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Regulation of Cytosolic Fructose-1,6-Bisphosphatase Under Water-Stressed Leaves of Sugar Beet: Protein Modification is not a Mechanism for Coarse Control

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A mechanism of coarse control of cytosolic FBPase under water stress has been investigated. Sugar beet plants grown in a green house were subjected to two different levels of water stress by withholding water: mild stress for 4 days and severe stress for 8 days. At mild stress, levels of transcript and protein in the leaves holding the water potential of -1.8 MPa were stable while the activity declined to 74% of control. At severe stress, the water potential in the leaves was approximately -4.0 MPa. The levels of the transcript and protein were 17% and 15% of the levels of control, respectively, suggesting the cytosolic FBPase gene was not expressed continuously in severe stress. However, the activity was still present although it is low (33% of control). Two dimensional protein gel was performed to find changes in the amino acid residues by charge modification under water stress. The isoelectric pH for the cytosolic FBPase on the Western blot was 5.4 at 37 kDa. The identical spot was found in different water stress levels indicating that protein modification of the cytosolic FBPase does not occur. Collectively, the regulation of cytosolic FBPase activity under water stress does not depend on the protein modification but depends on the biochemical levels at mild stress and the protein levels at severe stress.

Keywords: cytosolic FBPase, regulation, protein modification, water stress, sucrose biosynthesis

The rate of sucrose biosynthesis in the plant leaves is regulated by two key enzymes: cytosolic fructose-1, 6-bisphosphatase (FBPase) and sucrose-phosphate synthase (SPS) which are subject to multiple regulation (Huber *et al.*, 1989; Walker and Huber, 1989; Gerhardt *et al.*, 1987; Harn and Daie, 1992a; Daie, 1993; Harn and Daie, 1997). Until now, a great deal of progress has been made in understanding molecular regulation of SPS (Huber and Huber, 1996 and references therein) while limited information has been reported in regulation of the cytosolic FBPase with molecular approach. Generally, several mechanisms have been known to control these enzymes thus far: 1) fine control: allosteric control by metabolites and effector molecules; 2) coarse control: protein modification by phosphorylation/dephosphorylation and protein levels.

Above regulations would be changed or affected by limited water availability in plant, which is directly responsible for the rate of photosynthetic carbon fixation decreasing CO_2 assimilation rate (Kaiser, 1987). Consequently, water stress leads changes in carbon partitioning between sucrose and starch, decreasing starch and increasing sucrose levels in leaves (Quick *et al.*, 1989; Vassey and Sharkey, 1989; Dancer *et al.*, 1990; Harn and Daie, 1992). Water stress is also accompanied by raised levels of metabolites which allow to maintain cellular turgor potential so that osmotic adjustment is achieved for survival under lack of water contents (Meyer and Boyer, 1981). So far, researches toward understanding the effect of water stress on the enzymes involved in the sucrose biosynthesis have not been largely studied despite the fact that sucrose is a major energy source as well as a sole transporting sugar in plants. Quick *et al.* (1989), however, showed activation of SPS in detached spinach leaves as they lost 10% of their fresh weight. They proposed two factors affecting the increased

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SPS activity during water stress: 1) increase of the substrate concentration due to shrinking cell volume; 2) coarse control in the activation of SPS by protein modification of SPS. Recently, Harn and Daie (1992a) revealed a general profile of regulation of the cytosolic FBPase under water stress and suggested that the cytosolic FBPase of sugar beet leaves was regulated by coarse control: possibly posttranslational modification and protein levels. The main objective of this paper was to investigate the mechanism of coarse control of the cytosolic FBPase under drought conditions by detecting possible covalent modification which may change the protein structure. Here, this paper presents that the differential levels of water stress do not modify the cytosolic FBPase covalently.

MATERIALS AND METHODS

Plant Materials

Sugar beet plants (*Beta vulgaris* L.) were grown in 4 liter pots for three month in a green house. Drought stress was imposed by withholding water for 8 days. Control plants were watered daily. Five mature, photosynthetically active leaves of same age from five different plants were pooled as a replication and three independent replications from each condition were used. Water potential was measured on leaf discs (6 mm in diameter) by a thermocouple psychrometer at 2:00 PM of the day.

Cytosolic FBPase Activity

Cytosolic FBPase from sugar beet leaves was partially purified based on procedures previously published (Sharkey *et al.*, 1988) with slight modification (Harn and Daie, 1992a). Cytosolic FBPase was separated from the chloroplast isozyme by DEAE-sephadex A-50 (Pharmacia, Piscataway, NJ) ion-exchange batch chromatography. The enzyme activity of cytosolic FBPase was assayed spectrophotometrically by coupling the production of fructose 6-phosphate to the reduction of NADP using phosphoglucose isomerase and glucose 6-phosphate dehydrogenase (Kelly *et al.*, 1982).

Northern Blot Analysis

Total RNA was extracted as described by Chomczynski and Sacchi (1987). Running a electrophoresis and preparing a transferred blot were also described by Harn and Daie (1997). The membranes were hybridized by ^{32}P-labelled (1×10^6 dpm/mL) cDNA encoding the sugar beet cytosolic FBPase (Harn and Daie, 1992b) in a solution containing 50% deionized formamide, 5 X Denhardt's solution, 0.1% SDS, 100 μg/mL salmon sperm DNA and 5 X SSPE for 16 h at 42°C. After hybridization, the blots were washed three times in 1 X SSC-0.1% SDS at 42°C and again three times in 0.1 X-0.1% SDS after which they were exposed to X-ray film at -80°C.

Western Blot Analysis

Partially purified cytosolic FBPase (20 μg) was mixed with equal volume of 50 mM Tris-HCl buffer (pH 7.4) containing 2% SDS and 10% β-mercaptoethanol. Proteins were denatured in boiling water and separated on a 12% polyacrylamide slab gel (Laemmli, 1970) at 150 V for 4 h. Proteins were electroblotted to nitrocellulose membrane at 200 mA for 4 h at 4°C (Towbin *et al.*, 1979). The membrane blot was then soaked for 2 h in a blocking solution containing 1% BSA in TBS (10 mM Tris-HCl [pH 7.4], 0.15 M NaCl) and incubated overnight with primary antibody raised against the sugar beet cytosolic FBPase (Khayat *et al.*, 1993). The membrane blot was washed in TBS buffer three times and incubated for 2 h with alkaline phosphatase-conjugated goat anti-rabbit IgG (Bio-Rad). Cytosolic FBPase protein was detected by incubating the blot with alkaline phosphatase color development reagents, 0.015% BCIP and 0.03% NBT in carbonate buffer (0.1 M $NaHCO_3$, 1.0 mM $MgCl_2$, pH 9.8).

Two Dimensional Electrophoresis

The first dimension IEF was performed according to the method of O'Farrell *et al.* (1977). In the second dimension, tube gels were run on 10% polyacrylamide slab gels as described by Laemmli (1970). After electrophoresis, the proteins were transferred onto nitrocellulose membranes (Towbin *et al.*, 1979). Immunoblot was performed as described for Western blot analysis.

Densitometry

Signals on both Western and northern blots were quantified by densitometric scanning with an UltraScan XL Laser Densitometer (LKB, Sweden), using GELSCAN software (Pharmacia) to determine the area under the peak.

RESULTS

Water Potential and FBPase Activity

Leaf water potential of control plants (0 day) watered daily was -0.7±0.2 MPa (Table 1). Withholding water in green house for 4 days and 8 days resulted in -1.8±0.3 MPa and -4.0±0.3 MPa, respectively. Phenotypes of the plants of the 4 day and the 8 day stress represent a mild and a severe stress based on the observation by Harn and Daie (1992a). In fact, wilting became visually apparent after 4 days stress and the 8 days stress was the longest tolerated. Most of the plants at 9 days stress in green house did not recover from drought, despite rehydration. At 0 day control, the activity of partially purified cytosolic FBPase was 27±3 nmoles min^{-1} mg^{-1} protein and this activity was stable during stress experiments as long as the water potential was maintained at -0.7 ±0.2 MPa. At 4 days stress, the activity decreased to 74% of control. At 8 days stress, the activity maintained up to 33% of control indicating that cytosolic FBPase activity is detectably present even at the severe drought stress. The activity values at 8 days stress are authentic because the activity without the substrate was 2.1±1.1 nmoles min^{-1} mg^{-1} protein which is negligible (data not shown).

Northern and Western Analysis

To determine the effect of water stress on the transcript and protein levels of cytosolic FBPase, northern and Western blots were performed. Fig. 1 showed a single band of 1.3 kb cytosolic FBPase transcript present in leaf tissues. The steady-state levels of the cytosolic FBPase transcript slightly declined to 88% of control at 4 days stress and dramatically declined to 17% at 8 days stress (Fig. 1). The partially purified cytosolic FBPase was detected by the antibody raised against sugar beet cytosolic FBPase (Fig. 2). The levels of cytosolic FBPase protein of 4 days stress remained equivalent to the one of the control indicating that the protein is stable. At 8 days stress, however, the levels decreased to 15% of control. No degraded proteins were found on the Western blot either in mild or severe stressed samples. Collectively, the levels of protein and transcript were correlated at two different stresses. However, decline in cytosolic FBPase activity at 4 days stress may not be reflected by the stable levels of protein and the loss in activity at severe stress may reflect a decline in the protein levels.

Table 1. Changes in the activity of partially purified cytosolic FBPase in sugar beet leaves subjected to two different water stress levels. Data points are means of three replicates with SE. Numbers in parentheses represent the percentage of activity based on the control

Day	Water Potential, MPa	Activity, nmoles $min^{-1}mg^{-1}$ protein
0	-0.7±0.2	27±3 (100%)
4	-1.8±0.3	20±2 (74%)
8	-4.0±0.3	9±2 (33%)

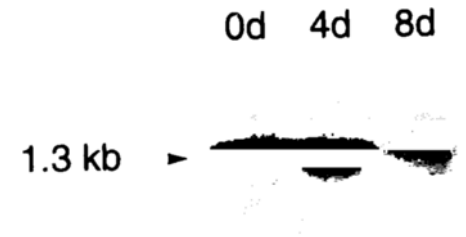

Fig. 1. Northern blot of the cytosolic FBPase in mature leaves subjected to 4 and 8 days water stress. Twenty µg of total RNA was loaded on 1% agarose gel, transferred onto nylon membrane and hybridized with ^{32}P-labelled sugar beet cytosolic FBPase cDNA. Intensity of bands was scanned by densitometer and the peak area of the samples was calculated as 100% for control, 88% for 4 days stress and 17% for 8 days stress.

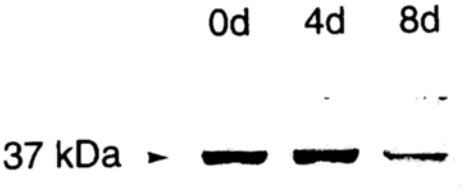

Fig. 2. Western blot of partially purified cytosolic FBPase from sugar beet leaves subjected to 4 and 8 days water stress. Twenty µg of the protein was loaded as described. Protein gel was transferred onto nitrocellulose and hybridized with antibody raised against a purified cytosolic FBPase. Intensity of bands was scanned by densitometer and the peak area of the samples was calculated as 100% for control, 102% for 4 days stress and 15% for 8 days stress.

Two Dimensional SDS-PAGE Analysis

To investigate the possibility that posttranslational changes by covalent modification in the existing protein could occur under water stress, partially purified proteins from above conditions were separated accord-

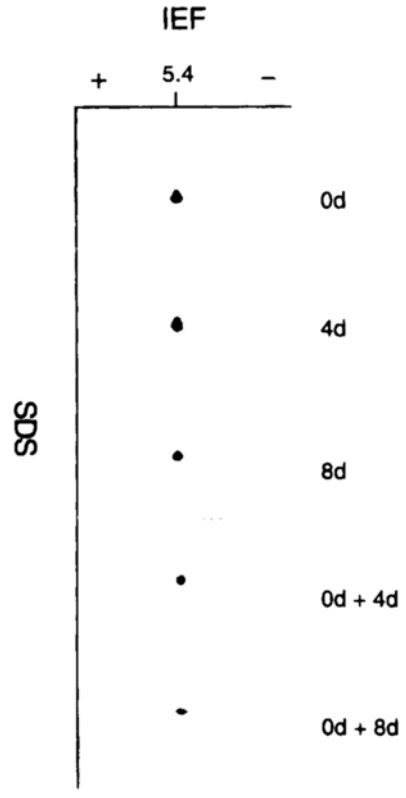

Fig. 3. Western blot of partially purified cytosolic FBPase from sugar beet leaves subjected to 4 and 8 days water stress. Partially purified protein extracts were loaded on two dimensional PAGE gels. Protein gels were transferred onto nitrocellulose membrane and hybridized with antibody raised against cytosolic FBPase of sugar beet leaves. Ten μg of protein from 0 d and 4 d was loaded; 40 μg from 8 d; 5 μg from each of 0 d and 4 d; 5 μg of 0 d mixed with 20 μg of 8 d. d: day.

ing to isoelectric point by isoelectric focusing in the first dimension, and according to molecular weight by SDS electrophoresis in the second dimension. The separated proteins on the two dimensional PAGE were transferred to nitrocellulose membrane and hybridized with antiserum raised against the cytosolic FBPase. Cytosolic FBPase proteins partially purified from water-stressed leaves of 0, 4 and 8 days stresses were used for the Western blots. Only one spot was detected on Western blots (Fig. 3). The isoelectric pH for the protein spot was obtained by cutting a tube gel to small pieces, measuring the pH value of the each gel piece and locating the pH value for the protein spot. All the protein spots correspond to 37 kDa at pH 5.4. The intensity of the protein spot at 0 day and 4 day is identical while the intensity of the 8 day is smaller than the one of 0 day although four times higher amount of proteins of 8 day was loaded. To make sure that only one polypeptide of 37 kDa is present at pH 5.4 under different stress conditions, the same amount of proteins from 0 day and 4 day, and from 0 day and 8 day was mixed and Western blot hybridizations were performed. The same spot was also found in the mixed samples indicating that the protein was not shifted on the 2D gel and no amino acid charge difference of the protein by covalent modification occurred.

DISCUSSION

Despite the substantial progress toward cloning the genes associated drought-tolerance in plants, little is known about molecular and biochemical regulation of the metabolic enzymes under a wide range of water deficit. Changes in enzyme activity under water stress can be reflected by three parameters: 1) levels of the enzyme amount; 2) modification of the enzyme; 3) metabolic effector molecules such as inhibitors or activators of the enzyme. Recently, cytosolic FBPase was found to be regulated by coarse control suggesting that the cytosolic FBPase of sugar beet leaves may be regulated by possibly posttranslational modification in mild stress as well as protein levels in severe water stress (Harn and Daie, 1992a). In this report here, in order to prove the posttranslational modification of the cytosolic FBPase under water stress, we focused on detecting any changes in amino acid charge difference of cytosolic FBPase by using two dimensional gel under two water stress levels, mild and severe (4 and 8 days, respectively). However, there was no apparent change in the isoelectric mobility of cytosolic FBPase protein under mild and severe drought-stress (Fig. 3).

Collectively, the regulation of cytosolic FBPase under water stress can be explicated at two levels of stress: mild and severe. Relatively stable transcript and protein coupled with the a decline in the activity at the mild stress (Table 1, Fig. 1 and 2) suggest that the regulation of the cytosolic FBPase is mostly at the biochemical levels, if any, rather than the protein levels. Due to shrinkage of the cell volume, increased concentration of solutes such as sucrose may inhibit the cytosolic FBPase activity as a feedback inhibition although it is speculative. During severe stress, however, the regulation of cytosolic FBPase appeared to involve drastic decline in the protein levels (Fig. 2). The cytosolic FBPase gene expression may not occur continuously during severe stress and this may override the biochemical regulation, if any.

Lack of water availability is a major environmental constraint that limits the crop productivity. It causes many changes in plant metabolism such as low photosynthetic rates decreasing carbon supply (Brunce, 1998; Vu *et al.*, 1987) and slow in growth and development (Manson *et al.*, 1988). Limited photoassimilates including sucrose produced in leaves may be utilized to support two mutually exclusive processes under water stress conditions: 1) assimilate export out of the leaf as an energy source; 2) osmotic adjustment within the leaf. In order to fulfill the both processes, the sucrose has to be synthesized. Indeed, water stress led a shift of carbon partitioning toward in favor of sucrose accumulation in sugar beet leaves by 30% and 300% of control at mild and severe stress, respectively (Harn and Daie, 1992a). Therefore, it is important to fully understand how drought-stressed plants regulate the rate of sucrose biosynthesis. Since the activity of cytosolic FBPase decreased during stress (Table 1), the sucrose synthesis rate would not be increased. Rather, it would be decreased based on the activity levels. Then, how was the sucrose accumulated under water-stressed sugar beet leaves? Three mechanisms on how sucrose levels in cytosol can be regulated during water stress: 1) continuous but low rate of sucrose synthesis for osmotic adjustment until 4 days stress where sugar beet leaves retain 74% of activity of the control (Table 1); 2) Inhibition of assimilate translocation in water-stressed leaves (Sung and Kriegh, 1979; Johnson and Moss, 1976; Quick *et al.*, 1992). In fact, the regulation may require decreased export because the sucrose not exported would contribute to osmotic adjustment in leaves, prolonging the survival of the plant; 3) inhibition of sucrose efflux by turgor loss at severe stress since the sucrose transport is known to be driven by turgor gradient (Geiger, 1975).

Other than the regulation of water stress, the cytosolic FBPase was regulated by light- dependent manner and the activity increased proportional to the light exposure time (Khayat *et al.*, 1993). In addition, the cytosolic FBPase was posttranslationally modified being inactive during a long-term dark incubation by possibly phosphorylating the enzyme (Harn and Daie, 1997). In case of SPS, it activated in spinach leaves (Quick *et al.*, 1989) and potato tubers (Reimholz *et al.*, 1994) under the hyperosmotic solutions of mannitol or sorbitol by dephosphorylation of the regulatory site. In this report, under mild and severe water stress, the protein structure of cytosolic FBPase seems to be not altered by covalent posttranslational modification. Therefore, the protein modification of cytosolic FBPase may occur depending on the different environmental conditions.

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