

Transgenic Arabidopsis Plants with Decreased Activity of Fructose-6-Phosphate,2-Kinase/Fru-2,6-Bisphosphatase Have Altered Carbon Partitioning¹

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The role of fructose-2,6-bisphosphate (Fru-2,6-P₂) as a regulatory metabolite in photosynthetic carbohydrate metabolism was studied in transgenic Arabidopsis plants with reduced activity of Fru-6-phosphate,2-kinase/Fru-2,6-bisphosphatase. A positive correlation was observed between the Fru-6-phosphate,2-kinase activity and the level of Fru-2,6-P₂ in the leaves. The partitioning of carbon was studied by ¹⁴CO₂ labeling of photosynthetic products. Plant lines with Fru-2,6-P₂ levels down to 5% of the levels observed in wild-type (WT) plants had significantly altered partitioning of carbon between sucrose (Suc) versus starch. The ratio of ¹⁴C incorporated into Suc and starch increased 2- to 3-fold in the plants with low levels of Fru-2,6-P₂ compared with WT. Transgenic plant lines with intermediate levels of Fru-2,6-P₂ compared with WT had a Suc-to-starch labeling ratio similar to the WT. Levels of sugars, starch, and phosphorylated intermediates in leaves were followed during the diurnal cycle. Plants with low levels of Fru-2,6-P₂ in leaves had high levels of Suc, glucose, and Fru and low levels of triose phosphates and glucose-1-P during the light period compared with WT. During the dark period these differences were eliminated. Our data provide direct evidence that Fru-2,6-P₂ affects photosynthetic carbon partitioning in Arabidopsis. Opposed to this, Fru-2,6-P₂ does not contribute significantly to regulation of metabolite levels in darkness.

In all eukaryotes Fru-2,6-bisphosphate (Fru-2,6-P₂) is a key regulator of carbohydrate metabolism. In plants, the knowledge about this regulatory molecule is based primarily on detailed biochemical studies of leaf metabolism and on in vitro characterization of the regulatory enzymes involved. According to our current understanding, Fru-2,6-P₂ plays an important role in the partitioning of fixed carbon between Suc and starch during photosynthesis. The concentration of Fru-2,6-P₂ in plants is determined by the relative activities of Fru-6-phosphate,2-kinase (F6P,2-K, EC 2.7.1.105) and Fru-2,6-bisphosphatase (F26BPase, EC 3.1.3.46). Kinetic characterization of partially purified Fru-6-phosphate,2-kinase/Fru-2,6-bisphosphatase (F2KP) from spinach showed that both activities are allosterically regulated by metabolites (Cséke and Buchanan, 1983; Cséke et al., 1983; Stitt et al., 1984; Larondelle et al., 1986). The F6P,2-K is activated by Fru-6-P and inorganic phosphate (P_i) and is inhibited by 3-phosphoglyceric acid and dihydroxyacetone phosphate (DHAP) whereas the F26BPase is inhibited by Fru-6-P and P_i. In plants Fru-2,6-P₂ operates as an allosteric regulator of two enzyme activities, cytosolic Fru-1,6-bisphosphatase (cyt-FBPase), which forms Fru-6-P, and pyrophosphate:Fru-6-phosphate, 1-phosphotransferase (PFPP), which catalyzes the reversible

interconversion between Fru-1,6-P₂ and Fru-6-P. The function of Fru-2,6-P₂ in photosynthetic carbon metabolism is primarily ascribed to inhibition of cyt-FBPase. Through this inhibition, Fru-2,6-P₂ contributes to regulation of the partitioning of fixed carbon between starch and Suc (Stitt, 1990). The physiological significance of Fru-2,6-P₂ as an activator of PFPP, and as a regulator of metabolism in sink tissue is still poorly understood (Stitt, 1998).

The effect of elevated concentration of Fru-2,6-P₂ on carbon metabolism has recently been studied in transgenic lines of tobacco and *Kalanchoë daigremontiana* expressing a modified mammalian gene encoding a F6P,2-K (Scott et al., 1995; Truesdale et al., 1999). The increased amount of Fru-2,6-P₂ (200%–350% of wild type [WT]) inhibited Suc synthesis and stimulated the accumulation of starch in tobacco and *K. daigremontiana*. In contrast, the expression in tobacco of the same mammalian gene, modified to encode only an active F26BPase, resulted in a reduction of the Fru-2,6-P₂ level down to 58% of WT (Kruger and Scott, 1995). In the beginning of the photoperiod these tobacco plants accumulated Suc more rapidly than WT plants, and the rate of starch synthesis was lower. These studies have been valuable in confirming that Fru-2,6-P₂ operates as a regulator in vivo. A complicating factor in these experiments is the presence of the native plant enzyme that, due to its regulatory properties, may operate to counteract the effect of the introduced mammalian activities.

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cDNA clones encoding F2KP have recently been isolated from plants, and for the Arabidopsis and potato cDNA clones, the F6P,2-K and F26BPase activities have been verified by *Escherichia coli* expression (Draborg et al., 1999; Villadsen et al., 2000). The molecular mass of F2KP from Arabidopsis, spinach, maize, and potato is 93 to 96 kD (Villadsen et al., 2000) and the enzyme comprises a catalytic COOH-terminal region and an NH₂-terminal region, which is unique for the plant F2KP proteins. The catalytic COOH-terminus of the plant enzyme is homologous to the mammalian F6P,2-K/F26BPases, consisting of two separate domains catalyzing the F6P,2-K and the F26BPase activity, respectively. The available cDNA clones allow for antisense and cosuppression techniques as an alternative approach to obtain plants with low levels of Fru-2,6-P₂.

In this study, transgenic Arabidopsis plants with reduced F2KP activity were produced by an antisense approach. The transgenic plant lines had strongly reduced levels of Fru-2,6-P₂ and changed carbon partitioning, which provide evidence that Fru-2,6-P₂ is a primary regulator of photosynthetic carbon metabolism.

RESULTS

Generation of Transgenic Arabidopsis Lines with Differing Levels of F2KP

To analyze the role of F2KP in carbon partitioning, transgenic plants with decreased F2KP were produced. A 930-bp fragment of the *AtF2KP* gene, encoding a part of the catalytic COOH-terminal region of the enzyme was, under the control of the cauliflower mosaic virus 35S promoter, introduced in antisense orientation into Arabidopsis. A total of 48 individual T₁ progeny lines were isolated and analyzed by measuring their F6P,2-K activity. Ten plant lines with F6P,2-K activities covering a range of 5% to 100% of WT were selected and homozygous plant lines were obtained by self-pollination. The homozygous plant lines were analyzed for F6P,2-K activity and transgene copy number. The plant lines contained one to six copies of the *AtF2KP* antisense construct in their genome. The copy number of *AtF2KP* of independent plant lines did not correlate to their respective levels of F6P,2-K activity (data not shown). The plants with strong reduction in F2KP had a similar size to WT plants and showed minor phenotypic differences, having more narrow leaves with a slightly darker coloration.

The reduced amount of F2KP protein in the Arabidopsis plants was detectable by immunoblotting (Fig. 1). F2KP could not be detected in the AS1 plant line, whereas an intermediate protein level was found in AS16 compared with WT level. The levels of F2KP protein detected by immunoblotting correlates to the enzyme activities determined for AS1, AS16, and WT (Figs. 2 and 3). Plant lines with severely

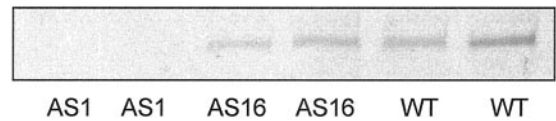


Figure 1. Immunoblot analysis of antisense F2KP and WT Arabidopsis plants. Transgenic plants lines with decreased F6P,2-K/F26BPase activity were analyzed by western blot and the 93-kD F2KP protein was detected in the plant extracts using polyclonal antibodies against the AtF2KP-phosphatase region. Each lane contained 6.5 μ g of protein.

reduced F6P,2-K activity had a low level of Fru-2,6-P₂ and plant lines with a partially reduced F6P,2-K activity had an intermediate level of Fru-2,6-P₂ (Fig. 2). In general, there was a good correlation between the F6P,2-K activity and the level of Fru-2,6-P₂ in the different plants. Only for plant line AS13 did some plants deviate from this correlation (Fig. 2). Reduced F6P,2-K activity was verified for all plant material used in later radiolabeling experiments (Figs. 3 and 4). Furthermore, the F26BPase activity of the plant lines AS1, AS13, and AS16 was between 5% and 80% of WT activity, in accordance with the level of F6P,2-K activity in the same lines (Fig. 3, A and B). The mono-functional F26BPase activity (MacDonald et al., 1989) could not be detected in any of the Arabidopsis plant lines, including the WT (data not shown). The mono-functional F26BPase activity was detectable in a control experiment with spinach leaves.

The activities of PFP, Fru-6-phosphate,1-kinase (PFK), and Suc-phosphate synthase (SPS) were analyzed in leaves of WT plants and in transformants with low and intermediate levels of Fru-2,6-P₂ (plant line AS1 and AS13, respectively). There were no significant differences in enzyme activities and SPS activation state between the plant lines (data not shown).

The Metabolism of ¹⁴CO₂

To show that Fru-2,6-P₂ in vivo operates as a regulator of assimilated carbon partitioning, plants were pulse labeled with ¹⁴CO₂. The experiments (Fig. 3D) were carried out during the last hour of the photoperiod and plants were harvested immediately after a 10-min ¹⁴CO₂-labeling period. The total incorporation of ¹⁴C was not significantly different between WT and transgenes. The distribution of ¹⁴C in the antisense plant lines AS1 and AS2, which have very low activities of F6P,2-K and F26BPase and low levels of Fru-2,6-P₂ (Fig. 3, A–C), show that carbon partitioning was changed in favor of synthesis of soluble sugars as compared with the WT (Fig. 3D). Separation of the neutral sugars by thin-layer chromatography showed that ¹⁴C was mainly incorporated into Suc, and the Suc to starch labeling-ratio was 3-fold higher in AS1 compared with WT. The majority of ¹⁴C was incorporated into Suc and starch, and less

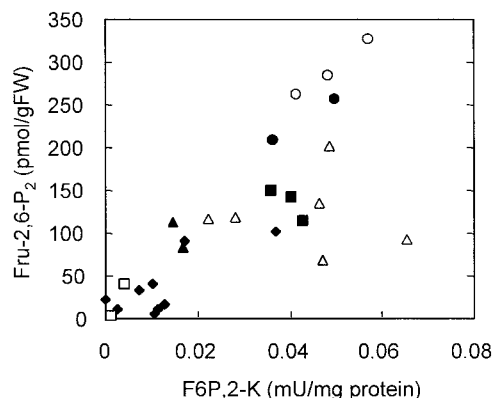


Figure 2. Relationship between F6P,2-K activity and the Fru-2,6-P₂ levels. The specific F6P,2-K activities are plotted against the Fru-2,6-P₂ levels in seven independent plant lines. Each point represents one plant. The plant lines are AS1 (◆), AS2 (□), AS6 (▲), AS13 (△), AS16 (■), control (●), and WT (○). Control is a plant line only transformed with an empty vector construct. All samples were harvested during the last hour of the photoperiod.

into other compounds. Incorporation of ¹⁴C for AS1 and AS2 was 47% ± 1% in soluble sugars, 38% ± 3% in starch, and 12% ± 4% in other compounds. The distribution of ¹⁴C in WT was 36% ± 2% in soluble sugars, 48% ± 2% in starch, and 16% ± 0.5% in other compounds. The plant lines AS13 and AS16 with intermediate levels of F2KP and Fru-2,6-P₂ did not change their sugars-to-starch labeling ratio compared with WT (Fig. 3D). Labeling experiments with a 10- or 30-min ¹⁴CO₂ pulse followed by a 10-min chase period gave similar results (data not shown).

The photosynthetic carbon partitioning may change during the photoperiod. Therefore, the distribution of carbon was studied at different time points at the start of the light period for plant line AS1 and WT. The plants were labeled for 10 min at the onset of the photoperiod or after 10 or 60 min of the light period (Fig. 4). For all three time points tested, more ¹⁴C was found in soluble sugars and less in starch in the antisense line than in WT plants. In the beginning of the photoperiod there was a higher incorporation of ¹⁴C into sugars than later in the photoperiod (Figs. 3 and 4).

Carbohydrates and Phosphorylated Metabolites

To investigate the carbon metabolism in more detail, the level of starch, sugars, and phosphorylated metabolites in the leaves were studied throughout the diurnal cycle. Upon illumination, the amount of Fru-2,6-P₂ in the leaves of WT decreased dramatically during the first 15 min. At the middle of the light period the plants had returned to about the same level of Fru-2,6-P₂ as in darkness. In AS1 leaves the Fru-2,6-P₂ level remained low throughout the diurnal period (Fig. 5A). At the start of the light period the level of Suc increased in leaves of WT and AS1 plants. However, the AS1 plants accumulated Suc

over a longer period and reached significantly higher levels than the WT (Fig. 5B). In general, Glc and Fru followed the same diurnal pattern as Suc, and the AS1 plants had higher levels of hexoses than the WT plants during the light period. These differences were eliminated during the dark period (Fig. 5, C and D). The amount of starch was always slightly lower (*t* test, *P* < 0.05) in the AS1 than in WT leaves (Fig. 5E). Starch accumulation was delayed in the AS1 plants compared with the WT plants, especially during the first hours of the light period. The levels of Fru-6-P and Glc-6-P in AS1 and WT leaves followed the same pattern throughout the diurnal period with slightly

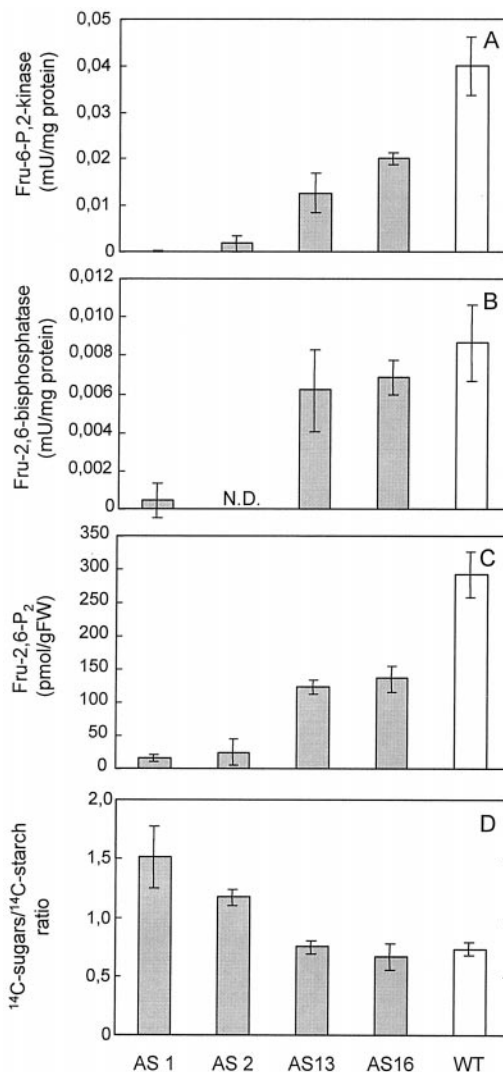


Figure 3. F6P,2-K and F26BPase activities, Fru-2,6-P₂ levels, and sugars-to-starch ¹⁴C-labeling ratio in independent plant lines. Samples were collected and analyzed for activity of F6P,2-K (A), F26BPase (B), and levels of Fru-2,6-P₂ (C). Similar plants were radiolabeled with ¹⁴CO₂ for 10 min and the ratio between ¹⁴C incorporated into sugars and starch was determined (D). Each value represents the means ± SD of four different plants. ND, Not determined. All samples were harvested and radiolabeling was performed during the last hour of the photoperiod.

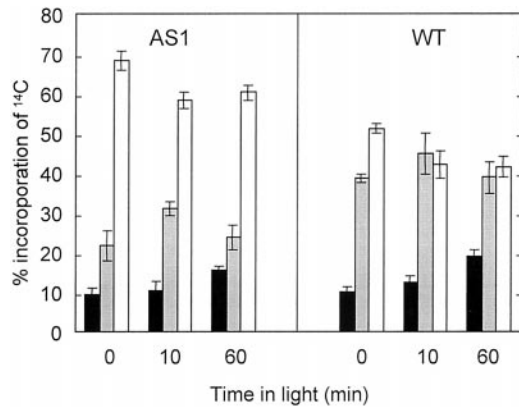


Figure 4. Photosynthetic carbon partitioning measured as ¹⁴C incorporation in WT and antisense plants. Plants with low levels of Fru-2,6-P₂ (AS1) and WT plants were photosynthetically labeled with ¹⁴CO₂ for 10 min from the beginning of the photoperiod (0) and after 10 min (10) and 60 min (60) of illumination. Each column represents the means ± SD of three individual plants. Black columns represent the sum of radioactivity incorporated into lipids, cationic, anionic, and insoluble compounds (excluding starch). Gray columns represent starch. White columns represent neutral compounds, mainly Suc.

lower levels of both hexose phosphates for the AS1 plants during darkness (Fig. 6, A and B). In contrast, the Glc-1-P content was lower in the AS1 leaves than in WT during the light period (Fig. 6C). During the dark period, the DHAP and glyceraldehyde-3-phosphate (GAP) contents were similar and low in WT and AS1 (Fig. 6, D and E). An accumulation of triose phosphates over the light period was observed for both plant lines. However, this accumulation was more rapid in WT and at the end of the light period, the WT had reached a significantly higher level of triose phosphates than AS1.

Photosynthetic Capacity of Plants with Low Levels of Fru-2,6-P₂

The photosynthetic oxygen evolution was determined at different light intensities (Fig. 7). Plants with low Fru-2,6-P₂ content had increased photosynthetic rates compared with WT at light intensities between 100 and 150 μmol m⁻² s⁻¹ photons. At light intensities above 240 μmol m⁻² s⁻¹ this difference was negligible.

DISCUSSION

The introduction into Arabidopsis of a part of the *F2KP* gene in antisense orientation resulted in plant lines with up to 95% reduction of the F6P₂-K activity compared with WT. F2KP is bifunctional (Villadsen et al., 2000) and, as expected, the F26BPase activity was also reduced (Fig. 3). Previous studies have shown the existence of a mono-functional F26BPase in spinach (Larondelle et al., 1986; MacDonald et al., 1989). Using the assay specifically designed to measure the mono-functional F26BPase in spinach (Mac-

Donald et al., 1989) we could not detect any activity in WT or in the transformants, suggesting that there is no mono-functional F26BPase in Arabidopsis leaves.

A positive correlation between the F6P₂-K activity and the level of Fru-2,6-P₂ was observed (Fig. 2). Plant lines with partially reduced F6P₂-K activity also showed intermediate levels of Fru-2,6-P₂. This is

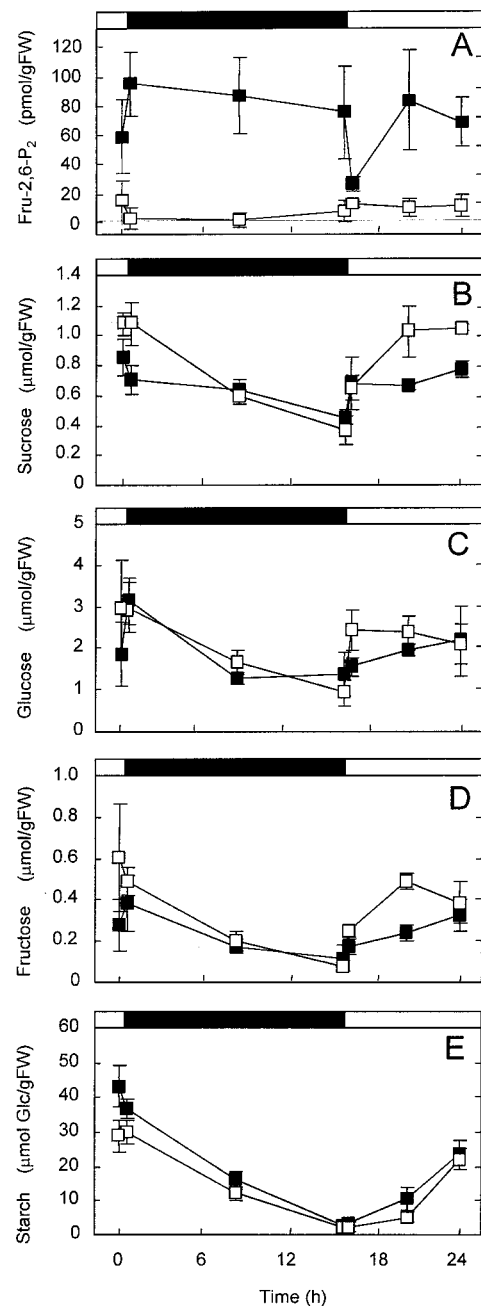


Figure 5. The level of Fru-2,6-P₂, sugars, and starch in WT Arabidopsis and antisense plants with low Fru-2,6-P₂ (AS1) during the diurnal cycle. Each point represents the means ± SD of four different plants. The bars at the top of the graphs indicate light (white bars) or dark (black bars). A, Fru-2,6-P₂; B, Suc; C, Glc; D, Fru; E, starch. White symbols, AS1; black symbols, WT.

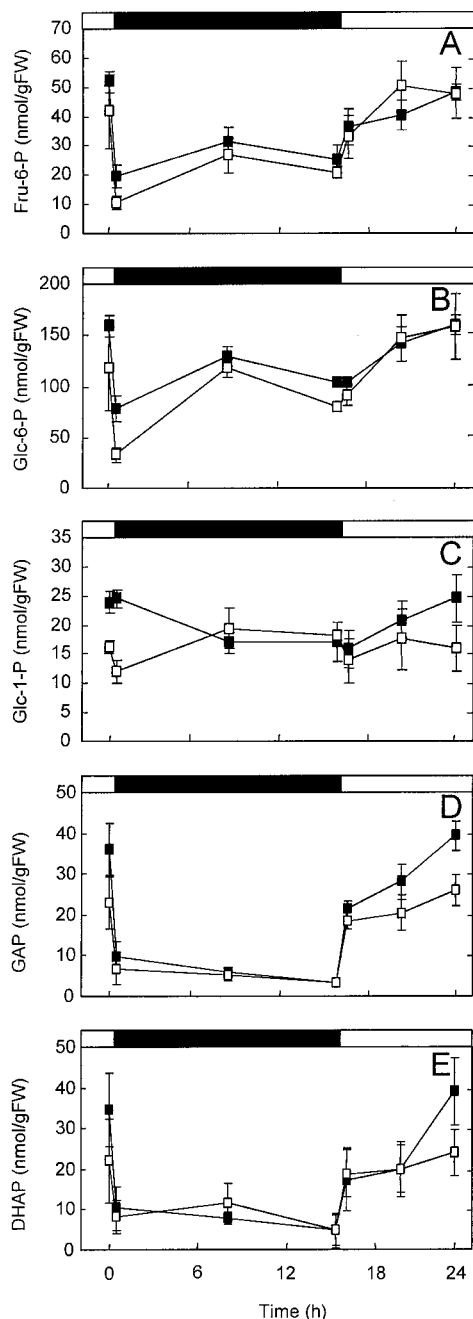


Figure 6. The hexose-phosphate and triose-phosphate content of WT plants and transgenic plants with low Fru-2,6-P₂. Each point represents the means \pm SD of four different plants. A, Fru-6-P; B, Glc-6-P; C, Glc-1-P; D, GAP; E, DHAP. White symbols, AS1; black symbols, WT.

notable for two reasons. First, a partial reduction of F6P₂-K and F26BPase activity might not result in a decrease of Fru-2,6-P₂ levels since, in theory, it is the balance between the two activities that determines the level of Fru-2,6-P₂. Second, F6P₂-K and F26BPase are allosterically regulated, and this could allow for a compensation for a reduced F2KP protein level. Thus, it might be expected that plants with intermediate F2KP levels would maintain the WT levels of

Fru-2,6-P₂. Our data show this not to be the case. The results could also be explained by the presence of a non-repressed mono-functional F26BPase. However, we found no indication of the monofunctional enzyme. It is clear that we are still missing information to fully understand how the concentration of Fru-2,6-P₂ in plant cells is regulated. It is possible that the system operates close to its maximal capacity and therefore cannot compensate for the introduced changes.

In AS1 plants having only 5% to 10% of WT level of Fru-2,6-P₂, ¹⁴C partitioning in favor of Suc formation was increased (Figs. 3E and 4). A 50% reduction of Fru-2,6-P₂ had little effect on the partitioning of photosynthetically fixed carbon. This suggests that the regulatory response of cyt-FBPase to Fru-2,6-P₂ is not linear within the range of Fru-2,6-P₂ observed in WT plants. A similar conclusion was reached by Stitt et al. (1987) based on extensive measurements of metabolite levels in subcellular compartments of spinach leaf cells and by modeling the rate of Suc synthesis as a response to changes in DHAP. The model predicted that Suc synthesis is only activated when a threshold level of DHAP is exceeded. Fru-2,6-P₂ is a key coordinating signal in this non-linear response of cyt-FBPase. Our data show that removal of Fru-2,6-P₂ changes the partitioning of carbon, implying that the WT levels of Fru-2,6-P₂ inhibits Suc synthesis. Therefore, we conclude that in the WT plants Fru-2,6-P₂ controls carbon flux by inhibiting cyt-FBPase. Only when the level of Fru-2,6-P₂ falls dramatically, as it does during the period immediately after dark to light transition, will the inhibition of cyt-FBPase be released. This is supported by our sugar measurements, which show a marked increase in levels of Suc and hexoses during the early light period.

The role of Fru-2,6-P₂ in carbohydrate metabolism has previously been studied by a different transgenic approach in tobacco and *K. daigremontiana* plants. In

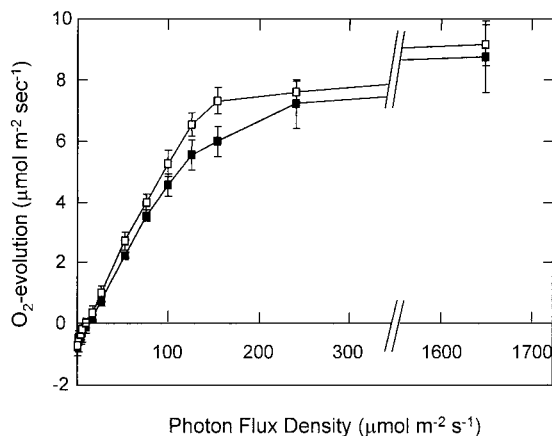


Figure 7. Light response curve for oxygen evolution in leaves of low Fru-2,6-P₂ plants (AS1) and for WT plants. Each point represents the means \pm SD of five independent plants, with three independent determinations of each. White symbols, AS1; black symbols, WT.

these plants the levels of Fru-2,6-P₂ were manipulated by the expression of modified versions of rat liver F6P₂-K/F26BPase, which then coexist with the native plant enzyme (Kruger and Scott, 1995; Scott et al., 1995; Truesdale et al., 1999). A decreased level of Fru-2,6-P₂ in tobacco resulted in a faster accumulation of Suc and a lower rate of starch synthesis compared with WT at the beginning of the photoperiod. This is similar to the effect on the partitioning observed for the AS1 and AS2 Arabidopsis lines in this study. However, when the tobacco plants are compared with the lines AS13 and AS16, which have a similar reduction of Fru-2,6-P₂ levels to the most extreme tobacco lines, we see no effect on metabolism in the Arabidopsis plants. This may reflect that the metabolic sensitivity to Fru-2,6-P₂ is different in the two species or that the different experimental growth conditions affected the response to the regulatory systems.

The levels of Fru-2,6-P₂ will depend on the metabolic activity of the leaf tissues. For the WT Arabidopsis plants, we observed a transient decrease of the level of Fru-2,6-P₂ at the beginning of the light period. During the rest of the light period the amount of Fru-2,6-P₂ returned to a higher level. A similar pattern has been reported for several other plant species, including spinach, soybean, tobacco, and potato (Gerhardt and Heldt, 1984; Kerr and Huber, 1987; Scott and Kruger, 1994). The transient drop in Fru-2,6-P₂ is likely to be caused by the rapid increase in levels of phosphorylated C-3 compounds (Fig. 6, D and E), which will inhibit the F6P₂-K activity. In the transgenic plants, the level of Fru-2,6-P₂ remained low throughout the diurnal period and, in summary, our data show that in light Fru-2,6-P₂ has a clear effect on the levels of Suc, hexoses, and starch in the leaf tissue (Fig. 5) and on the level of phosphorylated metabolic intermediates (Fig. 6). However, in darkness, the contribution of Fru-2,6-P₂ to metabolic regulation is less pronounced.

During the initial light period there was a significant increase in Suc levels in WT and transgenic plants. The WT plants quickly reached a steady level, whereas the transgenic plants with low level of Fru-2,6-P₂ accumulated Suc over a longer period and reached a 30% to 40% higher level than the WT (Fig. 5B). Thus, the changed fluxes of carbon observed by radiolabeling studies (Figs. 3D and 4) are reflected in increased absolute levels of Suc. The increased rates of Suc synthesis by resulting from low levels of Fru-2,6-P₂ also resulted in higher levels of hexoses and a delay in the accumulation of starch in the transgenic plant (Fig. 5, C–E). The data suggest that under the given environmental conditions, Fru-2,6-P₂ operates to define a set point for the level of Suc in WT plants.

A corresponding pattern was seen for triose-phosphates and Glc-1-P (Fig. 6, C–E). During the light period a significant difference between WT and the transgenic plants was built up. The lower levels of

triose phosphates in the transgenic plants can be explained by a release of cyt-FBPase inhibition, which allows for faster conversion of triose-phosphates into hexose phosphates. However, Fru-6-P and Glc-6-P are not accumulated (Fig. 6, A and B), which indicates that later steps of Suc synthesis are also up-regulated. Likewise, the reduction of Glc-1-P in the light period (Fig. 6C) is indicative of an up-regulation of the Suc synthesis in the transgenic plants, presumably by activation of SPS, which is known to be regulated in coordination with Fru-2,6-P₂ (Kerr and Huber, 1987; Stitt et al., 1987). The activation does not appear to be mediated by changes in Glc-6-P, known as an allosteric effector of SPS and SPS-kinase (Huber and Huber, 1996), since the concentrations are almost identical in the transgenic and WT plants in the light period (Fig. 6B). Also, no significant difference in SPS activity between WT and transformants was observed.

During the dark period the differences in sugars, starch, triose-phosphates, and Glc-1-P levels between WT and the transgenic plant line were all eliminated. This occurs even though a large difference in Fru-2,6-P₂ levels between WT and transgenic plants persisted throughout the dark period. We conclude that Fru-2,6-P₂ in the WT plants only has a limited effect on Suc synthesis during the dark period. Carbon derived from starch degradation during the dark period is exported from the chloroplast in the form of hexoses rather than as triose-phosphates (Schleucher et al., 1998; Weber et al., 2000). This implies that cyt-FBPase is not required for Suc synthesis in the dark, which has also been shown using transgenic potato plants with reduced levels of cyt-FBPase (Zrenner et al., 1996). In accordance with this, Strand et al. (2000) recently showed that transgenic Arabidopsis with decreased expression of cyt-FBPase compensates by increasing the Suc export during the night. As a consequence the regulatory power of Fru-2,6-P₂ over Suc synthesis is eliminated in the dark period, and our metabolite data are compatible with this model for carbon acquisition from leaf starch.

In Arabidopsis, initiation of starch degradation in the dark period may be delayed, as reflected in a temporary decrease in Suc levels (Caspar et al., 1991; Zeeman and ap Rees, 1999). In accordance with this we observed a rapid decrease in hexose phosphate levels in the initial dark period. Over the entire dark period, starch degradation (Fig. 5E) and levels of hexose phosphates were very similar in WT and transformants.

In opposition to this, Scott and Kruger (1995) observed that during the dark period, transgenic tobacco plants with increased levels of Fru-2,6-P₂ had decreased levels of hexose phosphates, increased levels of triose phosphates, and decreased rates of starch mobilization. The authors suggested that the changed starch mobilization was primarily due to an activation of PFP leading to an increased 3-phosphoglyceric ac-

id/ P_i ratio, which activates re-synthesis of starch. Our data are not compatible with changed carbon flux through PFP during the night period, since hexose phosphates and triose phosphates did not change significantly. The role of PFP in leaf metabolism remains elusive (Stitt, 1998). A more detailed analysis of leaf starch metabolism in plants with different levels of PFP and Fru-2,6- P_2 will be required.

In conclusion, we have successfully reduced the bifunctional enzyme F2KP in *Arabidopsis*, which results in plants with constitutive low levels of Fru-2,6- P_2 in leaf tissue. This results in altered carbon partitioning in favor of Suc formation during photosynthesis, indicating that WT levels of Fru-2,6- P_2 are inhibitory to Suc synthesis.

MATERIALS AND METHODS

Plant Material

Arabidopsis (cv Columbia) was used for all the experiments. Plants were grown in peat-soil in a controlled-climate chamber with mercury halide lamps supplemented with light from incandescent lamps at a photosynthetic flux of 120 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, a temperature of 20°C day/night, and 70% relative humidity. The photoperiod was 8 h for plants used for biochemical and physiological analysis and only fully developed vegetative rosettes were used. The photoperiod was 12 h for plants used for transformation and for seed production.

Vector Construction and Plant Transformation

A 930-bp DNA fragment corresponding to the *AtF2KP* gene (accession no. AF190739) encoding the COOH-terminal end of *Arabidopsis* F2KP (AtF2KP) was amplified by PCR using two gene-specific *Bam*HI-anchored primers (basepair positions 1,379–1,404, 5'-ATGATAGCTTGGATGCAAGAAGGTGG-3' and basepair positions 2,284–2,309, 5'-CTATTATAGTGTGAAGTGGCATCTCG-3') and an *AtF2KP* cDNA clone as template. The generated DNA fragment was cloned in antisense orientation between the enhanced cauliflower mosaic virus 35S promoter and the 35S terminator in the pPS48 plasmid (Kay et al., 1987). The promoter-antisense-terminator construct was excised with *Xba*I digestion and was ligated into the binary pPZP211 vector (Hajdukiewicz et al., 1994). The vector construct was introduced into *Agrobacterium tumefaciens*, and *Arabidopsis* plants were transformed by vacuum infiltration (Bechtold et al., 1993). Transformants were selected on Murashige-Skoog medium containing 50 mg L^{-1} kanamycin.

Immunoblotting

F2KP was detected in crude leaf extracts by antibodies raised against the phosphatase region of AtF2KP (Villadsen et al., 2000). Leaf material (0.4 g) of different plant lines was homogenized in 2 mL of buffer A (50 mM MOPS [3-(*N*-morpholino)-propanesulfonic acid]/KOH, pH 7.3, 5 mM MgCl_2 , 1 mM EDTA, 10% [v/v] ethylene glycol, 0.1% [v/v]

β -mercaptoethanol, 5 mM benzamidine, 1 mg mL^{-1} anti-pain, 1 mg mL^{-1} leupeptin, 2 mM phenylmethylsulfonyl fluoride, and 0.1% [v/v] Triton X-100). Total protein (6.5 μg) was separated by SDS-PAGE, blotted, and detected as in Nielsen (1994).

Enzyme Activities

The activities of F6P,2-K and bifunctional F26BPase in *Arabidopsis* were assayed by detection of the formation or degradation of Fru-2,6- P_2 as described by Nielsen (1992). The amount of Fru-2,6- P_2 was determined by following the activation of potato tuber PFP. One unit was defined as the conversion of 1 μmol of substrate per minute. The plant extracts were prepared in 5 volumes of buffer A (100 mg in 0.5 mL). The mono-functional F26BPase activity was determined from the rate of formation of Fru-6-P as according to MacDonald et al. (1989) and modified by Nielsen (1992). PFP and SPS activities were assayed as described by Nielsen et al. (1991) and Huber et al. (1989), respectively, and the activity of PFK was assayed as PFP, except that the reaction medium contained no Fru-2,6- P_2 or pyrophosphate, but did include 0.5 mM ATP. All enzymes were assayed at saturating substrate concentrations.

$^{14}\text{CO}_2$ Labeling of Intact Plants

Intact *Arabidopsis* plants were placed in an illuminated air-tight transparent plastic chamber. The photon flux was 280 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Immediately at the start of the photoperiod, 10 min into, 60 min into, or at the last hour of the photoperiod the plants were labeled by $^{14}\text{CO}_2$ released by acidification of sodium ^{14}C -bicarbonate. The concentration of CO_2 in the chamber was 0.03% (v/v), with a specific activity of 100 $\mu\text{Ci mmol}^{-1}$. After a 10-min pulse the aerial parts of the plants were immediately frozen in liquid nitrogen and were kept at -80°C . Plants were extracted at 80°C in 80% (v/v) ethanol. The ethanol-soluble and -insoluble fractions were prepared as described in Nielsen and Veierskov (1990). The amount of ^{14}C present in different fractions of the plant was determined by liquid scintillation counting. Neutral compounds representing soluble sugars were spotted on cellulose thin layer plates (Merck, Rahway, NJ) and were developed four times in ethylacetate:pyridine:water (20:7:5). After chromatography, radioactivity was visualized by phosphorimaging (Molecular Dynamics, Sunnyvale, CA).

Metabolites, Starch, and Soluble Sugars

Leaf material (50 mg) was ground in 2.5 mL (w/v) of ice-cold 10 mM KOH in a Duall glass homogenizer. The extract was centrifuged at 10,000g for 1 min and the supernatant was used for determination of Fru-2,6- P_2 by an assay based on activation of PFP from potato tubers (Van Schaftingen, 1984). The recovery of Fru-2,6- P_2 was 90%. Samples for measurement of hexoses were extracted in hot 80% (v/v) aqueous ethanol as described in Nielsen et al. (1991). The ethanol-soluble fraction was dried at room

temperature and redissolved in water (Nielsen et al., 1991). Starch in the ethanol-insoluble fraction was determined according to Nielsen et al. (1991). Suc, Glc, and Fru were determined by enzyme-linked reduction of NAD⁺ monitored spectrophotometrically at 340 nm. (Beutler, 1984; Kunst et al., 1984). To measure hexose- and triose-phosphates, frozen plant material was ground to a fine powder in liquid nitrogen and the metabolites were extracted in trichloroacetic acid according to Jelitto et al. (1992). Glc-1-P, Glc-6-P, Fru-6-P, DHAP, and GAP were determined by enzyme-linked oxidation of NADH according to Stitt et al. (1989).

Oxygen Evolution

The rate of photosynthetic oxygen exchange was determined with a leaf-disc oxygen electrode (model LD-2/2, Hansatech), and the varying light intensity was provided by a lamp (KL-1500, Schott, Glostrup, Denmark) fitted with neutral density filters as described by Haldrup et al. (1999). The leaf area was determined by Gel Doc 2000 (Bio-Rad, Hercules, CA) using Quantity One 4.0.2. software (Bio-Rad).

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