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Leaf responses of micropropagated apple plants to water stress: nonstructural carbohydrate composition and regulatory role of metabolic enzymes

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Summary We examined changes in nonstructural carbohydrate biosynthesis and activities of related enzymes in leaves of micropropagated apple plants (*Malus domestica* Borkh. cv. 'NaganoFuji') in response to water stress, with particular emphasis on the enzymes associated with sorbitol, sucrose and starch metabolism. Water stress resulted in the accumulation of photosynthates in leaves, mainly sorbitol, sucrose, glucose and fructose, accompanied by a reduction in starch concentration. Correlation and path analysis indicated that water stress affected the partitioning of newly fixed carbon among terminal products. In response to water stress, ADP-glucose-pyrophosphorylase (ADPGPPase) activity decreased, becoming a critical and limiting step in shifting partitioning of photosynthetically fixed carbon. Amylase and ADPGPPase affected sucrose and sorbitol metabolism, mainly by regulating substrate supply; however, competition for limited substrate had a greater effect on the biosynthesis of sorbitol than of sucrose. Starch metabolism was also strictly regulated by ADPGPPase and amylase, whereas other related enzymes were downstream of the pathway for synthesis and degradation of carbohydrates and thus had relatively little effect on starch metabolism. Sorbitol dehydrogenase and sucrose phosphate synthase were critical regulators of sorbitol and sucrose metabolism, respectively.

Keywords: carbohydrate metabolism, carbon partitioning, enzyme activity, path analysis.

Introduction

Water stress significantly affects carbohydrate-modulated gene expression in plants, thereby influencing vegetative growth and limiting crop productivity (Gifford and Evans 1981, Boyer 1982, Koch 1996, David et al. 1999). Plants adapt to water deficit by avoidance or tolerance mechanisms that reduce the possibility of cellular dehydration (Daie 1996, Ali et al. 1998, Xiong and Zhu 2002). Plants' tolerance of water deficit is largely dependent on their capacity for osmoregulation to maintain cell turgor through the accumulation of solutes (Morgan 1984, Loescher and Everard 1996).

Major end-products of photosynthesis in most species of the woody Rosaceae, e.g., *Prunus*, *Pyrus* and *Malus* species, are sorbitol, sucrose and starch (Wallaart 1980, Moing et al. 1992, Wang and Stutte 1992, Escobar-Gutiérrez and Gaudillère 1997). In response to water stress, apple (Wang and Stutte 1992, Xu et al. 2001), cherry (Ranney et al. 1991) and peach (Escobar-Gutiérrez et al. 1998) leaves show decreases in sucrose and starch, whereas sorbitol, glucose and fructose increase rapidly. The activity of aldose-6-phosphate reductase (A6PR), the key enzyme regulating sorbitol synthesis (Negm and Loescher 1981), increases markedly in apple leaves in response to water stress, perhaps indicating an increase in the partitioning of newly fixed carbon to sorbitol or transformation from other carbohydrates (Wang et al. 1996). In potted peach trees, drought-induced changes in carbohydrate concentrations were related to increased activities of enzymes associated with carbohydrate metabolism, such as amylase and A6PR, and decreased activity of sucrose phosphate synthase (SPS) (Chai et al. 2001). Analysis of correlations between changes in soluble sugar concentrations, including sorbitol, and changes in activities of metabolic enzymes offered insight into the effect of water stress on sugar metabolism (Moriguchi et al. 1990, Escobar-Gutiérrez et al. 1998, Chai et al. 2001). A simple correlation alone, however, may lead to erroneous conclusions about a specific enzyme's effect on carbohydrate metabolism because relationships between carbohydrate concentrations and metabolic enzyme activities may also be related either directly or indirectly.

Path analysis is a statistical method for determining the magnitude and direction of multiple effects on a complex process (McGiffen et al. 1994). The coefficients generated by path analysis are standardized partial regression coefficients (Afifi and Clark 1984). The variables may be related either directly or indirectly. In path analysis, the direct effects (calculated by the path coefficient) of independent variables are studied after removing the indirect effects. The indirect effect for each independent variable is calculated by multiplying the correlation between two independent variables by the direct effect of the opposite independent variable (Karlsson et al. 1988). Thus,

path analysis can help both quantify the effects of carbon metabolic enzyme activity on carbohydrate metabolism and elucidate the relative importance of certain enzymes in regulating carbohydrate metabolism under water-stress conditions.

Few studies have examined changes in carbohydrates in relation to the activities of related metabolic enzymes in water-stressed fruit trees. Moreover, fruit trees are large and experimental conditions are difficult to control in the field and even in pots. These difficulties can be overcome by inducing water deficits with polyethylene glycol 6000 (PEG-6000) in hydroponically grown micropropagated plants of uniform size. We investigated the effects of water stress on nonstructural carbohydrate metabolism of fruit trees by regulating the water status of 'Fuji' micropropagated apple plants by adding PEG-6000 to the nutrient solution. We also assessed the relative importance of enzymes associated with nonstructural carbohydrate metabolism by correlation and path analysis between nonstructural carbohydrate concentrations and various enzymatic activities in an attempt to identify the enzymatic reactions controlling carbon partitioning and carbohydrate metabolic pathways of the water-stressed fruit trees.

Materials and methods

Experimental site and materials

Micropropagated 'Fuji' apple plants (*Malus domestica* Borkh. cv. 'NaganoFuji') with heights of 20–30 cm were pre-cultured in pots with 1/2 strength Hoagland nutrient solution for 10 days. The culture conditions were 24–28 °C, 85% relative humidity and a 16-h photoperiod at 400 μmol m^{-2} s^{-1}. After 10 days, plants with uniform vegetative growth were selected for study.

Treatments

We subjected plants to two water-deficit treatments by regulating the osmotic potential of the nutrient solution by adding PEG-6000, as described by Hsiao (1973). Nutrient solutions of the mild stress (MIS) and severe stress (SES) treatments were adjusted to osmotic potentials of –0.75 and –1.5 MPa, respectively. The control plants received nutrient solution without PEG (CK). A randomized complete block design was used, with five replicates and 50 plants in each block. Leaf water potentials of fully expanded leaves on the upper part of each plant were measured with a Scholander pressure chamber (ZIZ-4, Lanzhou University, China). Leaf water potentials of CK plants remained at a stable high value (about –0.2 MPa) throughout the experiment, whereas leaf water potentials decreased progressively in water-stressed plants during the first 13 h of water stress and remained low thereafter: –0.5 to –0.7 MPa for MIS-treated plants and –1.0 to –1.2 MPa for SES-treated plants, when measured at the beginning of the photoperiod (Figure 1).

Leaf sampling for analysis of nonstructural carbohydrates and enzymatic activities

Micropropagated plants were destructively sampled at 1, 4, 8, 12, 24 and 48 h after the initiation of water-stress treatments.

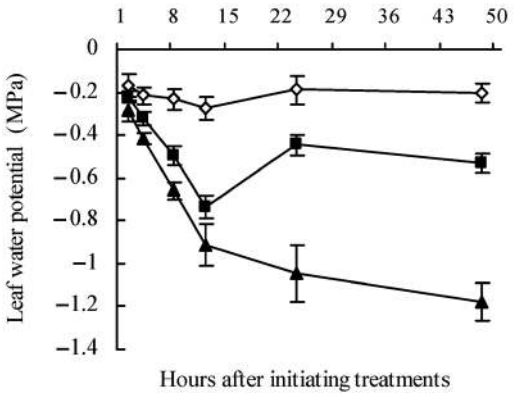

Figure 1. Response of leaf water potential of micropropagated apple plants to water stresses of different intensities. Each value is the mean of five observations; vertical bars indicate ± SE. Symbols: ◇ = normal water conditions; ■ = mild stress; and ▲ = severe stress.

Half of the fully expanded leaves collected were heat-killed in a drying oven at 108 °C for 0.5 h, dried to constant mass at 80 °C, ground to pass a 0.8-mm screen, and analyzed for nonstructural carbohydrates including sorbitol, sucrose, glucose and fructose. The remaining leaf samples were frozen immediately in liquid nitrogen and stored at –40 °C until assayed for enzymatic activities.

Nonstructural carbohydrate and enzyme activity analysis

To extract soluble carbohydrates, about 0.5 g of leaf dry mass was ground to a fine powder with a mortar and pestle, and extracted three times with 10 ml of 80% ethanol by incubating at 80 °C for 10 min followed by centrifugation at 2000 *g*. The pellet was retained for starch determination. The supernatants were combined and evaporated to dryness at 100 °C, then 5 ml of distilled water was added and the solution was filtered (0.45-μm). Total soluble carbohydrate (TSC) concentration of the filtrate was determined by the anthrone method (van Handel 1968).

Sorbitol, glucose, fructose and sucrose concentrations were determined by high-performance liquid chromatography (LC-10AD, Shimadzu, Kyoto, Japan) equipped with a refractive index detector (Rid-6A, Shimadzu). The compounds were separated on a 300 × 7.80 mm REZEX 8u 8% Ca Monos. column (Phenomenex, Torrance, CA) maintained at 85.5 °C and eluted with distilled water at a flow rate of 1.0 ml min^{-1}. Sorbitol, glucose, fructose and sucrose were identified and quantified by comparison with known standards (Moing et al. 1992).

After removing the soluble carbohydrates, starch in the pellet was solubilized by adding 0.2 ml of 0.5 M NaOH and incubating at 95 °C for 2 h, followed by neutralization with sodium acetate buffer and hydrolysis with amyloglucosidase (300 μg ml^{-1}) for 2 h at 38 °C. The glucose released was quantified by the glucose-peroxidase method with a test kit (Müller et al. 1994).

To prepare the enzyme extracts, 0.5 g of frozen leaves was ground to a fine powder in liquid nitrogen with quartz sand. The ground sample was then thoroughly mixed with extraction

buffer (Goldner and Glasziou 1991) containing 50 mM HEPES-NaOH (pH 7.5), 10 mM $MgCl_2$, 1 mM EDTA, 2.5 mM DTT, 0.05% Triton X-100 and 0.1% BSA. The homogenate was passed through four layers of gauze and the filtrate was centrifuged at 13,000 *g* for 10 min at 2 °C. The supernatant was dialyzed immediately against a tenfold volume of diluted extraction buffer (except for Triton X-100) for 20 h, and the dialyzate was changed once. The dialyzed enzyme extracts were then assayed. For each enzyme, three independent extractions per sample were performed, and the data presented correspond to the mean of five samples.

Aldose-6-phosphate reductase (EC 1.1.1.200) activity was assayed in the direction of synthesis by following the oxidation of NADPH in the presence of glucose-6-phosphate at 340 nm, as described by Merlo and Passera (1991). The reaction mixture contained 0.1 M Tris-HCl (pH 8.8), 0.1 mM NADPH, 50 mM glucose-6-phosphate and 50 µl of extract. One unit of A6PR is defined as the amount of enzyme catalyzing the oxidation of 1 µmol NADPH min^{-1} at 25 °C under standard assay conditions. The activity of sorbitol dehydrogenase (SDH, EC 1.1.1.14) was assayed by following the reduction of NAD^+ in the presence of D-sorbitol at 340 nm, according to the method described by Negm and Loescher (1979). The reaction mixture contained 0.1 M Tris-HCl (pH 9.0), 1 mM NAD^+, 0.5 M D-sorbitol and 0.1 ml of extract. Sorbitol and glucose-6-phosphate, dissolved in the same buffer as in the assay, started the reactions.

Sucrose-phosphate synthase (EC 2.4.1.14) activity was determined by measuring fructose-6-phosphate formation of sucrose-phosphate, according to the method described by Huber and Israel (1982), with some changes. The assay mixtures (140 µl) contained 50 mM HEPES-NaOH (pH 7.5), 10 mM $MgCl_2$, 5 mM NaF, 25 mM uridine diphosphoglucose, 10 mM fructose-6-phosphate and 80 µl of extract. The reaction was stopped after 40 min at 25 °C by addition of 140 µl of 1 M NaOH. Unreacted fructose-6-phosphate was destroyed by placing the tubes in boiling water for 10 min. After cooling, 0.5 ml of 0.1% resorcinol in 95% ethanol and 1.5 ml of 30% HCl were added, the tubes were incubated at 80 °C for 8 min, and the absorbance at 520 nm measured. Because SPS is a labile enzyme, precautions were taken to minimize potential loss in activity before and during extraction (Hubbard et al. 1989).

We assayed ADP-glucose-pyrophosphorylase (ADPGPPase, EC 2.7.7.27) by measuring pyrophosphate-dependent glucose-1-phosphate formation from ADP-glucose at 25 °C in a 1 ml reaction mixture, according to the procedures described by Rufty et al. (1983). Reactions were started by addition of ADP-glucose to a final concentration of 5 mM and production of NADPH was monitored at 340 nm.

Acid invertase (AI, EC 3.2.1.26) was assayed in a reaction mixture (1 ml) containing 0.1 M sodium acetate (pH 4.8), 0.1 M sucrose and 0.2 ml of extract. After incubation for 40 min at 37 °C, the reactions were stopped by adding 1 ml of dinitrosalicylic acid reagent. The reducing sugars released from sucrose were determined according to Miller (1959). Total amylase (EC 3.2.1.1 and EC 3.2.1.2) was assayed in a reaction mixture (1 ml) containing 0.1 M sodium acetate (pH 6.5), 1.5 mM NaF, 5 mM $CaNO_3$, 0.5% soluble starch and 0.2 ml of extract. After 40 min at 30 °C, the reactions were terminated, and the release of reducing groups was determined as described for the invertase assay (Merlo and Passera 1991). For the invertase and amylase assays, blanks contained reaction mixtures incubated with dinitrosalicylic acid reagent. All chemicals were purchased from Sigma (St. Louis, MO).

Statistical analysis

Nonstructural carbohydrate concentrations and enzymatic activities were subjected to analysis of variance with SAS software (SAS Institute, Cary, NC).

Path and correlation coefficients, determined with EQS software (Version 5.7 for Windows, Multivariate Software, Encino, CA; Bentler 1998), were analyzed between nonstructural carbohydrates, including only sorbitol, sucrose and starch, and activities of metabolic enzymes. The analysis of other soluble sugars (glucose and fructose) and enzyme activities are not reported in this paper because of the relatively low concentrations of glucose and fructose in apple leaves (Figures 2d and 2e), and the absence of clear and regular changes in those correlations in response to water stress. The normal equations used in the path analysis were:

$$r_{x_1y} = P_{x_1y} + r_{x_1x_2}P_{x_2y} + r_{x_1x_3}P_{x_3y} + r_{x_1x_4}P_{x_4y} + r_{x_1x_5}P_{x_5y} + r_{x_1x_6}P_{x_6y} \quad (1)$$

$$r_{x_2y} = r_{x_1x_2}P_{x_1y} + P_{x_2y} + r_{x_2x_3}P_{x_3y} + r_{x_2x_4}P_{x_4y} + r_{x_2x_5}P_{x_5y} + r_{x_2x_6}P_{x_6y} \quad (2)$$

$$r_{x_3y} = r_{x_1x_3}P_{x_1y} + r_{x_2x_3}P_{x_2y} + P_{x_3y} + r_{x_3x_4}P_{x_4y} + r_{x_3x_5}P_{x_5y} + r_{x_3x_6}P_{x_6y} \quad (3)$$

$$r_{x_4y} = r_{x_1x_4}P_{x_1y} + r_{x_2x_4}P_{x_2y} + r_{x_3x_4}P_{x_3y} + P_{x_4y} + r_{x_4x_5}P_{x_5y} + r_{x_4x_6}P_{x_6y} \quad (4)$$

$$r_{x_5y} = r_{x_1x_5}P_{x_1y} + r_{x_2x_5}P_{x_2y} + r_{x_3x_5}P_{x_3y} + r_{x_4x_5}P_{x_4y} + P_{x_5y} + r_{x_5x_6}P_{x_6y} \quad (5)$$

$$r_{x_6y} = r_{x_1x_6}P_{x_1y} + r_{x_2x_6}P_{x_2y} + r_{x_3x_6}P_{x_3y} + r_{x_4x_6}P_{x_4y} + r_{x_5x_6}P_{x_5y} + P_{x_6y} \quad (6)$$

where P_{x_ny} and r_{x_ny} represent path coefficients and simple correlation coefficients, respectively. The simple correlation coefficient in each normal equation was composed of direct (measured by the path coefficient) and indirect effects (measured by correlation coefficients). The subscripts x_1–x_6 in the normal equations represent six variables (activities of six metabolic enzymes), and subscript y represents the various carbohydrates (sorbitol, sucrose and starch); e.g., P_{17} was the direct effect of A6PR activity (subscript 1) on sorbitol concentration (subscript 7).

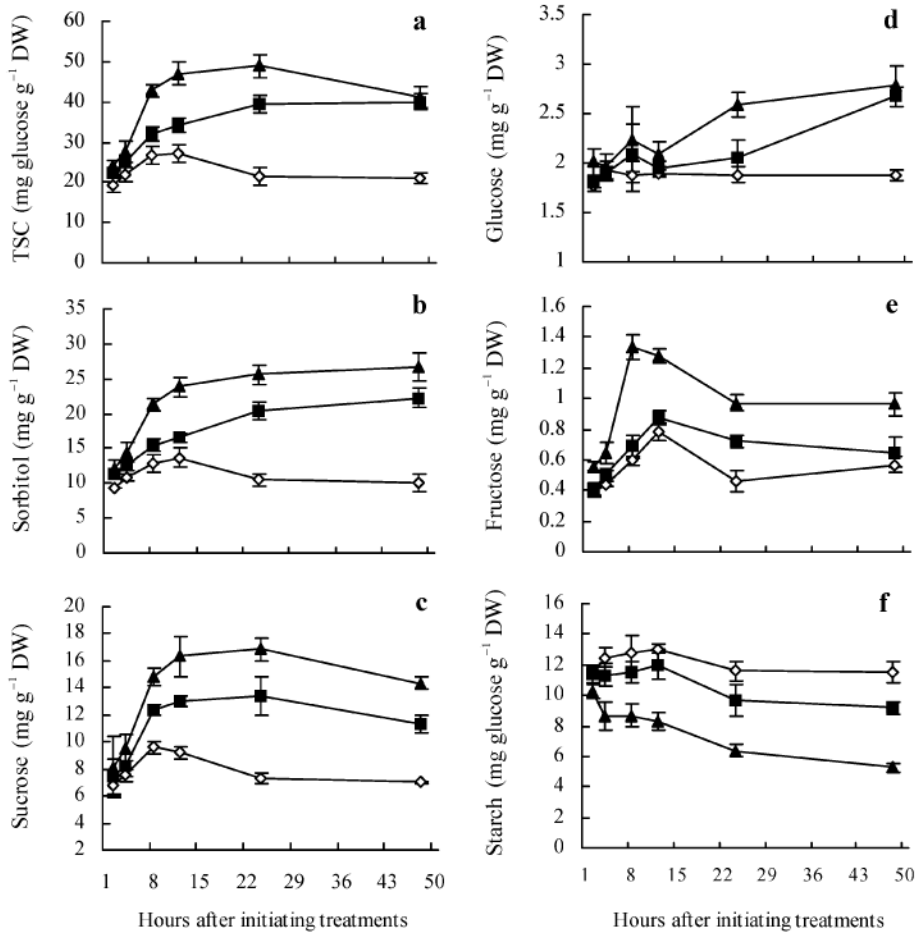

Figure 2. Concentrations of total soluble carbohydrate (TSC, a), sorbitol (b), sucrose (c), glucose (d), fructose (e) and starch (f) in fully expanded leaves of micropropagated 'Fuji' apple plants subjected to different intensities of water stress. Values are means of five observations; vertical bars indicate ± SE. Symbols: ◇ = normal water conditions; ■ = mild stress; and ▲= severe stress.

Results

Nonstructural carbohydrate concentration

Total soluble carboydrates accumulated in leaves within 8 h after initiating the SES treatment and 24 h after initiating the MIS treatment, and a high TSC concentration remained in the leaves to the end of the experiment (Figure 2a). A significantly higher foliar TSC concentration was observed 2 h after initiating the SES treatment and 8 h after initiating the MIS treatment compared with CK values. Foliar TSC concentration was much higher in SES-treated plants than in MIS-treated plants between 8 and 24 h after initiating the treatments, indicating that accumulation of TSC increased with increasing water deficit intensity.

Concentrations of sorbitol and sucrose were around 10 and 5 mg g^{-1} DW, respectively, in CK plants, and increased significantly during the first 12 h after initiating the SES and MIS treatments and remained high throughout the study (Figures 2b and 2c). At the end of the experiment, foliar sorbitol concentrations were 2.21 and 2.66 times higher, and foliar sucrose concentrations were 1.61 and 2.04 times higher in MIS- and SES-treated plants, respectively, than control values. Significantly higher concentrations of sorbitol and sucrose were found in SES leaves than in MIS leaves 8 h after initiating the treatments, and these differences were maintained throughout the experiment.

Water stress had a smaller effect on glucose concentration compared with sorbitol and sucrose concentrations, with significantly higher foliar glucose concentrations in the SES- and MIS-treated plants becoming apparent only at 24 and 48 h after initiation of the treatments, respectively (Figure 2d). The effect of water stress on foliar fructose concentration differed from its effects on the other soluble sugars (Figure 2e). Foliar fructose concentration increased significantly within 1 h after initiation of the SES treatment compared with control values, and the concentration had almost tripled after 8 h of SES treatment. The concentration of fructose then decreased slightly and after 24 h of treatment it remained stable until the end of the experiment. There was no significant effect of the MIS treatment on foliar fructose concentration during the experiment.

Foliar starch concentrations decreased significantly in response to water stress (Figure 2f), with the reduction increasing with severity of the water stress treatment. At the end of the experiment, foliar starch concentrations in the MIS- and SES-treated plants were 79.71 and 45.57% of the control value, respectively.

Activities of metabolic enzymes

Water stress significantly increased A6PR, SPS and amylase activities (Figures 3a–c), and significantly decreased SDH, AI and ADPGPPase activities (Figures 3d–f) compared with control values. Changes in enzymatic activities mainly occurred between 12 and 24 h after initiating the MIS and SES treatments, with the activities remaining relatively stable thereafter.

The activities of the carbon metabolic enzymes studied showed varied sensitivities to water stress. Although the SES and MIS treatments resulted in similar increases in A6PR activity and similar decreases in ADPGPPase activity throughout the experiment, the SES treatment had a greater effect on the activities of the other enzymes studied than the MIS treatment. The activities of A6PR and ADPGPPase were more sensitive to water stress than were the activities of the other enzymes.

Correlation and path analysis of sorbitol concentration and metabolic enzyme activities

In CK plants, foliar sorbitol concentration was significantly positively correlated with the activities of A6PR ($r_{17CK} = 0.60$), SPS ($r_{37CK} = 0.70$) and amylase ($r_{67CK} = 0.56$), and negatively correlated with the activities of AI ($r_{47CK} = -0.61$) and ADPGPPase ($r_{57CK} = -0.55$) (Table 1). No significant correlation was found between sorbitol concentration and SDH activity ($r_{27CK} = 0.33$). Path analysis showed that, among the enzymes studied, the direct path coefficient of A6PR (P_{17CK}) was the greatest (about 0.46) and SPS activity had an important direct effect on foliar sorbitol concentration ($P_{37CK} = 0.32$). Sorbitol deydrogenase activity had a strong negative direct effect on sorbitol concentration ($P_{27CK} = -0.31$), combined with positive indirect effects mediated through SPS activity ($r_{23}P_{37CK} = 0.49$). The direct path coefficients of AI, ADPGPPase and amylase on sorbitol concentrations were small (Table 1), as a result of greater negative or positive indirect effects mediated through SPS ($r_{34}P_{37CK} = -0.57$, $r_{35}P_{37CK} = -0.53$, $r_{36}P_{37CK} = 0.34$, respectively).

In water-stressed plants, there were significant positive correlations between foliar sorbitol concentrations and the activities of A6PR (except A6PR in the SES treatment), SPS and amylase, and significant negative correlations between foliar sorbitol concentrations and the activities of SDH, AI and ADPGPPase (Table 1). Path analysis showed that the stress-induced changes in A6PR ($P_{17MIS} = 0.25$, $P_{17SES} = 0.17$, respectively) and amylase ($P_{67MIS} = 0.49$, $P_{67SES} = 0.29$, respectively)

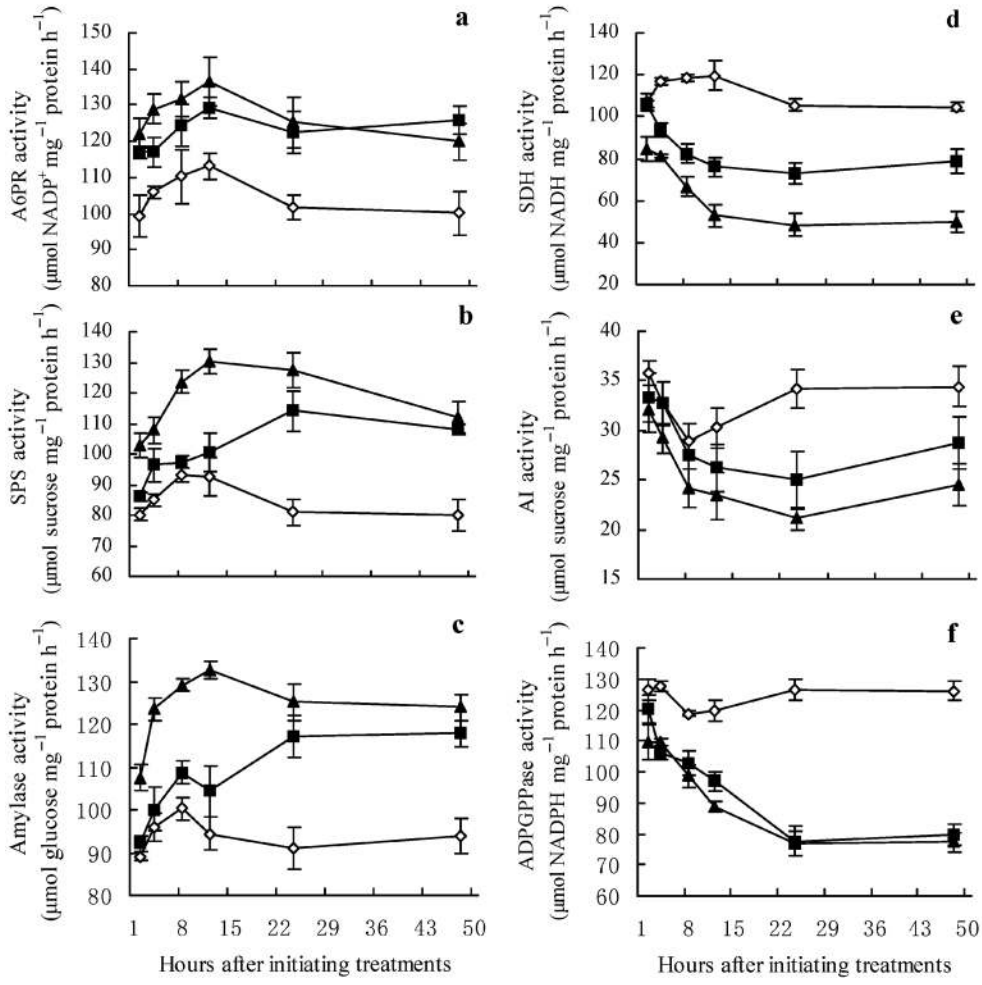

Figure 3. Activities of aldose-6-phosphate reductase (A6PR, a); sucrose phosphate synthase (SPS, b); amylase (c); sorbitol dehydrogenase (SDH, d); acid invertase (e); and ADP-glucose-pyrophosporylase (ADPGPPase, f), in fully expanded leaves of micropropagated apple plants subjected to different intensities of water stress. Values are means of five observations; vertical bars indicate ± SE. Symbols: ◇ = normal water conditions; ■ = mild stress; and ▲ = severe stress.

Table 1. Direct (bolded) and indirect effects of the activities of metabolic enzymes (aldose-6-phosphate reductase (A6PR), sorbitol dehydrogenase (SDH), sucrose phosphate synthase (SPS), acid invertase (AI), ADPglucose-pyrophosphorylase (ADPGPPase) and amylase) on sorbitol concentration (7) in the fully expanded leaves of micropropagated 'Fuji' apple plants subjected to mild water stress (MIS), severe water stress (SES) and control conditions (CK), and their correlations. The asterisks * and ** indicate significance at 0.05 and 0.01, respectively, and the regression equation was determined by an *F*-test.

Enzyme	Stress treatment	Correlation coefficient	A6PR (1)	SDH (2)	SPS (3)	AI (4)	ADPGPPase (5)	Amylase (6)
A6PR	CK	0.60 **	**0.46**	–0.13	0.26	0.00	–0.09	0.11
(1)	MIS	0.52 *	**0.25**	0.01	–0.05	0.00	0.29	0.02
	SES	0.16	**0.17**	0.01	–0.16	0.05	–0.06	0.15
SDH	CK	0.33	0.11	**–0.31**	0.49	0.00	–0.05	0.10
(2)	MIS	–0.80 **	–0.01	**–0.37 ***	0.12	0.01	–0.53	–0.02
	SES	–0.91 **	–0.02	**–0.18 ***	0.24	–0.22	–0.68	–0.05
SPS	CK	0.70 **	0.17	–0.21	**0.32**	–0.01	–0.10	0.13
(3)	MIS	0.83 **	0.01	0.02	**–0.16**	–0.01	0.56	0.41
	SES	0.59 *	0.08	0.03	**–0.34**	0.20	0.40	0.22
AI	CK	–0.61 **	–0.13	0.18	–0.57	**0.01**	0.06	–0.17
(4)	MIS	–0.62 **	–0.01	–0.02	0.11	**0.01**	–0.42	–0.30
	SES	–0.82 **	–0.03	–0.04	0.25	**–0.27**	–0.54	–0.20
ADPGPPase	CK	–0.55 *	–0.18	0.12	–0.53	0.00	**0.14**	–0.11
(5)	MIS	–0.93 **	–0.01	–0.02	0.14	0.01	**–0.63**	–0.42
	SES	–0.90 **	0.01	–0.04	0.19	–0.20	**–0.73**	–0.13
Amylase	CK	0.56 *	0.11	–0.11	0.34	–0.00	–0.05	**0.28**
(6)	MIS	0.91 **	0.01	0.02	–0.13	–0.00	0.54	**0.49**
	SES	0.67 **	0.09	0.03	–0.25	0.18	0.32	**0.29**

activities had direct positive effects, and that ADPGPPase, SDH and SPS contributed to the negative direct effects on foliar sorbitol concentrations of water-stressed plants (Table 1). Among the enzymes studied, only the direct effects of SDH activity on sorbitol concentration of water-stressed plants were significant ($P_{27MIS} = -0.37$, $P_{27SES} = -0.18$).

Although ADPGPPase had the largest negative effects on foliar sorbitol concentrations of water-stressed plants ($P_{57MIS} = -0.63$, $P_{57SES} = -0.73$), the direct effects were not statistically significant. The direct effects of SPS activity on foliar sorbitol concentration of water-stressed plants ($P_{37MIS} = -0.16$, $P_{37SES} = -0.34$) suggested that the increase in SPS activity had a negative effect on sorbitol accumulation in leaves of water-stressed plants. The direct effects of AI activities on foliar sorbitol concentrations increased with increasing severity of the water-stress treatment ($P_{47MIS} = 0.01$, $P_{47SES} = -0.27$, respectively). The indirect effects of ADPGPPase ($r_{35}P_{57MIS} = 0.56$, $r_{35}P_{57SES} = 0.40$) and amylase ($r_{36}P_{67MIS} = 0.41$, $r_{36}P_{67SES} = 0.22$), mediated through SPS activity, also had important effects on foliar sorbitol concentrations of water-stressed plants.

Correlation and path analysis of sucrose concentration and metabolic enzyme activities

In micropropagated CK plants, correlations between the activities of all enzymes studied and foliar sucrose concentrations were significantly positive (A6PR, SDH, SPS and amylase) or negative (AI and ADPGPPase) (Table 2). Among the positive effects of enzymes, SPS activity had a significant direct effect on sucrose concentration ($P_{38CK} = 0.35$), and A6PR activity also had a large direct positive effect on sucrose concentration ($P_{18CK} = 0.29$). In contrast, the activities of AI and ADPGPPase had large negative path coefficients ($P_{48CK} = -0.32$, $P_{58CK} = -0.40$, respectively) and effects on sucrose concentrations.

There were significant direct path coefficients of SPS activities on foliar sucrose concentrations of MIS- and SES-treated plants ($P_{38MIS} = 0.55$, $P_{38SES} = 0.36$, respectively) (Table 2). Variations in AI and ADPGPPase activities had important effects on foliar sucrose concentrations and their direct negative path coefficients were between –0.29 to –0.53. Activity of A6PR had weak direct effects on foliar sucrose concentrations of water-stressed plants ($P_{18MIS} = 0.02$, $P_{18SES} = 0.07$, respectively). The negative correlation and path coefficients of SDH activities with sucrose concentrations in water-stressed plants (all $r = -0.89$; $P_{28MIS} = -0.72$, $P_{28SES} = -0.10$, respectively) compared with the positive values in CK plants ($r_{28CK} = 0.55$, $P_{28CK} = 0.07$, respectively) might be associated with direct or indirect effects mediated by other metabolic enzymes; however, no consistent evidence was found to support this supposition.

Correlation and path analysis of starch concentration and metabolic enzyme activities

Starch concentrations correlated significantly with the activities of all metabolic enzymes studied except amylase activity in leaves of CK plants (Table 3). The direct effects of A6PR ($P_{19CK} = 0.16$), SPS ($P_{39CK} = -0.13$), ADPGPPase ($P_{59CK} = -0.05$) and amylase ($P_{69CK} = 0.09$) activities on foliar starch concentrations were significant.

Table 2. Direct (bolded) and indirect effects of the activities of metabolic enzymes (aldose-6-phosphate reductase (A6PR), sorbitol dehydrogenase (SDH), sucrose phosphate synthase (SPS), acid invertase (AI), ADP-glucose-pyrophosphorylase (ADPGPPase) and amylase) on sucrose concentration (8) in fully expanded leaves of micropropagated apple plants subjected to mild water stress (MIS), severe water stress (SES) and control conditions (CK), and their correlations. The asterisks * and ** indicate significance at 0.05 or 0.01, respectively, and the regression equation was determined by an *F*-test.

Enzyme	Stress treatment	Correlation coefficient	A6PR (1)	SDH (2)	SPS (3)	AI (4)	ADPGPPase (5)	Amylase (6)
A6PR	CK	0.81 **	**0.29**	0.03	–0.03	0.15	0.27	0.03
(1)	MIS	0.58 *	**0.02**	0.43	0.05	0.27	–0.14	–0.05
	SES	0.31	**0.07**	–0.01	0.17	0.02	–0.03	0.07
SDH	CK	0.55 *	0.15	**0.07**	–0.03	0.19	0.15	0.03
(2)	MIS	–0.89 **	–0.01	**–0.72**	–0.11	–0.38	0.25	0.08
	SES	–0.89 **	–0.01	**–0.10**	–0.25	–0.07	–0.37	–0.09
SPS	CK	0.81 **	0.22	0.05	**0.35 ***	0.26	–0.05	0.04
(3)	MIS	0.72 **	0.01	0.14	**0.55 ***	0.37	–0.27	–0.09
	SES	0.85 **	0.03	0.07	**0.36 ***	0.06	0.22	0.11
AI	CK	–0.72 **	–0.17	–0.04	0.04	**–0.32**	–0.18	–0.05
(4)	MIS	–0.89 **	–0.01	–0.50	–0.10	**–0.53**	–0.20	0.06
	SES	–0.84 **	–0.01	–0.08	–0.08	**–0.29**	–0.27	–0.10
ADPGPPase	CK	–0.80 **	–0.24	–0.03	0.04	–0.14	**–0.40**	–0.03
(5)	MIS	–0.71 **	–0.01	–0.36	–0.13	0.30	**–0.45**	0.09
	SES	–0.80 **	0.01	–0.10	–0.20	–0.06	**–0.40**	–0.06
Amylase	CK	0.56 *	0.08	0.02	–0.02	0.19	0.15	**0.14**
(6)	MIS	0.65 **	0.01	0.55	–0.10	0.33	–0.26	**0.12**
	SES	0.74 **	0.04	0.16	–0.17	0.16	0.18	**0.14**

Of the metabolic enzymes studied in water-stressed plants, ADPGPPase had the largest positive path coefficients (P_{59MIS} = 0.63, P_{59SES} = 0.46, respectively) and highly significant correlation coefficients (r_{59MIS} = 0.64, r_{59SES} = 0.83, respectively), whereas amylase activities had the largest negative path coefficients (P_{69MIS} = –0.77, P_{69SES} = –0.45, respectively) and correlations (r_{69MIS} = –0.73, r_{69SES} = –0.41, respectively) on foliar starch concentrations. The influences of the other metabolic enzymes on starch concentrations were variable and inconsistent between the MIS- and SES-treated plants.

Discussion

Influence of physiological properties of experimental materials in response to water stress

The observed increase in sorbitol concentration and decrease in starch concentration in leaves in response to water stress (Figures 2b and 2f) were in agreement with several previous studies (Hansen 1970, Grant and Ap Rees 1981, Ranney et al. 1991, Wang and Stutte 1992, Escobar-Gutiérrez et al. 1998). However, the accumulation of sucrose in the leaves in response to water stress (Figure 2c) contrasted with the findings of Escobar-Gutiérrez et al. (1998) and Rodrigues et al. (1993), who reported that sucrose concentration decreased in peach and grapevine leaves under water-stress conditions. Sucrose concentrations increased in young leaves of 'Jonathan' apple (Wang et al. 1995) and maize (Kim et al. 2000*a*) in response to water stress, indicating that the physiological properties of leaves have an important influence on their physiological response to drought. Our micropropagated plants were relatively young, and the leaf cuticle was thinner than in leaves of field-grown trees, so the physiological properties of the leaves in our study would be similar to those of young leaves of field-grown trees or plants that have a high capacity for osmotic adjustment through the accumulation of sucrose in response to water stress.

Partitioning of newly fixed carbon in leaves of micropropagated apple plants in response to water stress

Photosynthetically fixed carbon is mainly distributed to sorbitol, sucrose and starch in the woody Rosaceae (Wang and Stutte 1992, Escobar-Gutiérrez et al. 1998). It is therefore of interest to determine if the decreased starch (Figure 2f) and increased sorbitol and sucrose concentrations in leaves (Figures 2b and 2c) in response to water stress are associated with an effect of water stress on the partitioning of photosynthetically fixed carbon.

The primary regulatory enzyme in the starch biosynthetic pathway is ADPGPPase (Merlo and Passera 1991, Preiss and Sivak 1996). After 24 h of water stress, ADPGPPase activity in leaves decreased by about 50% compared with the control value (Figure 3f), and foliar starch concentration was significantly correlated with ADPGPPase activity (Table 3). Path analysis results showed that, among the enzymes studied, ADPGPPase had the largest positive direct effects on foliar starch concentrations (Table 3), indicating that decreased ADPGPPase activity resulted in decreased synthesis of starch in our water-stressed plants.

Table 3. Direct (bolded) and indirect effects of the activities of metabolic enzymes (aldose-6-phosphate reductase (A6PR), sorbitol dehydrogenase (SDH), sucrose phosphate synthase (SPS), acid invertase (AI), ADP-glucose-pyrophosphorylase (ADPGPPase) and amylase) on starch concentration (9) in the fully expanded leaves of micropropagated apple plants subjected to mild water stress (MIS), severe water stress (SES) and control conditions (CK), and their correlations. The asterisks * and ** indicate significance at 0.05 and 0.01, respectively, and the regression equation was determined by an *F*-test.

Enzyme	Stress treatment	Correlation coefficient	A6PR (1)	SDH (2)	SPS (3)	AI (4)	ADPGPPase (5)	Amylase (6)
A6PR	CK	0.50 *	**0.16 ***	0.14	–0.08	0.20	0.06	0.02
(1)	MIS	–0.18	**–0.06**	0.27	–0.06	0.36	–0.29	–0.40
	SES	0.28	**0.30**	–0.05	0.21	0.01	0.04	–0.23
SDH	CK	0.61 **	0.07	**0.34**	–0.09	0.24	0.03	0.02
(2)	MIS	0.36	0.04	**–0.45**	0.15	–0.50	0.53	0.59
	SES	0.79 **	–0.03	**0.48**	–0.31	–0.05	0.43	0.28
SPS	CK	0.62 **	0.10	0.23	**–0.13 ***	0.33	0.06	0.02
(3)	MIS	–0.58 *	–0.02	0.35	**–0.20**	0.49	–0.57	–0.64
	SES	–0.28	0.14	–0.33	**0.45**	0.05	–0.25	–0.33
AI	CK	–0.66 *	–0.08	–0.20	0.10	**–0.42**	–0.04	–0.03
(4)	MIS	0.03	0.03	–0.34	0.14	**–0.71**	0.42	0.47
	SES	0.58 *	–0.06	0.40	–0.34	**–0.06**	0.34	0.31
ADPGPPase	CK	0.45 *	–0.11	–0.13	0.10	–0.19	**–0.05 ***	–0.02
(5)	MIS	0.64 **	0.03	–0.38	0.18	–0.47	**0.63**	0.66
	SES	0.83 **	0.03	0.45	–0.24	–0.05	**0.46**	0.20
Amylase	CK	–0.43	0.06	0.12	–0.06	0.25	0.03	**0.09 ***
(6)	MIS	–0.73 **	–0.03	0.35	–0.16	0.43	–0.54	**–0.77**
	SES	–0.41	0.16	–0.30	0.33	0.04	–0.20	**–0.45**

Decreased biosynthesis of newly fixed carbon into starch would favor the sorbitol- and sucrose-synthesis pathways (Wang et al. 1996). In apple leaves, sorbitol and sucrose are synthesized through A6PR (Negm and Loescher 1981, Loescher and Everard 1996) and SPS (Bruneau et al. 1991, Hawker et al. 1991), respectively, from the same precursor, glucose-6-phosphate (G6P). Sucrose phosphate synthase had highly significant correlations and positive direct effects on foliar sucrose concentration during water stress (Table 2). The A6PR activities had direct positive effects on sorbitol concentrations (Table 1); however, a nonsignificant correlation was found between A6PR activities and sorbitol concentrations in SES-treated plants. In contrast, A6PR activities had direct positive effects on sorbitol concentrations during water stress (Table 1). These results indicate that competition for the limited supply of G6P favored sucrose biosynthesis over sorbitol biosynthesis in our water-stressed plants. Therefore, we conclude that water stress affected the partitioning of newly fixed carbon among terminal productions in leaves of micropropagated apple plants, and that the decrease in ADPGPPase activity probably became a critical and limiting step resulting in a shift in the partitioning of photosynthetically fixed carbon.

Enzymatic regulation of nonstructural carbohydrate metabolism under water-stress conditions

Nonstructural carbohydrate biosynthesis is regulated by metabolic enzymes in the leaves. The discovery of at least three SPS gene families (Lunn and MacRae 2003) and four invertase genes (Taliercio et al. 1999, Kim et al. 2000*b*) shows that these enzymes may be present in multiple forms in higher plants. However, we measured carbon metabolic enzymatic activities in crude extracts, so our data represent the total activity that results from several isoforms of SPS and acid invertase.

Enzymatic regulation of sorbitol metabolism The key enzyme in the sorbitol biosynthetic pathway is A6PR, and its activity increased rapidly in leaves as soon as the MIS and SES treatments were initiated (Figure 3a). However, the correlation between foliar sorbitol concentration and A6PR activity was not significant and the positive effect of A6PR activity on sorbitol accumulation was limited to SES-treated plants (Table 1). Therefore, we conclude that increased activity of A6PR was not the main cause of sorbitol accumulation in our water-stressed plants. Foliar sorbitol concentration could have increased as a result of decreased activity of SDH (Figure 3d), the key enzyme that catalyzes the oxidation of sorbitol to fructose (Loescher 1987, Yamaguchi et al. 1994). Significant negative correlations were obtained between sorbitol concentration and SDH activities, and path analysis between foliar sorbitol concentrations and the six metabolic enzymes studied showed that only SDH had significant direct effects on foliar sorbitol concentrations in both MIS- and SES-treated plants (Table 1). The enzyme ADPGPPase, which is involved in starch degradation (Hawker et al. 1991, Sarikaya et al. 2000), may also play a role in the accumulation of sorbitol because it has a strong negative direct effect on sorbitol concentration (Table 1). Our results indicated that SDH activity was a critical factor in regulating

sorbitol metabolism and that reduced degradation of sorbitol could account for the increased accumulation of sorbitol in leaves of water-stressed plants.

Enzymatic regulation of sucrose metabolism Sucrose phosphate synthase, a regulatory enzyme in the sucrose biosynthetic pathway (Hawker 1985, Lunn and MacRae 2003), had highly significant correlations and positive direct effects on foliar sucrose concentrations in water-stressed plants (Table 2). The correlation and path analysis of sucrose concentration and activities of related enzymes indicated that sucrose biosynthesis was strictly regulated by SPS, and that increased SPS activity (Figure 3b) mainly accounted for the accumulation of sucrose in leaves of water-stressed plants. Highly significant correlations were found between sucrose concentrations and the activity of AI, the irreversible regulator of sucrose hydrolysis (Merlo and Passera 1991, Kim et al. 2000*a*), but the larger negative direct effects of AI resulted mainly from large indirect effects of ADPGPPase (Table 2). Accumulation of sucrose in response to mild water stress was poorly correlated with AI activity in maize (Kim et al. 2000*a*). We conclude that AI activity was not the key factor contributing to the increase in foliar sucrose concentration in water-stressed plants. Based on the highly significant negative correlation between ADPGPPase activity and foliar sucrose concentration and its large direct effects on sucrose concentrations in water-stressed plants (Table 2), we conclude that a decrease in ADPGPPase activity (Figure 3f) may play an important role in foliar sucrose accumulation in water-stressed plants as a result of the reduced partitioning of newly fixed carbon to starch which would favor the sucrose synthetic pathways (Wang et al. 1996). We tested if sucrose synthase (SS) and neutral invertase, which converts sucrose to glucose and fructose (Zhu et al. 1997, Sturm 1999), influence the sucrose concentration in apple leaves. We found that SS and neutral invertase activities in leaves of water-stressed micropropagated apple plants were low, about 0.41–0.53 and 4.01–4.57 μmol sucrose mg^{-1} protein h^{-1}, respectively (data not shown). Moreover, no significant correlations were observed between sucrose concentration and activity of SS or neutral invertase in our experiment. Thus, the contributions of SS and neutral invertase to sucrose metabolism in leaves of micropropagated apple plants appear to be negligible.

Enzymatic regulation of starch metabolism Foliar starch concentrations decreased in response to water stress (Figure 2f), and were accompanied by a reduction in ADPGPPase activity and an increase in amylase activity (Figures 3c and 3f). Activities of ADPGPPase and amylase consistently had the largest positive or negative direct effects on foliar starch concentrations in MIS- and SES-treated plants (Table 3), resulting in decreased foliar starch concentrations in leaves of water-stressed micropropagated plants. Because the other carbohydrate metabolic enzyme activities assayed showed no consistent correlations or effects on foliar starch concentrations, we conclude that they were in downstream positions of the key regulatory steps in the synthesis and degradation of carbohydrates and so had relatively little effect on starch metabolism.

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