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Carbon-Partitioning in *Arabidopsis* Is Regulated by the Fructose 6-Phosphate, 2-Kinase/Fructose 2,6-Bisphosphatase Enzyme

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To further elucidate the mechanisms underlying carbon-partitioning in plants, we established an experimental system by generating transgenic *Arabidopsis* lines that overexpress both the fructose 6-phosphate, 2-kinase (F6P,2-K) and the fructose 2,6-bisphosphatase (F26BPase) domains. We also produced knockout transgenic plants for these domains via RNAi and T-DNA tagging. In F6P,2-K overexpressing transgenics, F6P,2-K activity increased slightly and Fru-2,6-P_2 levels were elevated by 80%, compared with the wild type (WT). F26BPase activity was similar between the WT and transgenic plants. However, when that domain was overexpressed, F26BPase activity was increased by 70% compared with the WT, whereas F6P,2-K activity was reduced to 85% of the WT level. In knockout and RNAi mutant lines that showed reduced F6P,2-K and F26BPase activities, levels of Fru-2,6-P_2 were only between 3 to 7% of those for the WT. In F6P,2-K overexpressing transgenic lines, the levels of starch, hexose, and triose phosphates slightly increased, while sucrose content was marginally reduced. In F26BPase overexpressing plants, however, the levels of soluble sugars and hexose phosphates were slightly increased, but starch and triose phosphate contents declined. Furthermore, compared with the WT, the levels of soluble sugars rose while starch and hexose phosphate quantities decreased in 2-kinase/fructose-2,6-bisphophatase knockout mutants. Therefore, our data reaffirms that Fru-2,6-P_2 contributes to the regulation of photosynthetic carbon-partitioning between starch and sucrose in *Arabidopsis* leaves by limiting sucrose synthesis.

Keywords: *Arabidopsis*, carbohydrate metabolites, carbon-partitioning, fructose 2,6-bisphosphate, fructose 6-phosphate, 2-kinase/fructose 2,6-bisphosphatase

In animals and yeast, fructose 2,6-bisphosphate (Fru-2,6-P_2) regulates both glycolysis and gluconeogenesis (van Shaftingen, 1984). In plants, it functions as a key regulator of photosynthetic carbon metabolism through the allosteric inhibition of cytosolic fructose 1,6-bisphosphatase (cFBPase). This reaction is responsible for the conversion of fructose 1,6-bisphosphate to fructose 6-phosphate during the formation of sucrose from triose phosphates (Stitt, 1990). Plant Fru-2,6-P_2 also acts as an allosteric regulator of fructose 6-phosphate, 1-phosphotransferase (PFP), which catalyzes the reversible interconversion between Fru-1,6-P_2 and Fru-6-P (Draborg et al., 2001). In leaves, Fru-2,6-P_2 regulates the coordination rates of CO_2 assimilation and sucrose synthesis, and also controls carbon-partitioning between sucrose and starch (Rowntree and Kruger, 1995; Scott et al., 2000; Trevanion, 2000). In transgenic plants, decreasing the levels of Fru-2,6-P_2 in leaves results in a rise in the carbon flux towards sucrose (Scott et al., 2000; Draborg et al., 2001), whereas increases in Fru-2,6-P_2 cause starch levels to be elevated (Scott et al., 1995).

The synthesis and degradation of Fru-2,6-P_2 are separately catalyzed by the activities of two specific bifunctional enzymes of the fructose-6-phosphate, 2-kinase/fructose-2,6-bisphosphatase protein (F2KP): fructose 6-phosphate, 2-kinase (F6P,2-K) and fructose-2,6-bisphosphatase (F26BPase). In leaves, each of these activities is reciprocally controlled by metabolic intermediates of the pathway. Kinetic characterization of partially purified spinach F2KP has shown that both activities are allosterically regulated by such metabolites (Cséke and Buchanan, 1983; Larondelle et al., 1986). F6P,2-K is activated by Fru-6-P and inorganic phosphates (P_i), but is inhibited by 3-phosphoglycerate and dihydroxyacetone phosphates (DHAP), whereas F26BPase is inhibited by Fru-6-P and P_i. Several tissue-specific isoforms of these enzymes have also been identified. The animal enzymes comprise a dimer of two identical subunits, each containing a highly conserved catalytic region and both N- and C-terminal extensions. These isoforms are, in fact, regulated by the reversible phosphorylation of amino acid residues in either the N- or C-terminal extensions (Pilkis et al., 1995).

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Plant F2KP enzymes have comparatively long N-terminal regions compared with those of animals. In general, their catalytic component is highly conserved between species (e.g., 88% identity between *Arabidopsis* and spinach, 81% between *Arabidopsis* and maize, and 88% between *Arabidopsis* and potato). The exception to this is the N-terminus, which has more variable sequences (59% identity between *Arabidopsis* and spinach, 58% between *Arabidopsis* and potato) (Villadsen and Nielsen, 2001; Markham and Kruger, 2002). While the exact function of the F2KP N-terminus is unknown, this region may be involved in modulating the ratio of kinase/phosphatase activities (Villadsen and Nielsen, 2001). An enzyme with F2KP-like activities, which is a homotetramer of a single 90-kDa polypeptide (Villadsen et al., 2000; Markham and Kruger, 2002), has been purified and partially characterized from spinach leaves (Larondelle et al., 1986; Macdonald et al., 1989). cDNAs of F2KP have also been isolated from several plant species, and F2KP activity in *Arabidopsis* and potato has been confirmed through an *Escherichia coli* expression system (Draborg et al., 1999; Villadsen et al., 2000). In addition, transgenic *Arabidopsis* and potato plants have been constructed by repressing endogenous F2KP by sense/antisense constructs, which results in strongly decreased levels of Fru-2,6-P_2 in leaves (Draborg et al., 2001; Rung et al., 2004). Modified versions of rat liver F6P, 2-K/F26BPase have also been expressed in tobacco and *Kalanchoe daigremontiana* (Scott and Kruger, 1994; Scott et al., 1995). In animals, the overexpression of rat liver fructose 2,6-bisphosphatase leads to a decrease in glycolysis and a delay in cell-cycle progression (Perez et al., 2000).

Here, we studied the function of F2KP in *Arabidopsis*, constructing transgenic plants that separately overexpressed the fructose-6-phosphate, 2-kinase (NK) and fructose-2,6-bisphosphatase domains (NP) of *Arabidopsis* F2KP. We also generated F2KP-RNAi and F2KP-KO plants. Our objective was to examine the relationships among carbon flux, sucrose biosynthesis, and hexose phosphate accumulation in leaves with reduced levels of Fru-2,6-P_2.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis (cv. Columbia) seeds were purchased from Lehle Seeds (http://www.arabidopsis.com); those of the heterozygous mutant line F2KP-KO (SALK 016314) were obtained from the SALK Institute, USA. Their resultant plants were grown in a 2:1:1 mix of topsoil, perlite, and vermiculite in a controlled-climate chamber at 23°C and 50% relative humidity, and under a 16-h photoperiod from incandescent lamps with a photosynthetic flux of 100 µmol photons $m^{-2}\,s^{-1}$.

Cloning the Kinase Domain (NK) and Phosphatase Domain (NP) of F2KP

A 1629-bp cDNA fragment encompassing both the N-terminal and kinase domains of *Arabidopsis* F2KP (NM100584) was PCR-amplified. The sense and antisense primer sequences included: 5′-GTCGACATGGGGGTCAGGTGCATGC-3′ (*Sal*I site is underlined) and 5′-TCTAGATCAGTCCATGAGTTTGTAG-3′ (*Xba*I site is underlined), respectively. The PCR product was then inserted into the pPZP211 vector (Hajdukiewicz et al., 1994) and designated as pNK14 (Fig. 1B). To construct a 1617-bp cDNA fragment containing the N-terminal and the phosphatase domains of F2KP, we used overlapping PCR technology. First, a 1058-bp cDNA fragment was PCR-amplified with sense and antisense primers 5′-GTCGACATGGGGTCAGGTGCATGC-3′ and 5′-TCACTCATCATAACCAGCTGCTAGATGTCTAT-3′, respectively. Second, a 586-bp cDNA fragment covering the phosphatase domain was amplified using primers 5′-GCTAATTATGATGAGTGACGGCATGGAGAAAG-3′ and 5′-TCTAGATCAGTCCATGAGTTTGTAG-3′. The two overlapping PCR fragments that resulted were used as a template for a third round of PCR with primer pairs 5′-GTCGACATGGGGGTCAGGTGCATGC-3′ and 5′-TCTAGATCAGTCCATGAGTTTGTAG-3′. The amplified DNA fragment was then inserted into the pPZP211 vector and designated as pNP7 (Fig. 1B).

RNAi Transgene Constructs

To generate a F2KP RNAi transgenic line, a 507-bp cDNA fragment was PCR-amplified with the primers 5′-GCTCTAGAGGCGCGCCTTGGATGCAAGAAGGTGG -3′ (*Xba*I and *Asc*I sites are underlined) and 5′-CGGGATCCATTTAAATCTGGCAAGTTTCTTTGCG-3′ (*Bam*HI and *Swa*I sites are underlined). The resulting PCR product was inserted into pFGC5941 (http://www.chromdb.org), which is a binary vector that contains a chalcone synthase intron designed to produce double-stranded RNA under the control of the CaMV 35S promoter in plants. F2KP cDNA fragments were first cloned in the sense orientation between the *Asc*I

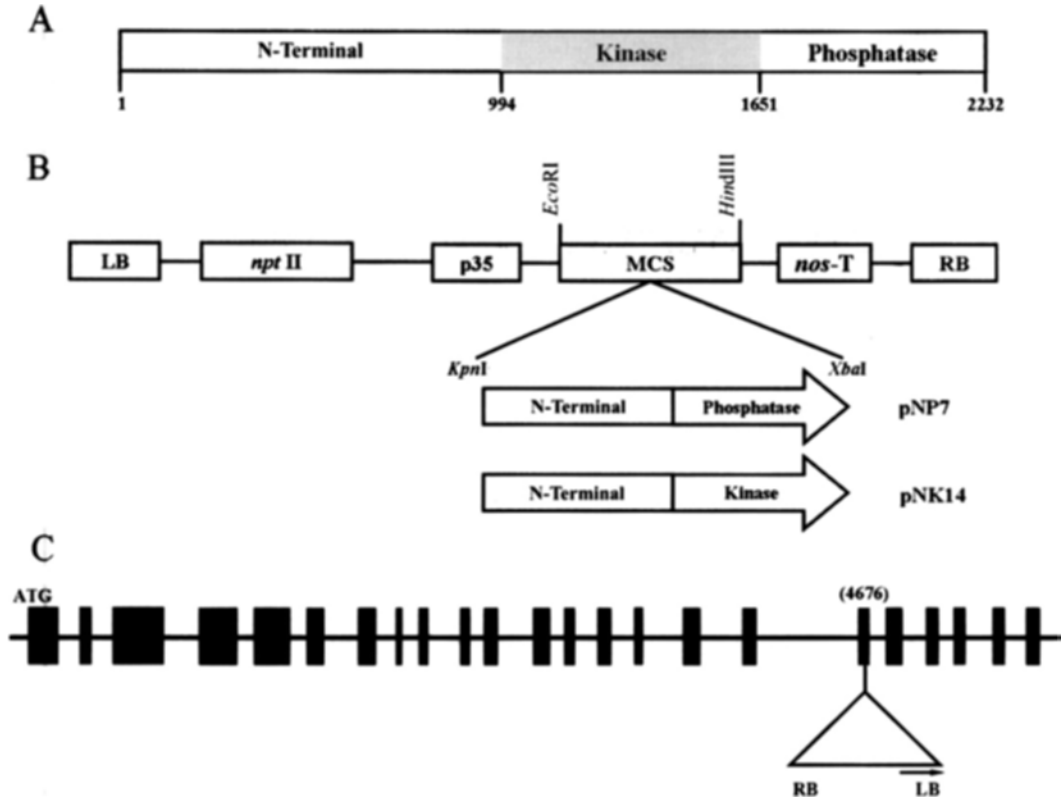

Figure 1. A, Structure of 6-phosphate, 2-kinase/fructose 2,6-bisphosphatase (F2KP) cDNA. **B,** Diagrammatic representation of the T-DNA region of binary plasmid vectors pNP7 and pNK14. **C,** Exon/intron structures of F2KP gene showing location of T-DNA insertion. Plasmid vector was derived from pPZP110. RB, LB, Right and left borders of T-DNA; *npt* II, neomycin phosphotransferase II gene; p35, CaMV 35S promoter; *nos*-T, nopaline synthase gene terminator; MCS, multiple cloning sites.

and *Swa*I sites, and then in the antisense orientation between the *Bam*HI and *Xba*I sites. *Agrobacterium tumefaciens* strain GV3101 carried this construct for the subsequent transformation of *Arabidopsis* via floral-dipping (Clough and Bent, 1998), and transgenic plants were selected via the BASTA herbicide (0.05%, v/v).

Isolation of Plants Containing Homozygous Recessive Alleles of F2KP

F2KP-KO plants were identified by the presence of a homozygous mutation in the F2KP gene, as detected through PCR-screening, by using gene-specific and T-DNA-specific primers according to the method of Krysan et al. (1999). Primer sequences were as follows: F2KP-KO, 5′-TCAGTCCATGAGTTTGTAGCGTTTCTC-3′; T-DNA, 5′-TGGTTCACGTGATGGGCCATCG-3′.

Agrobacterium-Mediated Transformation

Target DNA was transformed into the *A. tumefaciens* GV3101 strain via electroporation by first mixing it with *Agrobacterium* competent cells that were then pulsed at 1800 volts for 5 msec. These pulsed cells were added to 500 μL LB broth and incubated at 28°C for 3 h. Afterward, the cells were grown in LB agar plates containing spectinomycin (50 μg mL^{-1}). The floral-dip method was used for *Arabidopsis* transformation (Clough and Bent, 1998). F2KP overexpressing lines were selected on GM media containing kanamycin (25 μg mL^{-1}) and carbenicillin (50 μg mL^{-1}). F2KP-RNAi plants were grown in topsoil, perlite, and vermiculite (2:1:1) for about 2 weeks, and then selected with BASTA.

DNA Gel-Blot Analysis

Genomic DNA from *Arabidopsis* leaf tissue was isolated, digested for 8 h with *Eco*RI, and resolved on a 1% agarose gel. This separated DNA was then blotted onto a Hybond-N^+ membrane (Amersham Pharmacia Biotech, USA), using 0.4 N NaOH transfer buffer. The 500-bp (kinase domain) and 496-bp (phos-

phatase domain) fragments were PCR-amplified from plasmids pNK14 and pNP7, respectively. Sense and antisense primers included: for the kinase domain, 5'-CACTCTCACCGTTTTCAGCAG-3' and 5'-CCGTCGA-ACTCGTGTGCTAT-3'; and for the phosphatase domain, 5'-GTAAACTTTACGCAAAGAAACTTGC-3' and 5'-TCCATGAGTTTGTAGCGTTTC-3'. The resulting PCR products and a 1.39-kb *Nco*I/*Bam*HI fragment (*Chs*A intron) of pFGC5941 (RNAi) were ^{32}P-labelled by random priming and added to the filters in a hybridization solution containing 1% (w/v) BSA, 7% (w/v) SDS, 20 mM sodium phosphate (pH 7.2) and 0.5 mM EDTA at 65°C before being exposed on a phosphoimaging plate.

RNA Gel-Blot Analysis

Total RNA was prepared from *Arabidopsis* leaves by extraction with TRIzol reagent (Invitrogen, USA). Briefly, 30 μg of RNA was denatured, electrophoresed on an agarose/formaldehyde gel, and transferred onto a Hybond-N$^+$ membrane using 25 mM sodium phosphate. Hybridization was performed with domain-specific probes as described for our DNA gel-blot analysis.

Enzyme Activity Assays

F6P,2-K activity in *Arabidopsis* leaves was detected through the formation of Fru-2,6-P_2, as previously described by Nielsen (1992). Briefly, 50 mM MOPS-KOH buffer (pH 7.3) containing 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} antipain, 1 mg mL^{-1} leupeptin, 2 mM phenylmethylsulfonylfluoride, and 0.1% [v/v] Triton X-100 was used as the extraction buffer for this assay. Leaf samples (0.2 to 0.4 g) were homogenized in 5 volumes of extraction buffer, and the extract was then de-salted on a Sephadex G-25 column. Afterward, 20 mL of the de-salting extract was added to a 180 mL stock solution, and the reaction was stopped by the addition of 100 μL 0.1 M KOH after 10 min. The final concentration of the 100 μL reaction volume contained 50 mM MOPS-KOH (pH 7.3), 7.5 mM $MgCl_2$, 1 mM EDTA, 5 mM K-phosphate, 5 mM Fru-6-P, 20 mM Glc-6-P, 2.5 mM ATP, 5 mM phosphocreatine, and 4 U creatine phosphate. F26BPase activity was defined by the formation of Fru-6-P, also according to Nielsen (1992). F26BPase was extracted as described for F6P,2-K, and was de-salted into a similar buffer without $MgCl_2$. The assay was started by adding 5 μL of the extract to 275 mL of reaction mixture, and was stopped after 5 min by the addition of 300 μL of 0.1 M KOH. The total reaction mixture contained 5 μL extract, 0.1 μM Fru-2,6-P_2, 50 mM MOPS-KOH, 5 mM K-phosphate, 5 mM Fru-6-P, 20 mM Glc-6-P, 7.5 mM $MgCl_2$, 2.5 mM ATP, 5 mM phosphocreatine, and 4 U mL^{-1} creatine phosphokinase. The level of Fru-2,6-P_2 was detected by the activation of PFP, according to the method of van Schaftingen (1984). Fru-2,6-P_2 was extracted in the same manner as for F6P,2-K, and was de-salted into the same type of buffer. The assay mixture, in a total volume of 1 mL, contained 50 μL extract, 1 M Tris/acetate (pH 8.0), 500 mM MgOAc, 100 mM Fru-6-P, 40 mM NADH, 1 U aldolase, 10 U triose phosphate isomerase, 0.5 U Glc-6-P DH, and 0.005 U PFP. The reaction was started by the addition of 50 μL 10 mM pyrophosphate solution.

Extraction and Measurement of Metabolite Levels

Arabidopsis leaves (0.1-0.2 g) were ground in liquid nitrogen to fine powder, then extracted with 80% ethanol and 5% formic acid at 80°C for 20 min. This procedure was slightly modified from the protocol described by Lu and Sharkey (2004). The extraction solvents were evaporated in a 5301 Concentrator (Eppendorf, Germany). The pellets were then re-extracted with H_2O and centrifuged to determine their starch content. The aqueous phase was used to assay for soluble sugars and metabolic intermediates. Starch and soluble sugar contents were determined according to Yoon et al. (2004). Enzymes for the metabolite determination were purchased from Sigma-Aldrich (USA). Soluble sugar determinations were made with a CARY 300 Bio (Varian, USA) and a microplate reader (Tecan, Switzerland).

Gas-Exchange Measurements

CO_2 fixation under various intercellular CO_2 concentrations and illumination levels was measured in individual rosette leaves with an LI-6400 portable photosynthesis monitor (Li-Cor, USA). Gas was supplied by an automatic injector, then the intercellular concentrations and rates of assimilation were calculated as described (von Caemmerer and Farquhar, 1981). All photosynthesis measurements were made from at least five leaves in each mutant line. Light was supplied by an LED source attached to the leaf chamber. The gas humidity was sustained at approximately 10-30%, and the leaf temperature was maintained at 22-24°C.

RESULTS

Construction of Transgenic *Arabidopsis* Lines Possessing a Modified F2KP Gene

To analyze the role of the F2KP enzyme in carbon-partitioning, we used the *Agrobacterium*-mediated floral-dip method to introduce the N-terminally extended fructose-6-phosphate, 2-kinase (NK) and fructose-2,6-bisphosphatase (NP) domains of the *Arabidopsis* F2KP gene into wild-type *Arabidopsis* plants (Fig. 1A, 1B). A total of 12 NK and 10 NP homozygous lines were obtained and further analyzed by genomic DNA gel-blots to determine the copy number for each transgene. NK transgenic Lines 5, 9, and 14 and NP Lines 7 and 19 each had single insertions (Fig. 2A, 2B). Expression levels of the exogenous genes in the NK and NP transgenic lines were then examined by northern blot analysis (Fig. 3).

Transgenic *Arabidopsis* lines with reduced F2KP activities as well as a series of F2KP-RNAi plants were also produced. A total of 15 independent

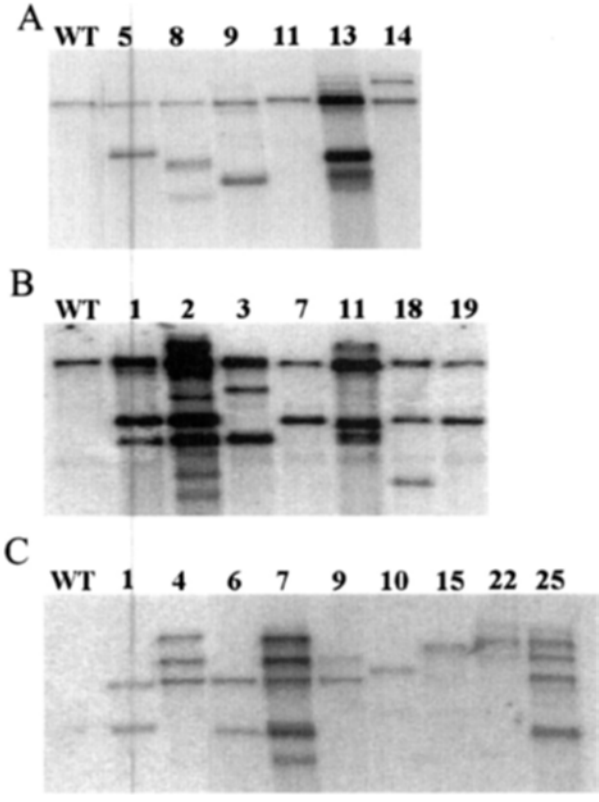

Figure 2. Southern blot analyses of transgenic plants harboring **(A)** fructose-6-phosphate, 2-kinase domain (NK), **(B)** fructose-2,6-bisphophatase domain (NP), and **(C)** F2KP RNAi vector.

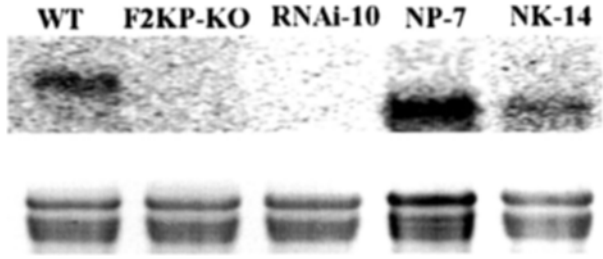

Figure 3. Northern blot analysis of F2KP transgenic *Arabidopsis*. Thirty μg of total RNA isolated from leaves was blotted onto nylon membrane and hybridized to [α-^{32}P] labeled domain-specific probe. Ethidium-bromide staining of rRNA is shown as a loading control.

transgenic plants from the NK-14, NP-7, and RNAi-10 lines were finally selected for further analysis, based on their single transgene copy numbers and expression levels (Fig. 2C, 3). In addition, we examined heterozygous T-DNA insertional mutants of *Arabidopsis* F2KP. This F2KP-KO line (SALK 016314) contains a T-DNA insertion at 4676 b downstream of the start ATG (Fig. 1C). We verified its structure by PCR-screening, using gene-specific and T-DNA-specific primers. RNA gel blot analysis to confirm the effects of the T-DNA insertion on F2KP expression (Fig. 3) revealed that this knockout mutant showed no detectable gene transcripts, similar to the F2KP-RNAi-10 line.

Determination of Fru-2,6-P_2 and F6P,2-K/Fru-2,6-BPase Levels in F2KP Transgenic *Arabidopsis* Lines

F6P,2-K activity was slightly increased in the transgenic *Arabidopsis* line overexpressing F6P,2-K (NK-14); levels of Fru-2,6-P_2 also increased in this line, by 70% compared with the wild-type (WT) plants (Fig. 4B, 4C). In contrast, the activity of F6P,2-K in the NP-7 F26BPase domain overexpressing line was reduced to 85% of the WT levels (Fig. 4A). Likewise, F26BPase activity of the NP-7 line was about 170% above the WT levels (Fig. 4A), whereas its activity in the knockout mutant lines was significantly reduced, to only 3 to 7% of that measured in the WT (Fig. 4A). The level of Fru-2,6-P_2 in knockout and RNAi mutant lines that showed reduced F6P,2-K activity was between 3% to 6% of the WT (Fig. 4C).

Changes in the Profiles of Carbohydrates and Phosphorylated Metabolites in F2KP Transgenic *Arabidopsis*

Levels of starch, sugars, and phosphorylated metab-

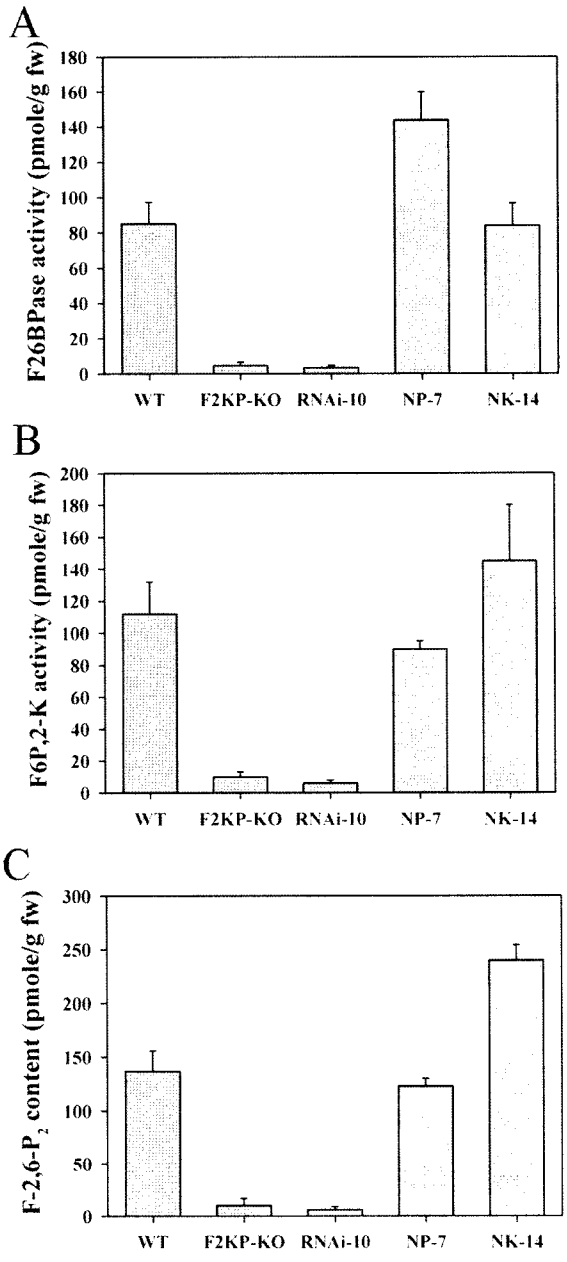

Figure 4. Activities of F26BPase **(A)** and F6P,2-K **(B)**, and levels of Fru-2,6-P2 **(C)** in transgenic *Arabidopsis*. Results are means ± SD (n = 5).

olites in the leaves of the transgenic *Arabidopsis* lines were analyzed throughout the diurnal cycle to examine the effects of F2KP disruption on carbon metabolism. Both glucose and sucrose contents in the NK-14 line were lower than in the WT over the cycle, with the level of fructose being even lower in that line as darkness ended (Fig. 5A-C). In contrast, the starch content of NK-14 was higher than in the WT (Fig. 5D). Glucose and fructose levels in the NP-7 line were slightly increased during the daytime but declined by the end of the day and were low in the dark (Fig. 5A, 5B). At the start of the light period, sucrose content rose in leaves from Line NP-7, and levels there were higher than in the WT (Fig. 5C). Starch quantities were slightly lower in NP-7 than in the WT during the light period (Fig. 5D).

The F2KP-KO and F2KP-RNAi mutants accumulated glucose, fructose, and sucrose over a longer period and at significantly higher levels than did the WT (Fig. 5A-C). However, the amount of starch was always lower in the F2KP-KO and F2KP-RNAi lines (Fig. 5D). Glc-6-P and Fru-6-P levels in NK-14 plants showed similar but slightly elevated carbohydrate patterns at the end of the day compared with the WT (Fig. 5E, 5F). Moreover, Glc-1-P and UDP-Glc contents were higher in the transgenic plants throughout the diurnal period (Fig. 5G, 5H). Finally, NP-7 mutants accumulated similar but slightly higher levels of hexose phosphate over the cycle than did WT plants.

During the light period, DHAP and glyceraldehyde-3-phosphate (GAP) contents were elevated in the NK-14 mutant (Fig. 5I, 5J). However, the accumulation of triose phosphates in the NP-7 line was lower than in the WT at the end of the day (Fig. 5I, 5J). Levels of hexose phosphate in both F2KP-KO and F2KP-RNAi plants were also lower in the light (Fig. 5E-H). During the dark period, the triose phosphate contents of F2KP-KO and F2KP-RNAi plants were similar to those of WT (Fig. 5I, 5J). Triose phosphate accumulation was more rapid in WT plants, however, and at the end of the day period its level was greater in WT plants than in the F2KP-KO and F2KP-RNAi lines.

Photosynthetic Rates

Photosynthetic activity was measured in leaves from NK-5, NP-14, F2KP-KO, RNAi-10 and WT *Arabidopsis* plants that were grown under different external CO_2 concentrations (0-600 ppm) and varying light intensities (0-1200 μmol m^{-2} s^{-1}). For NK-14, activity was similar to the WT at an intercellular CO_2 concentration above 500 ppm and an irradiance level of 300 μmol m^{-2} s^{-1} (Fig. 6A). However, the photosynthetic activity of NP-7 was lower than the WT at a CO_2 concentration above 500 ppm and irradiance level of 200 μmol m^{-2} s^{-1} (Fig. 6A, 6B). In addition, the F2KP-KO and RNAi-10 plants, with low Fru-2,6-P_2 contents, had increased photosynthetic rates compared with the

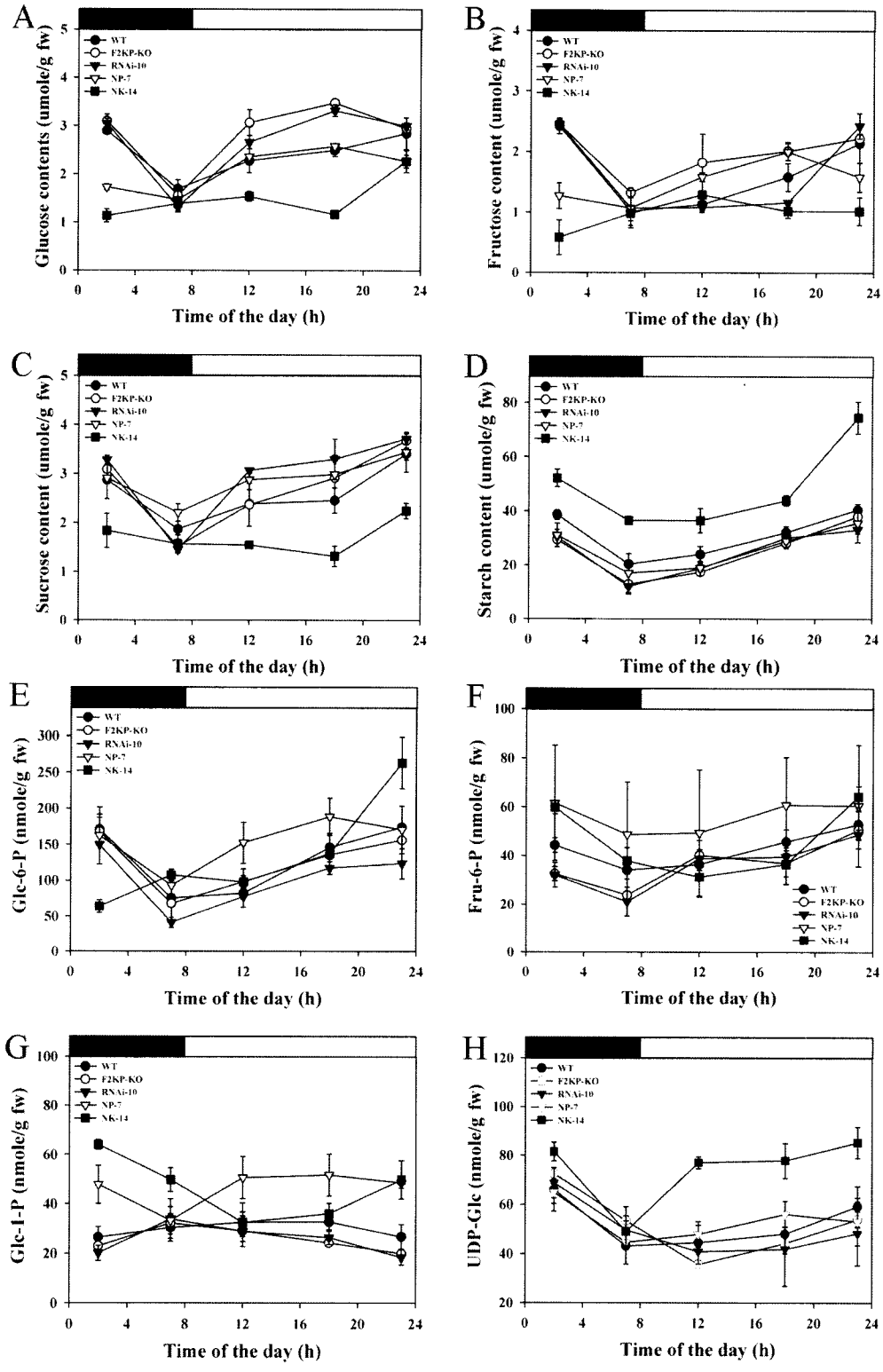

Figure 5. Determination of metabolite contents in F2KP transgenic *Arabidopsis*. **(A)** Glucose, **(B)** Fructose, **(C)** Sucrose, **(D)** Starch, **(E)** Glucose 6-phosphate, **(F)** Fructose 6-phosphate, **(G)** Glucose 1-phosphate, **(H)** UDP-glucose, **(I)** DHAP, and **(J)** Glyceraldehyde 3-phosphate. Values were determined from rosette leaves of wild type (●), F2KP-KO (○), RNAi-10 (▼), NP-7 (▽), and NK-14 (■) transgenic *Arabidopsis* during diurnal light/dark cycle (16 h/8 h). Results are means ± SD (n = 5). Black bars indicate dark period.

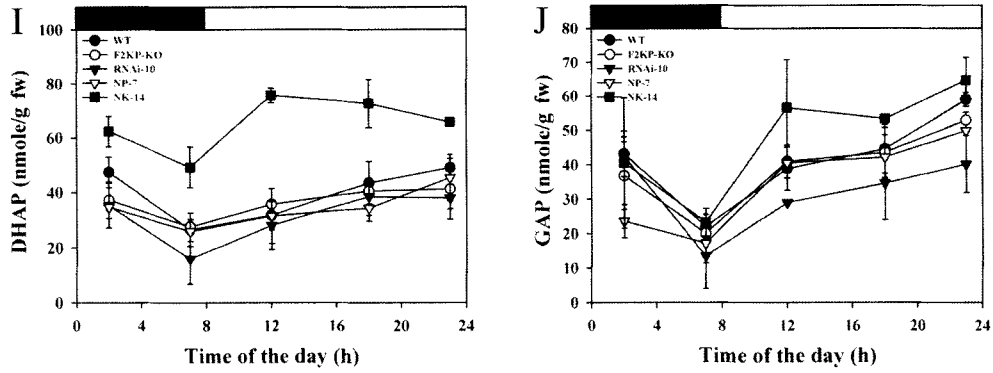

Figure 5. Continued.

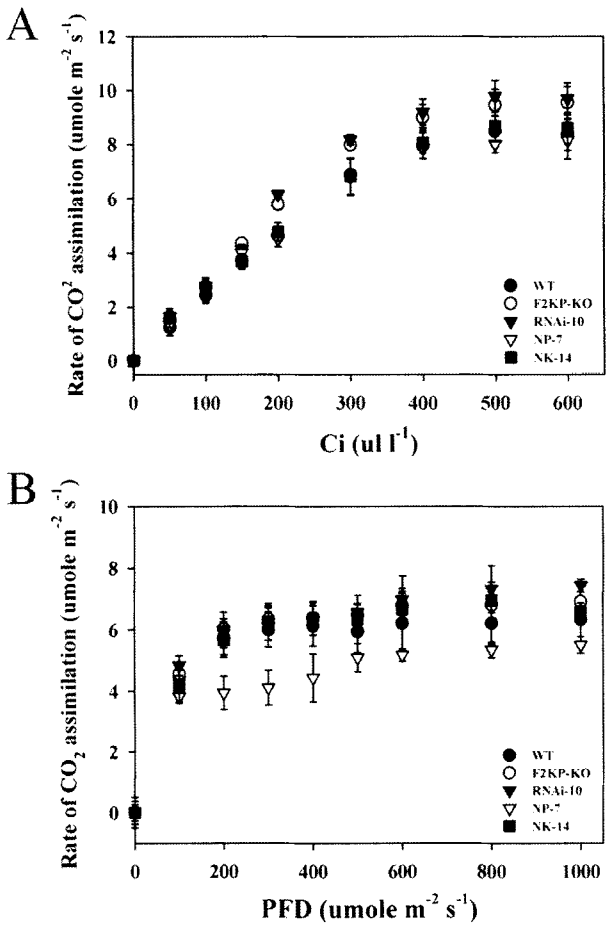

Figure 6. Photosynthetic activity in WT and transgenic *Arabidopsis*. **(A)** Dependencies of CO_2 assimilation rates under various intercellular CO_2 concentrations (Ci) at a photon flux density (PFD) of 500 μmol m^{-2} s^{-1}. **(B)** Dependencies of CO_2 assimilation rates under increasing irradiance in gas mixture containing 600 ppm CO_2. ● , wild type; ○ , F2KP-KO; ▼ , RNAi-10; ▽ , NP-7; ■ , NK-14. Each point represents mean (± SE) from at least 4 different plants within each line.

WT at a CO_2 concentration above 200 ppm and an irradiance level of 300 μmol m^{-2} s^{-1} (Fig. 6B).

DISCUSSION

To analyze its functioning in *Arabidopsis*, we constructed transgenic plants that had altered activities of the *F2KP* gene. To our knowledge, this is the first study to investigate the effects of F2KP activity on photosynthetic carbon-partitioning following the transgenic modulation of both F2KP kinase and phosphatase activities. This was achieved via the overexpression of the fructose-6-phosphate, 2-kinase (NK) and fructose-2,6-bisphosphatase (NP) domains of F2KP, and also by gene knockdown (F2KP-RNAi and F2KP-KO). Altered expression in these mutant lines was confirmed by Southern and RNA gel blot analyses, and by enzyme activity assays (Fig. 2-4). In plants, Fru-2,6-P_2 is an allosteric inhibitor of cFBPase, and exchanges Fru-1,6-P_2 to form sucrose in the cytosol (Stitt, 1990). Its role in carbohydrate metabolism in tobacco had also been examined, with levels of fructose-2,6-phosphate being affected by the expression of rat liver F6P,2-K/F26BPase (Scott et al., 1995, 2000). Down-regulated Fru-2,6-P_2 is associated with more rapid accumulation of sucrose whereas its up-regulation causes faster starch accumulation than in WT plants. Hence, decreased levels of F6P,2-K and Fru-2,6-P_2, and increased levels of F26BPase, result in higher sucrose and lower starch synthesis than can be measured in the WT of both *Arabidopsis* and potato (Draborg et al., 2001; Rung et al., 2004).

Here, the amount of Fru-2,6-P_2 in knockout mutant *Arabidopsis* lines with reduced F6P,2-K activity was between 3% and 6% of the WT levels (Fig. 4C). Furthermore, in our F2KP knockout mutants, and in F26BPase overexpressing plants with a low Fru-2,6-P_2 content, sucrose contents were higher than in the WT (Fig. 4, 5C). In contrast, starch content was reduced in F26BPase overexpressing and F2KP knockout plants. In F6P,2-K overexpressing transgenic lines, the level of

sucrose decreased but that of starch rose during photosynthesis. Altogether, our findings provide useful confirmation that Fru-2,6-P_2 plays a role in limiting sucrose synthesis during the diurnal cycle.

Hexose phosphate levels seemed to fluctuate in F6P,2-K overexpressing mutant lines at the end of the light period (Fig. 5E-H). Furthermore, an increase in Fru-2,6-P_2 content was associated with the accumulation of triose phosphates during the same period (Fig. 5I, 5J). These changes may be attributed to the action of Fru-2,6-P_2 via the inhibition of cFBPase. Zrenner et al. (1996) have studied transgenic potato lines in which a reduction in cFBPase activity is facilitated by antisense inhibition of cFBPase, and have demonstrated a large increase in triose phosphate levels. We also observed a decline in triose phosphate content and an increase in hexose phosphate quantities in F26BPase overexpressing *Arabidopsis* (Fig. 5), a result consistent with a decrease in Fru-2,6-P_2 concentration and a consequent lowering of cFBPase inhibition. This increase in Fru-6-P and Glc-6-P hexose phosphates would have favored sucrose production, leading to more rapid accumulation of this sugar during the photoperiod (Fig. 5C). Moreover, the drop in triose phosphates likely reduced starch synthesis because this action would have stimulated their export from the chloroplasts to the cytosol via the triose-phosphate phosphate translocator (TPT), thereby exhausting the availability of substrates. A decrease in triose phosphate also reduces the chloroplastic 3PGA/Pi ratio, inhibiting ADP-glucose pyrophosphorylase (AGPase) (Copeland and Preiss, 1981) and causing a decrease in starch synthesis.

The lower levels of triose phosphates in our F2KP knockout plants can be explained by a reversal in cFBPase inhibition, which allowed for more rapid conversion of triose phosphates into hexose phosphates (Fig. 5I, 5J). However, the levels of Glc-6-P and Fru-6-P did not accumulate to any great extent (Fig. 5E, 5F), thus prompting a more rapid accumulation of sucrose (Fig. 5C). Similarly, the decrease in Glc-1-P levels observed during the light period (Fig. 5G) suggests that sucrose synthesis was faster in the F2KP knock-out plants than in the WT, presumably because of the activation of sucrose-phosphate synthase (SPS), which is coordinated with Fru-2,6-P_2 (Kerr and Huber, 1987; Stitt et al., 1989).

Photosynthetic capacity also was affected in our F2KP transgenic *Arabidopsis* lines. F26BPase overexpressing mutant lines showed a 39% decrease in photosynthesis, whereas in the F2KP knockout plants, capacity increased by 15%. However, the F6P,2-K overexpressing lines showed no changes. In the case of the F26BPase overexpressing mutants, the drop in Fru-2,6-P_2 was accompanied by a decline in the rate of CO_2 assimilation (Fig. 6B), which is perhaps why cFBPase was activated in these lines and the export of plastidic C_3 intermediates was promoted by the TPT in the chloroplasts. Excessive movement of triose phosphate might be suppressed by the build-up of Calvin-cycle intermediates and by the prevention of photosynthesis (Scott et al., 2000), resulting in a higher sucrose content and lower starch accumulation (Draborg et al., 2001).

In summary, we constructed transgenic *Arabidopsis* plants with altered F2KP activities to study the possible roles of this enzyme in plant metabolism. Both metabolite content and photosynthetic capacity were analyzed. Transgenic leaves with reduced Fru-2,6-P_2 displayed carbon-partitioning that was favorable to sucrose synthesis during photosynthesis. Moreover, although metabolite levels and photosynthetic rates fluctuated, overexpression of the F6P,2-K and F26BPase domains did not result in significant changes in F2KP enzymatic activity, Fru-2,6-P_2 levels, or the overall plant phenotype. Thus, transgenic introduction of these domains into plants with an F2KP knock-out background in order to observe the effects on Fru-2,6-P_2 levels and the consequences for carbon-partitioning should provide interesting insights into plant metabolism. Other useful experiments would analyze F2KP transgenic lines under various growing conditions, including situations of high light intensity.

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