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Enzymatic Regulation of Sorbitol Metabolism in Micropropagated Apple Plants in Response to Water Stress

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Summary

Micropropagated 'Red Fuji' apple plants undergone three levels of water stress were studied to assess the effects on sorbitol metabolism. Sorbitol was the major soluble carbohydrate (47.5 % in leaves and 65.0 % in roots, respectively) and accumulated under treatments of mild stress (MIS) or moderate stress (MOS), while it decreased under severe stress (SES) treatment. Aldose-6-phosphate reductase (A6PR) activity increased rapidly, but the correlation between sorbitol content and A6PR activity was non significant and the positive effect of it on sorbitol accumulation was limited under MOS treatment in apple leaves; significant negative correlations were obtained between sorbitol content and sorbitol dehydrogenase (SDH) activity, and only SDH had a significant direct effect on sorbitol content under MIS and MOS treatments; the regulatory enzymes in the sucrose metabolism affected sorbitol content mainly through the indirect effects of ADP-glucose pyrophosphorylase (ADPGPPase) and amylase; and the rapid decreased of ADPGPPase activity and increased of amylase activity would be benefited for increase of sorbitol synthesis. No A6PR activity was detected in apple roots where SDH activity was present, the increase of sorbitol contents in roots under MIS and MOS treatments were caused by the more proportion of assimilates distributing and inhibition of sorbitol degradation. Reason of the decreased sorbitol contents under severe stress might be resulted by inhibition of both numerous synthetic and degrading enzymes of carbohydrates.

Key words. biosynthesis – correlation analysis – degradation – enzyme activity – path analysis

Introduction

Water deficiency stress is one of the most important environmental constraints that limit plant productivity (Gifford and Evans 1981; Boyer 1982). Many plants develop physiological adaptations to water stress, for example, plants achieve higher degrees of drought tolerance by accumulation of solutes which results in lowered cellular osmotic potential (Daie 1996). In previous studies, the partitioning of newly-fixed carbon in mature leaves was affected by water stress (Wang and Stutte 1992; Escobar-Gutiérrez and Gaudillère 1997), with the general trend of changes being that monosaccharides (i.e., glucose and fructose) or sugar alcohols (sorbitol, inositol et al.) accumulate while polysaccharide like starch is depleted (Wang and Stutte 1992; Keller and Ludlow 1993; Lombardinges et al. 1997). Sorbitol is a principal photosynthestic product and, together with sucrose, represents the main form of carbon translocated in phloem sap of most species of the *Rosaceae*, including the economically important genera *Malus*, *Pyrus* and *Prunus* (Webb and Burley 1962; Ranney et al. 1991; Escobar-Gutiérrez et al. 1998). The sorbitol to sucrose ratio in mature peach leaves varies with the origin of cultivars, and sorbitol synthesis may account for more than half of the newly fixed CO_2 (Escobar-Gutiérrez and Gaudillère 1997). In apple, sorbitol accounts for about 60–80 % of carbohydrates in the mature leaves, and it is transported to various sink tissues (Webb and Burley 1962; Bieleski and Redgwell 1985). Several studies indicate that water stress increases sorbitol accumulation, and the changes of carbohydrate metabolic enzyme activities might play important roles in this process (Escobar-Gutiérrez et al. 1998; Lo Bianco et al. 2000). The activity of A6PR, the key enzyme regulating sorbitol synthesis (Negm and Loescher 1981), tended to increase linearly in response to drought stress in mature peach leaves, and there was no significant effect on sucrose-phosphase synthase (SPS) activity (Escobar-Gutiérrez et al. 1998). Contrasting results were reported for peach leaves in Lo Bianco et al. (2000), where both SDH and A6PR activities were reduced, while only sucrose synthase (SS) was also affected by drought stress, among the sucrose enzymes. The different results between the two studies might be due to the difference of the length of the drought treatment, the method of drought imposition, and the light conditions.

Correlation analysis between the changes of the content of soluble sugars, including sugar alcohols, and the activities of metabolic enzymes was used to provide insight into carbohydrate metabolism of a plant under water stress (Moriguchi et al. 1990; Escobar-Gutiérrez et al.

1998; Lo Bianco et al. 2000). Simple correlation alone would lead to the erroneous conclusions about a specific enzymes' effect on sorbitol metabolism, because either direct or indirect influence existed on relationships between sorbitol content and metabolic enzyme activities. Path analysis is an important analytical tool for quantifying a perceived biological relationship through partitioning of correlation coefficients into direct and indirect effects (Sokal and Rohlf 1995; Ssabgo et al. 2004). If the variables are indirectly related, path analysis elucidates how other variables mediate the relationships (Everitt and Dunn 1992; Mcgiffen et al. 1994). Thus, path analysis can help both quantify the effects of carbon metabolic enzyme activity on sorbitol metabolism and elucidate the relative important of certain enzymes in regulating sorbitol metabolism under water deficient stress status.

The aim of this study was to investigate the effect of water deficiency stress on sorbitol synthesis and degradation in leaves and roots of micropropagated 'Fuji' apple plants of the same genotype, and to examine the correlations between sorbitol contents and metabolic enzymes by partitioning the correlations into direct effects (path coefficients) and indirect effects through the use of path analysis. We expected that these results would provide a substantial base for further studies on the enzymological mechanism of sorbitol metabolic modulation under water stress.

Materials and Methods

Abbreviations

ADPG, adenosine diphosphate glucose; ADPGPPase, ADPG-pyrophosphorylase; A6PR, aldose-6-phosphate reductase; SDH, sorbitol dehydrogenase; SPS, sucrose phosphate synthase; SS, sucrose synthase; AI, acid invertase; NI, neutral invertase; UDPG, uridine diphosphate glucose; MIS, mild stress; MOS, moderate stress; SES, severe stress; CK, normal water condition.

Plant material preparation and water stress application

Micropropagated 'Fuji' apple plants (*Malus domestica* Borkh. cv. 'NaganoFuji') with heights of 20 to 30 cm were pre-cultured in pots with 1/2 strength Hoagland nutrient solution for 10 days. The culturing conditions were 24–28±2 °C room temperature, and 85 % relative humidity under a 16/8 (h) light/dark cycle. Illumination was provided by cool white fluorescent lights with intensity of 600 μmol photons m^{-2} s^{-1}. The apple plants were transferred into the Hoagland solution for 10 d. Plants with uniform vegetative growth were selected for the study. With the method of Hsiao (1973), treatments of three different water deficit intensities were performed by regulating the osmotic potential of the solution using PEG-6000. Mild stress (MIS, solution with osmotic potential of −0.75 MPa), moderate stress (MOS, solution with osmotic potential of −1.5 MPa), and severe stress (SES, solution with osmotic potential of −2.5 MPa), were compared with the control under normal water conditions (CK). A randomized complete block design was used with 5 replicates, and 50 plants in each block.

Measurement of leaf water potential

The measure method of leaf water potential had been described in Li et al. (2003), CK plants kept a high and stable level about −0.2 MPa throughout the experiment, whereas leaf water potentials decreased progressively in the water stressed plants during the first 13 h and remained at low levels thereafter, −0.5 to −0.7 MPa for MIS, −1.0 to −1.2 MPa for MOS, and −1.4 to −1.7 MPa for SES, respectively, when measured at the beginning of the photoperiod.

Soluble carbohydrate and sorbitol analyses

Leaves and roots of the micropropagated apple plants were sampled at 2, 4, 8, 12, 24, 48 h after initiating the water stress treatments. The samples (including leaves and roots) were divided into two parts. One part was dried in an oven at 105 °C for 0.5 h, to constant weight at 80 °C, and ground to pass through a 0.8 mm screen, then used for analyses of soluble carbohydrate and sorbitol. The other part of the samples was frozen in liquid nitrogen and stored at −40 °C for analysis of the enzyme activities.

For soluble carbohydrate and sorbitol extraction, 0.25 g dried leaves or 0.5 g dried root powder was weighed and then extracted 3 times each with 10 ml 80 % (v/v) ethanol, in water at 80 °C for 10 min and centrifuged at 1.500 g for 10 min. The supernatants were combined and evaporated in a freeze dryer (FD-1, Rikakikai Co. Ltd, Tokyo, Japan), and the evaporated extract was diluted to appropriate volume with distilled water, and then centrifuged to remove the insoluble materials. After being treated with amberlite (0.3 g ml^{-1}) and filtered with a cellulose membrane filter (0.4 μm, Shanghai, China) (Suwignyo et al. 1995), the resulting solution was used for determination of soluble carbohydrate and sorbitol with high performance liquid chromatography (HPLC, LC-10AD; Shimadzu Corporation, Kyoto, Japan), equipped with a refractive index detector (Rid-6A; Shimadzu, Japan). A 10 μl sample was injected into the HPLC and sugar was separated by use of a 300×7.80 mm REZEX 8u 8 % Ca. Monos. column (Phenomenex) maintained at 85.5 °C. Distilled water was used as the mobile phase at a flow rate of 1.0 ml min^{-1}. Sucrose, glucose, fructose, and sorbitol were identified and quantified by comparison with a known standard sample (Moing et al. 1992) (Data of sucrose, glucose and fructose contents not shown). The total soluble carbohydrate was equal to the sum of sucrose, glucose, fructose, and sorbitol.

Enzyme activity analyses

Frozen leaf tissue (0.25 g fresh weight) or root tissue (0.50 g fresh weight) was ground in a chilled mortar using a 1:5 tissue-to-buffer ratio. The extraction buffer contained 50 mmol l^{-1} Hepes-NaOH (pH 7.5, at 25 °C), 5 mmol l^{-1} $MgCl_2$, 1 mmol l^{-1} EDTA, 2.5 mmol l^{-1} dithiothreitol (DTT), 0.05 % (v/v) TritonX-100 and 0.5 mmol l^{-1} BSA. In all cases, the tissue was ground in buffer and sand using a pre-cooled (2–4 °C) mortar and pestle. The homogenate was filtered and centrifuged at 13.000 g for 10 min at 2 °C. The supernatant was dialyzed immediately by 10-fold diluted extraction buffer (v/v, without Triton-100) for 20 h, changing the dialyzation once in the middle of the process. The dialyzed enzyme extract was used to assay the activities of all enzymes.

A6PR (EC 1.1.1.200) activity was assayed in the direction of synthesis following the oxidation of NADPH in the presence of glucose-6-P at 340 nm, as described by Merlo and Passera (1991). The reaction mixture contained 0.1 mol l^{-1} Tris-HCl (pH 8.8), 0.1 mmol l^{-1} NADPH, 50 mmol l^{-1} glucose-6-P and 50 µl extract. The activity of SDH (EC 1.1.1.14) was assayed by following the reduction of NAD^+ in the presence of D-sorbitol at 340 nm (Negm and Loescher 1979). The reaction mixture contained 0.1 mol l^{-1} Tris-HCl (pH 9.0), 1 mmol l^{-1} NAD^+, 0.5 mol l^{-1} D-sorbitol and 0.1 ml extract. Sorbitol and glucose-6-P, dissolved in the same buffer as used in the assay, were used to start the reactions.

SPS (EC 2.4.1.14) and SS (EC 2.4.1.13) activities were determined according to Hubbard et al. (1989). Reaction mixtures (70 µl) to determine SPS activity contained 50 mmol l^{-1} Hepes-NaOH (pH 7.5), 15 mmol l^{-1} $MgCl_2$, 5 mmol l^{-1} fructose 6-P, 15 mmol l^{-1} glucose 6-P, 10 mmol l^{-1} UDPG, 45 µl extract. Reaction mixtures were incubated at 25 °C and terminated at 0 and 20 min with 70 µl of 30 % KOH. Tubes were placed in boiling water for 10 min to destroy any unreacted fructose or fructose 6-P. After cooling, 1 ml of a mixture of 0.14 % anthrone in 13.8 mol l^{-1} H_2SO_4 was added and incubated in a 40 °C water bath for 20 min. Also after cooling, colour development was measured at 620 nm. The procedure for the sucrose synthase assay was identical to that of SPS except the reaction mixtures contained 10 mmol l^{-1} fructose and did not contain fructose 6-P or glucose 6-P.

ADPGPPase (EC 2.7.7.27) was determined by measuring pyrophosphate-dependent glucose-1-P formation from ADPG at 25 °C in a total 1 ml reaction mixture, according to the procedures described by Ou-Lee and Setter (1985). Reaction was started by addition of ADPG to a final concentration of 5.0 mmol l^{-1} and production of NADPH was monitored at 340 nm.

Acid invertase (AI; EC 3.2.1.26) and neutral invertase (NI; EC 3.2.1.27) were assayed in a reaction mixture (1 ml) containing 0.1 mol l^{-1} Na-acetate (pH 4.8 for acid invertase) or 0.1 mol l^{-1} K_2HPO_4 0.1 mol l^{-1} Na-citrate (pH 7.2 for neutral invertase), 0.1 mol l^{-1} sucrose and 0.2 ml extract. After incubation for 40 min at 37 °C, the reactions were stopped by addition of 1 ml dinitrosalicylic acid reagent, and reducing sugars released from sucrose were determined according to Merlo and Passera (1991). Total amylase activity (EC 3.2.1.1. and EC 3.2.1.2) was assayed in a reaction mixture (1 ml) containing 0.1 mol l^{-1} Na-acetate (pH 6.5), 1.5 mmol l^{-1} NaF, 5 mmol l^{-1} $CaNO_3$, 0.5 % soluble starch and 0.2 ml extract. After 40 min at 30 °C the reaction was terminated, and the release of reducing groups was determined as in the invertase assay. In both invertase and amylase assays, blanks contained reaction mixtures incubated with dinitrosalicylic acid reagent. All chemicals were purchased from Sigma (St. Louis, MO).

Statistical analyses were performed using the Statistical Analysis System software (Sas Institute 1985). Path coefficients and correlations, determined by using the software package EQS (Version 5.7 for Windows, Multivariate Software, Encino, CA) (Benlter 1998), were analyzed between sorbitol and activities of relative carbon metabolic enzymes. Since no A6PR activity was detected and there was no sorbitol synthesis in the apple roots, the influence of other relative enzymes (such as SPS, SS, AI, NI, ADPGPPase and amylase) on sorbitol metabolism was neglected.

Results

Influence of water stress on total soluble carbohydrate content

The total soluble carbohydrate contents in leaves of micropropagated apple were significantly affected by water stress (Fig. 1A). After 2 h of MOS treatment or 8 h of MIS treatment, the content of total soluble carbohydrate in leaves was significantly higher than that of the control, and total soluble carbohydrate accumulated faster under MOS than under MIS within 24 h, but there was no sig-

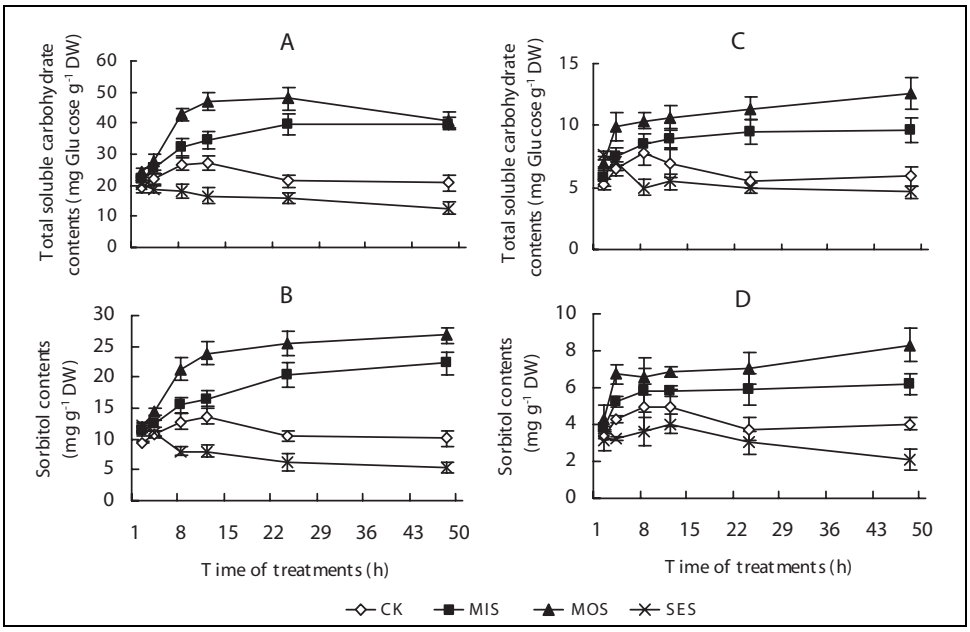


Fig. 1. The changes of total soluble carbohydrate content (A), and sorbitol content (B) in leaves, total soluble carbohydrate content (C), and sorbitol content (D) in roots of micropropagated apple plants in response to water stress at different intensity: Mild stress (MIS), moderate stress (MOS), and severe stress (SES). CK is the control under a normal water condition. The results are given as mean ± SE of 5 replicate samples.

nificant difference between MIS and MOS at the end of the treatment. A slight increase of total soluble carbohydrate content was observed in a short-term after initiating the SES treatment (2 h), and then significantly decreased, compared with CK.

Comparatively, the accumulation of total soluble carbohydrates in the roots of micropropagated apple plants was lower than that in the leaves (Fig. 1C). The contents of total soluble carbohydrate also became significantly higher than that of control when treated by MIS and MOS, and more affected by MOS. There was a similar pattern on change of the soluble carbohydrate as in leaves and kept the lowest level during the most of the experiment time when treated by SES.

Influence of water deficiency stress on sorbitol content

Under normal water conditions, the sorbitol content in apple leaves was around 10 mg g^{-1} DW. Water stress significantly increased the sorbitol content and the effect of MOS was more significant than that of MIS. At the end of treatment, the sorbitol content in apple leaves of MIS and MOS treatments were 2.21 times and 2.66 times the control, respectively (Fig. 1B). Sorbitol content increased significantly in a short-term after treated (2 h) by SES, then quickly decreased, being kept the lowest level during the experiment.

Sorbitol contents of MIS and MOS treatments were significantly higher than that of the control throughout most of the treatment (Fig. 1D) in roots of micropropagated apple plants. As treated by SES, sorbitol content decreased to half of control at the end of the experiment.

Influence of water stress on metabolic enzyme activity

Under MIS and MOS treatment, A6PR activity in leaves increased rapidly and was significantly higher compared with the control. But the increase of A6PR activity be-

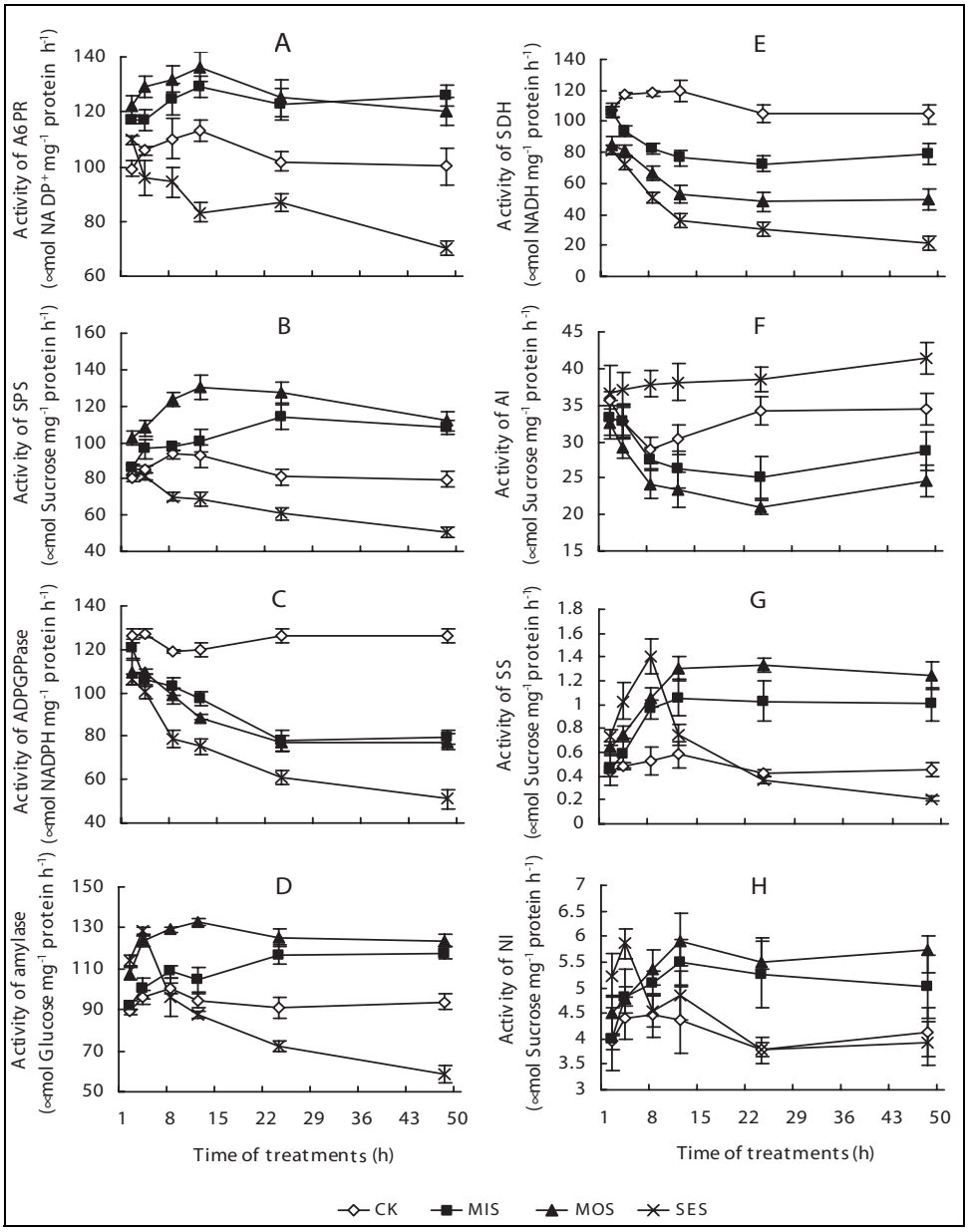


Fig. 2. The activity of aldose-6-phosphate reductase (A6PR, A), sucrose phosphate synthase (SPS, B), ADPglucose-pyrophosphorylase (ADPGPPase, C), amylase (D). sorbitol dehydrogenase (SDH, E), acid invertase (AI, F), sucrose synthase (SS, G), neutral invertase (NI, H) of micropropagated apple leaves in response to water stress at different intensity: mild stress (MIS), moderate stress (MOS), and severe stress (SES), and CK is the control under a normal water condition. The results are given as mean ± SE of 5 replicate samples.

tween MIS and MOS was not significant (Fig. 2A). As regarded to SES treatment, the activity of A6PR significantly increased during the first 2 h and then rapidly decreased; significantly lower than that CK after 8 h after initiating the treatment. In roots, no A6PR activity was detected, which suggested that the roots of micropropagated apple plants were not able to synthesize sorbitol.

The activity of SDH decreased significantly under water stress in leaves, and was related to the degrees of water stress. At the end of the experiment, SDH activity in leaves of MIS, MOS and SES plants decreased to 75.32 %, 47.91 % and 25.30 % of that of the control plants, respectively (Fig. 2E). The activity of SDH in roots was low (Fig. 3), only about 20 % of that in the leaves, and significantly decreased as treated by different degrees of water stress in 2 h of the treatments. The difference of the MIS and MOS treatments disappeared after 24 h of treatment.

SPS activity significantly increased 2 h after the MIS and MOS treatment, and remained at a higher level compared with the CK (Fig. 2B). A little change in the activity of SPS in response to early period of SES treatment, but decreased rapidly after 4 h. There was a difference in changes between the activity of invertases and SS, the activity of AI decreased (Fig. 2F) as the activity of SS and NI increased significantly as treated by MIS and MOS (Fig. 2G and 2H), but the activity of AI significantly increased under SES treatment.

The activity of ADPGPPase decreased significantly, there was no significant difference between MOS and MIS during most of the experiment (Fig. 2C), and much more affected by SES. In the present study, amylase activity of the control remained relatively stable (Fig. 2D), it increased rapidly under MIS and MOS treatment, with greater significant effect under MOS treatment, but it significantly decreased after 4 h SES treatment.

Correlation and path analysis between sorbitol content and metabolic enzyme activity in leaves

Under normal water status, sorbitol content was significantly positively correlated with the activity of A6PR (r_{1yCK}=0.60), SPS (r_{3yCK}=0.70) and amylase (r_{8yCK}=0.56), negatively correlated with the activity of AI (r_{6yCK}=−0.61) and ADPGPPase (r_{7yCK}=−0.55) (Table 1). However, the correlations between the sorbitol content with the activity of SDH (r_{2yCK}=0.33), SS (r_{4yCK}=0.29) and NI (r_{5yCK}=0.26) were not significant. Further path analysis showed that the direct path coefficient of A6PR (P_{1yCK}) was the biggest, about 0.32. SPS, ADPGPPase and amylase had important direct effects on the sorbitol content (P_{3yCK}=0.29, P_{7yCK}=0.26, P_{8yCK}=0.30, respectively), too. SDH activity had a strongly negative direct effect on sorbitol content (P_{2yCK}=−0.25), combined with the positive indirect effects via SPS activity ($r_{23}P_{3yCK}$=0.54). The direct path coefficients of SS, NI and AI on sorbitol contents were weaker (Table 1), and these were due to greater positive or negative indirect effects via SPS ($r_{43}P_{3yCK}$=0.54, $r_{53}P_{3yCK}$=0.46, $r_{63}P_{3yCK}$=−0.63, respectively).

In the case of the water deficiency stress treatment, there were significant positive correlations between sorbitol contents and the activities of A6PR (except MOS treatment), SPS, SS, NI (except MIS treatment) or amylase, and significant negative correlations with the activities of SDH, AI or ADPGPPase (Table 1). Path analyses showed that A6PR (P_{1yMIS}=0.29, P_{1yMOS}=0.21, respectively) and amylase (P_{8yMIS}=0.50, P_{8yMOS}=0.23, respectively) still exhibited relatively larger direct positive effects on sorbitol contents. SDH, SPS, SS, AI, and ADPGPPase contributed to the negative direct effects on sorbitol contents in leaves under water stress (Table 1). Only the direct effects of SDH activity on sorbitol contents were significant (P_{2yMIS}=−0.41, P_{2yMOS}=−0.32, respectively).

Except AI, activities of the related metabolic enzymes were significantly lowered under SES, having the reverse changes with those under MIS or MOS at the end of the experiment, by exception of ADPGPPase activity. The results indicated that disorders of metabolic functions of the enzymes had been caused by SES. Thus, both correlation and path analysis would not be conducted on either sorbitol content or the enzyme under SES in this experiment.

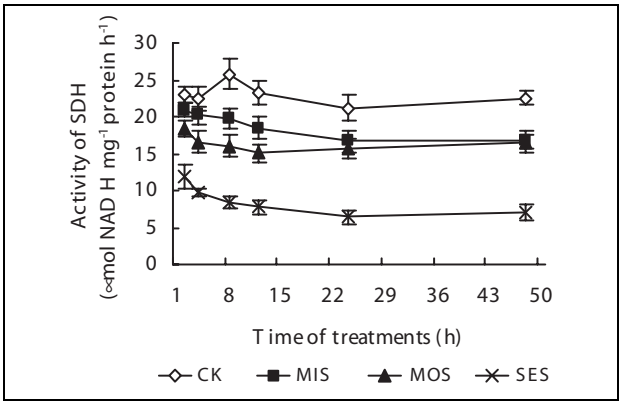


Fig. 3. The activity of sorbitol dehydrogenase (SDH) in micropropagated apple roots in response to water stress at different intensity: mild stress (MIS), moderate stress (MOS), severe stress (SES). CK is the control under a normal water condition. The results are given as mean ± SE of 5 replicate samples.

Correlation between sorbitol content and SDH activity in roots

No A6PR activity was detected in roots of micropropagated apple plants where SDH activity was present, which indicated that sorbitol degradation existed in apple roots, and without sorbitol synthesis process. SDH activity showed negative correlations with sorbitol contents under different water status (r_{CK}=−0.49, r_{MIS}=−0.58, r_{MOS}=−0.50, respectively), being suggested that sorbitol accumulated with a decrease of SDH activity (Fig. 4). The severe water stress made the correlation between sorbitol content and the activity of SDH disappeared (r_{SES}=0.01).

Discussion

The changes of sorbitol content in response to water stress

In the present work, sorbitol content of micropropagated apple plants accounted for over 47.5 % of soluble carbohydrates in leaves and as high as 65.0 % in roots (Fig. 1), and was sensitive to water stress. The observed increase in sorbitol content in leaves and roots in response to MIS

Table 1. Correlations and path analyses between sorbitol content (y)and activity of Aldose-6-phosphate reductase (A6PR, x1), sorbitol dehydrogenase (SDH, x2), sucrose phosphate synthase (SPS, x3), sucrose synthase (SS, x4), neutral invertase (NI, x5), acid invertase (AI, x6), ADPglucose-pyrophosphorylase (ADPGPPase, x7), and amylase (x8.) in the leaves of micropropagated apple plants under different water deficiency stress: mild stress (MIS), moderate stress (MOS), and severe stress (SES). CK is the control under a normal water condition. * and ** indicate that the regression equation is significant different at P<0.05 or at P<0.01 level, respectively.

Factor	Treatment	Correlation coefficient (r_{iy})	Direct path coefficient (P_{iy})	Indirect path coefficient ($r_{ij}P_{iy}$)							
				x1	x2	x3	x4	x5	x6	x7	x8
A6PR (x1)	CK	0.60**	0.32		−0.11	0.51	0.11	−0.00	0.02	−0.17	0.12
	MIS	0.52*	0.29		0.05	−0.06	−0.07	0.01	0.02	0.04	0.26
	MOS	0.16	0.21		−0.01	−0.13	−0.02	0.02	0.05	−0.07	0.12
	SES	0.86**	0.41		−0.59	−0.02	0.05	0.07	0.08	1.37	−0.50
SDH (x2)	CK	0.33	−0.25	0.14		0.54	0.08	−0.20	0.02	−0.10	0.10
	MIS	−0.80**	−0.41*	−0.02		0.13	0.10	−0.02	−0.02	−0.52	−0.38
	MOS	−0.91**	−0.32*	−0.02		0.20	0.08	−0.11	−0.21	−0.82	−0.14
	SES	0.84	−0.66	0.37		−0.02	0.03	0.05	0.07	1.49	−0.54
SPS (x3)	CK	0.70**	0.29	0.20	−0.17		0.12	−0.23	0.03	−0.19	0.14
	MIS	0.83**	−0.18	0.01	0.06		−0.08	0.01	0.02	0.56	0.41
	MOS	0.59*	−0.29	0.10	−0.08		−0.07	0.08	0.19	0.48	0.17
	SES	0.84**	−0.02	0.37	−0.58		0.03	0.08	0.08	1.43	−0.55
SS (x4)	CK	0.29	0.19	0.21	−0.11	0.54		−0.27	0.02	−0.10	0.04
	MIS	0.70**	−0.12	0.02	0.07	−0.12		0.02	0.02	0.46	0.35
	MOS	0.86**	−0.09	0.05	−0.11	−0.22		0.11	0.21	0.75	0.16
	SES	0.33	0.15	0.13	−0.12	−0.01		−0.07	0.04	0.16	−0.10
NI (x5)	CK	0.26	−0.19	0.16	−0.18	0.46	0.12		0.02	−0.06	0.07
	MIS	0.44	0.02	0.01	0.05	−0.11	−0.08		0.02	0.33	0.20
	MOS	0.71**	0.14	0.03	−0.08	−0.16	−0.07		0.18	0.51	0.16
	SES	−0.25	−0.27	−0.11	0.13	0.01	0.04		−0.04	−0.34	0.17
AI (x6)	CK	−0.61**	−0.03	−0.15	0.15	−0.63	−0.10	0.21		0.11	−0.18
	MIS	−0.62**	−0.03	−0.02	−0.06	0.12	0.09	−0.01		−0.42	−0.31
	MOS	−0.82**	−0.26	−0.04	0.09	0.22	0.07	−0.10		−0.65	−0.19
	SES	0.83**	−0.15	−0.21	0.29	0.01	−0.04	−0.06		−0.71	0.29
ADPGPPase (x7)	CK	−0.41	0.26	−0.22	0. 10	−0.58	−0.07	0.09	−0.01		−0.12
	MIS	−0.92**	−0.63	−0.02	−0.07	0.16	0.09	−0.01	−0.02		−0.42
	MOS	−0.90**	−0.60	−0.02	0.10	0.16	0.08	−0.08	−0.19		−0.10
	SES	0.87**	1.55	0.36	−0.63	−0.02	0.02	0.06	0.07		−0.56
Amylase (x8)	CK	0.56*	0.30	0.13	−0.09	0.38	0.02	−0.09	0.02	−0.10	
	MIS	0.91**	0.50	0.02	0.06	−0.15	−0.08	0.01	0.02	0.54	
	MOS	0.67**	0.23	0.11	−0.07	−0.21	−0.06	0.10	0.18	0.39	
	SES	0.78**	−0.60	0.33	−0.59	−0.02	0.03	0.08	0.07	1.44	

and MOS treatments (Fig. 1B and 1D) were in agreement with several previous studies (Wang and Stutte 1992; Escobar-Gutiérrez et al. 1998; Lo Bianco et al. 2000). Further, as considering the ratio of sorbitol/total soluble carbohydrate, the stress-inducing changes of this index were related to the intensity and duration of these two degrees water stress, therefore, sorbitol accumulation became the primary cause of solute increase under mild and moderate water stress treatments. However, sorbitol content significantly decreased by SES, which might be relate to the significant reduce of photosynthesis rates (Li et al. 2003), and furthermore would be related to disorders of metabolic functions of the related enzymes to carbohydrates in our experiment.

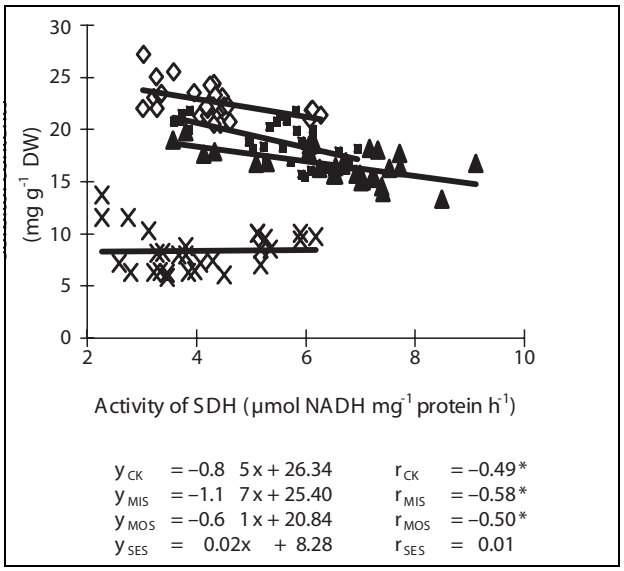


Fig. 4. Correlation analyses between sorbitol content and sorbitol dehydrogenase (SDH) activity in roots of micropropagated apple plants under different intensity of water stress. Mild stress (MIS ■), moderate stress (MOS ▲), severe stress (SES ×), and CK (◇) is the control under a normal water condition. * indicate that the regression equation is significant at P<0.05 level.

Enzymatic pathways of sorbitol metabolism in leaves under water stress

As path analysis can be employed to partition the correlation between activity of metabolic enzymes and sorbitol content into direct and indirect effects, it also can elucidate the variables that mediate the relationships (Mcgiffen et al. 1994). Conflicting results on A6PR activity under water stress have been obtained. A6PR activity in peach leaves either increased (Escobar-Gutiérrez et al. 1998) or decreased (Lo Bianco et al. 2000) under water stress. In our experiment, A6PR activity increased rapidly in leaves as soon as the micropropagated apple plants were subjected to water stress (Fig. 2A). However, the correlation between sorbitol content and the A6PR activity was non significant and the positive effect of A6PR activity on sorbitol accumulation was limited under MOS treatment (r_{1yMOS}=0.16, P_{1yMOS}=0.21, respectively) (Table 1). So the increased activity of A6PR might not be the main cause of sorbitol accumulation. The sorbitol accumulation might result from decreased activity of SDH (Fig. 2E), which oxidizes sorbitol to fructose (Loescher 1987). Significant negative correlations were obtained between sorbitol content and SDH activity, and only SDH had a significant direct effect on sorbitol content under water stress (P_{2yMIS}=–0.41, P_{2yMOS}=–0.32, respectively) (Table 1). These results suggest that SDH played a critical role in regulating sorbitol metabolism, and reduced degradation of sorbitol could be the primary case for sorbitol accumulation in leaves of micropropagated apple plants during the period of water stress.

SPS had a larger, directly positive effect on sorbitol content under normal water status (Table 1). Glucose 6-P is the common immediate precursor of sucrose biosynthesis through SPS (Bruneau et al. 1991, Lunn and Macrae 2003) and sorbitol biosynthesis through A6PR (Negm and Loescher 1981; Loescher and Everard 1996). Both sucrose and sorbitol are synthesized in the cytoplasm (Daie 1996; Loescher and Everard 1996), so sorbitol and sucrose could be independently biosynthesised with no substrate competition as there was a rich existing carbon source. Under water stress treatment, SPS exhibited negative direct effects on sorbitol content (P_{3yMIS}=–0.18, P_{3yMOS}=–0.29, respectively), suggesting that competition existed for limited substrate between sorbitol and sucrose under water stress (Li and Li 2005).

Because the negative direct effects of SPS on sorbitol contents mainly originated from the indirect effects of ADPGPPase ($r_{37}P_{7yMIS}$=0.56, $r_{37}P_{7yMOS}$=0.48, respectively) and amylase ($r_{38}P_{8yMIS}$=0.41, $r_{38}P_{8yMOS}$=0.17, respectively), the key enzymes involved in starch metabolism (Preiss 1982; Merlo and Passera 1991; Sarikaya et al. 2000) (Table 1), so the rapid decrease of ADPGPPase activity and increase of amylase activity (Fig. 2C and 2D) lead to an accumulation of glucose (Dancer et al. 1990; Wang et al. 1996), and decrease of the starch contents under water stress (Li and Li 2005), which benefited for increase of sorbitol synthesis.

The trends of activity of enzymes involved in sucrose catabolic pathway, such as AI, NI and SS (Hawker et al. 1987; Merlo and Passera 1991; Lunn and Macrae 2003) were not uniform under MIS and MOS treatments. Although they had significant or highly significant correlations with sorbitol content (Table 1), yet they mainly affected sorbitol content indirectly through ADPGPPase or amylase, so their direct effects on sorbitol metabolism were limited.

Role of SDH in sorbitol accumulation in roots under water stress

The activity of A6PR was not detectable in roots of micropropagated apple plants, suggesting that the roots could not synthesize sorbitol. Therefore, sorbitol in roots must have been transported from leaves through the phloem (Loescher and Everard 1996). This result was in partial agreement with previous studies that found root tissues have little or no capacity to synthesize sugar alcohols (Grant and Ap Rees 1981; Lewis 1984; Stoop and Pharr 1992). On the other hand, the roots contained SDH activity, and significant negative correlation with sorbitol content was observed under MIS and MOS treatments (r_{CK}=–0.49, r_{MIS}=–0.68, r_{MOS}=–0.50, respectively). Under MIS and MOS treatments, SDH activity significant decrease in water-stressed roots, and less degradation of sorbitol induced by water stress in roots of micropropagated apple plants. Meanwhile, the accumulation of sorbitol was also due to the higher proportion of assimilates distributed (Li et al. 2003).

In conclusion, sorbitol metabolism was sensitive to water stress and subjected to close metabolic control. SDH played a critical role in regulating sorbitol metabolism, and the changes in starch metabolism became a critical and, probably limiting step in regulating the sorbitol metabolism through the substrate level in leaves of micropropagated apple plants under water stress, and there was a competition between sorbitol and sucrose as the substrate was limited. Meanwhile, the accumulation of sorbitol in roots was mainly due to the higher proportion of assimilates distributed and the lower degradation of sorbitol induced by water stress.

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