transcriptional level under this kind of stress. Under bicarbonate stress, the changes also took place at the genes transcriptional level encoding *PPi-PFK, ATP-PFK,* and *G6PDH*. This reflects that bicarbonate stress provides a way to stimulate mRNA expression of genes encoding *PPi-PFK, ATP-PFK,* and *G6PDH*. The following decline of mRNA level of genes encoding those enzymes could be due to the detrimental effects of long time stress to plants. At last, the mRNA level of genes encoding those enzymes was even slightly lower under 30 mM $NaHCO_3$ treatment than that under control and might also be a consequence of overall reduction in biochemical activities of the plant cells under this stress.

Moreover, it was noticed that the mRNA level of *PFK* and *G6PDH* did not always correlate with enzymatic activity. This may have been due to post-transcriptional and post-translational modifications, compartmentalization, metabolite stability, substrate availability, etc. The changes in the abundance of transcripts are not necessarily translated into changes in metabolite levels [7]. The activity of the G6PDH enzyme under PEG treatment increased significantly on the 2d day (Fig. 4), but the mRNA of *G6PDH* genes had no obvious change (Fig. 5c). This may have been because of the plant can adapt to its environment by rapid metabolic modulations. According to previous reports, the adjustment of plant metabolism may occur even before the change in plant water status under stress [30]. Plant stress responses are dynamic and involve cross-talk complex between different regulatory levels. The mechanisms of action of different regulators are complicated, and it will be interesting to analyze how different kinds of stresses may influence metabolic processes, transcriptional levels, and adaptation to stresses.

Last of all, although mulberry seedlings had stopped growing at same time under two kinds of stresses, the relationship between of PFK activity and G6PDH activity is not a simply negative correlation and the changes of mRNA levels of *PFK* and *G6PDH* genes and their activities are so different under two kinds of stresses.

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