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Molecules and Cells



# Altered Expression of Pyrophosphate: Fructose-6-Phosphate 1-Phosphotransferase Affects the Growth of Transgenic *Arabidopsis* Plants

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**Pyrophosphate:fructose-6-phosphate 1-phosphotransferase (PFP) catalyzes the reversible interconversion of fructose-6-phosphate and fructose-1,6-bisphosphate, a key step in the regulation of the metabolic flux toward glycolysis or gluconeogenesis. To examine the role of PFP in plant growth, we have generated transgenic *Arabidopsis* plants that either overexpress or repress *Arabidopsis* PFP subunit genes. The overexpressing lines displayed increased PFP activity and slightly faster growth relative to wild type plants, although their photosynthetic activities and the levels of metabolites appeared not to have significantly changed. In contrast, the RNAi lines showed significantly retarded growth in parallel with the reduced PFP activity. Analysis of photosynthetic activity revealed that the growth retardation phenotype of the RNAi lines was accompanied by the reduced rates of $CO_2$ assimilation. Microarray analysis of our transgenic plants further revealed that the altered expression of *AtPFPβ* affects the expression of several genes involved in diverse physiological processes. Our current data thus suggest that PFP is important in carbohydrate metabolism and other cellular processes.**

## INTRODUCTION

Photoassimilated carbons exported from chloroplasts are oxidized to generate energy or mobilized in heterotrophic organs where they are either used as an energy source or stored for further use. In plants, glycolysis is the predominant pathway of respiration and provides the ATP, reductants and precursors required for plant growth and development (Plaxton, 1996). A distinct property of plant glycolysis as compared with this pathway in animal cells is the existence of a metabolic enzyme, pyrophosphate:fructose-6-phosphate 1-phosphotransferse (PFP) (Plaxton, 1996; Stitt, 1990). PFP catalyzes the reversible interconversion of fructose-6-phosphate (Fru-6-P) and fructose-1,6-bisphosphate (Fru-1,6-$P_2$), which is an important regulatory branch point of primary carbon metabolism toward glycolysis or gluconeogenesis (Nielsen et al., 2004; Plaxton, 1996; Stitt, 1990). In animal and yeast cells, this step is catalyzed by ATP-dependent phosphofructokinase (PFK) and cytosolic fructose-1,6-bisphosphatase (cFBPase) in the glycolytic and gluconeogenic directions, respectively, which is controlled by a regulatory metabolite, fructose-2,6-bisphosphate (Fru-2,6-$P_2$) (Cseke et al., 1982; Hue and Rider, 1987; Van Schaftingen, 1987). In plants, in the place of PFK, PFP is allosterically activated by Fru-2,6-$P_2$ and participates in the regulation of carbon flux (Nielsen et al., 2004; Stitt, 1990).

Since PFP catalyzed reactions are readily reversible and exist at near equilibrium *in vivo* (Kubota and Ashihara, 1990; Stitt, 1990; Weiner et al., 1987), it is possible that this enzyme participates in glycolysis and gluconeogenesis as well as in pyrophosphate ($PP_i$) formation and removal (Nielsen et al., 2004; Stitt, 1990). Previous biochemical studies (ap Rees et al., 1985; Carnal and Black, 1989; Hatzfeld et al., 1989; Mertens et al., 1990; Nakamura et al., 1992; Smyth and Black, 1984; Theodorou et al., 1992) and studies of transgenic plants (Hajirezaei et al., 1994; Nielsen and Stitt, 2001; Paul et al., 1995) have provided evidence that the plant PFP enzyme indeed plays a role in glycolysis. Moreover, PFP uses $PP_i$ as its phosphoryl donor in place of ATP during the phosphorylation of Fru-6-P to Fru-1,6-$P_2$ (Plaxton, 1996; Stitt, 1990) and this consequently gives an energetic advantage to plants (Mertens et al., 1990; Plaxton, 1996).

To better understand the role of PFP during plant growth, transgenic *Arabidopsis* plants with increased or decreased *AtPFP* expression were generated and their growth phenotypes were examined. Our analysis of these transgenic plants demonstrates that altered PFP expression leads to the changes in the growth phenotypes. In the present study also, we analyzed global changes in gene expression in response to altered PFP expression.

## MATERIALS AND METHODS

### Plant materials and growth conditions

Sterilized seeds of *Arabidopsis* (*Arabidopsis thaliana* ecotype Columbia) were germinated on agar plates of Gamborg B5 and MS media, respectively, supplemented with 2% sucrose, in a

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growth chamber (23°C, 16 h light/8 h dark cycle, light intensity of 100 μmol $m^{-2}$ $s^{-1}$). After germination, seedlings were transferred to pots containing commercial potting mix, Sunshine mix 5 (Sungro, Canada), and further incubated in a growth chamber.

**Vector construction**

To generate *Arabidopsis* PFP overexpression transformants, full-length cDNAs of the *AtPFPα1* (At1g20950) and *AtPFPβ1* (At1g12000) genes were amplified from an *Arabidopsis* leaf cDNA library and subcloned into the pGEM-T Easy vector (Promega, USA). The primers used for PCR amplification were 5′-GGTACCATGGATTCAGATTTCGGAATC-3′ and 5′-GGATCCTCATTGACCACCATTTGAAGA-3′ for *AtPFPα1*, containing *Kpn*I and *Bam*HI restriction sites, and 5′-GGTACCATGGCCCCTGCTCTCGCCGTTAC-3′ and 5′-TCTAGATTATTGAGCCCCGAGTTCAAG-3′ for *AtPFPβ1*, containing *Kpn*I and *Xba*I sites. After sequence confirmation, full-length cDNAs of either *AtPFPα1* or *AtPFPβ1* were individually cloned into the plant expression vector pPZP211-Ex which was constructed by introducing the *CaMV35S* promoter and *nos* terminator (*TNOS*) into the pPZP211 plasmid (Hajdukiewicz et al., 1994) containing the kanamycin resistant selection marker (Fig. 2A).

To generate *AtPFP* RNAi constructs, partial cDNA fragments for *AtPFPα1* (bp 551-1050 in the cDNA) and *AtPFPβ1* (bp 627-1126 in the cDNA) were amplified with the primers 5′-GCTCTAGAGGCGCGCCTTGTTATCATTGGAGGCG-3′ and 5′-CGGGATCCATTTAAATTACACCCTGCCTCAGCA-3′ for *AtPFPα1*, and 5′-GCTCTAGAGGCGCGCCTGAAAACTTCAGGAGTAA-3′ and 5′-CGGGATCCATTTAAATTCAGGGATTGCTCGGTG-3′ for *AtPFPβ1*. The forward primers harbor *Xba*I and *Asc*I sites, and the reverse primers contain *Bam*HI and *Swa*I sites. The resulting DNA fragments of either *AtPFPα1* or *AtPFPβ1* were inserted in both the sense and antisense orientations into the RNAi vector pFGC5941 (obtained from the Arabidopsis Biological Resource Center, USA) that contains the herbicide BASTA resistant (*Bar*) gene selection marker (Fig. 2B).

***Arabidopsis* transformation and screening**

The *AtPFP* constructs were used for *Agrobacterium*-mediated *Arabidopsis* transformation by the floral dip method (Clough and Bent, 1998). Transgenic *Arabidopsis* plants harboring the overexpression constructs for *AtPFP* genes were screened on agar plates containing kanamycin (25 mg/l). To screen *AtPFP* RNAi transformants, 0.05% BASTA was sprayed onto 10 day-old plants followed by two additional sprays at 5 day intervals. T2 plants which showed 100% viability in the T3 generation were selected as homozygous transgenic lines and used for further studies.

**Semiquantitative RT-PCR analysis**

Total RNAs were isolated from different tissues and developmental stages of *Arabidopsis* using TRIzol Reagent (Invitrogen, USA). Single-strand cDNAs were then synthesized from total RNAs (2 μg) using a first Strand cDNA Synthesis kit for RT-PCR (AMV) (Roche, Switzerland) with an oligo-dT primer according to the manufacturer's instructions. The resulting first-strand cDNAs were used in subsequent PCR reactions with gene-specific primers and control primers for β-tubulin. The gene-specific primers used were 5′-ACACTCGAAGTCTCACACCAC-3′ and 5′-TCATTGACCACCATTTGAAGA-3′ for *AtPFPα1*, 5′-ATCTTCTCTGAGTCTCTCTGA-3′ and 5′-TTAAGCAAATTGCTGACCGCT-3′ for *AtPFPα2*, 5′-TAGTCTCTCGACTAACCACC-3′ and 5′-CCTAATAGACCCGCAAAGGAC-3′ for *AtPFPβ1*, and 5′-TAAAGAATTTTGGTACTGTGAG-3′ and 5′-TGTATGAAATTGCGAAACTTT-3′ for *AtPFPβ2*. Primers used for the amplification of β-tubulin (*TUB2*) as a loading control (Baek et al., 2008; Snustad et al., 1992) were 5′-CTCAAGAGGTTCTCAGCAGTA-3′ and 5′-TCACCTTCTTCA-TCCGCAGTT-3′. The PCR conditions were as follows: 5 min at 95°C; followed by 27 cycles of 95°C for 1 min, 56°C for 1 min, 72°C for 1 min, and a final incubation at 72°C for 7 min.

**RNA gel blot analysis**

Total RNA was isolated from the leaves of the wild type and transgenic plants using TRIzol reagent. Total RNAs (40 μg) were separated in a denaturing formaldehyde agarose gel and blotted onto a Hybond-$N^+$ membrane (Amersham Biosciences, USA). The membranes were then hybridized with [α-$^{32}$P]-dCTP labeled probes in a hybridization solution containing 1% (w/v) BSA, 7% (w/v) SDS, 25 mM sodium phosphate (pH 7.2) and 1 mM EDTA at 65°C overnight. Full length cDNAs of *AtPFPα1* and *AtPFPβ1* were used as probes. After hybridization, the membranes were analyzed using a Typhoon 9210 phosphoimager (Amersham Biosciences, USA).

**PFP assay**

PFP activities of wild type and transgenic *Arabidopsis* in the glycolytic direction were determined by monitoring the formation of $NAD^+$ according to a previously described method (Hatzfeld et al., 1990). Briefly, crude proteins were extracted from the leaves of 24 day-old *Arabidopsis* plants. The formation of $NAD^+$ was then measured by the absorbance change at 340 nm using a CARY 300 Bio UV/Vis spectrophotometer (Varian, USA). One unit of PFP activity was defined as the formation of 1 μmol $NAD^+$ per minute. The results are shown as the mean ± SE from five individual plants.

**Extraction and measurement of metabolites**

Metabolites, including sucrose, glucose, fructose and starch, were extracted from 24 day-old *Arabidopsis* leaves by the method of Lu and Sharkey (2004) with minor modifications. Briefly, *Arabidopsis* leaves (0.2-0.3 g) were ground in liquid nitrogen until they formed a fine powder. To these pulverized tissues, 1 ml of killing solution (80% ethanol, 20% formic acid) was then added and the mixture was incubated at 80°C for 20 min. After centrifugation, the supernatants were evaporated in a concentrator (Eppendorf 5301, Eppendorf, Germany). The resulting residues were dissolved in 500 μl of $H_2O$ and then used to measure soluble sugar contents. To analyze the starch contents, the pellets from the previous centrifugation step were resuspended in 500 μl of water and autoclaved at 121°C for 3 h. An aliquot (50 μl) of these suspensions was adjusted to 20 mM Na-acetate (pH 4.8) and hydrolyzed with 30 U α-amylase and 25 U amyloglucosidase at 37°C overnight (Walters et al., 2004). The resulting hydrolyzates were then assayed to determine the starch contents. The carbohydrate and starch contents in the leaves of wild type and transgenic *Arabidopsis* were assayed enzymatically as described by Stitt et al. (1989) using a CARY 300 Bio UV/Vis spectrophotometer.

For the measurement of PPi contents, *Arabidopsis* leaves (0.1 g) were extracted in trichloroacetic acid using a method described by Weiner et al. (1987). The PPi contents in the extracts were then measured with pyrophosphate reagent (Sigma-Aldrich Co., USA) according to the manufacturer's protocol. Fru-2,6-$P_2$ was extracted using a a method described previously by Van Schaftingen et al. (1982). The Fru-2,6-$P_2$ levels were determined via stimulation with potato PFP. Each reaction mixture contained 2 mM Mg-acetate, 1 mM Fru-6-P, 0.15 mM NADH, 0.01 U potato PFP, 2 U aldolase, 4 U triosephosphate isomerase, 2 U glycerol-3-phosphate dehydrogenase and 75

mM Hepes buffer (pH 7.5) in a total volume of 1 ml. After 5 min preincubation, the reactions were initiated by adding 0.5 mM PPi and the absorbance changes were measured at 340 nm using a CARY 300 Bio UV/Vis spectrophotometer. Potato PFP, aldolase, triosephosphate isomerase and glycerol-3-phosphate dehydrogenase were purchased from Sigma-Aldrich Co.

**Gas-exchange measurements**

The rate of $CO_2$ assimilation under various intercellular $CO_2$ concentrations ($C_i$) and light intensities was measured with a LI-6400 portable photosynthesis measurement system (Li-Cor, USA). $CO_2$ was supplied by an automatic injector, after which the intercellular $CO_2$ concentrations and the rates of $CO_2$ assimilation were calculated as described by von Caemmerer and Farquhar (1981). Light was supplied from an LED source attached to the leaf chamber. The gas humidity was sustained at approximately 10-30% and the leaf temperature was maintained in the range of 22-24°C. All photosynthesis measurements were made on at least five plants of each mutant line.

**Microarray analysis**

For microarray analysis, total RNAs were isolated from the leaves of the wild type and transgenic plants and treated with a clean-up procedure using RNeasy Plant Mini Kit (Qiagen, USA). Microarray analyses of three biological replicates for each wild type and transgenic plant were performed by the NASC International Affymetrix Service (http://affymetrix.Arabidopsis.info) using the Arabidopsis ATH1 Affymetrix GeneChip. Normalized microarray data were obtained from the NASC Affymetrix facility and genes with a detection call 'present' were used for the expression analysis. Among these genes, those with a significant ($P < 0.05$, *t*-test) expression change of more than two-fold in the transgenic plants were defined as responsive. The responsive genes were functionally categorized using the GO terms at the TAIR (http://www.Arabidopsis.org/index.jsp).

## RESULTS

**Molecular characterization of the *AtPFPα* and *AtPFPβ* genes in *Arabidopsis***

Plant PFPs are primarily hetero-oligomers of two related α and β subunits (Kowalczyk, 1987; Kruger and Dennis, 1987; Nielsen, 1994; Stitt, 1990; Theodorou and Plaxton, 1996; Turner and Plaxton, 2003; Wong et al., 1990; Yan and Tao, 1984). The α-subunit probably exerts a regulatory function and the β-subunit is thought to be the catalytic subunit (Carlisle et al., 1990; Theodorou et al., 1992). In *Arabidopsis*, two PFP genes (*At1g20950* and *At1g76550* designated as *AtPFPα1* and *AtPFPα2*, respectively) encoding α-subunits and two β-subunit genes (*At1g12000* and *At4g04040* denoted *AtPFPβ1* and *AtPFPβ2*, respectively) have been reported (Nielsen et al., 2004). *AtPFPα1* comprises 19 exons in a similar manner to the *PFPα* gene from caster bean (Todd et al., 1995), whereas *AtPFPα2* is composed of 18 exons. The genes for both of the smaller AtPFP β-subunits contain 16 exons which is also equivalent to the caster bean (Todd et al., 1995). The alignment of the deduced amino acid sequences for each subunit of AtPFP with the corresponding amino acid sequences of known plant PFPs from potato (Carlisle et al., 1990) and castor bean (Todd et al., 1995) indicates that plant PFPs are well conserved among a variety of species, showing a high homology between 82 and 90% identity (90-94% similarity) within the α-subunits and 76 to 84% identity (84-89% similarity) among the β-subunits (data not shown).

The expression patterns of *AtPFP* genes in different tissues and at various developmental stages in *Arabidopsis* were examined by semiquantitative RT-PCR. *AtPFPα1* is expressed in leaves, flowers and roots, and *AtPFPα2* is also expressed in all of the tissues examined to a lesser extent (Fig. 1A). The *AtPFPβ1* and *AtPFPβ2* transcripts were detected in all tissues examined, with a high level of expression found for *AtPFPβ2* in the leaf tissue (Fig. 1A). On the other hand, the *AtPFP* genes, with the exception of *AtPFPβ2*, were observed to be consistently expressed throughout all of the developmental stages of *Arabidopsis* without any significant differences (Fig. 1B). *AtPFPβ2* is highly expressed during the early stages of development and shows its highest transcript levels in 15-day old seedlings (Fig. 1B).

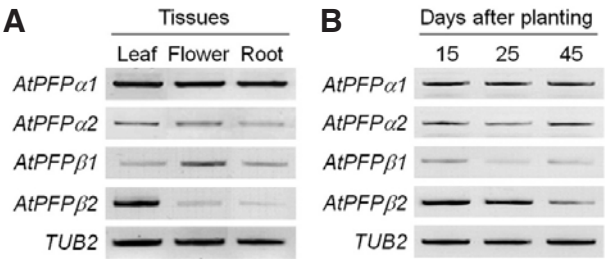


**Fig. 1.** Semiquantitative RT-PCR analysis of *AtPFP* expression in different tissues and at different developmental stages in wild type *Arabidopsis*. (A) Expression of *AtPFP* genes in the leaves, flowers and roots of 30 day-old *Arabidopsis* plants. *AtPFPα1* and *AtPFPα2* are expressed in all of the tissues examined with *AtPFPα2* detected at low levels and *AtPFPβ2* particularly evident in leaves. (B) Expression levels of *AtPFP* genes in both the seedlings (15 day-old) and leaves (25 and 45 day-old) of *Arabidopsis* plants. *AtPFPα1, AtPFPα2* and *AtPFPβ1* appeared to be expressed constitutively throughout each developmental stage, whereas *AtPFPβ2* is highly expressed at an early stage of development. *TUB2* is shown as a control.

**Generation and characterization of transgenic *Arabidopsis* plants that overexpress or repress their AtPFP genes**

Transgenic *Arabidopsis* plants that overexpress *AtPFP*s were generated by the introduction of constructs harboring the full length cDNAs of either *AtPFPα1* or *AtPFPβ1* under the control of the *CaMV35S* promoter (Fig. 2A). Among the homozygous overexpression transgenic plant lines that highly express either exogenous *AtPFPα1* or *AtPFPβ1*, two *AtPFPα1* overexpression lines (α1-7 and α1-19) and two *AtPFPβ1* overexpression lines (β1-5 and β1-16) were chosen for further analysis (Fig. 2C).

To generate transgenic *Arabidopsis* lines with reduced *AtPFP* expression, RNAi constructs targeting either *AtPFPα1* or *AtPFPβ1* (Fig. 2B) were transformed into *Arabidopsis* plants. We subsequently performed RNA gel blot analysis which revealed that several homozygous plants for each RNAi line showed no detectable levels of the corresponding genes (Fig. 2D). Of these, two *AtPFPα1*- (α1i-3 and α1i-10) and two *AtPFPβ1*-RNAi lines (β1i-21 and β1i-22) were selected for further analysis (Fig. 2D).

The PFP activity in the leaves of transgenic plants was next measured in the glycolytic direction. Consistent with the increased expression of *AtPFPβ1* (Fig. 2C), the PFP activity in the β1-5 and β1-16 transgenic lines was increased compared with wild type plants (Fig. 2E). In the α1-7 and α1-19 lines, the overexpression of the regulatory α subunit also led to increased PFP activity relative to wild type plants (Fig. 2E). Although the PFPs from many plants are composed of two different subunits,

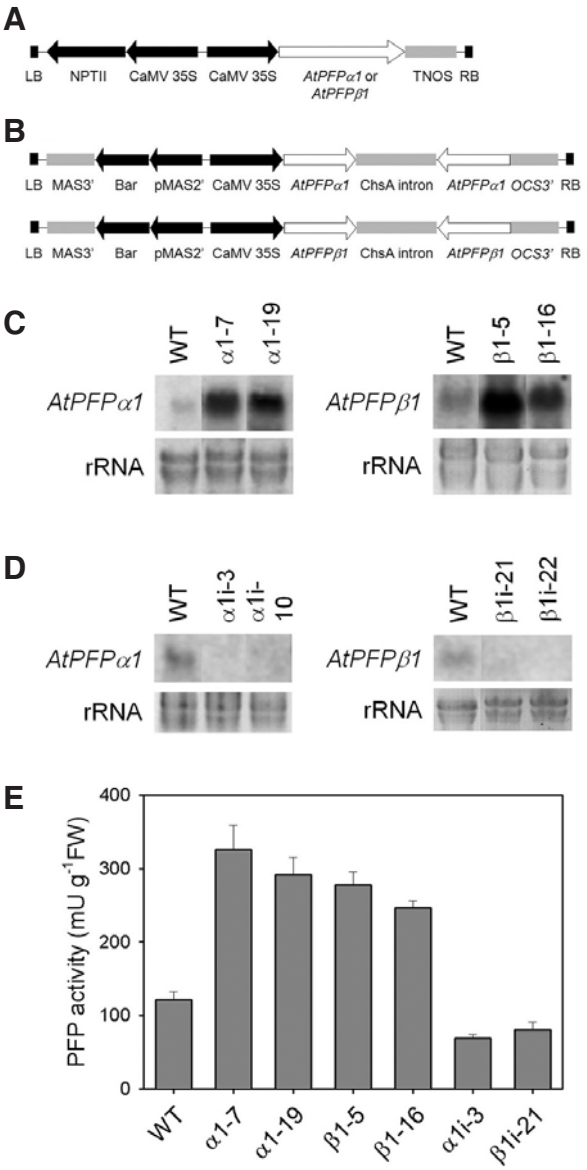


**Fig. 2.** Vector construction and characterization of the transgenic plants used in this study. (A) Constructs used for the overexpression of either *AtPFPα1* or *AtPFPβ1* in *Arabidopsis*, for which each full length cDNA was cloned into the plant expression vector pPZP211-Ex harboring a kanamycin resistance gene (NPTII). (B) For transgenic *Arabidopsis* with reduced *AtPFP* expression, the cDNA fragments for *AtPFPα1* (bp 551-1050) or *AtPFPβ1* (bp 627-1126) were inserted in both the sense and antisense orientations into the RNAi vector pFGC5941 (obtained from the Arabidopsis Biological Resource Center) which contains the herbicide BASTA resistant gene (*Bar*) as a selection marker. (C) The transgenic lines α1-7 and α1-19 harboring the *AtPFPα1* overexpression construct, and β1-5 and β1-16 containing the *AtPFPβ1* plasmid, show high levels of transgene expression. (D) In the RNAi lines for *AtPFPα1* (α1i-3 and α1i-10) and *AtPFPβ1* (β1i-21 and β1i-22), expression of the endogenous target genes is not detectable, whereas the wild type (WT) controls express these genes. Total RNAs were extracted from the leaves of each transgenic plant. EtBr-stained rRNA is shown as a RNA loading control. (E) PFP activities in the transgenic *Arabidopsis* lines were measured in the glycolytic direction. The results are given as the mean of five individual plants ± SE.

and typically form heterotetramers ($\alpha_2\beta_2$), several studies have demonstrated that they can be present in different molecular forms in plants (Nielsen, 1994; Theodorou and Plaxton, 1996; Theodorou et al., 1992; Turner and Plaxton, 2003; Wong et al., 1990; Yan and Tao, 1984). In some plant tissues, such as wheat seedlings, tomato fruit and black mustard suspension cells, PFPs comprising only the β-subunit are present (Theodorou et al., 1992; Wong et al., 1990; Yan and Tao, 1984). It has also been reported in black mustard suspension cells that an elevated PFP activity is coincident with an increased α/β ratio by $P_i$ starvation-inducible synthesis of the PFP α-subunit (Theodorou et al., 1992). Hence, although the conformation of *Arabidopsis* PFP has not been determined yet, it is likely that the enhancement of PFP activity in the α1-7 and α1-19 lines is due to an increase in the ratio of α-subunits in the PFP tetramers. This possibility is further supported by the evident decreases in PFP activity in the *AtPFPα1*-RNAi lines (Fig. 2E). The PFP activities in both α1i-3 and β1i-21 plants were decreased to 0.57 and 0.67 fold, respectively, relative to wild type plants (Fig. 2E).

The growth phenotypes of our transgenic *Arabidopsis* lines were carefully examined and it was observed that the altered expression of the *AtPFP* subunits affects their growth. Phenotypic analyses revealed that not only the *AtPFPβ1* overexpression lines (β1-5 and β1-16) but also the *AtPFPα1* regulatory subunit overexpression lines (α1-7 and α1-19) have a slightly higher growth rate than wild type plants (Fig. 3). The rosette diameters of these overexpression lines were also larger than those of the wild type plants (Figs. 3A and 3C). The fresh weights of the overexpression lines were also increased slightly compared with wild type plants (Fig. 3B). In contrast to the *PFP* overexpressing transgenic plants, the RNAi transgenic lines of *AtPFPα1* or *AtPFPβ1* showed the significant growth retardation and much smaller rosette leaves (Figs. 3A and 3C). The RNAi lines further displayed a significant decrease in their fresh weights compared with wild type plants (Fig. 3B). The transgenic PFP *Arabidopsis* plants also showed phenotypic differences in terms of their stem. Consistent with their vegetative growth phenotypes, the overexpression lines showed early onset of primary stems, whereas the RNAi mutant plants displayed delayed bolting compared with wild type plants (Fig. 3D).

**Photosynthetic activities and metabolite levels in the transgenic plants**

The rates of $CO_2$ assimilation in the mature rosette leaves of the transgenic *Arabidopsis* plants were measured under different light intensities to examine the effects of altered PFP expression upon photosynthetic activities. At an ambient $CO_2$ concentration (400 μmol $mol^{-1}$), the overexpression lines displayed photosynthetic rates that were comparable with those of wild type plants under both low (100 μmol $m^{-2}s^{-1}$) and high (500 μmol $m^{-2}s^{-1}$) light intensities (Fig. 4). The $CO_2$ assimilation rates, however, were significantly decreased in all PFP RNAi lines compared with wild type plants under both light conditions (Fig. 4). This indicates that the photosynthetic activities in the RNAi lines are limited by the decreased expression of the *AtPFP*s.

The levels of carbohydrates, such as sucrose, glucose, fructose and starch, in the leaves of our transgenic *Arabidopsis* plants were analyzed to examine the effects of altered PFP activity on carbon partitioning. Our analysis of the carbohydrate levels revealed a slight decrease in the sucrose levels in the α1-19, β1-16, α1i-3, α1i-10, β1i-21 and β1i-22 lines at the end of day (Fig. 5A), and a minor increase in the glucose levels in the overexpression lines (Fig. 5B). Nonetheless, a clear positive correlation between the altered expression of PFP subunits and

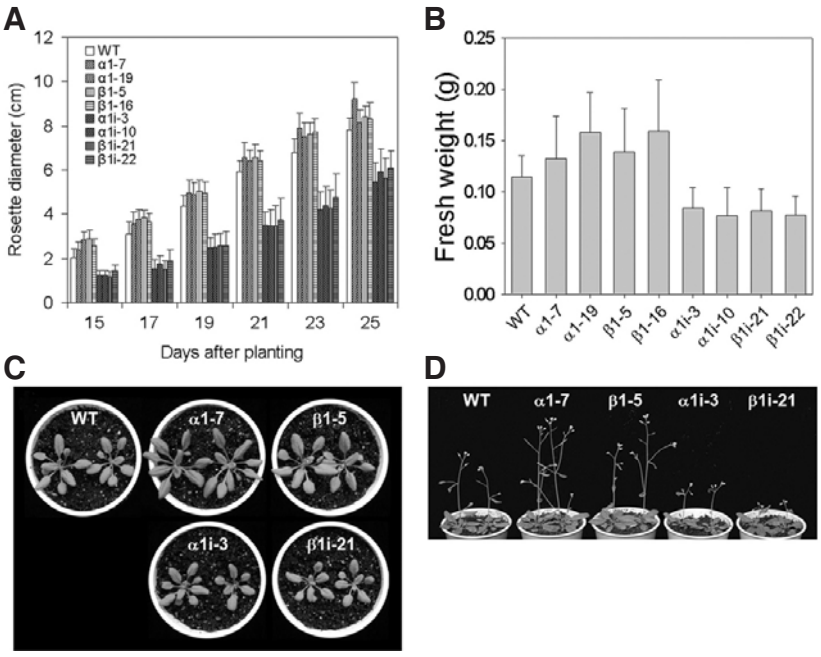


**Fig. 3.** Growth phenotype analysis and characterization of the transgenic *Arabidopsis* plants used in this study. (A) The growth of the transgenic plants was examined by measuring the rosette diameters during the vegetative growth period with a two day interval. The results shown are the mean rosette diameters ± SE from 24 individual plants for wild type and each transgenic line. The overexpression lines display slight growth enhancement, whereas the RNAi lines show significantly ($P < 0.05$, *t*-test) stunted growth compared with the wild type (WT) plants. (B) Fresh weights of 25-day old transgenic plants. The fresh weights of the overexpression lines were found to have increased, whereas those of all RNAi lines were decreased compared with the wild type plants. The results shown are mean fresh weight of the aerial parts ± SE from at least 24 individual plants for wild type and each transgenic line. (C) Representative growth phenotypes of the transgenic plants at 3 weeks after planting. (D) Growth phenotypes of the transgenic plants at 4 weeks after planting. Stem development and growth were also found to be enhanced in the overexpression lines but delayed in the RNAi lines.

the levels of carbohydrates examined was not evident. These results suggest that the altered expression and activity of PFP does not lead to detectable changes in the carbon partitioning profile in the leaves of the transgenic *Arabidopsis* plants. Consistently, severe decreases in the expression levels and activity of PFP have been previously shown to result in only minor changes in carbon partitioning in both transgenic potato and tobacco plants (Hajirezaei et al., 1994; Nielsen and Stitt, 2001).

Since PFP can participate in PPi formation and removal and is also activated by Fru-2,6-$P_2$ (Nielsen et al., 2004; Stitt, 1990), we examined the effects of altered *AtPFP* expression upon the PPi and Fru-2,6-$P_2$ contents in the leaves of the transgenic plants. The PPi contents were found to be slightly decreased in the leaves of all overexpression lines (Fig. 6A). However, we observed no correlation between the PPi levels and reduced *AtPFP* expression in the RNAi lines (Fig. 6A).

The levels of the regulatory metabolites, Fru-2,6-$P_2$, were also measured in the leaves of the transgenic *Arabidopsis* plants (Fig. 6B). The overexpression lines were found to similar Fru-2,6-$P_2$ levels to wild type plants. However, Fru-2,6-$P_2$ was increased in the leaves of most of the RNAi lines, except for β1i-22. This is quite consistent with previous results reported for transgenic tobacco plants (Nielson and Stitt, 2001; Paul et al., 1995).

**Altered *PFP* expression results in transcriptomic changes**

To examine the effects of altered PFP expression upon global gene expression, microarray analyses were performed using the transgenic lines, β1-5 and β1i-21. These experiments demonstrated that the expression levels of some genes change in response to the altered expression of *AtPFPβ1*. The genes significantly ($P < 0.05$) up- or down-regulated by more than 2-fold in the β1-5 and β1i-21 plants are listed in Tables 1 and 2, respectively. In the *AtPFPβ1* overexpression line β1-5, seven genes are induced and 13 genes are down-regulated (Table 1). Among the seven up-regulated genes, *At1g08940* (phosphoglycerate/bisphosphoglycerate mutase family protein) and

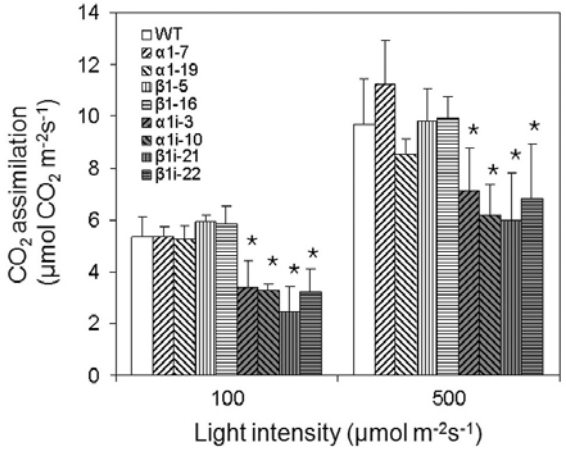


**Fig. 4.** Photosynthetic activities in the leaves of transgenic *Arabidopsis* plants with either increased or decreased expression of *AtPFP*s. The rate of $CO_2$ assimilation under different light conditions at an intercellular $CO_2$ concentration ($C_i$) of 400 μmol $mol^{-1}$ was measured. The photosynthetic rates of the RNAi lines were found to have significantly decreased compared with wild type plants. Asterisks indicate significant ($p < 0.05$, *t*-test) differences between the transgenic and wild type plants. The results shown are the mean ± SE of at least five plants for wild type and each transgenic line.

*At5g63580* (similar to flavonol synthase) are categorized via GeneOntology (GO) as 'other metabolic processes' by TAIR. *At4g33790* (acyl CoA reductase, putative) and *At5g65080* (encodes MADS-domain protein) are classified in this way as 'developmental processes'. A large portion of the down-regulated genes are also categorized by GO into 'other metabolic processes' (*At1g06100*, *At1g65470*, *At3g02820* and *At5g24240*) and 'cell organization and biogenesis' (*At1g65470*, *At5g47500* and *At5g5600*).

**Table 1.** Genes found to be significantly ($P < 0.05$) up- or down-regulated by > 2-fold in the *AtPFPβ1* overexpression line, β1-5

| Gene name | Description | Fold-change[a] | p-value |
|---|---|---|---|
| At5g63580 | Similar to flavonol synthase | 2.0207 | 0.0006 |
| At4g33790 | Acyl CoA reductase, putative | 2.7231 | 0.0015 |
| At1g43590 | Similar to unknown protein | 7.3715 | 0.0020 |
| At5g04950 | Nicotianamine synthase 1 | 2.4328 | 0.0149 |
| At5g65080 | Encodes MADS-domain protein. | 2.1627 | 0.0335 |
| At3g23550 | Similar to ALF5 (Aberrant lateral root formation 5) | 2.7524 | 0.0398 |
| At1g08940 | Similar to phosphoglycerate/bisphosphoglycerate mutase family protein | 3.2483 | 0.0436 |
| At5g66230 | Similar to sugar transporter superfamily | 0.3803 | 0.0031 |
| At1g65470 | Chromatin Assembly Factor-1 (CAF-1) p150 subunit | 0.4789 | 0.0057 |
| At5g50740 | Similar to ATFP3 (*Arabidopsis thaliana* farnesylated protein 3) | 0.4989 | 0.0076 |
| At5g47500 | Similar to pectinesterase family protein | 0.4954 | 0.0078 |
| At5g51600 | Mutant has defective roots. | 0.4353 | 0.0090 |
| At1g06100 | Delta-9 desaturase-like 2 protein | 0.4821 | 0.0106 |
| At4g13750 | Similar to ATP binding/DNA binding | 0.4860 | 0.0124 |
| At5g55720 | Putative pectate lyase 21 precursor | 0.3532 | 0.0133 |
| At5g17980 | Similar to C2 domain-containing protein | 0.4747 | 0.0161 |
| At2g22270 | Similar to unknown protein | 0.4602 | 0.0186 |
| At5g15780 | Similar to CWLP (Cell wall-plasma membrane linker protein) | 0.4038 | 0.0269 |
| At3g02820 | Similar to Zinc finger, CCHC-type | 0.4334 | 0.0302 |
| At5g24240 | Similar to phosphatidylinositol 3- and 4-kinase family protein | 0.0170 | 0.0329 |

[a]fold-change between the wild type and transgenic plants

**Table 2.** Genes found to be significantly ($P < 0.05$) up- or down-regulated by > 2-fold in the *AtPFPβ1* RNAi line, β1i-21

| Gene name | Description | Fold-change[a] | p-value |
|---|---|---|---|
| At4g05330 | A member of ARF GAP domain (AGD) | 2.0141 | 0.0079 |
| At1g26920 | Similar to unknown protein | 2.5329 | 0.0167 |
| At2g22980 | Similar to SNG1 (SINAPOYLGLUCOSE 1), serine carboxypeptidase | 2.1916 | 0.0431 |
| At4g19550 | Similar to RNA polymerase II transcription factor | 2.4394 | 0.0435 |
| At5g59670 | Similar to leucine-rich repeat protein kinase, putative | 0.4911 | 0.0001 |
| At1g33560 | Encodes a NBS-LRR disease resistance protein | 0.3121 | 0.0010 |
| At5g11650 | Similar to hydrolase, alpha/beta fold family protein | 0.3139 | 0.0048 |
| At5g15780 | Similar to CWLP (Cell wall-plasma membrane linker protein) | 0.2744 | 0.0059 |
| At1g48000 | Encodes a putative transcription factor (MYB112) | 0.4945 | 0.0073 |
| At5g10380 | RING-H2 finger protein ATL5F | 0.4872 | 0.0138 |
| At1g51660 | Encodes a mitogen-activated map kinase kinase | 0.3782 | 0.0146 |
| At1g72260 | Encodes a thionin | 0.3533 | 0.0219 |
| At1g78410 | Similar to Avr9/Cf-9 rapidly elicited protein 169 | 0.3825 | 0.0224 |
| At5g48540 | Similar to receptor protein kinase-related | 0.3775 | 0.0290 |
| At5g24240 | Similar to phosphatidylinositol 3- and 4-kinase family protein | 0.0120 | 0.0349 |
| At1g21120 | Similar to O-methyltransferase, putative | 0.3962 | 0.0375 |
| At2g37970 | Similar to SOUL heme-binding family protein | 0.4567 | 0.0384 |
| At1g06310 | Encodes a putative acyl-CoA oxidase | 0.4602 | 0.0422 |
| At3g22231 | Encodes a member of a novel 6 member *Arabidopsis* gene family | 0.4483 | 0.0432 |
| At2g47180 | Similar to ATGOLS2 (*Arabidopsis thaliana* galactinol synthase 2) | 0.4818 | 0.0484 |
| At5g46080 | Similar to ACR4 (*Arabidopsis* CRINKLY4) | 0.4584 | 0.0487 |
| At3g16530 | Lectin like protein | 0.4921 | 0.0498 |

[a]fold-change between the wild type and transgenic plants

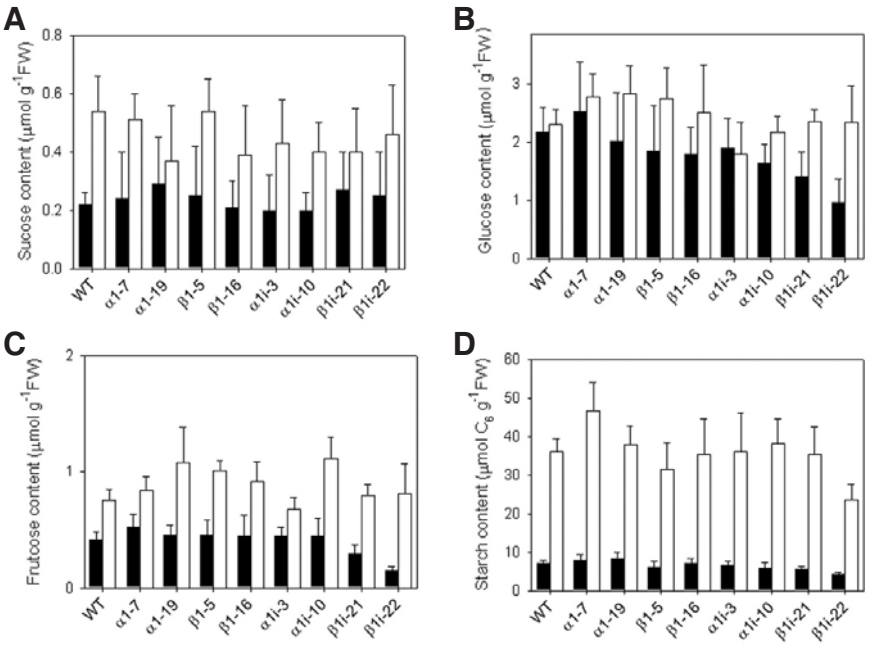


**Fig. 5.** Analysis of the carbohydrate contents in the leaves of both wild type and *AtPFP* transgenic *Arabidopsis* lines at the end of day (white bar) and at the end of night (black bar). (A) Sucrose, (B) glucose, (C) fructose, and (D) starch. The results shown are the mean ± SE of nine different measurements.

The reduced *AtPFPβ1* expression in the β1i-21 plants led to the down-regulation of 18 genes and the induction of only four genes (Table 2). The majority of the down-regulated genes are categorized by GO into 'other metabolic processes' (*At1g06310*, *At1g48000*, *At2g37970*, *At2g47180*, *At5g24240*, *At5g46080* and *At5g59670*), 'response to abiotic stress or biotic stimulus' (*At1g33560*, *At1g48000*, *At1g51660*, *At2g37970*, *At2g47180* and *At3g22231*) and 'response to stress' (*At1g33560*, *At1g 48000*, *At1g51660*, *At1g72260* and *At1g47180*). In contrast to β1-5, several genes involved in signal transduction were induced (*At4g05330*) or down-regulated (*At1g51660*, *At1g72260* and *At2g37970*) in the β1i-21 line.

These microarray data indicate that the altered expression of AtPFPs cause the transcriptomic changes in a substantial number of genes involved in various physiological processes in *Arabidopsis*. This suggests the possibility that PFPs participate in plant growth through the coordination with these responsive genes.

## DISCUSSION

PFP is a characteristic enzyme that in plants can be involved in both glycolysis and gluconeogenesis through its catalysis of the reversible interconversion between Fru-6-P and Fru-1,6-$P_2$ (Nielsen et al., 2004; Stitt, 1990). In plants, PFP activity occurs in different tissues and at various developmental stages (ap Rees et al., 1985; Carnal and Black, 1989; Cseke et al., 1982; Dennis and Greyson, 1987; Hatzfeld et al., 1989; Kowalczyk, 1987; Kruger and Dennis, 1987; Mertens et al., 1990; Nakamura et al., 1992; Nielsen, 1994; Theodorou and Plaxton, 1996; Theodorou et al., 1992; Turner and Plaxton, 2003; Wong et al., 1990; Yan and Tao, 1984), but is at its highest levels in young and actively growing tissues (Dennis and Greyson, 1987). In our present expression analysis of PFP in *Arabidopsis*, we demonstrate that the *AtPFP* genes are expressed in leaves and in other heterotrophic tissues, such as the flowers and roots, and are also detectable at all the developmental stages examined (Fig. 1). This suggests that PFP likely plays a role during growth and development from the early vegetative to reproductive stages of development in *Arabidopsis*.

In our current analyses of PFP transgenic *Arabidopsis* plants, we find that altered *AtPFP* expression correlates with the growth phenotypes (Figs. 2 and 3). Under normal growth conditions, only minor changes are evident in the photosynthetic activity (Fig. 4) and in the apparent levels of carbohydrates (Fig. 5) in the leaves of the transgenic plants overexpressing *AtPFP*s. Thus, we assume that the slight growth enhancement of the overexpression lines is in part likely due to the different usage of $PP_i$ by altered PFP activities (Fig. 2E). This possibility is supported by the decreased PPi contents in these overexpression lines (Fig. 6A). For the phosphorylation of Fru-6-P, PFP utilizes $PP_i$ a by-product of biosynthetic reactions as its phosphoryl donor instead of ATP (Plaxton, 1996; Stitt, 1990). Transgenic plants expressing *E. coli* pyrophosphatase also demonstrate that reduced $PP_i$ levels result in decreased ATP levels (Mustroph et al., 2005). The role of $PP_i$ as an energy donor has also been demonstrated in earlier studies of anaerobic microorganisms such as *Propionibacterium shermanii* and *Entamoeba histolytica* (Wood, 1985). In these organisms, PFP is responsible for the conversion of Fru-6-P to Fru-1,6-$P_2$. Owing to the use of $PP_i$ as a phosphoryl donor, these organisms derive considerable energetic advantages from PFP.

In contrast to the proposed role of PFP in carbohydrate metabolism, studies of transgenic potato (Hajirezaei et al., 1994), tobacco (Nielsen and Stitt, 2001; Paul et al., 1995) and sugarcane (Groenewald and Botha, 2008) plants with reduced PFP expression reported no visible growth phenotypes, which is not consistent with our present finding for PFP RNAi *Arabidopsis* lines that show retarded growth (Fig. 3). It has been reported that Fru-2,6-$P_2$ activates PFPs and is involved in carbon partitioning in plants (Lee et al., 2006; Nielsen et al., 2004; Park et al., 2007; Stitt, 1990). In transgenic tobacco plants with reduced PFP activities, the contents of Fru-2,6-$P_2$ in the actively growing tissues and source leaves were shown previously to be dramatically increased by more than 3-fold (Nielsen and Stitt, 2001; Paul et al., 1995). The Fru-2,6-$P_2$ contents were, however, only marginally increased (1.3 to 2-fold) or unaltered in the leaves of our PFP RNAi lines (Fig. 6B), suggesting that in

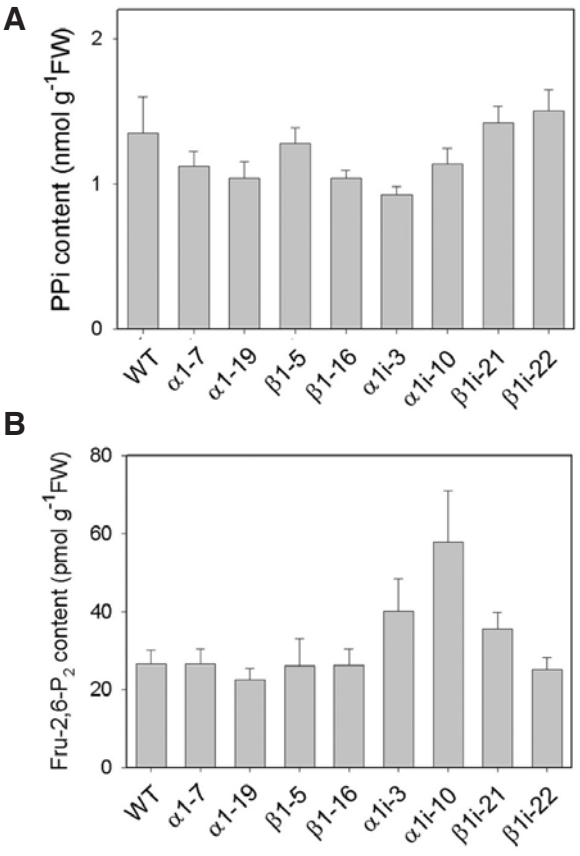


**Fig. 6.** Analysis of the PPi (A) and Fru-2,6-$P_2$ (B) contents in the leaves of *AtPFP* transgenic *Arabidopsis* lines and wild type plants in the middle of day. The results shown are the mean ± SE of three different measurements.

*Arabidopsis*, the reduced PFP activities are not fully compensated by increased Fru-2,6-$P_2$ levels. In addition, almost no activity changes of PFK and cFBPase that are involved in the same step as PFP were observed in our transgenic lines (data not shown). Hence, our current results indicate that the discrepancy between transgenic tobacco and *Arabidopsis* plants is possibly due to species difference.

The growth retardation of the transgenic lines in early growth period suggests that PFP plays an important role in actively growing plants. It has been reported that PFP is highly active in both heterotropic tissues (ap Rees et al., 1985; Nakamura et al., 1992) and actively growing tissues (Mertens et al., 1990; Nielsen, 1994). In addition, the rate of photosynthetic $CO_2$ assimilation in our current RNAi transgenic lines was found to be significantly reduced under ambient and high light conditions (Fig. 4). These observations suggest that PFP is likely to be involved in various developmental processes in addition to carbohydrate metabolism in source tissues. On the other hand, our microarray analyses demonstrate that altered *AtPFP* expression leads to changes in the transcript levels of some genes in the transgenic lines. The genes we found to be responsive to altered *AtPFP* expression have been reported previously to be involved in physiological processes such as flowering [At5g65080 (Ratcliffe et al., 2003)], cuticular wax formation [At4g33790 (Rowland et al., 2006)], disease resistance [At1g33560 (Grant et al., 2003)], and drought tolerance [At2g47180 (Taji et al., 2002)]. Hence, we speculate from our current data that PFP is possibly involved in various cellular processes in addition to carbon metabolism. To further understand the roles of PFPs in plant growth and development, the relationships between *AtPFP*s and these responsive genes will need to be further elucidated.

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