A Bifunctional Enzyme with L-Fucokinase and GDP-L-Fucose Pyrophosphorylase Activities Salvages Free L-Fucose in *Arabidopsis*

Toshihisa Kotake[a]*, Sachiko Hojo[a], Noriaki Tajima[b], Koji Matsuoka[c], Tetsuo Koyama[c], and Yoichi Tsumuraya[a]

From [a] Division of Life Science, Graduate School of Science and Engineering, and [b] Department of Biochemistry and Molecular Biology, Faculty of Science, [c]Division of Material Science, Graduate School of Science and Engineering, Saitama University, 255 Shimo-okubo, Sakura-ku, Saitama 338-8570, Japan.

Running head: Bifunctional L-Fucokinase/GDP-L-Fucose Pyrophosphorylase

*Address correspondence to: Toshihisa Kotake, Division of Life Science, Graduate School of Science and Engineering, Saitama University, 255 Shimo-okubo, Sakura-ku, Saitama 338-8570, Japan. Fax: +81-48-858-3384; E-mail: kotake@molbiol.saitama-u.ac.jp.

Monomeric sugars generated during the metabolism of polysaccharides, glycoproteins, and glycolipids are imported to the cytoplasm and converted to respective nucleotide sugars via monosaccharide 1-phosphates, to be reutilized as activated sugars. Since L-fucose (L-Fuc) is activated mainly in the form of GDP derivatives in seed plants, the salvage reactions for L-Fuc are expected to be independent from those for Glc, Gal, L-arabinose, and glucuronic acid, which are activated as UDP-sugars. For this study we have identified, in the genomic database of *Arabidopsis*, the gene (designated *AtFKGP*) of a bifunctional enzyme with similarity to both L-fucokinase and GDP-L-Fuc pyrophosphorylase. Recombinant AtFKGP (rAtFKGP) expressed in *Escherichia coli* showed both L-fucokinase and GDP-L-Fuc pyrophosphorylase activities, generating GDP-L-Fuc from L-Fuc, ATP, and GTP as the starting substrates. Point mutations in rAtFKGPs at either Gly^{133} or Gly^{830} caused loss of GDP-L-Fuc pyrophosphorylase and L-fucokinase activity, respectively. The apparent K_m values of L-fucokinase activity of rAtFKGP for L-Fuc and ATP were 1.0 and 0.45 mM, respectively, and those of GDP-L-Fuc pyrophosphorylase activity for L-Fuc 1-phosphate and GTP were 0.052 and 0.17 mM, respectively. The expression of *AtFKGP* was detected in most cell types of *Arabidopsis*, indicating that salvage reactions for free L-Fuc catalyzed by AtFKGP occur ubiquitously in *Arabidopsis*.

Loss-of-function mutants with T-DNA insertion in *AtFKGP* exhibited higher accumulation of free L-Fuc in the soluble fraction than the wild-type plant. These results indicate that AtFKGP is a bifunctional enzyme with L-fucokinase and GDP-L-Fuc pyrophosphorylase activities, which salvages free L-Fuc in *Arabidopsis*.

INTRODUCTION

The monosaccharide L-fucose (L-Fuc) is found as a constituent of cell wall polysaccharides and sugar moieties of glycoproteins and has important physiological functions in seed plants. L-Fucosyl residues, for example, occur as non-reducing terminal residues attached through α-(1→2)-linkages to penultimate sugar residues in xyloglucan and arabinogalactan-protein (1, 2). The L-fucosylated trisaccharide side chains of xyloglucan modulate the interaction of xyloglucan with cellulose microfibrils, thereby affecting the mechanical properties of plant cell walls (3, 4), while some *N*-glycans of plant glycoproteins have L-fucosyl residues attached through α-(1→3)-linkages to the proximal GlcNAc residues, which are characteristic of plants but not found in mammals and are responsible for the immunogenicity of plant-glycoproteins in mammals (5). L-Fucosyl residues are also attached through α-(1→4)-linkages to L-rhamnosyl residues in pectic rhamnogalacturonan II (RG-II) in seed plants (6). These L-fucosyl residues are transferred onto the glycoconjugates by actions of respective L-fucosyltransferases, which use GDP-L-Fuc as the L-fucosyl donor. Recent studies have identified a xyloglucan-specific L-fucosyltransferase, AtFUT1 (7), and the related L-fucosyltransferase genes (*AtFUT2-10*) have also been found in the genome of *Arabidopsis* (8).

GDP-L-Fuc, the activated form of L-Fuc, is generated through both *de novo* and salvage pathways (9, 10). Recently, the importance of levels of GDP-L-Fuc for the architecture of L-Fuc-containing cell wall polysaccharides was demonstrated through the study of the L-Fuc deficient mutant of *Arabidopsis*, *mur1*. The *mur1* mutant has reduced L-Fuc content in RG-II because of a defect in GDP-Man 4,6-dehydratase (EC 4.2.1.47) that catalyzes the first step of conversion of GDP-Man to GDP-L-Fuc in the *de novo* pathway (11, 12). The loss of L-fucosyl residues indispensable for the boron-mediated dimmer formation of pectic RG-II reduces the growth of rosette leaves in the mutant. The dwarf phenotype of *mur1*, however, can be rescued by exogenously applied monomeric L-Fuc, possibly because a compensating supply of GDP-L-Fuc is generated from L-Fuc via L-Fuc 1-phosphate (L-Fuc 1-P) in the salvage pathway (11). It is highly probable that such salvage reactions utilizing free L-Fuc released during degradation of cell wall polysaccharides and glycoproteins also occur in normal plants: however, little is known about the molecular mechanisms of salvage reactions for free L-Fuc in seed plants.

The salvage pathways convert free monosaccharides to nucleotide sugars as their activated forms and involve two reaction processes, phosphorylation of the monosaccharides and formation of nucleotide sugars from monosaccharide 1-phosphates (monosaccharide 1-Ps) and corresponding nucleoside triphosphates. Whereas Glc, Gal, glucuronic acid (GlcA), galacturonic acid (GalA), and L-arabinose (L-Ara) are activated

mainly as UDP derivatives, L-Fuc and Man appear as GDP derivatives in the salvage pathways (9). Recently, we have identified a UDP-sugar pyrophosphorylase (EC 2.7.7.64), designated PsUSP (*Pisum sativum* UDP-sugar pyrophosphorylase), with broad substrate specificity toward various monosaccharide 1-Ps in pea sprouts. Consistent with the activated forms of monosaccharides, PsUSP catalyzed the formation of UDP-Glc, UDP-Gal, UDP-GlcA, UDP-GalA, and UDP-L-Ara from respective monosaccharide 1-Ps and UTP, but failed to convert L-Fuc 1-P and Man 1-P to respective UDP-sugars (13, 14). This suggests that free L-Fuc and Man are converted to the respective GDP-sugars by specific enzymes distinct from those acting on Glc, Gal, GlcA, GalA, and L-Ara in the salvage pathways. The enzymes participating in the salvage reactions for free L-Fuc have been well characterized in mammals, particularly in pigs. A pig kidney L-fucokinase (EC 2.7.1.52) specifically converts free L-Fuc to L-Fuc 1-P using ATP as the phosphate donor (15). Based on the amino acid sequence, the L-fucokinase is categorized into the GHMP (Galactokinase, Homoserine kinase, Mevalonate kinase, Phosphomevalonate kinase) family, which includes galactokinase (EC 2.7.1.6) and L-arabinokinase (EC 2.7.1.46) found in seed plants (16, 17). GDP-L-Fuc pyrophosphorylase (EC 2.7.7.30) catalyzing the formation of GDP-L-Fuc from L-Fuc 1-P and GTP has also been characterized in the pig kidney (18). By the action of the GDP-L-Fuc pyrophosphorylase, L-Fuc 1-P, possibly formed by the L-fucokinase in intact cells, is converted to GDP-L-Fuc in the presence of GTP. In short, free L-Fuc is probably collaboratively salvaged by L-fucokinase and GDP-L-Fuc pyrophosphorylase in mammalian cells. On the other hand, a bifunctional enzyme with both L-fucokinase and GDP-L-Fuc pyrophosphorylase activities, designated Fkp, has been identified in *Bacteroides fragilis*, which mammals harbor as a symbiont in their intestines (19). Although several genes have been annotated as GHMP family proteins or nucleotide sugar pyrophosphorylases in the genomes of *Arabidopsis* and rice, neither L-fucokinase nor GDP-L-Fuc pyrophosphorylase has so far been identified in seed plants.

In order to clarify the molecular mechanism of free L-Fuc salvage in seed plants, we searched the genomic database and identified a gene that encodes a protein with similarity to both L-fucokinase and GDP-L-Fuc pyrophosphorylase in *Arabidopsis*. From the properties of the recombinant protein expressed in *Escherichia coli*, it can be inferred that the gene product has both L-fucokinase and GDP-L-Fuc pyrophosphorylase activities. Loss-of-function mutants were used to confirm the physiological functions of the gene product in *Arabidopsis*.

EXPERIMENTAL PROCEDURES

Materials—Arabidopsis thaliana ecotype Columbia (Col) was used in this study. The T-DNA insertion lines, SALK-012400 and SALK-053913 (genetic background, Col), and the *mur1-1* mutant (genetic background, Col) were provided by the Nottingham Arabidopsis Stock Center (NASC, Loughborough, UK). *Arabidopsis* plants were grown on rockwool fibers (Nittobo, Tokyo, Japan) under continuous light at 23°C for 35 days. The genotype of the At1g01220

gene in the T-DNA insertion lines was determined by genomic PCR using specific primers. A partial fragment derived from the wild-type At1g01220 gene including the T-DNA insertion site in SALK-012400 was amplified by genomic PCR with specific primers 012400-F-1 (5'-GGACGGACACTCCTTGACACTGGG-3') and 012400-R-1 (5'-GCCAATCATGCCTTGAAGGAACCC-3'), and a fragment of the wild-type At1g01220 gene in SALK-053913 was amplified with 053193-F-1 (5'-GTTGGAGCATAAAGTATGGGGAGC-3') and 053193-R-1 (5'-ATGTGTAGCTCGTTTCCAGCGTCG-3'). The T-DNA insertion was confirmed by amplifying a genomic fragment with LBb1 (5'-GCGTGGACCGCTTGCTGCAACT-3') and 012400-F-1 in SALK-012400, and LBb1 and 053193-F-1 in SALK-053913 using ExTaq (Takara Bio Inc., Otsu, Japan) under the following conditions: 0.5 min denaturing at 94˚C, 0.5 min annealing at 65˚C and 2.0 min amplification at 72˚C, 35 cycles.

ATP, CTP, GDP, GTP, ITP, UTP, PPi, L-Ara, L-Fuc, Gal, L-Gal, GalA, Glc, GlcA, GlcNAc, Man, L-rhamnose (L-Rha), Xyl, Gal 1-P, Glc 1-P, GlcA 1-P, GlcNAc 1-P, L-Fuc 1-P, Man 1-P, Xyl 1-P, and GDP-L-Fuc were purchased from Sigma-Aldrich Japan (Tokyo, Japan). L-Ara 1-P (β-L-arabinopyranose 1-P) was chemically synthesized according to the methods of Aspinall *et al*. (20) and MacDonald (21).

Protein Determination—The concentration of protein was determined by the method of Bradford (22) with bovine serum albumin (BSA) as the standard. In the analysis of the enzymatic properties, substrate specificities, and kinetic values, the concentration of the recombinant enzyme was determined based on the color intensity of the stain with Comassie Brilliant Blue R-250 (CBB R-250) on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (23) compared to the BSA standard.

cDNA Cloning—Total RNA was extracted from 2-week-old *Arabidopsis* seedlings containing cotyledons, true leaves, and roots. The seedlings were frozen in liquid nitrogen, and then homogenized with mortar and pestle. The RNA was extracted with an Isogen kit (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. Single strand cDNA was synthesized from 2 μg of total RNA from *Arabidopsis* seedlings using a reverse-transcriptase, ReverTra Ace-α- (Toyobo, Osaka, Japan), and an oligo(dT)-adaptor primer, (5'-GCGACATCATCGAATTCCGATGTTTTTTTTTTTTTTT-3'). For cloning of At1g01220 cDNA, a set of primers, At1g01220-F-1 (5'-CGAATTCATGTCTAAGCAGAGGAAG-3') and At1g01220-R-1 (5'-GGAATTCAAATACAGATGCTCC-3'), was designed based on the genomic database of *Arabidopsis*. The PCR was performed with proofreading polymerase (KOD-Plus-, Toyobo) and the set of primers, using the single strand cDNA as a template under the following conditions: 0.5 min denaturing at 94˚C, 0.5 min annealing at 55˚C, and 3.0 min amplification at 68˚C, 35 cycles. The amplified cDNA fragment was treated with ExTaq at 72˚C for 5 min to allow the subsequent cloning into a pGEM T-Easy vector (Promega, Madison WI). Point mutations were introduced at Gly^{133} and Gly^{830} of At1g01220 into cDNA by PCR using sets of primers, FKGP-PM1-F (5'-AGGTGACTCCAAAAGGGTTC-3') and

FKGP-PM1-R (5'-GCAGCATGAAGCATCAATAC-3'), and FKGP-PM2-F (5'-TCTAGGAACCTCGAGCATTC-3') and FKGP-PM2-R (5'-GCACTGCCACGAGGAACATTG-3'), respectively. The nucleotide sequence of the cloned fragment was determined with an ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA).

Expression and Purification of Recombinant Enzyme—The coding region of the cloned At1g01220 cDNA was excised from the pGEM T-Easy vector, and then subcloned into an *Eco*RI site of a pET32a expression vector (Novagen, Madison, WI) to give rise to a plasmid construct, At1g01220/pET32a, which was designed to express the recombinant enzyme fused to thioredoxin and 6 x His-tags at the N-terminus. The plasmid construct was transfected into the BL21 (DE3) gold strain of *E. coli* (Stratagene, La Jolla, CA). The cells were grown at 10˚C, and the recombinant protein was induced by treatment with 1.0 mM isopropyl β-D-thiogalactopyranoside for 24 h. To stabilize the recombinant protein in *E. coli*, chaperone proteins, GroES and GroEL, were co-expressed using the pGro7 plasmid (Takara Bio Inc.). The cells were harvested and lysed in a buffer containing 50 mM Tris-HCl buffer, pH 8.0, 0.2 M NaCl, 20% (w/v) glycerol, 1.0 mM phenylmethylsulfonyl fluoride (PMSF), and 0.2% lysozyme from chicken egg (Wako, Osaka, Japan). The lysate was put on a 1.5 x 6.0-cm Chelating Sepharose FF column (GE Healthcare Bio-Sciences, Tokyo, Japan) that had been equilibrated with the buffer without PMSF and lysozyme. The column was washed with 50 mM Tris-HCl buffer, pH 7.5, 0.2 M NaCl, 20% (w/v) glycerol, and 50 mM imidazole, and then the bound protein was eluted with the same buffer containing 250 mM imidazole. The purified recombinant enzyme (0.2 mg from a 500-ml culture of *E. coli*) was digested with 1.0 unit of thrombin (Novagen) at 22˚C for 24 h in order to split off the fused thioredoxin and 6 x His-tags. The purified recombinant enzyme was examined for its purity, properties, and substrate specificity.

Enzyme Assay—The L-fucokinase activity of the recombinant enzyme was determined by monitoring the formation of L-Fuc 1-P in the presence of L-Fuc and ATP. The reaction mixture contained 50 mM Tris-HCl buffer, pH 7.5, 2 mM $MgCl_2$, 10 mM L-Fuc, 2 mM ATP, 5% (w/v) glycerol, and enzyme in a final volume of 0.2 ml. After incubation at 35˚C, the reaction was terminated by dipping the mixture in a boiling water bath for 2 min: this assay method was defined as the standard assay condition for L-fucokinase activity. The reaction products were detected using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) system equipped with a CarboPac PA-1 column (4 x 250 mm, Dionex Japan, Osaka, Japan). The column was eluted with a linear gradient of 0.1-0.2 M sodium acetate in 0.1 M NaOH (0-20 min), followed by isocratic elution with 0.5 M sodium acetate in 0.1 M NaOH (20-30 min) at a flow rate of 1 ml per min. The amount of L-Fuc 1-P produced was estimated from the peak area based on the curve of standard L-Fuc 1-P. One unit of L-fucokinase activity was defined as the amount capable to produce 1 μmol of L-Fuc 1-P from L-Fuc and ATP per min. Elution times of standard sugar and nucleotide were 2.3 min for L-Fuc and

10.3 min for L-Fuc 1-P (Supplementary Fig. 1A).

The GDP-L-Fuc pyrophosphorylase activity of the recombinant enzyme was determined using a reaction mixture consisting of 50 mM 3-morpholinopropanesulfonic acid (MOPS)-KOH buffer, pH 7.0, 2 mM $MgCl_2$, 0.25 mM L-Fuc 1-P, 1 mM GTP, and enzyme in a final volume of 40 µl. After incubation at 35°C, the reaction was terminated as above (this was defined as the standard assay condition for GDP-L-Fuc pyrophosphorylase activity), and the reaction products were analyzed according to the method described previously (13, 24). The reaction products were applied to a high-performance liquid chromatography (HPLC) system with a Shimadzu LC-10AD equipped with a CarboPac PA-1 column. The column was eluted with 0.05 M sodium acetate for the initial 2 min, then by a linear gradient of 0.05-1.0 M sodium acetate (2-40 min), followed by isocratic elution with 1 M sodium acetate (40-46 min) at a flow rate of 1 ml per min. The reaction products were monitored by measuring absorbance at 262 nm and the amount of GDP-sugar produced was estimated from the peak area based on the curve of standard GDP-L-Fuc. Elution times of the standards were as follows: GDP-L-Fuc, 30.2 min; GTP, 38.9 min (Supplementary Fig. 1B). One unit of GDP-L-Fuc pyrophosphorylase activity was defined as the amount capable of producing 1 µmol of GDP-L-Fuc from L-Fuc 1-P and GTP per min. The assay for pyrophospholysis activity of GDP-L-Fuc (reverse direction of the enzyme action) was performed in a 0.1 ml reaction mixture containing 50 mM MOPS-KOH buffer, pH 7.0, 2 mM $MgCl_2$, 1 mM GDP-L-Fuc, 1 mM PPi, and enzyme. Here, the enzyme activity was measured in a manner analogous to the above with GTP as the calibration standard and expressed as µmol of GTP produced per min.

Successive reactions of L-fucokinase and GDP-L-Fuc pyrohosphorylase activities were carried out using a reaction mixture consisting of 50 mM Tris-HCl buffer, pH 7.5, 1 mM $MgCl_2$, 20 mM L-Fuc, 2 mM ATP, 2 mM GTP, 5% (w/v) glycerol, and enzyme in a final volume of 100 µl. After incubation at 35°C, the reaction was terminated and the reaction products were analyzed as described above. To identify GDP-L-Fuc (the reaction product) formed from L-Fuc, ATP, and GTP by the recombinant enzyme, the product was purified by chromatography on a charcoal column (Wako), paper chromatography, then HPLC (the details of product preparation are given in the legend of Supplementary Fig. 2), and analyzed by NMR spectroscopy. The sample was dissolved in D_2O and ^{1}H-NMR spectra were recorded at 400 MHz and at room temperature with a Bruker DPX-400 spectrometer. HDO was used as the internal standard (4.78 ppm). Ring-proton assignments in NMR were made by first-order analysis of the spectra and confirmed by H-H Cosy experiments.

Quantitative Analysis of mRNA—The relative amount of At1g01220 mRNA was estimated by quantitative PCR. Single strand cDNA was synthesized from total RNA of the tissues or organs using oligo$(dT)_{12-18}$ primer. A set of specific primers for the At1g01220 gene, At1g01220-RTP-F (5'-GAACCAGACCTTTGGGTGAA-3') and At1g01220-RTP-R (5'-AAACCGTAGTTGCAGGGATG-3'), and for *ACTIN2* (*ACT2*), ACT2-RTP-F (5'-ACCTTGCTGGACGTGACCT-3') and

ACT2-RTP-R (5'-CACCAATCGTGATGACTTGC-3'), were designed using the Primer 3.0 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The PCR was performed with a SYBR Premix ExTaq kit (Takara Bio Inc.) under the following conditions: 10 sec denaturing at 95˚C, 30 sec annealing at 60˚C, and 20 sec amplification at 72˚C, 40 cycles. The PCR product was detected with Opticon 2 (Bio-Rad, Hercules, CA) and the relative amount of the mRNA to *ACT2* mRNA was calculated.

Determination of Free Monosaccharide Contents in Plants—A soluble fraction containing free monosaccharides was prepared from *Arabidopsis* plants grown for 35 days. Aerial parts of the plants were homogenized with mortar and pestle in water. The homogenate was centrifuged at 10,000 x *g* for 5 min and the supernatant was collected as the soluble fraction. To recover free monosaccharides completely, the precipitate was homogenized again and centrifuged, and the resulting supernatant was combined with the soluble fraction. The soluble fraction was applied to a 1.5 x 2.0-cm DEAE-Sephadex A-25 (GE Healthcare Bio-Sciences) column. Neutral monosaccharides were eluted with 40-ml water, concentrated by an evaporator, and then analyzed by HPAEC-PAD as described previously (25). Separation of standard monosaccharides is shown in Supplementary Fig. 3.

RESULTS

Identification of Bifunctional L-Fucokinase/GDP-L-Fucose Pyrophosphorylase in the Arabidopsis *Genome*—In order to elucidate the salvage pathway for free L-Fuc in plants, we searched for open reading frames (ORFs) with similarity to the mouse L-fucokinase gene (*Mus musculus*, AJ297482) (26) in the *Arabidopsis* genome. The BLAST search revealed that the protein encoded by ORF At1g01220 has the highest similarity (23% identical) with murine L-fucokinase. Although *Arabidopsis* contains several ORFs other than At1g01220 encoding GHMP kinase family proteins, including galactokinase (GAL1, At3g06580) (27) and L-arabinokinase (ARA1, At4g16130) (28), these ORFs did not show significant similarity to the L-fucokinase sequence. We also used the amino acid sequence of human (*homo sapiens*) GDP-L-Fuc pyrophosphorylase (AF017445) (18) for a BLAST search of the *Arabidopsis* genome. The protein encoded by At1g01220 appears to possess significant similarity (22% identical) to the GDP-L-Fuc pyrophosphorylase if one considers only the N-terminal half region (from Trp^{34} to Leu^{565}). Other ORFs possibly encoding nucleotide sugar pyrophosphorylases were less similar to the human GDP-L-Fuc pyrophosphorylase sequence. Since At1g01220 encodes a protein that shares similarities with both L-fucokinase and GDP-L-Fuc pyrophosphorylase, we designated it *AtFKGP* (*Arabidopsis thaliana* L-fucokinase/GDP-L-Fuc pyrophosphorylase). While the sequence similarity to the murine L-fucokinase was observed over nearly all of AtFKGP, that to the human GDP-L-Fuc pyrophosphorylase was limited to the N-terminal half region. At1g01220 also exhibited low but significant sequence similarity (17% identical) to a bacterial bifunctional L-fucokinase/GDP-L-Fuc pyrophosphorylase, Fkp, from *B. fragilis* (19).

The cDNA for *AtFKGP* was isolated by RT-PCR using cDNA from *Arabidopsis* seedlings as the template, and the nucleotide sequence of *AtFKGP* was determined (Supplementary Fig. 4). The comparison of the cDNA sequence with the genomic sequence of *Arabidopsis* (TAIR, http://www.arabidopsis.org/index.jsp) revealed that the *AtFKGP* gene consists of seven exons and six introns, which is in accordance with the computer-based annotation by the Arabidopsis Genome Initiative (AGI). *AtFKGP* encodes a polypeptide of 1,055 amino acids with a calculated molecular mass of 116,351.5 Da and a theoretical isoelectric point (pI) of 5.87. The peptide sequence of AtFKGP did not contain a secretory signal or transmembrane domain, suggesting that AtFKGP exists inside the cells. A BLAST search of the rice (*Oryza sativa*) genome identified an ORF closely related (62% identical) to *AtFKGP*, which we designated *OsFKGP* (*Oryza sativa* L-fucokinase/GDP-L-Fuc pyrophosphorylase) (Supplementary Fig. 4). Based on the amino acid sequence deduced from the cDNA, AtFKGP appeared to contain a region (from Leu^{129} to Lys^{146}) similar to the pyrophosphorylase consensus motif [$L(X)_2GXGTXM(X)_4PK$] conserved for nucleotide sugar pyrophosphorylases (13, 29). On the other hand, a region from Pro^{826} to Ser^{835} of AtFKGP was highly similar to the known ATP-binding motif [$PX_3GL(G/S)SSA$] that forms a phosphate-binding loop wrapping around the β-phosphate group of ATP in GHMP family proteins (Fig. 1A) (30). Both the pyrophosphorylase consensus and the ATP-binding motifs were also conserved in OsFKGP (Supplementary Fig. 4, Fig. 1A), suggesting that these motifs are important for the functions of FKGP proteins in seed plants. Phylogenetic analysis revealed that AtFKGP forms a plant FKGP family together with OsFKGP, which is apparently independent from the vertebrate L-fucokinase family and bacterial Fkp (19) (Fig. 1B).

Properties of the Recombinant Protein— To characterize the enzymatic properties of the gene product of *AtFKGP*, the cDNA fragment corresponding to the full-length AtFKGP was subcloned into an expression vector, and the recombinant AtFKGP (rAtFKGP) was expressed in *E. coli*. The recombinant protein was not stable in *E. coli* cells, accumulating as an inclusion body, but co-expression of the chaperone proteins GroEL and GroES remarkably improved the stability of rAtFKGP. The recombinant protein fused to thioredoxin and His-tags was partially purified by chelating chromatography (Table 1) and appeared as a protein with molecular mass of more than 120 kDa on SDS-PAGE (Fig. 2). Thrombin-digestion of the purified rAtFKGP decreased the relative molecular mass by elimination of the fused thioredoxin and 6 x His tags. The purified rAtFKGP still contained a considerable amount of other proteins including GroEL protein expressed by the pGro7 plasmid (asterisk in Fig. 2). The protein band with relative molecular mass of 70 kDa may correspond to an endogenous DnaK protein of *E. coli*. Second purification of rAtFKGP by chelating (Chelating Sepharose FF) and gel-permission (Sephacryl S-200) chromatography was performed, but these proteins could not be removed. The chaperone proteins seem to bind to rAtFKGP, maintaining its conformation.

In the presence of L-Fuc and ATP,

rAtFKGP formed L-Fuc 1-P and generated GDP-L-Fuc from L-Fuc 1-P and GTP, whereas in the control experiment, a thioredoxin-His-tags protein expressed by the empty pET32a vector showed neither enzyme activity. These results indicate that *AtFKGP* encodes a protein with L-fucokinase and GDP-L-Fuc pyrophosphorylase activities as expected from the amino acid sequence similarity discussed above. To confirm the bifunctionality of AtFKGP, point mutations were introduced at the Gly^{133} and Gly^{830} residues important for GDP-L-Fuc pyrophosphorylase and L-fucokinase activities of rAtFKGP, respectively, and the effect of the mutations on the activities was examined. The point mutation substituting Ala for Gly^{133} (designated PM1) abolished GDP-L-Fuc pyrophosphorylase activity of rAtFKGP, but hardly affected the L-fucokinase activity (Fig 3). On the other hand, the point mutation substituting Ala for Gly^{830} (PM2) reduced L-fucokinase activity by more than 90%, while preserving the GDP-L-Fuc pyrophosphorylase activity. The recombinant protein having both PM1 and PM2 lost both activities. These results indicate that AtFKGP has two distinct activities, L-fucokinase and GDP-L-Fuc pyrophosphorylase activity.

The recombinant AtFKGP was stable in 50% (w/v) glycerol, 1 mM PMSF, 0.2 M NaCl, 50 mM Tris-HCl buffer, pH 7.5 at -20˚C for one month, but more than half of the GDP-L-Fuc pyrophosphorylase activity was lost within one day when it was stored without glycerol at 4˚C. Thrombin digestion did not increase but rather decreased specific activity of rAtFKGP (Table 1), suggesting that the fused protein does not impair the enzyme activity of rAtFKGP. The purified rAtFKGP fused to thioredoxin and His-tags was therefore used in the following experiments.

Enzymatic Properties — The enzymatic properties, substrate specificities, and kinetics of rAtFKGP were determined. The specific activity of rAtFKGP for L-fucokinase was estimated at 0.17 unit/mg protein and that for GDP-L-Fuc pyrophosphorylase at 0.71 unit/mg protein (here the protein content was assessed based on the color intensity of rAtFKGP stained with CBB R-250 on SDS-PAGE). The specific activities of rAtFKGP apparently differ from those (1.27 and 0.36 unit/mg protein, respectively) of pig L-fucokinase (15) and GDP-L-Fuc pyrophosphorylase (18). As has been observed for many nucleotide sugar pyrophosphorylases from other sources, rAtFKGP catalyzed both synthesis of GDP-L-Fuc from L-Fuc 1-P and GTP (GTP:L-fucose-1-phosphate guanylyltransferase action, the forward direction), and pyrophospholysis producing L-Fuc 1-P and GTP from GDP-L-Fuc and PPi (GDP-L-Fuc pyrophosphorylase action, the reverse).

The L-fucokinase activity of rAtFKGP absolutely required divalent cations such as Mg^{2+} and Mn^{2+}, as had the pig L-fucokinase in (15). The divalent cations were also necessary for the GDP-L-Fuc pyrophosphorylase activity of rAtFKGP (Table 2). Although Mn^{2+} was a better cation than Mg^{2+} for the L-fucokinase activity, it had only a poor effect on the GDP-L-Fuc pyrophosphorylase activity of rAtFKGP compared with Mg^{2+}. The maximal L-fucokinase activity of the enzyme occurred at pH 10.5, whereas the maximal GDP-L-Fuc pyrophosphorylase activity of the enzyme was observed in the pH range from 6.5 to 8.0 (Fig. 4). Since the optimum pHs for L-

fucokinases from mammals are around 8.0 (15, 32), and since it is not likely that AtFKGP functions as L-fucokinase at pH 10.5 in *Arabidopsis* tissues, a reaction mixture buffered to pH 7.5 was used in the assays for L-fucokinase activity of rAtFKGP throughout the study. It is conceivable that the glycine-NaOH buffer used for the determination of the optimum in the high pH range affected the L-fucokinase activity. However, neither glycine nor sodium ion (50 mM) elevated the activity at low pH levels, indicating that the high pH optimum of the L-fucokinase activity of AtFKGP is attributable to the nature of the enzyme protein itself. The optimum temperature for L-fucokinase activity of AtFKGP was 40˚C, and the enzyme lost more than 99% of its activity at 50˚C (Fig. 5). GDP-L-Fuc pyrophosphorylase activity of rAtFKGP was maximal in the range from 30 to 45˚C (Fig. 5). The GDP-L-Fuc pyrophosphorylase activity was thus somewhat more stable than the L-fucokinase activity.

Substrate Specificity of L-Fucokinase Activity—Generally, monosaccharide kinases catalyze phosphorylation of monosaccharides using ATP as the phosphate donor. Our results above show that rAtFKGP possesses L-fucokinase activity, forming L-Fuc 1-P in the presence of L-Fuc and ATP. We examined whether rAtFKGP catalyzes other monosaccharide kinase reactions using 10 mM L-Ara, Glc, Gal, L-Gal, Man, Xyl, L-Rha, GalA, GlcA, and GlcNAc in the presence of 2 mM ATP under standard assay conditions. None of these monosaccharides served as a substrate for rAtFKGP at all. Although L-fucokinase purified from pig kidney shows weak activity on Glc, forming Glc 1-P (15), rAtFKGP did not act on Glc. The substrate specificity of the L-fucokinase activity toward nucleotide phosphate donors was also determined. The enzyme failed to form L-Fuc 1-P, when CTP, GTP, ITP, or UTP was added to the reaction mixture instead of ATP. Although GTP served as a nucleotide donor for the GDP-L-Fuc pyrophosphorylase activity of rAtFKGP (see below) it did not serve as phosphate donor for the L-fucokinase activity of rAtFKGP.

Substrate Specificity of GDP-L-Fuc Pyrophosphorylase Activity—To determine the substrate specificity of the GDP-L-Fuc pyrophosphorylase activity of rAtFKGP toward nucleotides, the effect of adding ATP, CTP, ITP, or UTP to the reaction mixtures on the formation of GDP-L-Fuc from L-Fuc 1-P and GTP was examined (Table 3). The formation of GDP-L-Fuc was barely inhibited by these nucleotides, and no product corresponding to nucleotide sugars other than GDP-L-Fuc was detected on HPLC analysis. Addition of PPi, however, strongly inhibited the formation of GDP-L-Fuc by rAtFKGP. The inhibition of nucleotide sugar synthesis by PPi has also been observed for a UDP-Glc pyrophosphorylase from *Acanthamoeba castellanii* (31) and PsUSP (13), where it is due to product inhibition. These results indicate that rAtFKGP specifically uses GTP as the nucleotide donor.

The specificity of the enzyme toward monosaccharide 1-Ps was determined using the following substrates: L-Ara 1-P, Gal 1-P, Glc 1-P, Man 1-P, Xyl 1-P, GlcA 1-P, and GlcNAc 1-P, together with L-Fuc 1-P. We found that rAtFKGP fails to utilize monosaccharide 1-Ps other than L-Fuc 1-P as the glycosyl donor. This strict substrate specificity of GDP-L-Fuc pyrophosphorylase toward L-Fuc 1-P has also been observed for

enzymes from other origins, such as pig kidney (18). Together with the substrate specificity of L-fucokinase activity of rAtFKGP toward monosaccharides, the specific action of GDP-L-Fuc pyrophosphorylase activity of rAtFKGP on L-Fuc 1-P indicates that AtFKGP specifically catalyzes conversion of L-Fuc to GDP-L-Fuc via L-Fuc 1-P, and is not involved in the salvage reactions for other monosaccharides in *Arabidopsis*.

Successive L-Fucokinase and GDP-L-Fuc Pyrophosphorylase Reactions — Generation of GDP-L-Fuc from L-Fuc via L-Fuc 1-P by successive L-fucokinase and GDP-L-Fuc pyrophosphorylase activities of rAtFKGP was performed in a reaction mixture containing L-Fuc, ATP, and GTP as the starting substrates (Fig. 6). The amount of GDP-L-Fuc in the reaction mixture increased proportionally to the reaction time. L-Fuc 1-P also accumulated in the reaction mixture, but the relative amount was very low compared with GDP-L-Fuc. The results indicate that AtFKGP catalyzes two successive reactions, phosphorylation of L-Fuc and generation of GDP-L-Fuc from L-Fuc 1-P and GTP in such a way that L-Fuc 1-P formed by the L-fucokinase activity is immediately consumed as the substrate for the GDP-L-Fuc pyrophosphorylase activity of AtFKGP. The GDP-L-Fuc generated from L-Fuc, ATP, and GTP by the successive reactions was purified and analyzed for its structure by ^{1}H-NMR spectroscopy (Supplementary Fig. 1). The signals of a doublet of doublets at δ 3.54 ppm ($J_{2"3"}$ 9.9 Hz, $J_{1"2"}$, 7.7 Hz, H-2 of L-Fuc), a doublet of doublets at δ 3.64 ppm ($J_{3"4"}$ 3.4 Hz, $J_{2"3"}$ 9.9 Hz, H-3 of L-Fuc), a doublet at δ 3.69 ppm ($J_{3"4"}$ 3.4 Hz, H-4 of L-Fuc), a quartet at δ 3.75 ppm (H-5 of L-Fuc), and a doublet having an area of three protons at δ 1.21 ppm ($J_{5"6"}$ 6.5 Hz, H-6 of L-Fuc) indicate the pyranose-type ring system of L-Fuc. The one-proton signal at δ 4.90 ppm as a doublet of doublets ($J_{1"2"}$ 7.7 Hz, $J_{P\text{-}2H1"}$ 8.1 Hz, H-1 of L-Fuc) indicates that the anomeric configuration of the L-fucopyranose is β. All other signals in the ^{1}H-NMR spectra were in accordance with those of GDP-L-Fuc chemically synthesized (33) and of GDP-L-Fuc enzymatically synthesized from GDP-Man (34). These results establish unambiguously that the product is GDP-β-L-fucopyranose, a metabolite in the pathway of nucleotide sugars in seed plants.

Kinetics of the Recombinant Enzyme — Effects of substrate concentration on the L-fucokinase activity of rAtFKGP were examined by measuring the activity with varying concentrations of L-Fuc and ATP. The resulting K_m and k_{cat} values are listed in Table 4. The K_m value (1.0 mM) of the L-fucokinase activity of rAtFKGP for L-Fuc was manifestly higher than those (0.027 and 0.12) for known L-fucokinases from pig kidney (15) and pig liver (32), respectively. The kinetic values of GDP-L-Fuc pyrophosphorylase were also determined. The protein exhibited high affinity to L-Fuc 1-P (K_m value, 0.052 mM). A similar K_m value (0.060) for L-Fuc 1-P has been reported for GDP-L-Fuc pyrophosphorylase from pig kidney (18). The low accumulation of L-Fuc 1-P in the successive reactions (Fig. 6) may be attributed to the low K_m value toward L-Fuc 1-P, which enables the enzyme to rapidly convert L-Fuc 1-P to GDP-L-Fuc. The k_{cat} values of GDP-L-Fuc pyrophosphorylase activity of rAtFKGP for GDP-L-Fuc (7.86 munit/mg protein) and PPi (8.02) in the reverse reaction (pyrophospholysis of GDP-

L-Fuc) were apparently higher than those for L-Fuc 1-P (1.59) and GTP (1.82) in the forward reaction. It would seem that the equilibrium of the nucleotide sugar synthesis and pyrophospholysis catalyzed by AtFKGP is controlled by the concentrations of L-Fuc 1-P, GTP, PPi, and GDP-L-Fuc in intact cells.

Expression of AtFKGP *in Tissues of* Arabidopsis — In order to analyze the expression pattern of *AtFKGP*, quantitative analyses of the mRNA were performed in various tissues of *Arabidopsis* (Fig. 7). The expression of *AtFKGP* was detected in all tissues examined, indicating that the salvage reactions for free L-Fuc catalyzed by AtFKGP occur ubiquitously in *Arabidopsis*. Free L-Fuc is likely released from L-Fuc-containing glycoconjugates by the action of α-L-fucosidases (EC 3.2.1.51). *AtFXG1* is a unique gene known to encode α-L-fucosidase (EC 3.2.1.51) in *Arabidopsis* (35). Whereas the expression of *AtFXG1* is reported to be high in young leaves and inflorescence of *Arabidopsis* (35), relatively high expression of *AtFKGP* was observed in flower buds.

L-Fuc Accumulation in the Mutant Plants —The dual activity of rAtFKGP as both L-fucokinase and GDP-L-Fuc pyrophosphorylase suggests that AtFKGP is involved in the salvage of free L-Fuc in intact plants. To address the physiological functions of AtFKGP in *Arabidopsis*, L-Fuc content in the plant tissues was measured in loss-of-function mutants of *AtFKGP*. There are two independent mutants with T-DNA insertion in the *AtFKGP* gene, namely SALK-012400 (designated *fkgp-1*) and SALK-0553913 (*fkgp-2*), in which the insertions occurred in the third and sixth exons of the *AtFKGP* gene, respectively (Fig. 8A). The genotypes of the mutants were confirmed by genomic PCR using specific primers (see EXPERIMENTAL PROCEDURES), and homozygous lines of *fkgp* mutants (*fkgp-1#1*, *fkgp-1#16*, *fkgp-2#1*, and *fkgp-2#14*) were isolated. The content of free L-Fuc in the soluble fraction extracted from the *Arabidopsis* plants was determined by HPAEC-PAD. Both *fkgp-1* and *-2* mutants appeared to accumulate more than 40 times as much free L-Fuc in the soluble fraction as the wild-type plants (Fig. 8B), whereas the content of the other monosaccharides (Glc, Gal, and L-Ara) detected in this experiment was hardly affected by the mutations (Supplementary Fig. 3). The results indicate that AtFKGP plays central roles in the salvage of free L-Fuc in *Arabidopsis*. Although the mutations abolish the function of AtFKGP completely, thus causing strong accumulation of free L-Fuc, the *fkgp* mutants did not show any altered visible phenotype. Moreover, the sugar composition of cell wall polysaccharides was not affected by the *fkgp* mutations (data not shown), whereas it has been reported that the *mur1* mutation results in a considerable reduction of L-Fuc content in cell wall polysaccharides (36). These results suggest that GDP-L-Fuc is mainly supplied through the *de novo* pathway as reported for mammalian cells (26), and that the services of AtFKGP are not necessarily required for the synthesis of L-Fuc-containing cell wall polysaccharides in *Arabidopsis*, at least under normal growth conditions.

DISCUSSION

Free L-Fuc released during metabolism of glycoconjugates is imported and converted to GDP-L-Fuc via L-Fuc 1-P as an intermediate metabolite in the salvage pathway in mammals (15, 18). In the symbiotic

bacterium *B. fragilis*, GDP-L-Fuc is generated from exogenous L-Fuc via L-Fuc 1-P by a bifunctional L-fucokinase/GDP-L-Fuc pyrophosphorylase, Fkp (19). L-Fucokinase and GDP-L-Fuc pyrophosphorylase activities of rAtFKGP expressed in *E. coli* and high accumulation of free L-Fuc in *fkgp* mutants demonstrate that seed plants also possess a salvage pathway for free L-Fuc similar to that of mammals and the bacterium. AtFKGP has weak but significant similarities to both mammalian L-fucokinase and GDP-L-Fuc pyrophosphorylase and contains ATP-binding and pyrophosphorylase consensus motifs. AtFKGP also has significant similarity to Fkp. These facts suggest that the *AtFKGP* gene has evolved from an ancestral gene common to mammalian L-fucokinase and bacterial *Fkp* genes. It is possible that the mammalian L-fucokinases have then lost their GDP-L-Fuc pyrophosphorylase activity in the evolutional process, while plant FKGP proteins and bacterial Fkp preserved both activities. The low sequence similarity among plant FKGP, bacterial Fkp, and mammalian L-fucokinases suggests that the horizontal gene spread is an old event.

In the *de novo* pathway, GDP-L-Fuc is generated from fructose 6-phosphate via four intermediate compounds, Man 6-phosphate, Man 1-P, GDP-Man, and GDP-4-keto-6-deoxy-Man by the actions of five different enzymes (9). On the other hand, GDP-L-Fuc is synthesized from L-Fuc through just two reactions catalyzed by a single AtFKGP protein in the salvage pathway in *Arabidopsis*. It would seem that the salvage of free L-Fuc should be advantageous for seed plants by efficiently generating GDP-L-Fuc that may serve as a donor substrate for L-fucosyltransferases. However, the loss-of-function mutants of *AtFKGP, fkgp-1* and *-2*, did show neither visible phenotype, such as a growth defect, nor a change in L-Fuc content in the cell wall polysaccharides, although they did accumulate large amounts of free L-Fuc. This indicates that GDP-L-Fuc is in general mainly supplied through the *de novo* pathway, as has been reported for mammalian cells (26), for the synthesis of L-Fuc-containing cell wall polysaccharides. However, the generation of GDP-L-Fuc through the salvage pathway by AtFKGP may be important for the synthesis of L-Fuc-containing glycoconjugates under severe growth conditions where GDP-L-Fuc can't be sufficiently obtained through the *de novo* pathway. The conservation of the salvage pathway for free L-Fuc in plants, mammals, and bacteria supports the idea that the pathway is physiologically important.

The bifunctional L-fucokinase/GDP-L-Fuc pyrophosphorylase may be the ultimate enzymatic form in plants to efficiently drive the sequential reactions to form GDP-L-Fuc, namely phosphorylation of L-Fuc and generation of GDP-L-Fuc from L-Fuc 1-P and GTP because L-Fuc 1-P formed by the L-fucokinase activity can be utilized as the immediate substrate for GDP-L-Fuc pyrophosphorylase activity without diffusion into the cytoplasm. We suggest that AtFKGP possesses two distinct sites for the L-fucokinase and the GDP-L-Fuc pyrophosphorylase activities, based on the following observations: (1) Gly^{133} and Gly^{830} were important for the GDP-L-Fuc pyrophosphorylase and L-fucokinase activities, respectively, and these two residues acted independently and did not affect the counter activity (Fig. 3); (2) properties of the two activities are different,

e.g., the optimal pH (10.5) for the L-fucokinase activity of rAtFKGP differs from that (6.5-8.0) for the GDP-L-Fuc pyrophosphorylase activity (Fig. 4); (3) in the sequential reaction, when L-Fuc, ATP, and GTP were present as substrates, L-Fuc 1-P accumulated, indicating that L-Fuc 1-P formed by the L-fucokinase activity was not retained by rAtFKGP (Fig. 6); (4) AtFKGP has a region with similarity to mammalian GDP-L-Fuc pyrophosphorylase in the N-terminal half region and contains an ATP-binding motif conserved for GHMP kinases in the C-terminal region. Tertiary structure prediction with the 3D-PSSM program (37) showed that the structure of the N-terminal half region (Ala^{123}-Lys^{522}) of AtFKGP has significant similarity (E-value, 2.71e-3) to a UDP-GlcNAc pyrophosphorylase from *E. coli* (38) and that the structure of the C-terminal region (Gly^{693}-Ile^{1055}) is closely related (E-value, 1.87e-31) to a galactokinase from *Lactococcus lactis* (39), suggesting that the catalytic sites for the two activities are located in distinct regions of AtFKGP. However, to clarify the spatial location of catalytic sites for the two activities, stereochemical analysis of the three-dimensional structure of AtFKGP would be necessary.

REFERENCES

1. Hayashi, T. (1989) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **40**, 139-168
2. Nothnagel, E. A. (1997) *Int. Rev. Cytol.* **174,** 195-291
3. Levy, S., York, W. S., Stuike-Prill, R., Meyer, B., and Staehelin, L. A. (1991) *Plant J.* **1**, 195-215
4. Levy, S., Maclachlan, G., and Staehelin, L. A. (1997) *Plant J.* **11**, 373-386
5. van Ree, R., Cabanes-Macheteau, M., Akkerdaas, J., Milazzo, J.-P., Loutelier-Bourhis, C., Rayon, C., Villalba, M., Koppelman, S., Aalberse, R., Rodriguez, R., Faye, L., and Lerouge, P. (2000) *J. Biol. Chem*. **275**, 11451-11458
6. O'Neill, M., Albersheim, P., and Darvill, A. (1990) The pectic polysaccharides of primary cell walls. In: *Methods in Plant Biochemistry* (Dey, P. M. ed), vol 2, pp. 415–441, Academic Press, London
7. Perrin, R. M., DeRocher, A. E., Bar-Peled, M., Zeng, W., Norambuena, L., Orellana, A., Raikhel, N. V., and Keegstra, K. (1999) *Science* **284**, 1976-1979
8. Sarria, R., Wagner, T. A., O'Neill, M. A., Faik, A., Wilkerson, C. G., Keegstra, K., and Raikhel, N. V. (2001) *Plant Physiol.* **127**, 1595-1606
9. Reiter, W.-D. and Vanzin, G. F. (2001) *Plant Mol. Biol.* **47**, 95-113
10. Bonin, C. P. and Reiter, W.-D. (2000) *Plant J.* **21**, 445-454
11. O'Neill, M. A., Eberhard, S., Albersheim, P., and Darvill, A. G. (2001) *Science* **294**, 846-849
12. Bonin, C. P., Freshour, G., Hahn, M. G., Vanzin, G. F., and Reiter, W.-D. (2003) *Plant Physiol.* **132**, 883-892
13. Kotake, T., Yamaguchi, D., Ohzono, H., Hojo, S., Kaneko, S., Ishida, H. K., and Tsumuraya, Y. (2004) *J. Biol. Chem.* **279**, 45728-45736

14. Ohashi, T., Cramer, N., Ishimizu, T., and Hase, S. (2006) *Anal. Biochem.* **352**, 182-187
15. Park, S. H., Pastuszak, I., Drake, R., and Elbein, A. D. (1998) *J. Biol. Chem*. **273**, 5685-5891
16. Tsay, Y. H. and Robinson, G. W. (1991) *Mol. Cell. Biol*. **11**, 620-631
17. Lange, B. M. and Croteau, R. (1999) *Proc. Natl. Acad. Sci. U.S.A*. **96,** 13714-13719
18. Pastuszak, I., Ketchum, C., Hermanson, G., Sjoberg, E. J., Drake, R., and Elbein, A. D. (1998) *J. Biol. Chem*. **273**, 30165-30174
19. Coyne, M. J., Reinap, B., Lee, M. M., and Comstock, L. E. (2005) *Science* **307**, 1778-1781
20. Aspinall, G. O., Cottrell, I. W., and Matheson, N. K. (1972) *Can. J. Biochem.* **50**, 574-580
21. MacDonald, D. L. (1962*) J. Org. Chem.* **27,** 1107-1109
22. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248-254
23. Laemmli, U. K. (1970) *Nature* **227**, 680-685
24. Pauly, M., Porchia, A., Olsen, C. E., Nunan, K. J., and Scheller, H. V. (2000) *Anal. Biochem.* **278**, 69-73
25. Ishikawa, M., Kuroyama, H., Takeuchi, Y., and Tsumuraya, Y. (2000) *Planta* **210**, 782-791
26. Niittymäki, J., Mattila, P., Roos, C., Huopaniemi, L., Sjöblom, S., and Renkonen, R. (2004) *Eur. J. Biochem*. **271**, 78-86
27. Kaplan, C. P., Tugal, H. B., and Baker, A. (1997) *Plant Mol. Biol.* **34**, 497-506
28. Sherson, S., Gy, I., Medd, J., Schmidt, R., Dean, C., Kreis, M., Lecharny, A., and Cobbett, C. (1999) *Plant Mol. Biol.* **39**, 1003-1012
29. Mio, T., Yabe, T., Arisawa, M., and Yamada-Okabe, H. (1998) *J. Biol. Chem.* **273**, 14392-14397
30. Hartley, A., Glynn, S. E., Barynin, V., Baker, P. J., Sedelnikova, S. E., Verhees, C., de Geus, D., van der Oost, J., Timson, D. J., Reece, R. J., and Rice, D. W. (2004) *J. Mol. Biol.* **337**, 387-398
31. Rudick, V. L. and Weisman, R. A. (1974) *J. Biol. Chem.* **249**, 7832-7840
32. Ishihara, H., Massaro, D. J., and Heath, E. C. (1968) *J. Biol. Chem.* **243**, 1103-1109
33. Schmidt, R. R., Wegmann, B., and Jung, K.-H. (1991) *Liebigs Ann. Chem.* 121-124
34. Albermann, C., Distler, J., and Piepersberg, W. (2000) *Glycobiology* **10,** 875-881
35. Iglesias, N., Abelenda, J. A., Rodiño, M., Sampedro, J., Revilla, G., and Zarra, I. (2006) *Plant. Cell. Physiol.* **47,** 55-63
36. Kelley, L. A., MacCallum, R. M., and Sternberg, M. J. (2000) *J. Mol. Biol*. **299**, 499-520
37. Reiter, W.-D., Chapple, C. C. S., and Somerville, C. R. (1993) *Science* **261,** 1032-1035
38. Brown, K., Pompeo, F., Dixon, S., Mengin-Lecreulx, D., Cambillau, C., and Bourne, Y. (1999) *EMBO J.* **18**, 4096-4107
39. Thoden, J. B. and Holden, H. M. (2003) *J. Biol. Chem.* **278**, 33305-33311

FOOTNOTES

This research was supported in part by a Grant for Ground Research for Space Utilization to T.K. from the Japan Space Forum and a Grant-in-Aid for Scientific Research to T.K. (No. 17770028) from Ministry of Education, Culture, Sports, Science and Technology, Japan.

The abbreviations used are: L-Ara, L-arabinose; AtFKGP, *Arabidopsis thaliana* L-fucokinase/GDP-L-Fuc pyrophosphorylase; BSA, bovine serum albumin; CBB R-250, Comassie Brilliant Blue R-250; L-Fuc, L-fucose; GalA, galacturonic acid; GlcA, glucuronic acid; HPEAC-PAD, high-performance anion-exchange chromatography with pulsed amperometric detection; HPLC, high-performance liquid chromatography; MES, 2-morpholinoethanesulfonic acid; monosaccharide 1-P, monosaccharide 1-phosphate; MOPS, 3-morpholinopropanesulfonic acid; OsFKGP, *Oryza sativa* L-fucokinase/GDP-L-Fuc pyrophosphorylase; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; PsUSP, *Pisum sativum* UDP-sugar pyrophosphorylase; L-Rha, L-rhamnose; RG-II, rhamnogalacturonan II; SDS, sodium dodecyl sulfate. Sugars mentioned in this paper belong to the D-series unless otherwise noted.

FIGURE LEGENDS

FIG. 1. **Amino acid sequence and conserved motifs of AtFKGP.** *A*, the putative pyrophosphorylase consensus and the ATP-binding motifs of AtFKGP were aligned with other proteins. Amino acid residues conserved for all proteins are highlighted in black; those for more than five proteins are in gray. Gaps (-) were introduced to achieve maximum similarity. Accession numbers for the clones are as follows: AtARA1, At4g16130.1; AtFKGP, At1g01220.1; AtGalK, At3g42850; AtUGP1, At2g35020.1; AtVTC1, At2g39770.1; HsAGX1 from human, Q16222; MmGFPP from mouse, AAI10553; HsGalK from human, AAB51607; HsUGP2 from human, AAH47004; MmFK from mouse (*Mus musculus*), AAP20647; OsFKGP from rice, Os03g0115100; PfGalK from *Pyrococcus furiosus*, AAL80569; PsUSP from pea (*Pisum sativum*), AB178642; ScGalK from *Saccharomyces cerevisiae*, CAA53677. *B*, phylogenetic relationships of AtFKGP with known L-fucokinases, a bacterial bifunctional L-fucokinase/GDP-L-Fuc pyrophosphorylase (Fkp), and related genes from *Caenorhabditis elegans*, *Dictyostelium discoideum*, *Leishmania major*, *Ostreococcus tauri*, and *Solibacter usitatus* were analyzed using ClustalW. The accession numbers and their origins (in parentheses) are shown. The percentages of bootstrap values for the respective branches are shown. The alignments were not manually adjusted. The *bar* indicates 0.1 substitutions per site. Asterisks indicate the residues that are substituted with Ala residues in the point mutation (PM1 and PM2) experiments shown in Fig. 3.

FIG. 2. **SDS-PAGE of rAtFKGP at different purification steps.** The rAtFKGP was expressed in *E. coli*. The protein (approximately 1 μg) obtained after different purification steps was analyzed by SDS-PAGE. Protein in the gel was stained with CBB R-250. *Lanes S*, molecular mass markers; *lane 1*, lysate of *E. coli*; *lane 2*, rAtFKGP purified on a chelating column; *lane 3*, thrombin-digested rAtFKGP. The *arrows* indicate rAtFKGP before and after thrombin digestion, and the *asterisk* indicates a chaperone protein, GroEL, expressed by the pGro7 plasmid.

FIG. 3. **Influence of point mutations on L-fucokinase and GDP-L-Fuc pyrophosphorylase activities of rAtFKGP.** L-Fucokinase (*open bar*) and GDP-L-Fuc pyrophosphorylase (*closed*

bar) activities of rAtFKGP with point mutation(s) at either Gly^{133} (substituted with Ala, PM1) or Gly^{830} (substituted with Ala, PM2), and both Gly^{133} and Gly^{830} (PM1/PM2) were measured. The activities are shown as a percentage of that of wild-type rAtFKGP. Data are averages of triplicate assays.

FIG. 4. **Effect of pH on L-fucokinase and GDP-L-Fuc pyrophosphorylase activities of rAtFKGP.** Activity-pH curves of L-fucokinase (*upper*) and GDP-L-Fuc pyrophosphorylase (*lower*) were obtained using buffers (50 mM) of differing pH. The enzyme reactions were performed under standard conditions for each enzyme activity except for the buffer (see EXPERIMENTAL PROCEDURES). Data are averages of triplicate assays.

FIG. 5. **Effect of temperature on L-fucokinase and GDP-L-Fuc pyrophosphorylase activities of rAtFKGP.** Activity-temperature curves of L-fucokinase (*upper*) and GDP-L-Fuc pyrophosphorylase (*lower*) result from incubation at different temperatures. The enzyme reactions were performed under standard conditions for each enzyme activity except for the reaction temperature (see EXPERIMENTAL PROCEDURES). Data are averages of triplicate assays.

FIG. 6. **Formation of GDP-L-Fuc in sequential reactions by L-fucokinase and GDP-L-Fuc pyrophosphorylase activities of rAtFKGP**. The sequential reactions were performed using a reaction mixture containing 20 mM L-Fuc, 2 mM ATP, and 2 mM GTP. The amounts of L-Fuc 1-P (*closed circle*) and GDP-L-Fuc (*open circles*) in the reaction mixture were determined by HPAEC-PAD and HPLC, respectively. Data are averages of triplicate assays.

FIG. 7. **Expression pattern of *AtFKGP*.** The relative amount of *AtFKGP* mRNA to *ACT2* mRNA was quantified by quantitative PCR. Data are averages of triplicate assays.

FIG. 8. **Accumulation of free L-Fuc in *fkgp* mutants.** *A*, a schematic diagram of T-DNA insertion sites in *fkgp* mutants is shown. The insertion sites of *fkgp* mutants were confirmed by genomic PCR. *B*, the contents of free L-Fuc in *fkgp* mutants were determined. Free monosaccharides were extracted and purified from wild-type plant (Col, *open bar*), *mur1-1* mutant (*closed bar*), and *fkgp* mutants (*fkgp-1*#1 and #16 and *fkgp-2*#1 and #14, *gray bars*) grown for 35 days. The monosaccharide contents were measured by HPAEC-PAD. Data are averages of triplicate assays.

Table 1

Purification of rAtFKGP expressed in E. coli

The data for rAtFKGP were obtained from an experiment using 500-ml culture medium as the starting material. For the control experiment 250-ml medium were used.

	Total protein	Total activity[a]	Specific activity	Purification	Yield
	mg	*munits*	*munits/mg protein*	*-fold*	*%*
rAtFKGP					
Lysate	27.8	69.6 (-[b])	2.5 (-)	1.0	100.0
Ni-Sepharose FF	0.23	29.1 (9.0)	127 (39.1)	50.8	41.8
Thrombin digestion	0.24	19.9 (4.1)	82.9 (17.1)	33.2	28.6
Control (pET32a vector)					
Lysate	12.3	0.0 (-)	0.0 (-)	-	-
Ni-Sepharose FF	0.43	0.0 (0.0)	0.0 (0.0)	-	-

[a] Assay was done for GDP-L-Fuc pyrophosphorylase activity; L-fucokinase activity is shown in parentheses.

[b] Not determined.

Table 2

Effect of metal ions on activities of L-fucokinase and GDP-L-Fuc pyrophosphorylase of rAtFKGP

Metal ion[a]	L-Fucokinase		GDP-L-Fuc pyrophosphorylase	
	munit/mg protein	*%*[b]	*munit/mg protein*	*%*[b]
None	0 ± 0	(0)	0 ± 0	(0)
Mg^{2+}	119 ± 3	(100)	616 ± 27	(100)
Mn^{2+}	145 ± 6	(122)	163 ± 15	(27)
Co^{2+}	0 ± 0	(0)	38 ± 2	(6)
Ba^{2+}	0 ± 0	(0)	0 ± 0	(0)
Ca^{2+}	0 ± 0	(0)	0 ± 0	(0)
Cu^{2+}	0 ± 0	(0)	0 ± 0	(0)
Hg^{2+}	0 ± 0	(0)	0 ± 0	(0)
Al^{3+}	0 ± 0	(0)	0 ± 0	(0)
Fe^{3+}	0 ± 0	(0)	0 ± 0	(0)

[a] The final concentration of metal ions was 2 mM.

[b] Relative activities are expressed as a percentage of that in the presence of 2 mM Mg^{2+}.

Table 3

Effect of nucleotide triphosphates on activity of GDP-L-Fuc pyrophosphorylase of rAtFKGP

Nucleoside triphosphate	Enzyme activity[a]	Relative activity[b]
	munit/mg protein	*%*[b]
None	457 ± 0	100
ATP	468 ± 6	102
CTP	431 ± 23	94
ITP	433 ± 14	95
UTP	477 ± 15	104
GDP	500 ± 11	109
PPi	164 ± 3	36

[a] GDP-L-Fuc pyrophosphorylase activity of rAtFKGP was determined in the presence of 1 mM of other nucleotides or PPi under standard assay conditions.

[b] Relative activities are expressed as a percentage of that without addition of these compounds.

Table 4

Kinetics of rAtFKGP

Substrate	K_m [c]	k_{cat}
	mM	*/s*
L-Fucokinase activity[a]		
L-Fuc	1.0	0.34
ATP	0.45	0.38
GDP- L-Fuc pyrophosphorylase activity[b]		
(GDP-L-Fuc formation)		
L-Fuc 1-P	0.052	1.59
GTP	0.17	1.82
(GDP-L-Fuc degradation)		
GDP-L-Fuc	0.40	7.86
PPi	0.18	8.02

[a] The L-fucokinase activity of rAtFKGP was measured under standard assay conditions with varying concentrations of L-Fuc (0.5-10 mM) and ATP (0.1-1.0 mM).

[a] The GDP-L-Fuc pyrophosphorylase activity in the forward direction (synthesis of GDP-L-Fuc) was measured under standard assay conditions by incubation with varying concentrations of L-Fuc 1-P (0.02-1.0 mM) and GTP (0.05-2.0 mM), and that in the reverse direction with varying concentrations of GDP-L-Fuc (0.05-1.0 mM) and PPi (0.1-2.0 mM).

[c] The K_m and k_{cat} values were calculated from a Hanes-Woolf plot using the obtained activities.

A

Pyrophosphorylase consensus motif

```
                  *
AtFKGP   VLMLHAGGDSKRV-PWANPMGKVFLPLP  152
OsFKGP   VLLLHAGGDSKRV-PWANPMGKAFLPLP  171
MmGFPP   ILLIHSGGYSQRL-PNASALGKIFTALP  127
AtVTC1   KALILVGGFGTRLRPLTLSFPKPLVDFA   29
PsUSP    AFVLVAGGLGERL---GYNGIKVALPAE  143
AtUGP1   VVLKLNGGLGTTM---GCTGPKSVIEVR  105
HsUGP2   VVVKLNGGLGTSM---GCKGPKSLIGVR  133
HsAGX1   AVLLLAGGQGTRL---GVAYPKGMYDVG  128
```

ATP-binding motif

```
                  *
AtFKGP   TWANVPRGSGLGTSSILAAA  840
OsFKGP   TWANVPRGSGLGTSSILAAA  855
MmFK     TWSELPHGSGLGTSSILAGA  849
AtARA1   VSSAVPEGKGVGSSAAVEVA  707
AtGalK   VSSTVPEGKGVGSSASVEVA  639
PfGalK   ITGDLPLGAGLSSSASFEVG  113
HsGalK   VVSSVPLGGGLSSSASLEVA  148
ScGalK   CEGDVPTGSGLSSSAAFICA  177
```

B

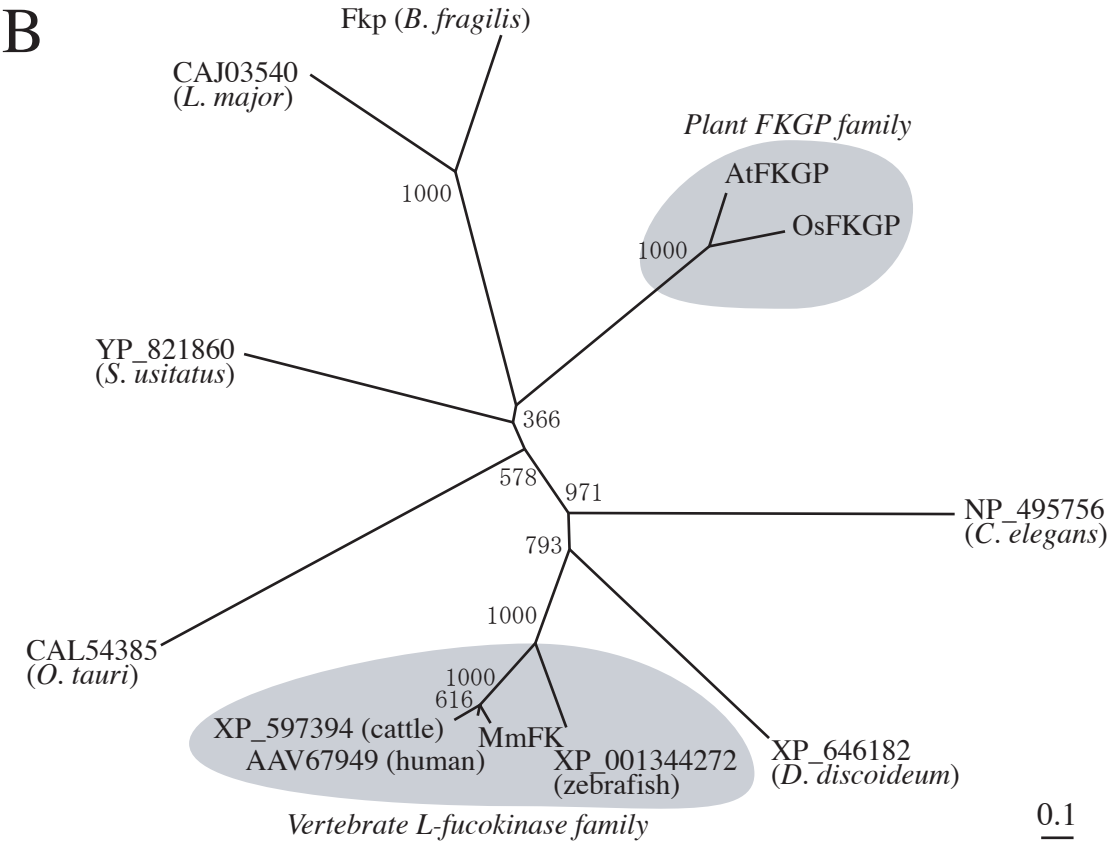


Fig. 1. Kotake et al.

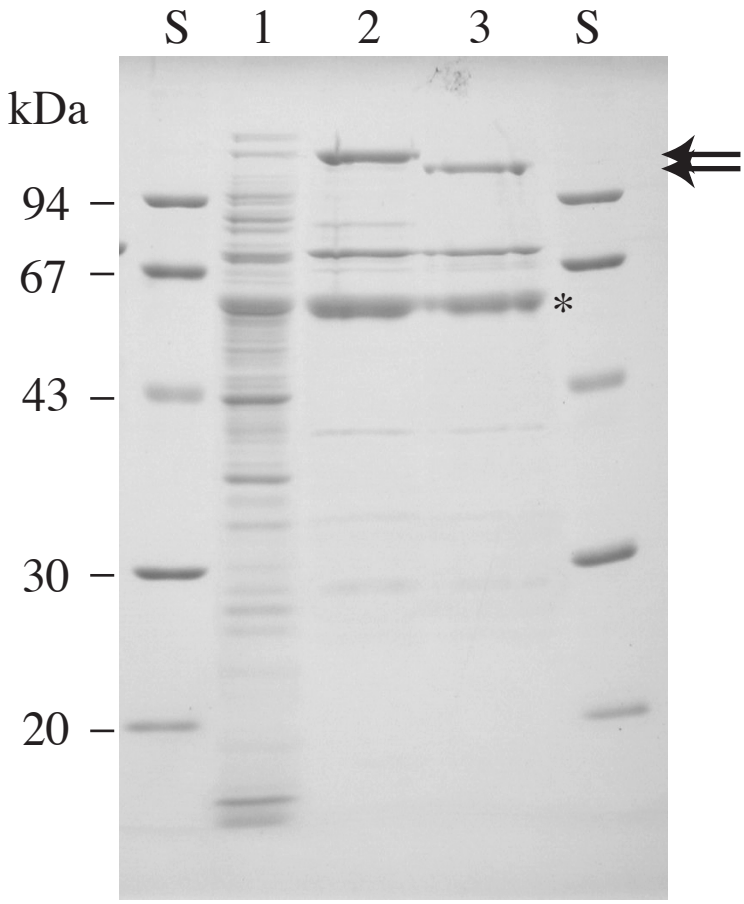


Fig. 2. Kotake et al.

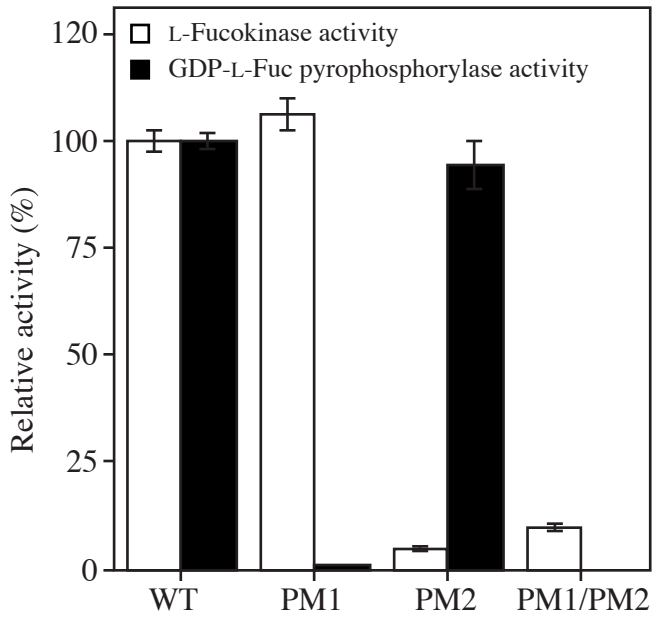


Fig. 3. Kotake et al.

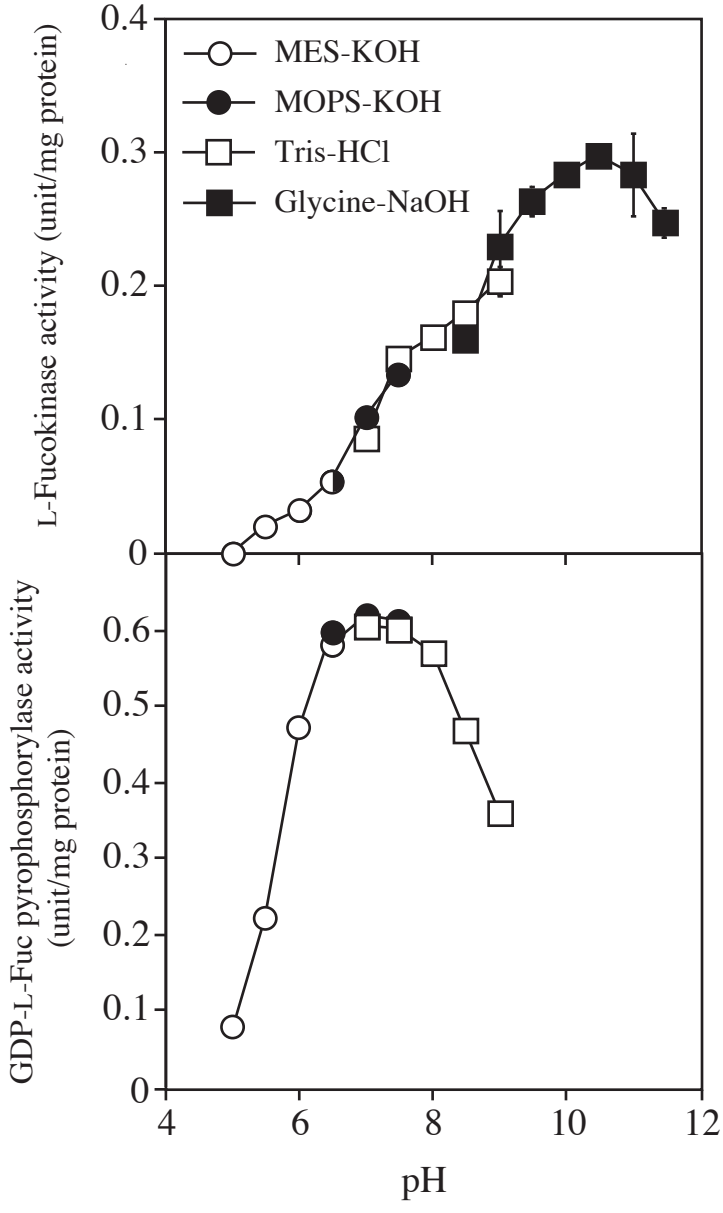


Fig. 4. Kotake et al.

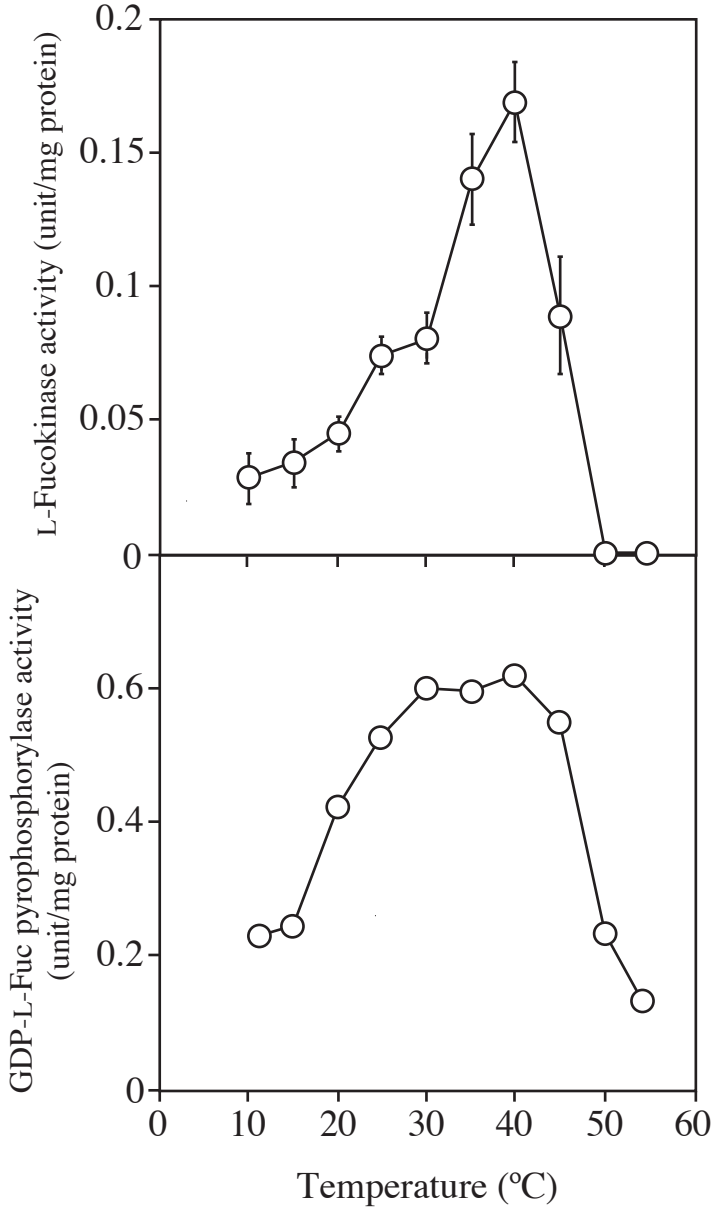


Fig. 5. Kotake et al.

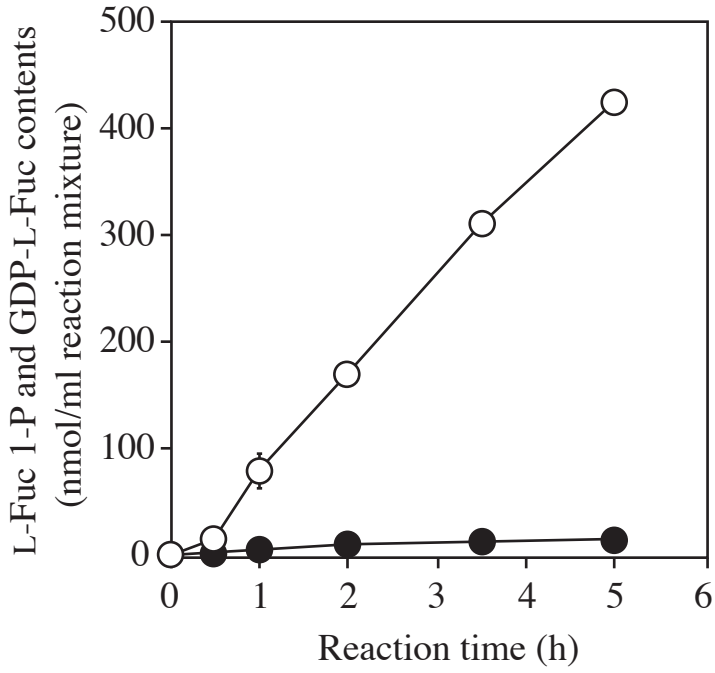


Fig. 6. Kotake et al.

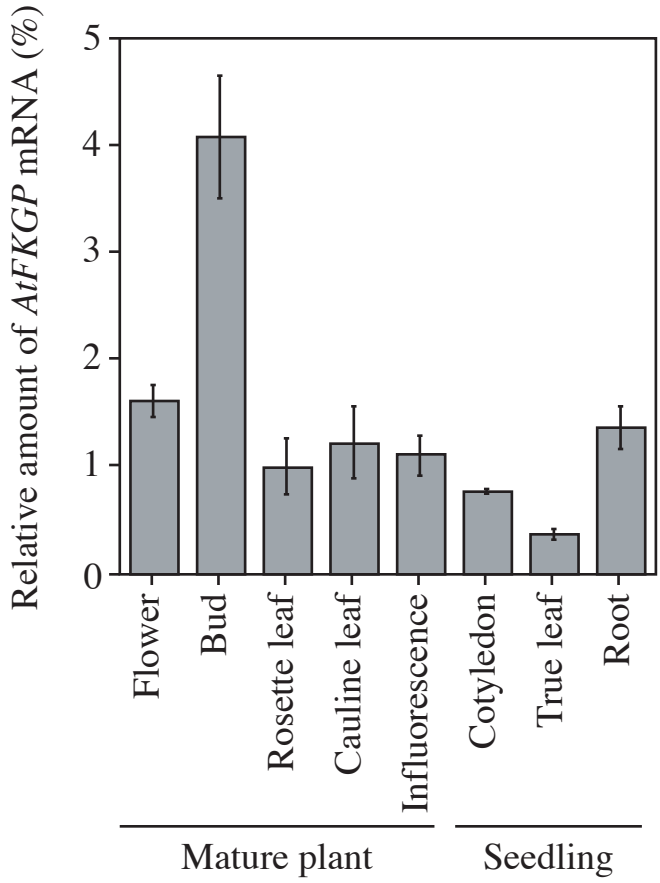


Fig. 7. Kotake et al.

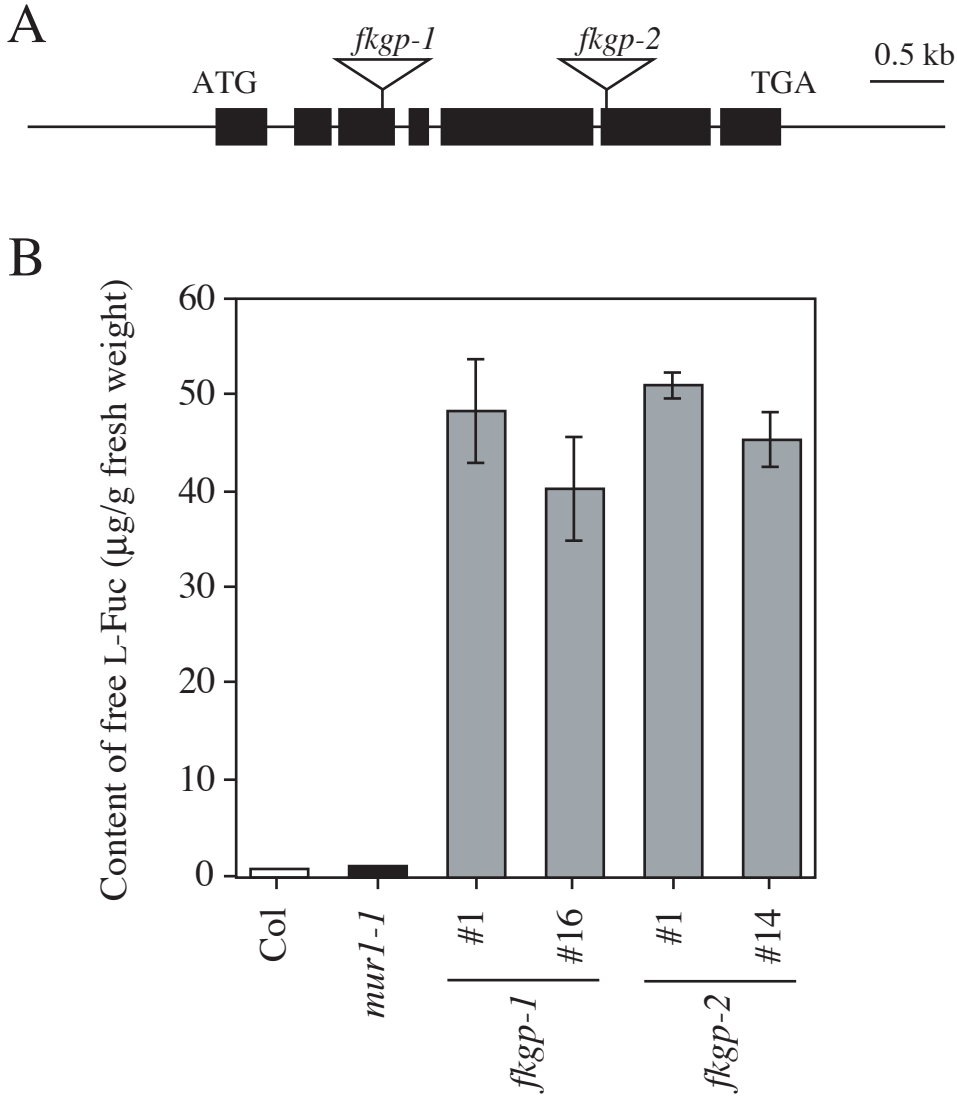


Fig. 8. Kotake et al.

Supplementary Fig. 1. **Chromatographic separation of nucleotides and nucleotide sugars on HPLC.** *A*, elution patterns of standard L-Fuc 1-P (retention time, 10.3 min) and L-Fuc (2.3 min) on HPAEC-PAD are shown. *B*, *C* and *D*, elusion patterns of standard GDP-sugars, ADP-Glc, UDP-sugars, and nucleotides. Elution times of standard specimens were as follows: GDP-L-Fuc (30.2 min), GDP-Glc (30.9), GTP (38.9), AGP-Glc (22.8), ADP (28.0), ATP (34.0), UDP-Gal (21.5), UDP-Glc (22.6), UDP-L-Ara (23.2), UDP-Xyl (23.7), UDP (27.4), UTP (32.7), UDP-GlcA (36.0), CTP (28.3), ITP (40.4).

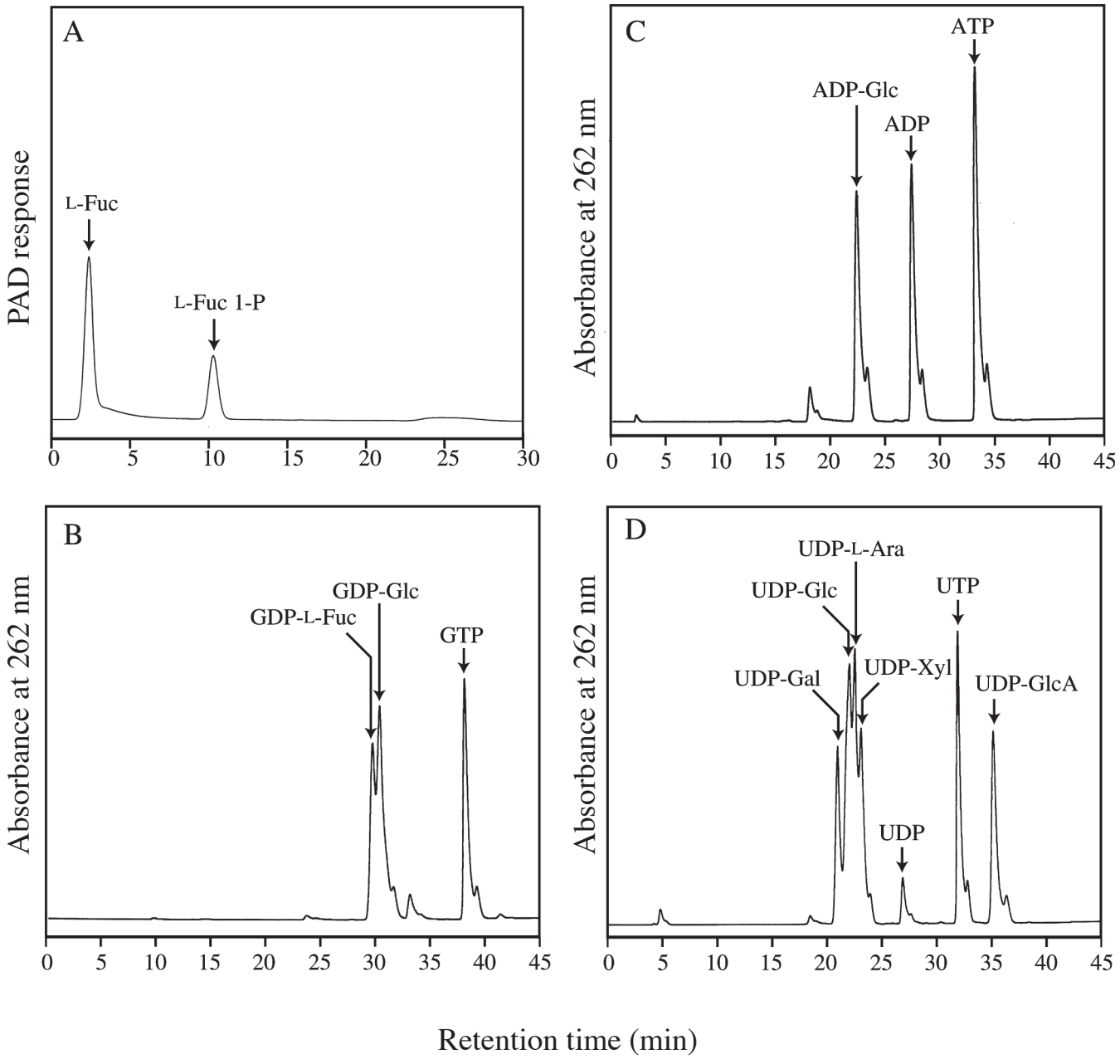
Supplementary Fig. 2. **Preparation and identification of GDP-L-Fuc generated from L-Fuc, ATP, and GTP by recombinant AtFKGP**. Successive reactions of L-fucokinase and GDP-L-Fuc pyrophosphorylase activities were carried out using a large-scale reaction mixture consisting of 50 mM Tris-HCl buffer, pH 7.5, 1 mM $MgCl_2$, 20 mM L-Fuc, 2 mM ATP, 2 mM GTP, 5% (w/v) glycerol, and the recombinant enzyme in a final volume of 20 ml. After incubation at 35°C for 24 h, the reaction products were applied onto a 2.2 x 14.5-cm charcoal column (Wako). After washing the column with water (200 ml), the adsorbed GDP-L-Fuc was eluted with 70% (v/v) ethanol containing 0.1 M ammonium hydroxide (300 ml), and monitored for total sugar and absorbance at 260 nm. To purify the GDP-L-Fuc, the eluted GDP-L-Fuc fraction was concentrated and then subjected to preparative paper chromatography using Whatman 3MM filter paper with 7:4:2 (v/v/v) 1-butanol/ethanol/water as the solvent (to wash out salts from the mixture), followed by 5:2 (v/v) ethanol/1 M ammonium acetate, pH 6.8 (to separate GDP-L-Fuc

from other reaction components). GDP-L-Fuc was extracted from the paper with water, and further purified by a HPLC as described for the analysis of GDP-L-Fuc in the EXPERIMENTAL PROCEDURES section. The ^{1}H-NMR spectra for the GDP-L-Fuc (A) were recorded, and compared with those of standard GDP-L-Fuc purchased from Sigma-Aldrich Japan (B). The signals assigned to respective H groups of the L-Fuc moieties are indicated.

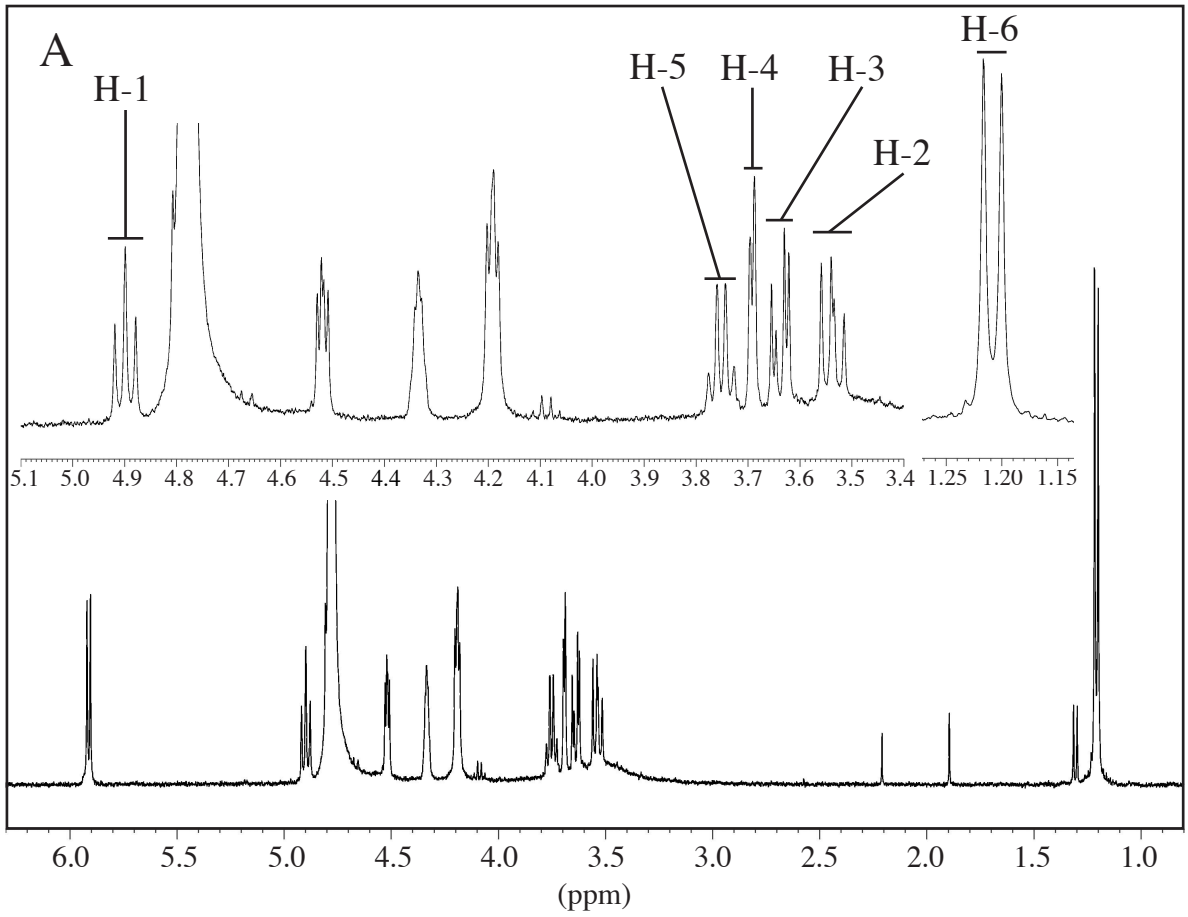
Supplementary Fig. 3. **Determination of L-Fuc content in the soluble fraction prepared from *Arabidopsis* plants.** The concentrations of free monosaccharides included in the soluble fraction prepared from wild-type (*A*) and *fkgp-1* (*B*) plants were determined using an HPAEC-PAD system. In the *fkgp-1* mutant, free L-Fuc accumulated whereas the contents of other monosaccharides such as L-Ara and Glc detected in this experiment were not affected. Elution times of standard monosaccharides (*C*) were: L-Fuc (4.7 min), L-Rha (7.7), L-Ara (8.5), Gal (11.0), Glc (12.0), Man (12.8), Xyl (13.5). Note that the elution protocol (25) used in the experiment was different from that for L-Fuc 1-P (see the EXPERIMENTAL PROCEDURES section).

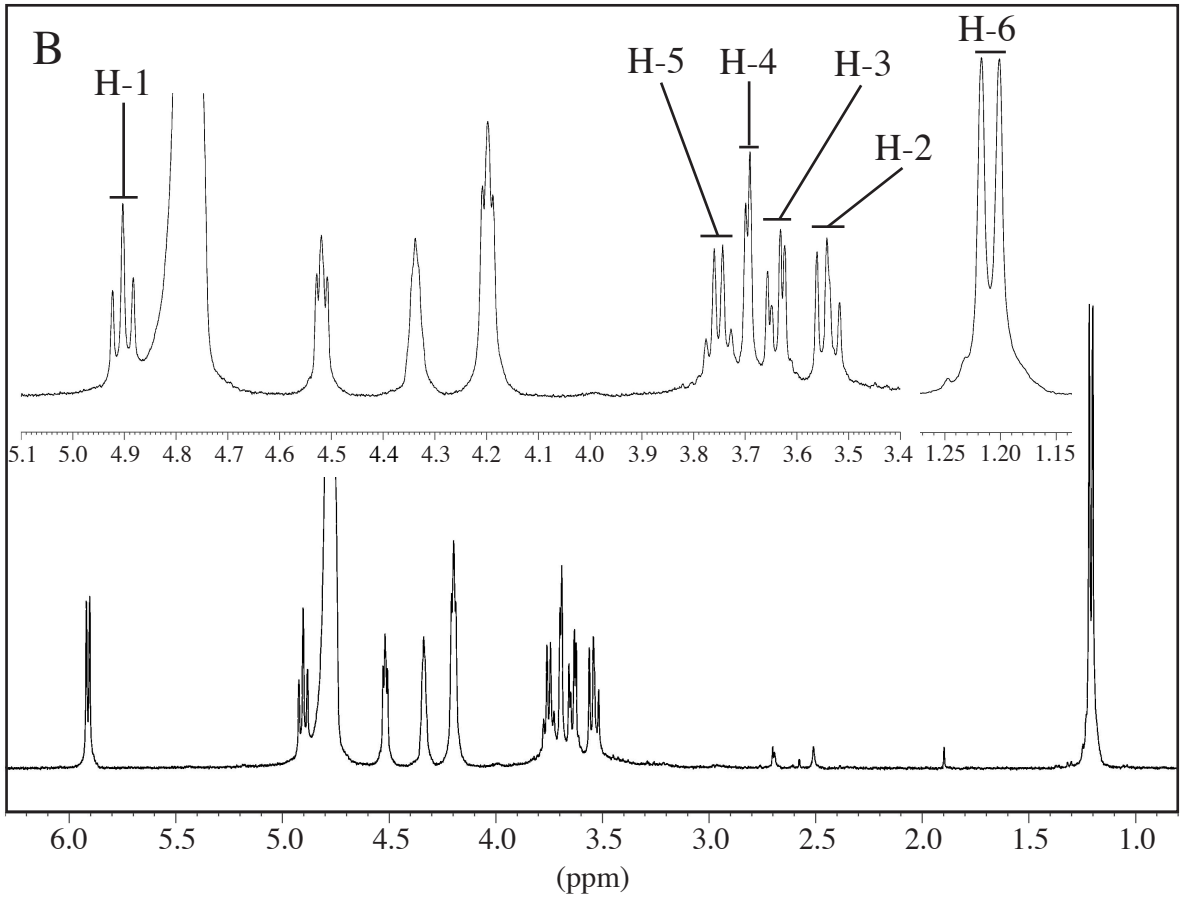
Supplementary Fig. 4. **Amino acid sequences of AtFKGP and OsFKGP.** The amino acid sequence of AtFKGP was aligned with OsFKGP by the pairwise method using the ClustalW program. The amino acid residues are numbered from the first methionine. Gaps (-) were introduced to achieve maximum similarity. Identical amino acid residues among between AtFKGP and OsFKGP are highlighted in black. The putative

pyrophosphorylase consensus motif is *underlined*, and the ATP-binding motif is *doubly-underlined*. Asterisks indicate the Gly residues of AtFKGP that are substituted with Ala residues in the point mutation (PM1 and PM2) experiments shown in Fig. 3.

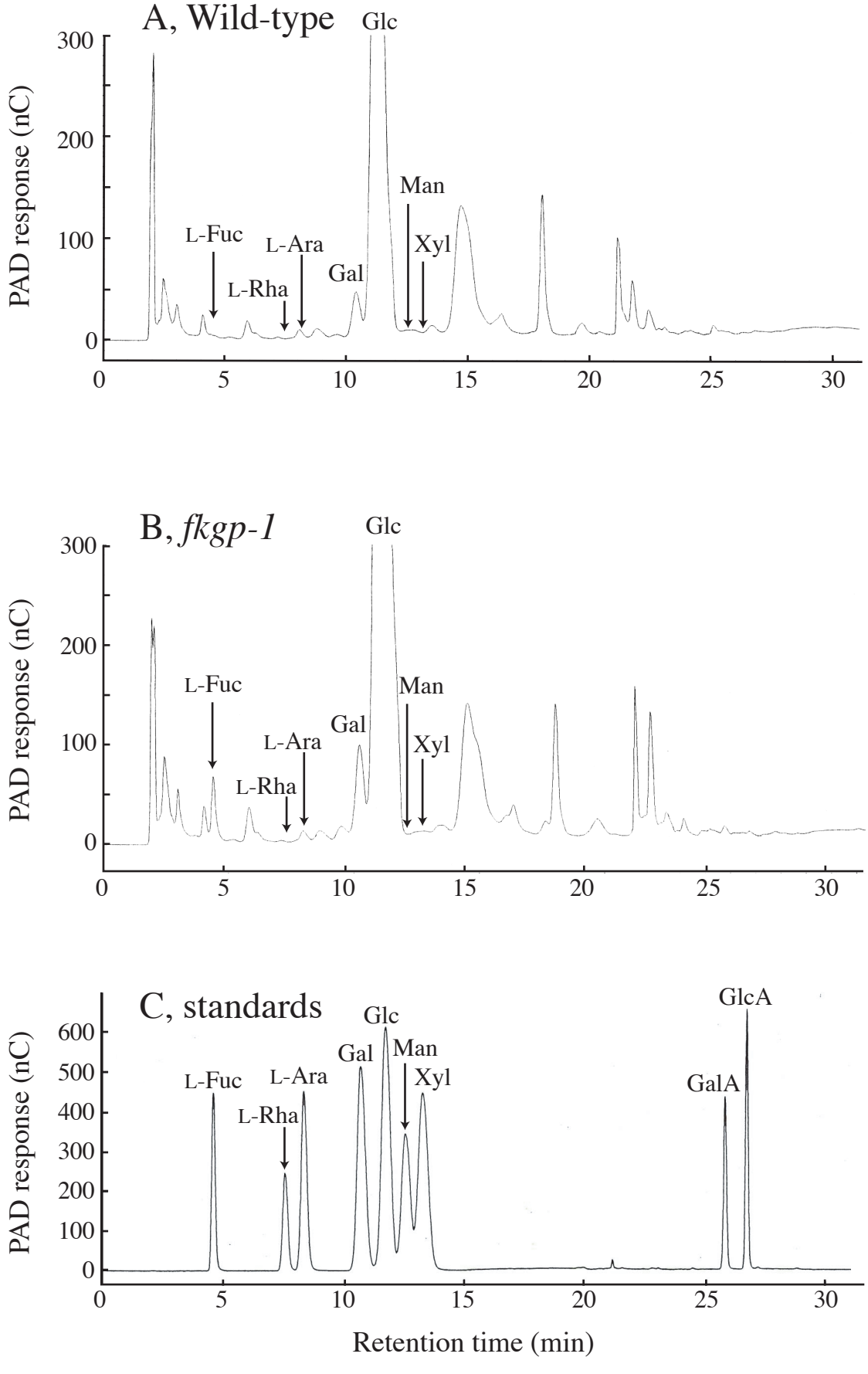


Supplementary Fig. 1. Kotake et al.

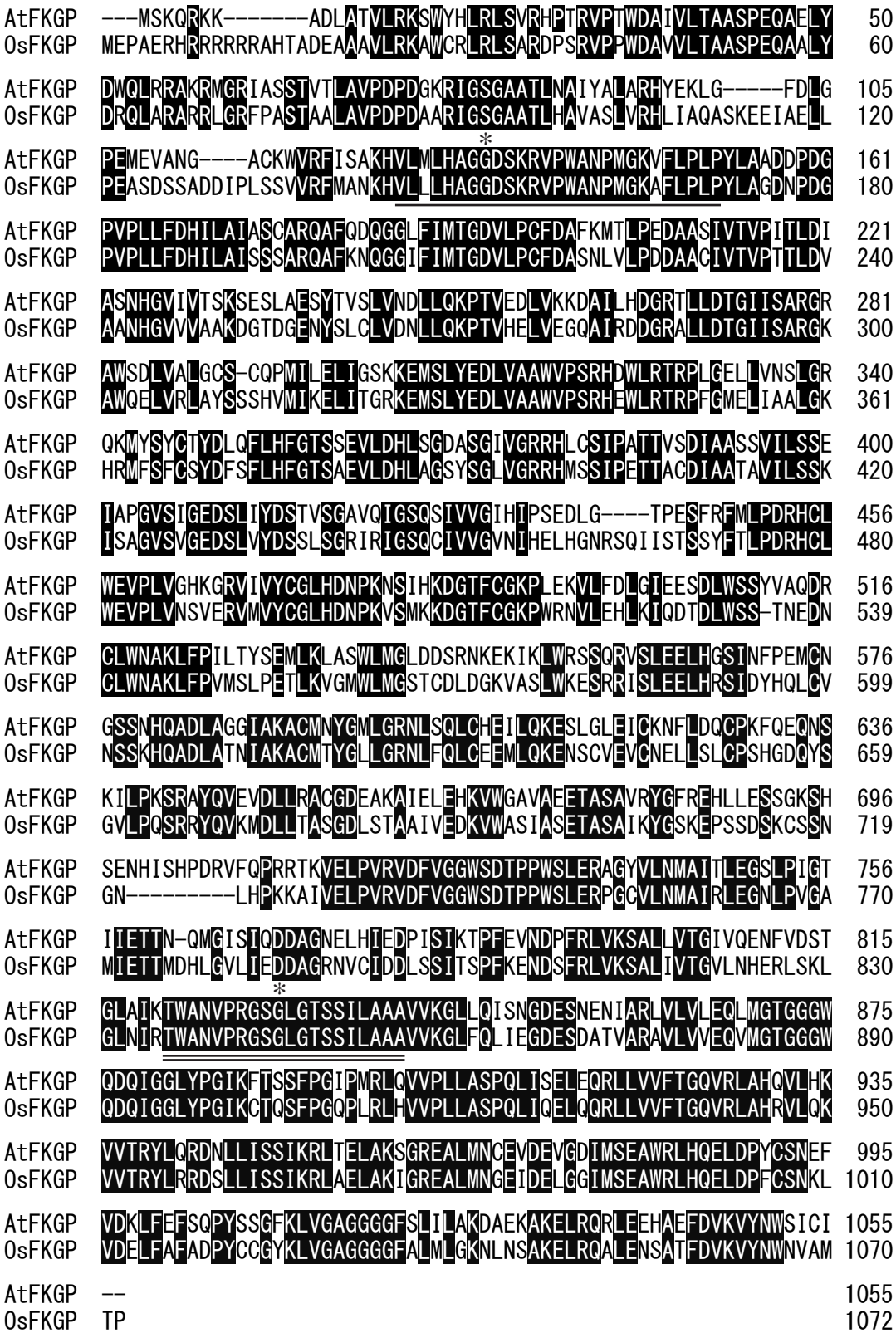




Supplementary Fig. 2. Kotake et al.



Supplementary Fig. 3. Kotake et al.



Supplementary Fig. 4. Kotake et al.