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Modification of Cytosolic Fructose-1,6-Bisphosphatase in Sugar Beet Leaves During Long-Term Dark Period

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Sugar beet plants (*Beta vulgaris L.*) were exposed to long-term incubation in light (34 h) and dark (78 h) and changes in enzyme activity, protein and transcripts levels of cytosolic fructose-1,6-bisphosphatase (FBPase) in leaves were measured. The enzyme activity in leaves exposed to 34 h light was three times higher than the activity in leaves subjected to 78 h dark condition. Surprisingly, the enzyme activity was still remained stable even in the long-term dark treatment although the activity is lower. The transcript levels of cytosolic FBPase in 78 h dark were expressed equivalent to 10% of the expression occurred in 34 h light. However, the protein levels remained unchanged in long-term incubation with either light or dark. The cytosolic FBPase protein was found to be modified during the long-term dark incubation. The isoelectric pH for the protein spot detected by western blot on the two dimensional PAGE is 5.4 in light sample while the pH value in dark sample is 6.0. Interestingly, the cytosolic FBPase in long-term dark treated leaves was activated 44% more by incubating in 50 mM mannose (sequestering agent of endogenous Pi) comparing to the activity with mannitol treatment as a control. Collectively, these data suggest that the cytosolic FBPase become less active by post-translational modification during a long-term dark period and this modification probably occurs due to the phosphorylation of the enzyme.

Key words: cytosolic FBPase, regulation, protein modification, sucrose, phosphorylation

Fructose-1,6-bisphosphatase (FBPase, EC 3.1.3.11) catalyses the hydrolysis of fructose-1,6-bisphosphate to yield fructose-6-phosphate and inorganic phosphate. This enzyme is present in a variety of tissues and micro-organisms and involved in one of key steps in the gluconeogenesis pathway. In plants, two isozymes have been found in two different locations: cytosol and chloroplast (Kelly *et al.*, 1982). Cytosolic FBPase is involved in sucrose biosynthesis whereas chloroplastic FBPase participates in reductive pentose-phosphate pathway. Both enzymes play key regulatory roles in their corresponding pathways but these two are different in allosteric control and kinetic properties (Daie, 1993 and references therein).

Light-induced transcription is one of the well known aspects of gene expression in photosynthetic-related genes of plants (Thompson, 1991; Deng, 1994). Expression of a gene encoding the chloroplastic FBPase has been known to be regulated by light in wheat and potato (Raines *et al.*, 1988; Lloyd *et al.*, 1991; Kossman *et al.*, 1992). However, the enzyme activity itself was regulated by light-modulated reduction of disulfide groups via the ferredoxin/thioredoxin system (Buchanan, 1980) which undergoes a modification of the enzyme. Therefore, there are two different regulations of the chloroplastic enzyme: one for transcription and the other for translation. Only a few have been equivocally reported about activation and inactivation of cytosolic FBPase activity under light/dark transition in the plant leaves. Foyer et al. (1982) showed no change in the *in vitro* activity of the cytosolic FBPase of dark treated spinach leaf protoplasts after illumination. On the other hand, chloroplastic FBPase activity and photosynthetic rate rapidly increased under the same conditions. Cheikh and Brenner (1992) also reported that there was no distinct diurnal change in the activity of cytosolic

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FBPase in soybean leaves of field grown plants. In contrast, cytosolic FBPase activity in leaves from a growth chamber grown plants manifested one peak during the first half of the light period indicating some kind of activation occurred during early light illumination. The first evidence of activation and inactivation of cytosolic FBPase activity by light and dark transition was revealed by Khayat *et al.* (1993). They reported that the activity of cytosolic FBPase was light-modulated during a normal diurnal cycle of 24 h. In addition, an interesting observation by Khayat *et al.* (1993) was that there was no change in the amount of cytosolic FBPase protein during diurnal cycle, suggesting a posttranslational modification.

The activity of cytosolic FBPase is subject to allosteric control by fructose-2,6-bisphosphate (F26BP) which is a potent inhibitor of cytosolic FBPase (Stitt, 1990). It is also inhibited by AMP (Herzog *et al.*, 1984; Stitt *et al.*, 1985). Despite the substantial progress toward understanding the metabolic regulation of cytosolic FBPase in higher plants, little is known about its molecular regulation. Here, we present an evidence that the cytosolic FBPase in sugar beet leaves is modified in long-term dark period and the modification is occurred possibly by phosphorylation of the protein.

MATERIALS AND METHODS

Plant Materials

Sugar beet plants (*Beta vulgaris L.*) were grown in a growth chamber under different conditions. For three months, they were kept under 12 h light period (500 $\mu E\ m^{-2}s^{-1}$) and 12 h dark period at 25°C, with a cycle of light-on at 6 AM and light-off at 6 PM. For the light samples, the plants were kept in light from 10 h after light-on to the next 24 h (total 34 h) while for the dark samples they were kept in dark from 6 h after light-off to the next 72 h (total 78 h). Fully expanded mature leaves of the same age from different plants were pooled, and three independent replications from each condition were used.

Cytosolic FBPase Activity

Cytosolic FBPase from sugar beet leaves were partially purified based on procedures previously published (Sharkey *et al.*, 1988; Harn and Daie, 1992a) with slight modification. Cytosolic FBPase was separated from the chloroplast isozyme by DEAE-sephadex A-50 (Pharmacia, Piscataway, NJ) ion-exchange batch chromatography. An aliquot (0.1 mL) of the enzyme extract was added to 1.0 mL of DEAE-Sephadex A-50 pre-equilibrated with elution buffer. The enzyme extract was allowed to equilibrate with the DEAE-Sephadex and sedimented for 3 min at 1,000×g in a microcentrifuge. The supernatant was saved and the enzyme activity of cytosolic FBPase from the supernatant 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). Twenty μg of total RNA (12 μL) was denatured with 25 μL formamide, 8 μL formaldehyde, and 5 μL of 10 X MOPS buffer (0.2 M MOPS-NaOH [pH 7.0], 0.05 M sodium acetate, and 0.01 M EDTA) incubated at 65°C for 10 min. RNA samples were loaded on 1% agarose gel containing I X MOPS buffer and 0.66 M formaldehyde. After electrophoresis at 80 V for 2 h, the gel was transferred to a nylon membrane in a blotting buffer (10 X SSC) using a Vacugene pump. 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% 2-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 partially purified proteins were precipitated in 100% ethanol, pelleted, dried and resuspended in lysis buffer (9.4 M urea, 2% NP-40, 5% 2-mercaptoethanol, 2% ampholines [3.5-10 and 5-7 pH range with 1:4 ratio]). The first dimension IEF was performed according to the method of O'Farrell *et al.* (1977). Tube gels (10 cm long and 1.5 mm diameter) containing 3.7% acrylamide, 0.21% bisacrylamide, 9.5 M urea, 2% NP-40, 5% 2-mercaptoethanol and 2% ampholines (3.5-10 and 5-7 pH range with 1:2 ratio) were pre-run for 15 min at 200 V, 30 min at 300 V and 30 min at 400 V. Equal protein amounts (10 μg) was loaded in cylindrical gels with an overlay solution (8 M urea, 5% NP-40, and 1% ampholines [3.5-10 and 5-7 pH range with 4:1 ratio]) and run for 16 h at 400 V followed by 2 h further at 800 V. The tube gels were equilibrated in sample buffer (10% glycerol, 2.3% SDS, 5% 2-mercaptoethanol, 0.0625 M Tris pH 6.8) for 40 min. In the second dimension, tube gels were run on 10% polyacrylamide slab gels (180 mm × 160 mm × 10 mm) for 4 h at 150 V. After electrophoresis, the proteins were transferred onto nitrocellulose membranes electroblotting for 4 h at 200 mA. Immunoblot was performed as described for western blot analysis.

Densitometry

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

RESULTS

Inactivation of Cytosolic FBPase in Dark Period

Cytosolic FBPase was partially purified from leaves sampled from two different time points, one with 34 h light period and the other with 78 h dark period. Fig. 1 showed that the cytosolic FBPase activity was

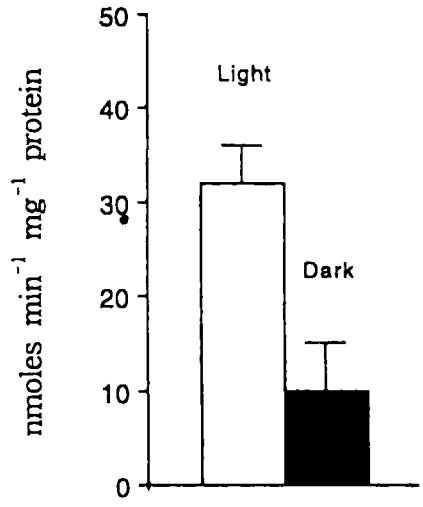

Fig. 1. Changes in the activity of partially purified cytosolic FBPase in sugar beet leaves exposured for 34 h light and 78 h dark. Data points are means of three replicates with SE.

10±5 nmoles min^{-1} mg^{-1} protein in 78 h dark period while the activity in 34 h light period was three times higher than the activity in dark. The magnitude of cytosolic FBPase detected in dark are authentic because the basal activity measured without the partially purified cytosolic FBPase is 2±2 nmoles min^{-1} mg^{-1} protein (data not shown). Therefore, the cytosolic FBPase activity is detectable even in a long-term dark duration although the activity is low.

Northern and Western Analysis

The steady-state mRNA levels of the cytosolic FBPase exposed to long-term light and dark periods were significantly different (Fig. 2A). The cytosolic FBPase gene was expressed highly during the light period showing a single band (1.3 kb) on the northern blot hybridized by ^{32}P-labelled cDNA encoding cytosolic FBPase of sugar beet. In contrast, the transcript levels during the dark period remained low, which is equivalent to 10% of the transcript levels occurred in the light period (Fig. 2B).

Cytosolic FBPase protein was partially purified, denatured, fractionated by SDS-PAGE and hybridized with an antibody raised against cytosolic FBPase protein of sugar beet. Interestingly, the cytosolic FBPase protein levels remained unchanged in light and even in long-term dark periods (Fig. 3A). By densitometric scanning, the band intensity of the dark sample is equivalent to 92% of the intensity of the light sample (Fig. 2B).

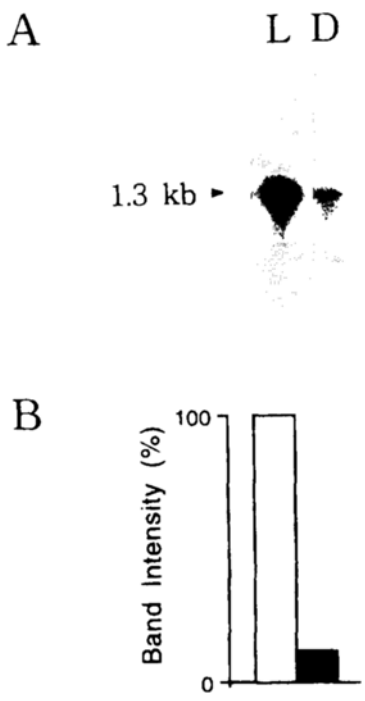

Fig. 2. A. Northern blot of the cytosolic FBPase in mature leaves subjected to 34 h light and 78 h dark conditions. Twenty μg of total RNA was loaded on 1% agarose gel, transferred onto nylon membrane and hybridized with ^{32}P-labelled sugar beet cytosolic FBPase cDNA. L: light, D: dark. B. Intensity of bands was scanned by densitometer and the peak area of the dark was calculated as 10% of the peak of the light.

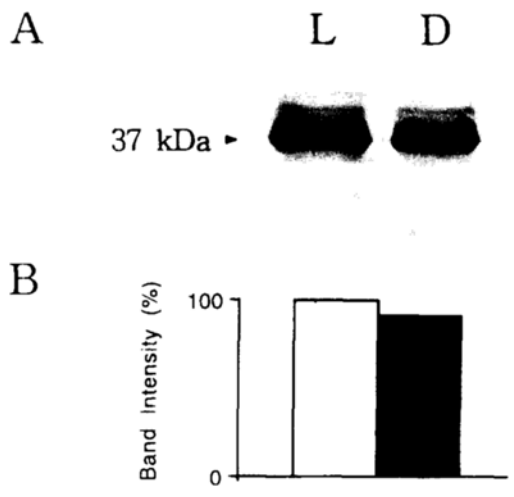

Fig. 3. A. Western blot of partially purified cytosolic FBPase from sugar beet leaves subjected to 34 h light and 78 h dark condition. Twenty μg of protein was loaded on a 12% acrylamide gel, transferred onto nitrocellulose and cross-reacted with antibody raised against cytosolic FBPase of sugar beet leaves. L: light, D: dark. B. Intensity of bands was scanned by densitometer and the peak area of the dark was calculated as 92% of the peak of the light.

Two Dimensional SDS PAGE Analysis

To investigate a possibility that posttranslational changes by covalent modification in the existing protein could occur under light and dark incubations, partially purified proteins from above conditions were separated according to isoelectric point by isoelectric focusing in the first dimension, and according to molecular weight by SDS electrophoresis in the second dimensions. The separated proteins on the two dimensional PAGE were transferred to nitrocellulose membrane and hybridized with antiserum raised against the cytosolic FBPase. Fig. 4 shows one major spot detected on Western blot. 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 corresponding to 37 kDa. The pH value of the protein spot in the light incubation is 5.4 while the value in the dark is about 6.0 at 37 kDa. Therefore, during a long-term dark condition, the iso-

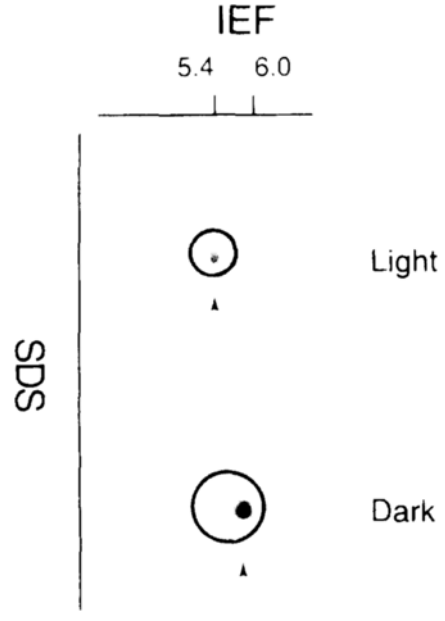

Fig. 4. Western blot of partially purified cytosolic FBPase from sugar beet leaves subjected to 34 h light and 78 h dark condition on two dimensional PAGE gels. Ten μg of the protein was loaded as described in Methods and Materials. Protein gel was transferred onto nitrocellulose and hybridized with antibody raised against cytosolic FBPase of sugar beet leaves. For the dark sample, the membrane was developed longer to find a trace amount of protein spot at pH 5.4.

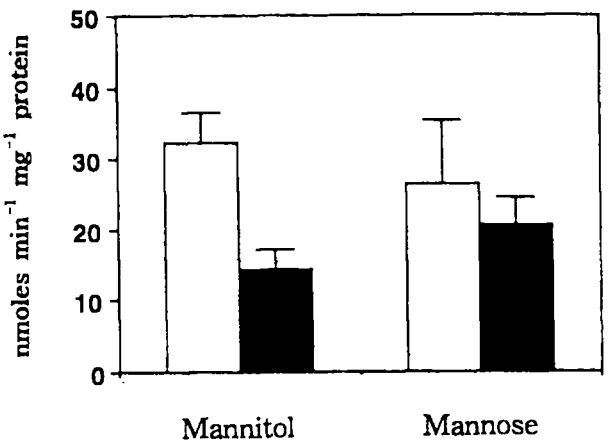

Fig. 5. The effect of 50 mM Mannose on the activity of the cytosolic FBPase in sugar beet leaves subjected to 34 h light (open bar) and 78 h dark (solid bar) condition. Data points are means of three replicates with SE.

electric mobility of cytosolic FBPase protein was shifted by possible modification of the protein.

Reactivation of Cytosolic FBPase Activity by Mannose

To examine a possible reversion of enzyme activity from a less active form to an active form in dark by sequestering endogenous Pi levels in cells, leaf discs (1cm in diameter) from light and dark exposed leaves were immersed in 50 mM mannose for 4 h in light and dark chamber, respectively. Interestingly, the cytosolic FBPase purified partially from the leaves of the long-term dark treatment was activated up to 44% higher by 50 mM mannose comparing to the activity with mannitol treatment (50 mM) as a control (Fig. 5). However, the activity of cytosolic FBPase from 34 h light treatment decreased about 18% by mannose. In addition, the enzyme activity of the cytosolic FBPase was not affected by mannitol as compared with the activity values shown in Fig. 1.

DISCUSSION

We have investigated a possible regulation of cytosolic FBPase under long-term light (34 h) and dark (78 h) treatments beyond a diurnal cycle. The activites of cytosolic FBPase are 32 ± 4 nmoles min^{-1} mg^{-1} protein and 10 ± 5 nmoles min^{-1} mg^{-1} protein in light and dark, respectively (Fig. 1). The activities obtained from long-term dark samples are similar to the data observed by Khayat *et al.* (1993) under a short-term dark period. It is surprising that the activity is still detectable in longer dark period although it is low. The activity present in dark is authentic value because without partially purified enzyme extract in the reaction the activity is close to zero (2 ± 2 nmoles min^{-1} mg^{-1} protein, data not shown). A possibility of inhibition by F26BP during the dark period can be ruled out because partial purification of the enzyme would have resulted in the removal of inhibitors. Like the enzyme activity, the transcript levels in 78 h dark period are also lower than the levels in 34 h light period. Therefore, levels of transcript and enzyme activity were correlated even in longer incubation of dark and light, which is a similar pattern with the data observed during a diurnal cycle (Khayat *et al.*, 1993).

Interestingly, the protein levels were not changed in long-term dark incubation (Fig. 3). This is a rather unusual finding considering that plants were kept such a long period in dark. However, a similar pattern was also reported with a diurnal cycle (Khayat *et al.*, 1993), which is a rather short-term incubation. Therefore, the cytosolic FBPase seems to be a stable protein *in vivo*.

The cytosolic FBPase was found to be posttranslationally modified in long-term dark. The mobility of the enzyme is shifted on the two dimensional gel (Fig. 4). Therefore, the protein modification of the cytosolic FBPase is an explanation for the discrepant levels between activity and protein. This is the first observation that the cytosolic FBPase is actually modified in plants. Although the exact nature of modification of the cytosolic enzyme is unclear, it is not like a case of chloroplastic FBPase whose activation by light occurs via the ferredoxin/thioredoxin system (Buchanan, 1980). This mechanism is lacking for the cytosolic enzyme because a unique sequence responsible for the light-dependent regulation of the chloroplastic isozyme is absent from the cytosolic FBPase gene (Harn and Daie, 1992b). In addition to posttranslational modification, a possibility of posttranscriptional regulation can not be ruled out due to the different levels of transcript and protein.

Several metabolic enzymes such as phosphoenolpyruvate carboxylase, nitrate reductase and sucrose phosphate synthase (PEPC, NR and SPS) are well known enzymes that are reversibly activated by phosphorylation and dephosphorylation, a very common form of covalent modification for many regulatory enzymes (Huber *et al.*, 1987; Walker and Huber, 1989; Stitt *et al.*, 1988; Jiao *et al.*, 1991; Huber *et al.*, 1994). NR and SPS are activated in light by dephosphorylation and inactivated in dark by phosphorylation.

However, light activation requires the process of photosynthesis, not just the presence of light. This was further substantiated by the observation that treatment of spinach leaf discs with mannose, a phosphate sequestering agent (Herold *et al.*, 1976), activated SPS in darkness (Stitt *et al.*, 1988; Huber *et al.*, 1989). The cytosolic FBPase isolated from leaf discs fed by mannose was also activated 44% more than the activity of control, suggesting that the internal Pi levels was sequestered allowing the enzyme to be less phosphorylated. Therefore, the molecular regulation of cytosolic FBPase of sugar beet leaves under long-term dark may occur by phosphorylating the enzyme being inactive while the enzyme may be activated by dephosphorylation upon light. It is, however, hard to understand why the mannose treatment of the light samples caused 18% lower activity than the activity with control.

A posttranslational modification of phosphorylation/dephosphorylation has been reported for the yeast FBPase (Mazon *et al.*, 1982). In yeast, phosphorylation of the FBPase was accompanied by a decrease in the enzymatic activity and FBPase became activated when the enzyme released the phosphate. However, it should be noted that phosphorylation/dephosphorylation is not universal for all FBPases. Rabbit liver and pig kidney FBPases are not phosphorylated (Meek and Nimmo, 1984). In addition, light activation of SPS is usually complete within 5 to 15 min, and dark inactivation requires about 20 to 30 min to reach completion. However, cytosolic FBPase was not completely activated within 30 min (Khayat *et al.*, 1993). Rather, the activity increased as the exposure time in the light increased. Since the requirement time for modification of cytosolic FBPase in dark duration is not known, further studies such as a precise time of modification under light/dark modulation as well as a mechanism of molecular regulation of the presumed phosphorylation and dephosphorylation of cytosolic FBPase are yet to be determined.

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