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# *In Vivo* Phosphorylation of *Arabidopsis thaliana* 6-phosphofructo-2-kinase/fructose-2,6-bisphosphate 2-phosphatase

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### Introduction

Cellular levels of F2,6BP are regulated by synthesis and degradation, both of which are catalyzed by a bifunctional enzyme, 6-phosphofructo-2-kinase (EC 2.7.1.105; PFK 2)/fructose-2,6-bisphosphate 2-phosphatase (EC 3.1.3.46; FBPase 2). In mammals, there are several PFK 2/FBPase 2 isozymes, which are regulated organ-specifically by protein phosphorylation in their N-terminal and/or C-terminal short extension sequences (Pilkis et al. 1995). In plants, regulation of PFK 2/FBPase 2 by several metabolites has been reported (Stitt 1990a). However, conflicting results have been obtained in the last couple of decades as to the occurrence of phosphorylated PFK 2/FBPase 2. Early studies by Walker and Huber (1987a, 1987b) implicated a possible involvement of protein phosphorylation in the regulation of PFK 2/FBPase 2. These authors demonstrated that *in vitro* Mg-ATP treatment affected chromatographic behavior as well as the relative activity of partially purified spinach PFK 2/FBPase 2. On the other hand, protein kinase A treatment, which is known to promote phosphorylation of PFK 2/FBPase 2 in animal cells (Pilkis et al. 1995), had no influence on spinach PFK 2/FBPase 2 activities *in vitro* (Cseke and Buchanan 1983). Furthermore, a phosphorylated protein intermediate is known to occur in the FBPase 2 reaction of PFK 2/FBPase 2 (Pilkis et al. 1995). Accordingly, no direct evidence for the phosphorylation of PFK 2/FBPase 2 has been obtained to date. In the present study, we address the question of whether protein phosphorylation of PFK 2/FBPase 2 occur in plant cells.

### Materials and methods

Seeds of *Arabidopsis thaliana* (ecotype Columbia) were grown for 19 to 22 d at 23°C under conditions of 16-h light and 8-h darkness, using a photon flux density of 80 μmol $m^{-2}$ $s^{-1}$ during the light period. Nineteen-day-old *Arabidopsis* plants were individually fed four times with 500 μl each of 10 μM [$^{32}$P]Pi (18.5 MBq; Amersham Pharmacia) at 09:00 hours and 21:00 hours on the first and second days of labeling. Labeled plants were homogenized at 07:00 hours on the third day of labeling, and the crude protein extracts were incubated with purified specific antibody at room temperature for 1 h. The resulting immunoconjugates were precipitated with Protein A Sepharose (Amersham Pharmacia) and samples were processed for immunoblot analysis. The distribution of the radioactivity on the membrane was measured with a Bio-Imaging Analyzer (BAS2000, Fuji Photo Film, Tokyo, Japan).

## Results

Immunoblotting with antisera specific to *Arabidopsis* PFK 2/FBPase 2 detected 96-kDa and 92-kDa bands in the crude protein extracts from rosette leaves of *Arabidopsis*. Incubation of protein samples with alkaline phosphatase before SDS-PAGE erased the 96-kDa band with concomitant increase of the 92-kDa band, suggesting that the former is a phosphorylated form of the latter. Therefore, PFK 2/FBPase 2 proteins were radio-labeled in [$^{32}$P]Pi-fed plants and then precipitated as immunoconjugates with purified specific antibodies. Immunoblot analysis verified that both 96-kDa and 92-kDa bands were successfully precipitated in the immunoconjugates (Fig. 1, lane 1), and the radioactivity on the immunoblot membrane showed that the 96-kDa protein was strongly radio-labeled, whereas the 92-kDa protein was less radio-labeled (Fig. 1, lane 2).

To exclude the possibility that the 96-kDa band represented a phosphorylated catalytic intermediate of PFK 2/FBPase 2 in the FBPase 2 reaction, the phospho-amino acids derived from the [$^{32}$P]-labeled 96-kDa band were determined. The results showed that phospho-serine and phospho-threonine were labeled to a major extent and to a minor extent, respectively (data not shown). These data suggest that *Arabidopsis* PFK 2/FBPase 2 is phosphorylated *in vivo* by protein kinases.

We further examined the phosphorylation status of PFK 2/FBPase 2 in rosettes of *Arabidopsis* under different physiological conditions. As shown in Fig. 2A, the 92-kDa and 96-kDa bands in the extracts from whole rosettes reached their maximal levels at 15:00 and 23:00 hours, respectively, and the relative ratio of 96-kDa and 92-kDa bands in whole rosette extracts became largest at 23:00 hours. Using rosette leaves harvested at 15:00 hours, we further compared the phosphorylation status of PFK 2/FBPase 2 in different rosette leaves (Fig. 2B). The crude protein extracts were collected according to leaf age, *i.e.,* 1st–2nd leaves, 3rd–4th leaves, 5th–7th leaves and leaves younger than 8th leaves. The 96-kDa band was enriched in leaves younger than the 8th leaves (lane 5), in which the level of the 96-kDa band was 2.5-folds higher that that of 92-kDa band. By contrast, both 96-kDa and 92-kDa bands occurred at comparable levels in leaves older than 4th leaves (lanes 2 and 3), and the 96-kDa band became slightly enriched in 5th–7th leaves.

## Discussion

Our evidence showed that *Arabidopsis* PFK 2/FBPase is phosphorylated *in vivo* by protein kinases. We detected 96-kDa and 92-kDa bands as heavily-phosphorylated and less-phosphorylated forms of *Arabidopsis* PFK 2/FBPase, respectively. The relative levels of 96-kDa and 92-kDa bands in whole rosette leaves were not constant throughout the daytime, and the ratio of the 96-kDa band over the 92-kDa band was highest at the beginning to nighttime and also in very young rosette leaves. These results suggest that the phosphorylation status is regulated physiologically and developmentally. As described above conflicting results were obtained as to occurrence of the phosphorylated protein in the past decades. Because the phosphorylation status could change under different physiological conditions, variation in other reports may reflect differences of their experimental conditions. Physiological consequences of the phosphorylation of PFK 2/FBPase 2, such as the effects of phosphorylation on PFK 2 and FBPase 2 activities of the bifunctional enzyme, cellular F2,6BP levels and on cellular carbon metabolism, remain to be clarified in future research.

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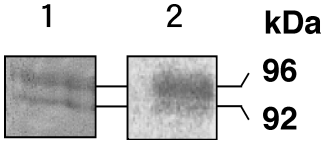


**Fig. 1** *In vivo* phosphorylation of PFK 2/FBPase 2. Immunoblot analysis (lane 1) and distribution of the radioactivity on the membrane (lane 2).

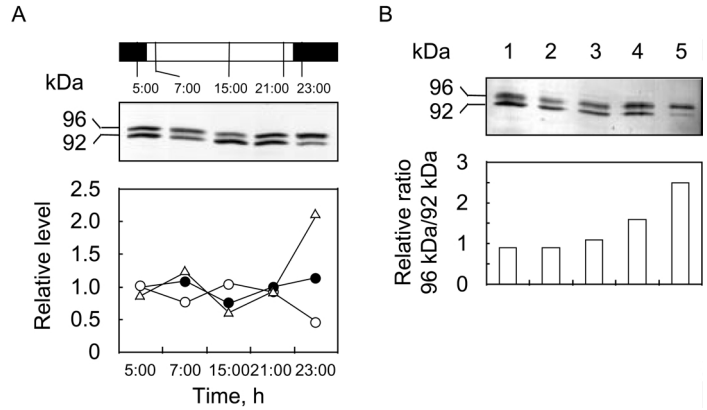


**Fig. 2** The phosphorylation status of PFK 2/FBPase 2 in *Arabidopsis*. A, Black and white bars above the sampling time represent nighttime and daytime, respectively. A graph shows relative levels of 96-kDa (●) and 92-kDa (○) bands and the ratio of the 96-kDa band over the 92-kDa band (□). B, Crude protein extracts (50 μg) were prepared at 15:00 hours according to the leaf age: lane 1, whole rosette leaves; lane 2, 1st–2nd leaves; lane 3, 3rd–4th leaves; lane 4, 5th–7th leaves; and lane 5, leaves younger than 8th leaves. A graph shows the ratio of the 96-kDa band over the 92-kDa band.