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Research article

Response of sugar metabolism in apple leaves subjected to short-term drought stress

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ABSTRACT

For a comprehensive understanding of gene expression, enzyme activity and sugar concentrations in response to short-term water deficit in apple (Greensleeves), sugar-modulated gene expression and enzyme activities were analyzed. Water stress resulted in the accumulation of sorbitol, glucose, fructose, galactose and starch, accompanied by a significant reduction in photosynthesis and sucrose concentration. In response to short-term water deficits, the activities of aldose-6-phosphate reductase (A6PR; EC 1.1.1.200), sorbitol dehydrogenase (SDH; EC 1.1.1.14), neutral invertase (NINV; EC 3.2.1.26), sucrose synthase (SUSY; EC 2.4.1.13), and fructokinase (FK; EC 2.7.1.4) were higher, whereas cell wall invertase (CWINV; EC 3.2.1.26) and hexokinase (HK; EC 2.7.1.1) activities were lower. In addition, sucrose phosphate synthase (SPS; EC 2.4.1.14) activity increased during the initial stages of dehydration and then decreased as the drought strengthened. Transcript levels of *MdA6PR*, *MdSDH1/2*, *MdNINV1/2*, *MdSUSY3*, *MdFK1/2/4*, *MdSOT1/2*, *MdSUC1-3*, *MdTMT2/3*, *MdvGT1*, *MdpGlcT1-4* were upregulated, whereas transcript levels of *MdCWINV1/2*, *MdHK1/2/3/5*, *and MdTMT1* were downregulated after 6 days of water stress. These findings suggest that the sorbitol metabolism pathway is induced and high levels of hexose derived from photosynthetic products are transported into vacuoles for adjustment to the water deficit. Our results provide insights into the relationships between sugar levels and sugar-modulated gene and enzyme activity in response to the imposition of short-term water stress.

1. Introduction

Carbohydrates produced from photosynthesis in plant leaves provide energy and building blocks for plant growth and productivity. In addition, soluble carbohydrates (e.g., sucrose, fructose, glucose and sorbitol) are known to act as important osmoregulation substances to maintain cell turgor under osmotic stress (Subbarao et al., 2000), e.g., drought and salt. Drought stress significantly affects carbohydrate-modulated gene expression in plant cells (Gifford and Bremner, 1981; Boyer, 1982; Koch, 1996; Bartels and Sunkar, 2005), and changed soluble sugars under drought stress act as signal molecules to regulate the expression of many key genes involved in plant defense responses and metabolic processes, consequently controlling plant resistance and growth (Rosa et al., 2009). Regulation of soluble carbohydrate concentrations in plant cells is an important pathway of plant adaptation or resistance to water deficit, but the understanding of this pathway is still limited.

Soluble carbohydrate concentrations in leaves are highly regulated by the balance between synthesis, degradation and export. Sucrose is a main soluble product of photosynthesis in most plants and is synthesized in green leaves and transported into sink tissues or cell compartments by sucrose transporters (Ruan, 2014). One of the key enzymes involved in sucrose synthesis is sucrose-phosphate synthase (SPS) (Huber and Huber, 1992). Sucrose can be converted to fructose (Fru) and glucose (Glc) by invertase or to Fru and UDP-glucose (UDPG) by sucrose synthase (SUSY) (Ruan, 2014). Although SUSY can catalyze both sucrose synthesis and decomposition, the latter is its main role (Geigenberger and Stitt, 1993). The resulting Glc and Fru are then phosphorylated to glucose-6-phosphate (G6P) and fructose-6-phosphate (F6P), respectively, by hexokinase (HK) or fructokinase (FK) (Granot,

Abbreviations: A6PR, aldose-6-phosphate reductase; AINV, vacuolar acid invertase; CWINV, cell wall invertase; DTT, dithiothreitol; FC, field capacity; FK, fructokinase; Fru, fructose; F6P, fructose-6-phosphate; Glc, glucose; HK, hexokinase; NINV, neutral invertase; PPFD, photosynthetic photon flux density; PVPP, polyvinylpolypyrrolidone; SDH, sorbitol dehydrogenase; Suc, sucrose; SUSY, sucrose synthase; SPS, sucrose phosphate synthase; SPP, sucrose-phosphate phosphatase; UDPG, UDP-glucose

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2007).

The interconversions between F6P, G6P, G1P, and UDPG are enzymatically catalyzed in readily reversible reactions. Both F6P and UDPG produced in sugar metabolism can be combined to resynthesize Suc via sucrose phosphate synthase (SPS) and sucrose-phosphate phosphatase (SPP, EC 3.1.3.24) (Hoffmann-Thoma et al., 2010). Suc, Glc and Fru and other soluble sugars that have not been metabolized are transported into vacuoles by special transporter proteins located on the vacuole membrane. Once inside the vacuole, Suc can also be converted to Glc and Fru by vacuolar acid invertase (AINV) (Ruan, 2014). This sugar metabolism system in cells regulates sugar concentrations and maintains the balance in osmotic potential and turgor between the cytosol and other subcellular compartments.

Most stress conditions (especially drought) lead to the accumulation of carbohydrates in leaves, which can play an important role in osmoprotection, osmotic adjustment, carbon storage and radical scavenging in plants (Parvaiz and Satyawati, 2008). Under stress conditions, a change in the sugar concentrations would be a result of the regulation of sugar metabolism and export. Despite the reduced carbon fixation in drought-stressed leaves, plants accumulate a large amount of water-soluble carbohydrates such as glucose, fructose, sucrose, stachyose, mannitol and pinitol; the types of soluble carbohydrates vary among species (Bohnert et al., 1995; Pinheiro et al., 2001; Bartels and Salamini, 2001; Villadsen et al., 2005; Valliyodan and Nguyen, 2006). The accumulation of water-soluble carbohydrates is widely regarded as an adaptive response of plants to drought stress. These soluble carbohydrates are not only used as osmolytes for maintaining leaf cell turgor and protecting membrane integrity (Bartels and Sunkar, 2005; Verslues et al., 2006) but also act as nutrient and metabolite signaling molecules, modulating the expression of a large number of metabolic genes through sugar-sensing mechanisms (Ho et al., 2001; Price et al., 2004). The accumulation of these sugars represents extensive carbon redistribution in plants when carbon fixation is reduced. To elucidate the regulation of carbohydrate metabolism involved in carbon redistribution for the accumulation of soluble sugars in plant leaves under drought stress, a systematic metabolic pathway-based analysis would provide insight into the roles of carbohydrate metabolism in response to drought stress. Although sugar contents and enzyme activity in pathways of carbohydrate metabolism have been investigated under drought stress in many plants (Ranney et al., 1991; Wang and Stutte, 1992; Escobar-Gutiérrez et al., 1998; Xu et al., 2001), a systematic understanding of gene expression, enzyme activity and sugar concentration is limited, and it is not well known which genes are important in regulating sugar metabolism under drought stress as several isoenzymes are coded by different homologous genes (Li et al., 2018).

In apple and many other *Rosaceae* fruit trees, sorbitol is a primary end product of photosynthates produced in leaves (Beruter, 2004). It was suggested that water stress favors a functional role for sorbitol as an osmoticum in apple (Wang and Stutte, 1992; Bianco et al., 2012; Wu et al., 2014), whereas other soluble sugars exhibited different responses to drought stress in different apple cultivars (Wang et al., 1995; Li et al., 2005). However, our knowledge is very limited to why these carbohydrate contents in apple leaves would change to adapt to drought stress.

The objectives of this study were to understand how apple plants adjust carbohydrate metabolic flow to increase sugar osmolytes under the conditions of reduced photosynthesis and to identify which genes are involved in the regulation of sorbitol and sugar metabolism in apple leaves during drought stress and after re-watering. We focused mainly on the regulation of enzyme activities and gene expression levels involved in the synthesis of sorbitol and the subsequent conversion to sucrose and hexoses in drought-stressed apple leaves using a systematic metabolic pathway-based expression analysis. These analyses provide a complete picture of the regulation and relationships of various genes and enzymes involved in these metabolic pathways and the role of important isoenzymes in the alteration of carbohydrate metabolism in drought-stressed apple leaves.

2. Materials and methods

2.1. Plant materials

One-year-old 'Greensleeves' apple (*M. domestica* Borkh.) trees grafted on seedling rootstocks (*M. hupehensis* (Pamp) Rehd.var.pinyiensis Jiang, which is an apomictic species) were used in this study. Grafted plants were planted in plastic pots (28 × 21 cm) that were filled with a mixture of sand, organic-eral fertilizer, and soil (1:1:3, v:v:v). All pots initially weighed the same (9.5 kg) to facilitate calculations and to maintain a specified field capacity via the weighing method. All trees were grown in the greenhouse at Northwest A&F University, Yangling (34°20′N, 108°24′E), Shaanxi, China. They were watered daily and were supplied weekly with Hoagland's solution (pH 6.5 ± 0.1) during the growing season. In August, uniform and healthy plants (with a height of 1.2 m) were moved into the glasshouse. Growing conditions included a temperature range of 25 °C (night) to 32 °C (day), a relative humidity range of 70–80% and a mid-day photosynthetic photon flux density (PPFD) of approximately 2000 photons m^{-2} s^{-1}. After 2 weeks, the 102 plants were separated into eight groups (one group consisted of at least 12 plants as one biological replicate); four groups were well watered as the control to maintain the soil water content at a.m. 75–80% field capacity (FC), and the remaining groups (drought-treated) were watered once to 75–80% FC at the beginning of the experiment. At 2, 4 and 6 days after watering ceased, the mature leaves of the control and treated plants were sampled between 10:00 and 11:00 a.m., and the leaf water potential (Ψw) was measured using a pressure chamber (Model 1000, PMS Instrument Co., Corvallis, OR, USA). Six days after watering ceased, the upper leaves wilted, so the drought plants were re-watered after sampling and the soil water conditions were maintained for two days before these watered samples were harvested. Once collected from the plants, the leaf samples were immediately frozen in liquid nitrogen and were stored at −80 °C until use.

2.2. Photosynthesis and relative electrolyte leakage

Before leaves were harvested to determine the relative electrolyte leakage, net photosynthesis was measured between 9:30 and 11:00 with a Li-Cor 6400 portable photosynthesis system (Li-Cor Inc., Lincoln, NE, USA). The relative electrolyte leakage of fresh leaves was measured in the laboratory according to Wu et al. (2014) using an electrical conductivity meter.

2.3. Measurements of soluble sugars and starch

Soluble sugars were extracted and derivatized according to Li et al. (2016). Briefly, 0.1 g of leaf samples was extracted in 1.4 ml 75% methanol, and ribitol added as an internal standard. After fractionation of non-polar metabolites in chloroform, 2 and 50 µl of the polar phase of each sample were collected and transferred into 2.0-ml Eppendorf vials to determine highly abundant metabolites (such as Sor and Suc) and less abundant metabolites (such as Glc, Fru, and Gal), respectively. The samples were dried under vacuum without heating and were then derivatized sequentially with methoxyamine hydrochloride and N-methyl-N-trimethylsilyl-trifluoroacetamide (Yang et al., 2018). After derivatization, the metabolites were analyzed with a Shimadzu GCMS-2010SE (Shimadzu Corporation, Tyoto, Japan). The metabolites were identified by comparing their fragmentation patterns with those from a mass spectral library generated on our GC/MS system and from an annotated quadrupole GC–MS spectral library downloaded from the Golm Metabolome Database (http://csbdb.mpimp-golm.mpg.de/csbdb/gmd/msri/gmd_msri.html). Quantifications were based on standard curves generated for each metabolite and internal standard.

The tissue residue after 75% methanol extraction for GC-MS analysis was re-extracted with 80% (v/v) ethanol at 80 °C three times, and the pellet was retained for the determination of starch. After digesting the residue with 30 U of amyloglucosidase (EC 3.2.1.3) at pH 4.5 overnight, starch was determined enzymatically as glucose equivalents (Li et al., 2016).

2.4. Assay of enzyme activities

The activities of SDH, SPS, SUSY, CWINV, NINV, AINV, FK, and HK were assayed according to our methods used for apple fruit (Li et al., 2018) with some modifications.

For SDH, a 0.60-g sample was homogenized in 4 ml of 100 mM potassium phosphate (pH 7.8) buffer containing 1 mM dithiothreitol (DTT), 1 mM EDTA, 1% BSA, 0.2% Triton X-100 and 2% (w/v) insoluble polyvinylpolypyrrolidone (PVPP). The extract was then centrifuged at 16, 000 *g* for 10 min at 4 °C, after which 1 ml of the supernatant was immediately desalted in a Sephadex G25 PD-10 column (Amersham BioSciences, Piscatway, NJ, USA). SDH activity was assayed in a 1.0 ml reaction mixture containing 300 mM Sor, 1 mM NAD^+, and 0.2 ml of the desalted extract in 100 mM Tris-HCl (pH 9.6), and NADH production was determined at 340 nm. SDH activity was calculated in terms of 1 μmol of NAD^+ reduced per hour under the assay conditions.

To extract A6PR, SPS, SUSY, CWINV, NINV, AINV, FK, and HK, each sample (0.60 g) was homogenized in 4 ml of 200 mM Hepes-KOH (pH 8.0) buffer containing 2 mM EDTA, 2.5 mM DTT, 5 mM $MgCl_2$, 2 mM benzamidine, 0.1 mM leupeptin, 0.1% BSA, 2% glycerol, 1% Triton X-100 and 2% PVPP, similar to that used by Li et al. (2012). The homogenate was centrifuged at 16, 000 *g* for 20 min at 4 °C, and 1 ml of the supernatant was desalted in a Sephadex G25 PD-10 column (Amersham BioSciences, Piscatway, NJ, USA) equilibrated with the extraction buffer at the concentration of 50 mM Hepes-(KOH) (pH 7.4) but without Triton X-100 or DTT.

A6PR activity was assayed according to Negm and Loescher (1981) with some modifications. Briefly, the 1 ml reaction mixture contained 0.1 M Tris-HCl (pH 9.0), 0.15 mM NADPH, 50 mM G6P and 50 μl leaf extract. The mixture was incubated at 27 °C, and NADPH reduction was determined at 340 nm A6PR activity was calculated in terms of 1 μmol of NADPH oxidation per hour under the assay conditions.

CWINV and AINV were assayed at 37 °C for 60 min in a 200 μl assay mixture that contained 100 mM phosphate-citrate buffer (pH 7.2), 100 mM sucrose, and 50 μl of the desalted extract or denatured extract (as a blank). The assay was stopped by boiling for 3 min before adding 0.75 M Tris-HCl buffer (pH 8.5). The blanks contained the same mixture, but the extract was boiled for 5 min before being mixed. The assay conditions for NINV were the same except that the assay mixture contained Hepes-KOH (pH 7.2) as a buffer. The level of glucose generated from sucrose was determined by the enzyme-coupling method. Invertase activity was presented as the amount of enzyme that produced 1 μmol glucose per hour under the assay conditions.

We determined the activity of SPS as described by Li et al. (2018). The assay mixture (200 μl) contained 50 mM Hepes-KOH (pH 7.4), 4 mM $MgCl_2$, 1 mM EDTA, 4 mM F6P, 3 mM UDP-glucose, 20 mM G6P and 100 μl of the sample. The reaction was carried out at 27 °C for 30 min followed by boiling in water for 3 min. A blank was run for each assay by adding denatured extracts. After centrifugation for 2 min at 12, 000 g, 75 μl of the reaction mixture was used for UDP measurements in a spectrophotometric assay. The assay mixture (1.0 ml) contained 50 mM Hepes-KOH (pH 7.0), 0.8 mM phosphoenolpyruvate, 0.3 mM NADH, 5 mM $MgCl_2$, 14 U of lactate dehydrogenase, and 4 U of pyruvate kinase (to start the reaction).

SUSY activity was measured following the procedure of Dancer et al. (1990). The enzyme extract (20 μl) was incubated at 27 °C for 30 min in a 100 μl final volume of assay medium containing 20 mM Hepes-KOH (pH 7.0), 100 mM sucrose and 4 mM UDP. The reaction was stopped by boiling in water for 3 min. Blanks contained the same assay mixture, but denatured extract was used. The UDPG content produced in the assay was measured spectrophotometrically following the reduction of NAD^+ coupled with UDPG dehydrogenase activity in a reaction mixture (1.0 ml) containing 5 mM $MgCl_2$, 2 mM NAD^+ and 0.02 U UDPG dehydrogenase, and 100 μl of the reaction mixture in 200 mM glycine (pH 8.9) was used for SUSY. The mixture was incubated at 27 °C for 30 min, and NADH production was determined at 340 nm.

HK and FK activities were assayed via a continuous spectrophotometric assay as described by Li et al. (2018) with minor modifications. For HK, the 0.5 ml assay mixture contained 50 mM Tris-HCl (pH 8.0), 2.5 mM ATP, 4 mM $MgCl_2$, 1 U of G6P dehydrogenase, 0.33 mM NAD^+, 1 mM glucose and 25 ml of desalted extract. For FK, one unit of phosphoglucoisomerase was also added, and 0.4 mM fructose was used instead of glucose.

2.5. RNA extraction and qRT-PCR assays

Total RNA was extracted from samples using an RNAprep plant kit (Tiangen, Beijing, China) according to the manufacturer's instructions. The RNA was then reverse-transcribed into cDNA using a PrimeScript™ RT reagent kit and gDNA Eraser to avoid possible genomic DNA contamination (TaKaRa, Dalian, China). The primer sequences used for quantitative real-time polymerase chain reaction (qRT-PCR) assays are presented in Supplementary Table 1. *Mdactin* (CN938023) was chosen as an internal control for data normalization. qRT-PCR was performed on an ABI 7300 Real-Time PCR instrument (Thermo Fisher Scientific) using a SYBR Green Premix Ex Taq Kit (Takara, Kyoto, Japan). The PCR conditions were as follows: pre-denaturing at 94 °C °C °C.

2.6. Statistical analysis

Data were subjected to repeated measures ANOVA with SAS statistical software (Version 9.2, SAS Institute, Cary, NC, USA). Data are presented as the mean ± SD of four biological replicates. LSD values were calculated in cases where significant variance was found at $P < 0.05$.

3. Results

3.1. Water stress and Pn

Midday leaf water potentials decreased in drought-stressed trees as the water withholding duration increased, whereas controls exhibited constant leaf water potentials (Fig. 1). According to Hsiao et al. (1976), water-stressed plants show a mild water deficit after 2 days without watering, moderate stress after 4 days without watering, and severe stress after 6 days without watering, as indicated by their leaf water potential, compared to well-watered controls. Two days after the drought-stressed trees were rewatered, the leaf water potential returned to the level measured under the mild water deficit (Fig. 1). The leaves showed a continuous decrease in Pn with drought stress, and a low value was measured after 6 days without watering, whereas Pn recovered significantly 2 days after rewatering.

3.2. Soluble carbohydrates

The carbohydrates measured in the apple tree leaves were sorbitol, sucrose, fructose, glucose, galactose and starch (Fig. 2). Sorbitol, a main photosynthetic assimilate in apple leaves, increased significantly above the control levels after 4 and 6 days without watering, even though net photosynthesis decreased at those times. In addition, the concentration of sorbitol was significantly lower than that measured in the control 2 days after rewatering. Sucrose concentration in drought-stressed leaves was much lower than those in the control leaves after 2 days without watering and was significantly lower 2 days after rewatering. However,

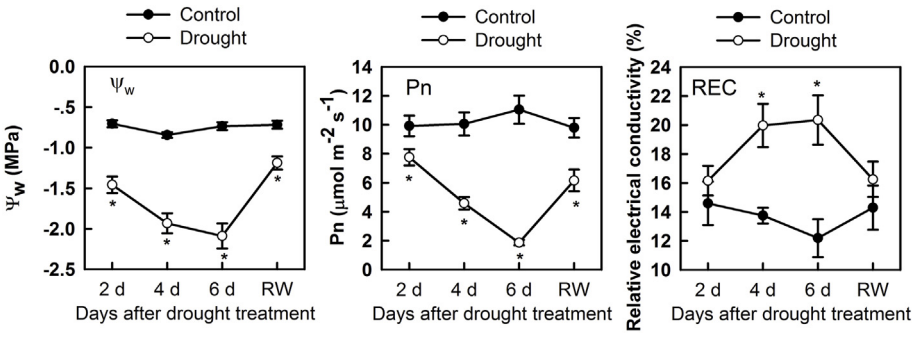

Fig. 1. Changes in water potential (ψ_w), net photosynthesis (Pn) and relative electrical conductivity (REC) in apple leaves at different days after drought treatment and at 2 days after recovery water (RW). Each point is mean ± SD for four biological replicate samples. The star at each sampling point is the least significant difference at the 0.05 level between drought treatment and control samples.

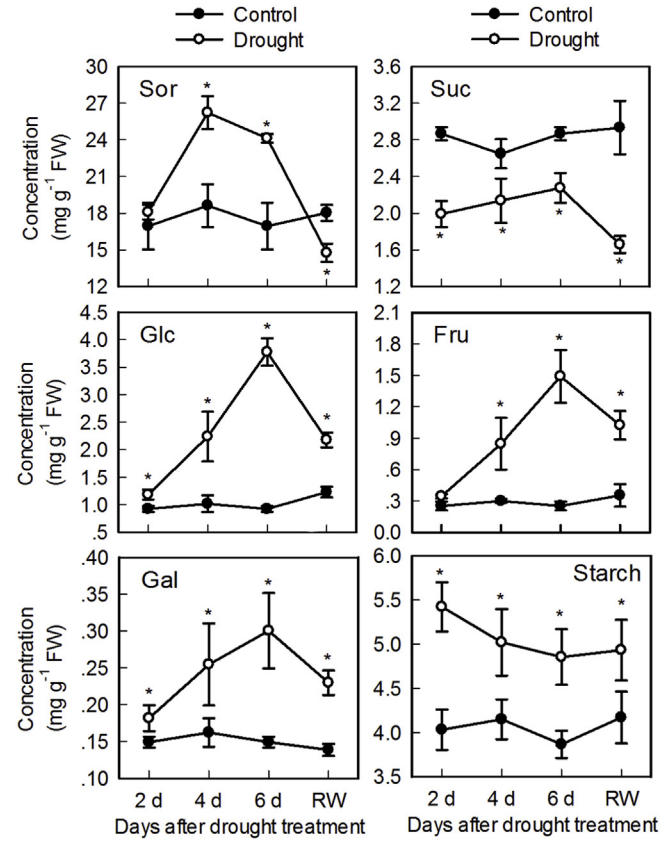

Fig. 2. Changes in concentrations of sorbitol (Sor), sucrose (Suc), glucose (Glc), fructose (Fru), galactose (Gal) and starch in apple leaves at different days after drought treatment and at 2 days after recovery water (RW). Each point is mean ± SD for four biological replicate samples. The star at each sampling point is the least significant difference at the 0.05 level between drought treatment and control samples.

the fructose, glucose and galactose concentrations increased with increased drought stress compared with the control levels, and their levels partly recovered 2 days after rewatering. Starch concentrations peaked after 2 days without watering and were higher than the control levels even after rewatering.

3.3. Enzyme activity and gene expression of A6PR and SPS

A6PR, an enzyme involved in the limiting step of sorbitol synthesis, showed significantly increased activity above the control levels after 2 days without watering, whereas A6PR activity decreased on the following day but returned to the control level 2 days after rewatering (Fig. 3). *MdA6PR* mRNA expression showed similar change patterns as its enzyme activity (Fig. 5).

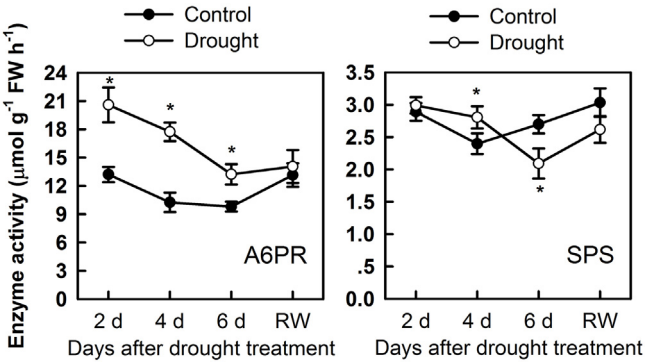

Fig. 3. Changes in enzyme activities of aldose-6-phosphate reductase (A6PR), sucrose phosphate synthase (SPS) in apple leaves at different days after drought treatment and at 2 days after recovery water (RW). Each point is mean ± SD for four biological replicate samples. The star at each sampling point is the least significant difference at the 0.05 level between drought treatment and control samples.

Compared with the control, the enzyme activity of SPS obviously increased after 4 days without watering but decreased after 6 days without watering, while its activity nearly returned to the control level after rewatering (Fig. 3). Six homology genes encoding SPS showed different responses to drought stress. The expression abundances of *MdSPS1* and *MdSPS5* were obviously downregulated in stressed leaves compared with those in the control, but *MdSPS2* and especially *MdSPS3* and *MdSPS4* increased under drought stress (Fig. 5).

3.4. The activity and gene expression of enzymes related to sugar metabolism

SUSY mainly catalyze both sucrose synthesis and decomposition, and its activity was significantly higher than the control level, not only after 4 and 6 days without watering, but also 2 days after rewatering; the peak activity was measured after 4 days without watering (Fig. 4). Among 5 *MdSUSY* genes, *MdSUSY2* and *MdSUSY4* showed decreased expression in drought-stressed leaves after 6 days without watering (Fig. 5). The expression levels of *MdSUSY1*, *MdSUSY3* and *MdSUSY5* were upregulated by drought stress, and the peak was detected after 4 days without watering. Even if stressed plants were rewatered for 2 days, the expression levels of both *MdSUSY3* and *MdSUSY5* were 2-fold higher than those measured in the control (Fig. 5).

With respect to the enzymes related to sugar metabolism, the activities of SDH and FK increased at 2–3 time points of drought stress and returned to the control levels 2 days after rewatering, whereas NINV activities in stressed leaves were significantly higher after 4 and 6 days without watering compared with those measured in the control (Fig. 4). However, under drought stress conditions, CWINV and HK in apple leaves showed significantly decreased activities, whereas AINV activities were unchanged (Fig. 4).

Both *MdSDH1* and *MdSDH2* expression levels increased under

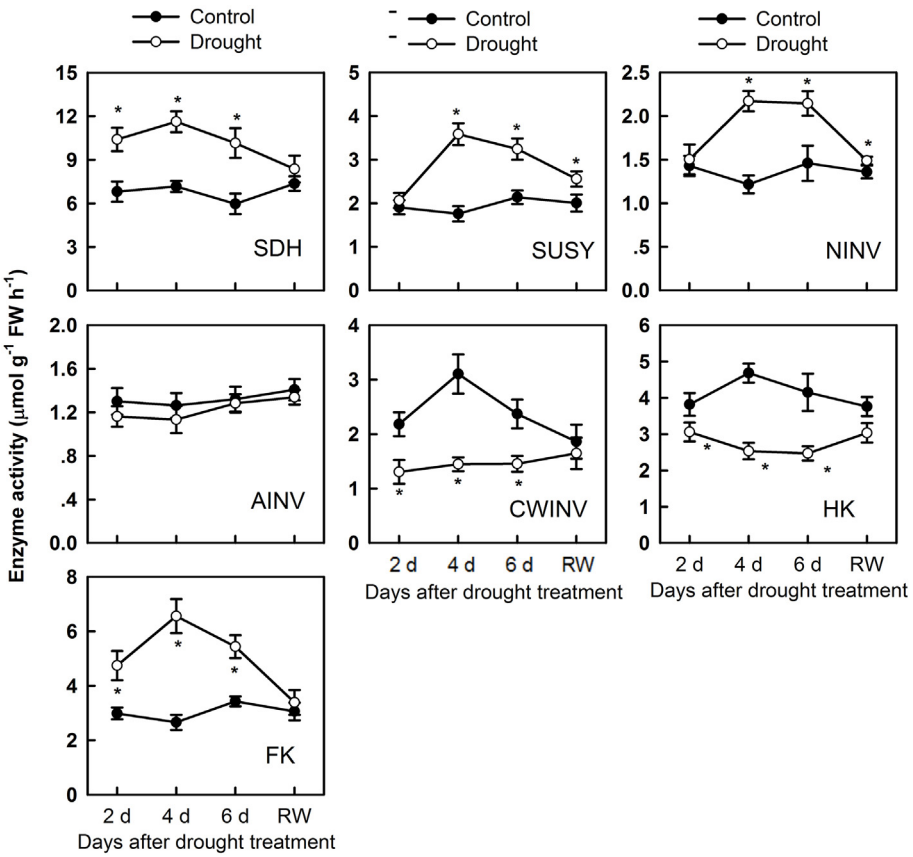

Fig. 4. Changes in enzyme activities of sorbitol dehydrogenase (SDH), sucrose synthase (SUSY), cell wall invertase (CWINV), neutral invertase (NINV), acid invertase (AINV), hexokinase (HK), and fructokinase (FK) in apple leaves at different days after drought treatment and at 2 days after recovery water (RW). Each point is mean ± SD for four biological replicate samples. The star at each sampling point is the least significant difference at the 0.05 level between drought treatment and control samples.

drought stress and reached a peak after 6 days without watering (Fig. 5). Moreover, *MdSDH2* had higher expression 2 days after rewatering compared with the control. The expression levels of 2 *MdCWINVs* (especially *MdCWINV2*) and 4 *MdHKs* were downregulated in apple leaves by drought stress even after rewatering for 2 days, whereas 2 of 3 *MdNINVs* (*MdNINV1/2*) and 3 of 4 MdFKs (*MdFK1/2/4*) in drought-stressed leaves showed increased expression, with peak expression after 4 days without watering (Fig. 5). Additionally, *MdAINV2* had decreased expression after 2 and 4 days without watering, but the expression increased after 6 days without watering (Fig. 5).

3.5. Expression profiling of genes related to sugar transport

To further understand the influence of drought stress on sugar transport in apple leaf cells, the mRNA expression of the main sugar transporters was detected in drought-stressed leaves. The expression levels of sorbitol transporters *MdSOT1* and *MdSOT2* were increased under water-stress conditions, and *MdSOT1* had a significant peak after 4 days without watering. Three sucrose carriers (or transporters), *MdSUC1/2/3*, showed increased trends with drought stress, and *MdSUC1* expression in the rewatered leaves remained higher than that in the control, while *MdSUC3* expression peaked after 4 days without watering (Fig. 6).

Tonoplast sugar transporter *MdTMT1* expression was inhibited by drought stress even after rewatering, but *MdTMT2* had obviously increased expression after 4 days without watering and showed the highest expression after 6 days without watering (Fig. 6). By contrast, *MdTMT3* expression in the treated leaves was more than 2-fold higher than that in the control after 6 days without watering and 2 days after rewatering (Fig. 6).

Putative vacuole glucose transporter *MdvGT1* expression was increased in the treated leaves even after 2 days of rewatering, but *MdvGT2* showed adverse changes (Fig. 6). The expression abundance of putative 4 plastid glucose transporter *MdpGlcTs* increased under drought stress, especially after 6 days without watering. However, 2 days after rewatering, treated leaves still had higher expression levels of *MdpGlcT1* compared with the control, but the expression abundances of *MdpGlcT2* and *MdpGlcT3* were lower (Fig. 6).

4. Discussion

Leaf water potential has been used to determine the degree of plant water deficit or water status, and osmotic adjustment is an adaptive process that greatly assists in the maintenance of turgor under water deficit (Turner et al., 1978). The significant decrease of leaf water potential and continued increase of relative electrical conductivity during the experimental treatments (Fig. 1) suggest that the trees were subjected to an increasing degree of drought stress. As the leaf water potential decreased, photosynthesis was significantly inhibited in water-limited apple plants (Fig. 1).

It is well established that high soluble carbohydrate accumulation contributes to the adaptive mechanism in response to water deficit in woody plants (Kozlowski, 1981; Jones et al., 1985). In our study, the short-term drought stress provoked a significant increase in sorbitol, glucose, fructose, galactose and starch concentrations, while sucrose concentration was significantly lower in the drought-stressed one-year old 'Greensleeves' trees than in well-watered one-year old 'Greensleeves' trees (Fig. 2). These results are in agreement with reports on apple (Wang and Stutte, 1992; Xu et al., 2001), cherry (Ranney et al., 1991) and peach (Escobar-Gutiérrez et al., 1998). The rapid increase in sorbitol and the slow rate at which it disappeared strongly suggests that the prime role of sorbitol in these one-year old 'Greensleeves' trees is the storage of carbon. By contrast, the behavior of sucrose during the drought phase suggests that it was more available than sorbitol, which was formed at the same time.

The responses of foliar starch concentrations to water deficit (Fig. 2)

	2D	4D	6D	RW
A6PR	0.58	0.88	0.47	0.05
SPS1	-0.23	-0.49	-0.57	-0.33
SPS2	0.44	1.86	0.72	0.15
SPS3	0.36	3.40	3.08	1.11
SPS4	1.94	3.67	6.11	2.65
SPS5	0.15	-0.26	-0.57	-0.28
SPS6	0.96	0.14	-0.24	-0.32
SUSY1	0.31	1.09	0.45	0.23
SUSY2	0.07	-0.26	-0.50	-0.37
SUSY3	0.62	1.59	1.50	1.01
SUSY4	0.15	-0.06	-0.30	-0.03
SUSY5	0.78	6.03	1.24	1.01
SDH1	0.46	1.40	1.66	-0.08
SDH2	0.22	1.23	3.01	1.41
CWINV1	-0.16	-0.19	-0.42	-0.21
CWINV2	-0.34	-0.66	-0.77	-0.54
NINV1	0.60	2.34	1.48	0.60
NINV2	0.25	0.72	1.01	-0.04
NINV3	-0.26	-0.28	-0.48	-0.12
AINV1	-0.29	-0.18	0.17	-0.32
AINV2	-0.65	-0.50	0.81	0.29
AINV3	-0.22	0.09	0.39	-0.10
HK1	-0.34	-0.41	-0.43	-0.11
HK2	-0.35	-0.50	-0.54	-0.38
HK3	-0.39	-0.50	-0.68	-0.54
HK5	-0.35	-0.42	-0.47	-0.11
FK1	0.50	1.85	1.35	0.20
FK2	0.45	0.94	0.75	0.51
FK3	-0.18	-0.24	-0.53	-0.25
FK4	0.05	0.88	0.45	0.31

Fig. 5. Change folds in transcript level of genes encoding the enzyme related with sugar synthesis and metabolism in apple leaves compared to these in the control samples at different days after drought treatment and at 2 days after recovery water (RW). Relative expression abundances based on qRT-PCR are shown in Supplementary Table 1. Change fold at each sample point is counted as following method: change folds = (drought value – control value)/control value. In above figure, the red setting at each sampling point is the least significant upregulation at the 0.05 level in drought treatment compared with control samples, whereas green setting means signicant downregulation, and the gray is unsignicant. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

were not compatible with those reported for apple (Wang and Stutte, 1992; Xu et al., 2001; Li et al., 2005); these authors observed decreases in the starch concentration in apple trees under water stress. We believe that this difference might be due to the different treatment methods of drought stress applied to the apple trees. In our study, the one-year old 'Greensleeves' trees directly experienced a short-term drought stress, while drought was imposed in several cycles before the initiation of water stress in other experiments (Wang and Stutte, 1992; Xu et al., 2001; Li et al., 2005). Under sudden drought stress, although photosynthetic rate is reduced, the translocations of sucrose and sorbitol would be slowed from source leaves to sink tissue because of reduced water movement and inhibited growth of active sink cells. In addition, synthesis capabilities of sorbitol and sucrose were increased under drought stress. As results, the significantly increased concentrations of sorbitol and hexoses in cytosol would inhibit Triose phosphate (TP) input from chloroplasts, and excessive TP in the chloroplast would be used for starch synthesis. We propose that starch accumulation was a short-term adjustment to the sudden water deficit in our study.

As reported by Li et al. (2005), both the expression and activity of

	2D	4D	6D	RW
SOT1	0.49	1.32	0.32	0.31
SOT2	0.88	1.28	1.24	0.03
SUC1	0.47	1.87	1.92	0.46
SUC2	0.49	0.54	0.95	-0.10
SUC3	0.60	0.87	0.38	-0.01
TMT1	-0.31	-0.70	-0.65	-0.50
TMT2	-0.04	0.97	2.96	0.90
TMT3	-0.22	-0.15	1.21	1.26
vGT1	0.43	1.05	1.31	1.08
vGT2	-0.44	-0.41	-0.24	-0.32
pGlcT1	0.28	0.70	2.08	0.52
pGlcT2	0.18	0.11	0.95	-0.36
pGlcT3	-0.03	0.10	0.79	-0.47
pGlcT4	0.07	0.72	0.67	-0.08

Fig. 6. Change times in transcript level of genes encoding sugar transporters in apple leaves compared to these in the control samples at different days after drought treatment and at 2 days after recovery water (RW). Relative expression abundances based on qRT-PCR are shown in Supplementary Table 1. Change time at each sample point is counted as following method: change time = (drought value – control value)/control value. In above figure, the red setting at each sampling point is the least significant upregulation at the 0.05 level in drought treatment compared with control samples, whereas green setting means signicant downregulation, and the gray is unsignicant. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

A6PR, the key enzyme that regulates sorbitol synthesis (Cheng et al., 2005), were markedly higher in water-stressed leaves than in normal watered leaves (Fig. 3). In response to increased sorbitol synthesis and accumulation, both the transcript level and activity of SDH also increased in the water-stressed leaves (Fig. 3), which is consistent with previous findings in transgenic apple leaves overexpressing *MdFK2* (Yang et al., 2018). As fructose in leaves could be converted from sorbitol by SDH in cells (Li et al., 2016), a significantly higher fructose level was observed in leaves after water stress (Fig. 2). The significant increase in *FK1/2/4* expression and FK activity are important to ensure the phosphorylation of high levels of fructose and that more carbon is allocated to TCA or glycolysis for energy production.

The modulation of SPS activity was not correlated with changes in the sucrose concentration (Figs. 2 and 4). However, this observation is in disagreement with the results of Li et al. (2005), who showed that SPS had highly significant correlations with and positive effects on foliar sucrose concentration in water-stressed apple plants. Drought induced an increase in the expression of *MdNINV1/2* and the activity of NINV (Figs. 4 and 5), which converts one molecule of sucrose into two hexose molecules (Zhu et al., 1997) and generates higher hexose molarity to maintain sufficient turgor. We believe that the active sucrose metabolism catalyzed by NINV was the key factor that contributed to the decrease in foliar sucrose concentration under water deprivation. Increased HK activity was predicted in water-stressed plants based on significantly increased glucose concentration generated through sucrose metabolism. However, *MdHK1-5* expression and HK activity were obviously lower than those observed under normal watering and showed gradually decreased activity (Figs. 2 and 5). Renz and Stitt (1993) showed that both *SlHK1* and *SlHK2* were inhibited by ADP acting competitively against ATP, and *SlHK1* was also inhibited by glucose-6-phosphate (G-6-P) in potato. Although we did not detect F6P, we expected to observe an increase in F6P and ADP flux because of significantly elevated fructose phosphorylation via FK. This means that more F6P would be reversibly converted to G6P. Thus, it is likely that HK expression and activity are subject to product inhibition by excessive G6P or ADP generated from the phosphorylation of fructose via FK.

In source leaves, the small amount of sucrose in extracellular spaces can be hydrolyzed by cell wall invertase or imported into the cells by SUC (Ruan, 2014; Wei et al., 2014), while sorbitol in extracellular spaces can be imported into the cells by SOT (Wei et al., 2014). Water stress provoked a sharp decrease in cell wall invertase activity (Fig. 2) but elevated *MdSUC1-3* expression and *MdSOT1/2* expression (Fig. 2), indicating that more sucrose and sorbitol were imported from extracellular spaces into the cytosol for regulation of osmotic potential and turgor among intracellular compartments.

Tonoplast transporters play functional roles in osmotic balance, sugar storage and homeostasis in the cytosol by importing or exporting sugars in the vacuoles. Apple vacuolar glucose transporters *(MdvGTs*) transport vacuolar glucose, and apple tonoplast membrane transporters (*MdTMTs*) are involved in fructose, glucose or sucrose transport (Li et al., 2018). After 6 days of drought stress, the transcript levels of *MdTMT2/3* and *MdvGT1*, which can transport fructose and glucose into the vacuoles, were significantly upregulated (Fig. 6), indicating that more hexoses derived from sorbitol and sucrose were transported into the vacuoles to regulate the water potential and maintain the normal volume of the cells. This is consistent with the sharply elevated glucose and fructose concentrations under water-stress conditions (Fig. 2). Thus far, the exact transport function of *MdTMT1* remains unclear, but the orthologous gene *AtTMT1* can transport sucrose across the tonoplast membrane into the vacuoles in *Arabidopsis* (Schulz et al., 2011), and the expression patterns of *MdTMT1* are in general agreement with that of Suc accumulation in fruit (Li et al., 2016). Thus, the downregulation of *MdTMT1* could be a result of decreased sucrose concentration in the cytosol. However, the transcription and activity of *MdAINV* was not significantly altered, although the sucrose concentration decreased (Fig. 5). Elevated expression of *MdpGlcT1-4*, which plays a role in the efflux of glucose from plastids (Wei et al., 2014), and the slowly reduced starch content (Fig. 2), indicate that stored starch was degraded to supply energy.

In our experiments, short-term drought invoked stress in the one-year old 'Greensleeves' trees. In response to the water deficit, sorbitol synthesis was upregulated, whereas the breakdown of sucrose was upregulated to generate more hexose, and high levels of fructose were phosphorylated for energy production. This altered sugar metabolism, together with corresponding changes in the sugar transport system, contributed to carbohydrate osmotic adjustment and apple drought tolerance.

Author contributions

JJY and JZ performed the majority of experiments. CL performed essential experiments. ZZ analysed and discussed data. FWM and MJL supervised work. All authors contributed to final manuscript.

Conflicts of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

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