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

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Monomeric sugars generated during the metabolism of polysaccharides, glycoproteins, and glycolipids are imported into the cytoplasm and converted to respective nucleotide sugars via monosaccharide 1-phosphates, to be reutilized as activated sugars. Because 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 data base of Arabidopsis, the gene (designated AtFKGP) of a bifunctional enzyme with 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 rAtFKGP showed either Gly233 or Gly330 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 tDNA 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 salvage free L-Fuc in Arabidopsis.

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 nonreducing terminal residues attached through α-(1→2)-linkages to penultimate sugar residues in xyloligucan and arabinoalgalactan protein (1, 2). The L-fucosylated trisaccharide side chains of xyloligucan modulate the interaction of xyloligucan with cellulose microfibrils, thereby affecting the mechanical properties of plant cell walls (3, 4), whereas some N-glycans of plant glycoproteins have L-fucosyl residues attached through α-(1→3)-linkages to the proximal GlcNAc residues (5). The L-fucosylated trisaccharide is 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 xyloligucan-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 dimer 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

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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 (\textit{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 UDP 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 GDP-\( l \)-Fuc 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 \textit{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 \textit{Arabidopsis} and rice, neither \( l \)-fucokinase nor GDP-\( l \)-Fuc pyrophosphorylase has been identified so far in seed plants.

To clarify the molecular mechanism of free \( l \)-Fuc salvage in seed plants, we searched the genomic data base and identified a gene that encodes a protein with similarity to both \( l \)-fucokinase and GDP-\( l \)-Fuc pyrophosphorylase in \textit{Arabidopsis}. From the properties of the recombinant protein expressed in \textit{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 \textit{Arabidopsis}.

**EXPERIMENTAL PROCEDURES**

\textit{Materials—} \textit{Arabidopsis thaliana} ecotype Columbia (Col) was used in this study. The TDNA insertion lines, SALK-012400 and SALK-053913 (genetic background, Col), and the \textit{mur1-1} mutant (genetic background, Col) were provided by the Nottingham Arabidopsis Stock Center (Loughborough, UK). \textit{Arabidopsis} plants were grown on rockwool fibers (Nittobo, Tokyo, Japan) under continuous light at 23 °C for 35 days. The geno-type of the At1g01220 gene in the tDNA insertion lines was determined by genomic PCR using specific primers. A partial fragment derived from the wild-type At1g01220 gene, including the tDNA insertion site in SALK-012400, was amplified by genomic PCR with specific primers 012400-F-1 (5’-GGACGCACTCTCCTTGACACTGGG-3’) and 012400-R-1 (5’-GCCATCATGCTTGAAAGAACCC-3’), and a fragment of the wild-type At1g01220 gene in SALK-053913 was amplified with 053913-F-1 (5’-GTTGAGCATAAAAGTATGGGGACG-3’) and 053913-R-1 (5’-ATGTGTAGCTGTATTTCCAGCTG-3’). The tDNA insertion was confirmed by amplifying a genomic fragment with LBb1 (5’-GCCGACCCGCTTCTGCAACT-3’) and 012400-F-1 in SALK-012400 and LBb1 and 053913-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.

\textit{ATP, CTP, GDP, GTP, ITP, UTP, PPI, \( \textit{L}\)-Ara, \( \textit{L}\)-Fuc, Gal, \( \textit{L}\)-Gal, GalA, Glc, GlcA, GlcNAc, Man, \( \textit{L}\)-Rha, Xyl, Gal-1-P, Glic-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. \( l \)-Ara-1-P (\( \textit{L}\)-arabinopyranose 1-P) was chemically synthesized according to the methods of Aspinall et al. (20) and MacDonald (21).

\textit{Protein Determination—} The concentration of protein was determined by the method of Bradford (22) with bovine serum albumin 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 Coomassie Brilliant Blue R-250 on SDS-PAGE (23) compared with the bovine serum albumin standard.

\textit{cDNA Cloning—} Total RNA was extracted from 2-week-old \textit{Arabidopsis} seedlings containing cotyledons, true leaves, and roots. The seedlings were frozen in liquid nitrogen and then homogenized with a 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 \textit{Arabidopsis} seedlings using a reverse transcriptase, ReverTra Ace-α (Toyobo, Osaka, Japan), and an oligo(dT) adaptor primer (5’-GGCAATCATCATGGAATTCGAGATTCCCGATTTTTTTTTTTTTTTTT-3’). For cloning of At1g01220 cDNA, a set of primers, At1g01220-F-1 (5’-GGCAATTCTACGTCTCAAGCAGAGGAG-3’) and At1g01220-R-1 (5’-GGAATTCAATACAGTACCCT-3’), was designed based on the genomic data base of \textit{Arabidopsis}. The PCR was performed with proofreading polymerase (KOD-Plus, Toyobo) and the set of primers, using the single strand cDNA as a tem-
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 EcoRI 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 His\(_6\) tags at the N terminus. The plasmid construct designed to express the recombinant enzyme fused to thioredoxin was excised from 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 His\(_6\) 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 harvested and lysed in a buffer containing 0.5 mM Tris-HCl, 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 × 60-cm chelating Sepharose FF column (GE Healthcare, 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 the bound protein was then 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 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′-GCACGTAAGACCTATCTAC-3′), and FKGP-PM2-F (5′-TCTAGGAACCTCCGAGCATC-3′) and FKGP-PM2-R (5′-GCACGTCAGGGACAGATTG-3′), respectively. The nucleotide sequence of the cloned fragment was determined with an ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA).

Enzyme Assay—The L-fucokinase activity of the recombinant enzyme was determined using a reaction mixture consisting of 50 mM 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 mM sodium acetate for the initial 2 min, then by a linear gradient of 0.05–1.0 mM sodium acetate (2–40 min), followed by isocratic elution with 1 mM 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 (supplemental Fig. S1B). 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 pyrophosphorolysis activity of GDP-L-Fuc (reverse direction of the enzyme action) was performed in a 0.1-mM reaction mixture containing 50 mM MOPS-KOH buffer, pH 7.0, 2 mM MgCl\(_2\), 1 mM GDP-L-Fuc, 1 mM PP\(_i\), 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 pyrophosphorylase 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 supplemental Fig. S2), and analyzed by NMR spectroscopy. The sample was dissolved in D\(_2\)O, and 1H 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 (d\(_T\)12–18 primer. A set of specific primers for the At1g01220 gene, At1g01220-RTP-F (5′-GAACCAAGACCTTTT-
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GGGTGAA-3’ and At1g01220-RTP-R (5’-AAACCGTACTGCTCAGGATG-3’) and for ACTIN2 (ACT2), ACT2-RTP-F (5’-ACCTTGGCTGGACGTGACCT-3’) and ACT2-RTP-R (5’-CACAATCGTGTAGACTTGCG-3’), were designed using the Primer 3.0 program. The PCR was performed with a SYBR Premix ExTaq kit (Takara Bio Inc.) under the following conditions: 10 s denaturing at 95 °C, 30 s annealing at 60 °C, and 20 s amplification at 72 °C, 40 cycles. The PCR product was detected with Opticon 2 (Bio-Rad), 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 a mortar and pestle in water. The homogenate was centrifuged at 10,000 \( g \) 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. 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-cm DEAE-Sephadex A-25 (GE Healthcare) column. Neutral monosaccharides were eluted with 40 ml of water, concentrated by an evaporator, and then analyzed by HPAEC-PAD as described previously (25). Separation of standard monosaccharides is shown in supplemental Fig. S3.

**RESULTS**

Identification of Bifunctional \( L \)-Fucokinase/GDP-\( L \)-Fucose Pyrophosphorylase in the *Arabidopsis* Genome—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 Trp34 to Leu160). Other ORFs possibly encoding nucleotide sugar pyrophosphorylases were less similar to the human GDP-\( L \)-Fuc pyrophosphorylase sequence. Because At1g01220 encodes a protein that shares similarities with both \( L \)-fucokinase and GDP-\( L \)-Fuc pyrophosphorylase, we designated it as *AtFKGP* (*Arabidopsis thaliana* \( L \)-fucokinase/GDP-\( L \)-Fuc pyrophosphorylase). Although 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 reverse transcription-PCR using cDNA from *Arabidopsis* seedlings as the template, and the nucleotide sequence of *AtFKGP* was determined (supplemental Fig. S4). The comparison of the cDNA sequence with the genomic sequence of *Arabidopsis* (TAIR) 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. *AtFKGP* encodes a polypeptide of 1,055 amino acids with a calculated molecular mass of 116,351.5 Da and a theoretical isoelectric point (pl) 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) (supplemental Fig. S4). Based on the amino acid sequence deduced from the cDNA, *AtFKGP* appeared to contain a region (from Leu129 to Lys146) similar to the pyrophosphorylase consensus motif (*L*(*X*4)*G*(*X*2)*G*(*X*)4*PK*) conserved for nucleotide sugar pyrophosphorylases (13, 29). On the other hand, a region from *AtFKGP* was highly similar to the known ATP-binding motif (P(*X*2)*G*(*X*)4*SSA*) that forms a phosphate-binding loop wrapping around the \( \beta \)-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* (supplemental Fig. S4 and 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 a 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 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 a 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 tag pro-
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AtFKGP was expressed in E. coli. The protein (~1 μg) obtained after different purification steps was analyzed by SDS-PAGE. Protein in the gel was stained with Coomassie Brilliant Blue R-250. Lanes S, molecular mass markers; lane 1, lysate of E. coli; lane 2, AtFKGP purified on a chelating column; lane 3, thrombin-digested AtFKGP. The arrows indicate AtFKGP before and after thrombin digestion, and the asterisk indicates a chaperone protein, GroEL, expressed by the pGro7 plasmid.
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 1 month, but more than half of the GDP-1-Fuc pyrophosphorylase activity was lost within 1 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 1-Fucokinase was estimated at 0.17 unit/mg protein and that for GDP-1-Fuc pyrophosphorylase at 0.71 unit/mg protein (here the protein content was assessed based on the color intensity of rAtFKGP stained with Coomassie Brilliant Blue R-250 on SDS-PAGE). The specific activities of rAtFKGP apparently differ from those (1.27 and 0.36 units/mg protein, respectively) of pig 1-fucokinase (15) and GDP-1-Fuc pyrophosphorylase (18). As has been observed for many nucleotide sugar pyrophosphorylases from other sources, rAtFKGP catalyzed both synthesis of GDP-1-Fuc from 1-Fuc phosphate guanylyltransferase action, the forward direction), and pyrophosphorolysis producing 1-Fuc-1-P and GDP from GDP-1-Fuc and PPi (GDP-1-Fuc pyrophosphorylase action, the reverse).

The 1-fucokinase activity of rAtFKGP absolutely required divalent cations such as Mg²⁺ and Mn²⁺, as had the pig 1-fucokinase in Ref. 15. The divalent cations were also necessary for the GDP-1-Fuc pyrophosphorylase activity of rAtFKGP (Table 2). Although Mn²⁺ was a better cation than Mg²⁺ for the 1-fucokinase activity, it had only a poor effect on the GDP-1-Fuc pyrophosphorylase activity of rAtFKGP compared with Mg²⁺.

The maximal 1-fucokinase activity of the enzyme occurred at pH 10.5, whereas the maximal GDP-1-Fuc pyrophosphorylase activity of the enzyme was observed in the pH range from 6.5 to 7.5. The activities are shown as a percentage of that of wild-type (WT) rAtFKGP. Data are averages of triplicate assays.

| Metal ion | 1-Fucokinase | GDP-1-Fuc pyrophosphorylase |
|----------|--------------|-----------------------------|
| None     | 0 ± 0        | 0 ± 0                       |
| Mg²⁺     | 119 ± 3      | 616 ± 27                    |
| Mn²⁺     | 145 ± 6      | 163 ± 15                    |
| Ca²⁺     | 0 ± 0        | 0 ± 0                       |
| Ba²⁺     | 0 ± 0        | 0 ± 0                       |
| Cu²⁺     | 0 ± 0        | 0 ± 0                       |
| Hg²⁺     | 0 ± 0        | 0 ± 0                       |
| Ag⁺⁺     | 0 ± 0        | 0 ± 0                       |
| Fe³⁺     | 0 ± 0        | 0 ± 0                       |

* The final concentration of metal ions was 2 mM.
* Relative activities are expressed as a percentage of that in the presence of 2 mM Mg²⁺.
charides 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 cata-

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**TABLE 3**

| Nucleotide triphosphate | Enzyme activity\(^a\) (milliunits/mg protein) | Relative activity\(^b\) % |
|-------------------------|---------------------------------------------|--------------------------|
| None                    | 457 ± 0                                     | 100                      |
| ATP                     | 468 ± 6                                     | 102                      |
| CTP                     | 433 ± 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.
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FIGURE 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 circle) in the reaction mixture were determined by HPAEC-PAD and HPLC, respectively. Data are averages of triplicate assays.

lyzes conversion of l-Fuc to GDP-l-Fuc via l-Fuc-1-P, and it 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 1H NMR spectroscopy (supplemental Fig. S1). The signals of a doublet of doublets at δ 3.54 ppm (J2,3 9.9 Hz, J1,2 7.7 Hz, H-2 of l-Fuc), a doublet of doublets at δ 3.64 ppm (J5,6 3.4 Hz, J3,4 9.9 Hz, H-3 of l-Fuc), a doublet at δ 3.69 ppm (J5,6 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 (J6,7 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 (J1,2 7.7 Hz, J3,4 8.1 Hz, H-1 of l-Fuc) indicates that the anomeric configuration of the l-fucopyranose is β. All other signals in the 1H 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 Km and kcat values were calculated from a Hanes-Woolf plot using the obtained activities. 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). 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).

Expression of AtFKGP in Tissues of Arabidopsis—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 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.

TABLE 4

| Substrate         | Km  | kcat |
|-------------------|-----|------|
|                   | mM  | s    |
| l-Fuc             | 0.95| 1.42 |
| ATP               | 0.65| 1.55 |
| GDP-l-Fuc         | 0.86| 2.35 |
| GDP-l-Fuc         | 0.53| 3.15 |
| l-Fuc-1-P         | 0.91| 1.53 |
| GTP               | 1.07| 1.44 |
| GDP-l-Fuc         | 0.56| 3.01 |
| PPi               | 1.12| 1.45 |

a The Km and kcat values were calculated from a Hanes-Woolf plot using the obtained activities.

b 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).

c 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).
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 tDNA insertion in the AtFKGP gene, namely SALK-012400 (designated fkgp-1) and SALK-053913 (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 (supplemental Fig. S3). 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-fucokinasenases have then lost their GDP-l-Fuc pyrophosphorylase activity in the evolutionary process, whereas plant FKGP proteins and bacterial Fkp preserved both activities. The low sequence sim-
licity among plant FKG, 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, 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 not 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-fucose 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 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) Gly133 and Gly830 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) The 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 (Ala123–Lys522) of AtFKGP has significant similarity to a UDP-GlcNAc pyrophosphorylase from E. coli (38) and that the structure of the C-terminal region (Gly1993–Ile1995) is closely related 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.

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