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Characterization of a Fungal L-fucokinase involved in *Mortierella alpina* GDP-L-fucose Salvage Pathway

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Abstract

GDP-L-fucose functions as a biological donor for fucosyltransferases, which are required for the catalysis of L-fucose to various acceptor molecules including oligosaccharides, glycoproteins, and glycolipids. *Mortierella alpina* is one of the highest lipid-producing fungi and can biosynthesis GDP-L-fucose in the *de novo* pathway. Analysis of the *M. alpina* genome suggests that there is a gene encoding L-fucokinase (FUK) for the conversion of fucose to L-fucose-1-phosphate in the GDP-L-fucose salvage pathway, which has never been found in fungi before. This gene was characterized to explore its role in GDP-L-fucose synthesis. The yield of GDP-L-fucose is relatively higher in lipid accumulation phase (0.096 mg per g cell) than that in cell multiplication phase (0.074 mg per g cell) of *M. alpina*. Additionally, the transcript level of FUK is up regulated by nitrogen exhaustion when *M. alpina* starts to accumulate lipid, highlights the functional significance of FUK in the GDP-L-fucose biosynthesis in *M. alpina*. Gene encoding FUK was expressed heterologously in *Escherichia coli* and the resulting protein was purified to homogeneity. The product of FUK reaction was analyzed by liquid chromatography and mass spectrometry. Kinetic parameters and other properties of FUK were investigated. Comparative analyses between the FUK protein and other homologous proteins were performed. To our knowledge, this study is the first to report a comprehensive characterization of FUK in a fungus. *M. alpina* could be used as an alternative source for the production of GDP-L-fucose.

Keywords: Characterization/ GDP-L-fucose synthesis/ L-fucokinase/ *Mortierella alpina*/ salvage pathway

Introduction

L-fucose (6-deoxy-L-galactose) is an important monosaccharide in complex carbohydrates and is frequently found at the non-reducing end of oligosaccharide chains (Munro, J.R., et al. 1975). It is a constituent of cell wall polysaccharides in plants and some fungi of specific taxa (Bartnicki-Garcia, S. 1968, Levy, S., et al. 1997, Pengra, R.M., et al. 1969, Van Laere, A.J., et al. 1977), and a sugar moiety of glycoconjugates including glycoproteins and glycolipids (Ma, B., et al. 2006). Fucosylated carbohydrate structures are involved in a variety of biological and pathological processes in eukaryotic organisms, such as tissue development, angiogenesis, fertilization, cell adhesion, inflammation, and tumor metastasis (Ma, B., et al. 2006). Fucosylation is carried out by fucosyltransferases, which require the activated nucleotide form of L-fucose (GDP-L-fucose) as the L-fucosyl donor.

Currently GDP-L-fucose is still a relatively rare nucleotide sugar and thus laboratories working in the field of fucosylation would benefit of more accessible sources. As chemical synthesis of GDP-L-fucose is relatively complex and costly, microorganisms could be used as an alternative source of this molecule. In higher organisms, GDP-L-fucose can be generated in the cytoplasm from GDP-mannose via a *de novo* pathway or from L-fucose via a salvage pathway (Fig. 1) (Becker, D.J. and Lowe, J.B. 2003). The *de novo* synthesis of GDP-L-fucose is catalyzed by GDP-D-mannose 4, 6-dehydratase (GMD) and GDP-keto-6-deoxymannose 3, 5-epimerase/4-reductase (GMER). This pathway is present in most animal cells and tissues, as well as in plants and microorganisms. The salvage pathway utilizes fucose obtained from extracellular origin or from intracellular degradation of glycoproteins and glycolipids in specific circumstances (Niittymaki, J., et al. 2004). In the salvage biosynthetic pathway,

L-fucose is converted to L-fucose-1-phosphate by L-fucokinase (FUK). GDP-L-fucose pyrophosphorylase (GFPP) further catalyzes the formation of GDP-L-fucose from L-fucose-1-phosphate and GTP. FUK was identified in the humans (Hinderlich, S., et al. 2002), pigs (Park, S.H., et al. 1998), mice (Niittymaki, J., et al. 2004), *Arabidopsis* (Kotake, T., et al. 2008a) and *Bacteroides fragilis* (Liu, T.W., et al. 2011). Little is known about the molecular mechanisms of salvage reactions for free L-fucose in fungi. The yeast S*accharomyces cerevisiae* has been used to expressed the exogenous de novo and salvage pathway enzymes for the synthesis of GDP-L-fucose. Since most fungi cannot synthesis GDP-L-fucose themselves (Ren, Y., et al. 2010), fungi are not a traditional source for the production of GDP-L-fucose.

Mortierella alpina is a well-known polyunsaturated fatty acids (PUFA)-producing oleaginous fungi, which is one of the most important industrial species for the production of PUFAs (Wang, H., et al. 2013). After nitrogen exhaustion, cell multiplication stopped and cells now switched to lipid accumulation (Wynn, J.P., et al. 1999). The nitrogen metabolism is also suggested to be connected to nucleotide sugar metabolism in *Streptococcus thermophilusis* (Svensson, M., et al. 2007). The GDP-L-fucose *de novo* synthesis pathway in *M. alpina* was first purified and characterized in fungi in our laboratory (Ren, Y., et al. 2010), indicating that *M. alpina* could be used for the production of GDP-L-fucose. We have also sequenced the whole genome of *M. alpina* (ATCC 32222) (Wang, L., et al. 2011). Apart from GMD and GMER genes for the *de novo* pathway, a gene encoding FUK required for the free fucose-dependent salvage pathway was also found in *M. alpina*'s genome. Thus, *M. alpina* provides an interesting model for studying the mechanism of GDP-L-fucose biosynthesis in

fungi.

To clarify the molecular mechanism of free L-fucose salvage in fungi, we characterized the function of the gene encoding FUK in the GDP-L-fucose salvage pathway in *M. alpina in vitro*. Kinetic parameters and the effects of temperature, pH and metal ions on the activity of FUK were investigated. Multiple sequence alignment and phylogenetic analysis of the FUK protein with homologous proteins were also performed.

Results

Gene searching. The *M. alpina* genomic sequence was completed in our laboratory and deposited as GenBank accession number ADAG00000000 (Wang, L., et al. 2011). According to the genome searching, a putative FUK gene was found in the *M. alpina* genome (GenBank BankIt submission number 1825638). The searching results are shown in Table S1.

GDP-L-fucose content in *M. alpina*. GDP-L-fucose was extracted from the mycelia and analyzed by liquid chromatography and mass spectrometry (LC-MS) (Fig. 2B). The retention times of both the purified product (Fig. 2B) and commercial GDP-L-fucose standard (Fig. 2A) were 17.5 min, indicating GDP-L-fucose was successfully extracted from *M. alpina*. The amount of GDP- L-fucose was calculated from the peak areas by reference to an external standard GDP-L-fucose (Table. S4). In lipid accumulation phase (69 h), the amount of GDP-L-fucose in cell after purification was 0.096 mg per g cell, which is relatively higher than that in cell multiplication phase (18.5 h, 0.074 mg per g cell). Thus, *M. alpina* could be used as an alternative source for the production of GDP-L-fucose. In the future study, the amount of GDP- L-fucose can be calculated by reference to an internal standard such as

isotope labeling GDP- L-fucose to support reliability of the experiment.

Expression levels of genes involved in GDP-L-fucose biosynthesis. Real-time quantitative PCR (qRT-PCR) was performed to investigate the changes in the transcript levels of genes involved in GDP-L-fucose biosynthesis during the course of lipid accumulation, which was induced by nitrogen exhaustion (Wynn, J.P., et al. 1999). Total RNA samples were extracted from the mycelia collected from cultures in different growth phases. The mycelia samples were collected during cell multiplication stage (18.5 h), just before nitrogen was used up completely (20 h, and 22 h), and once the lipid had accumulated for a long period of time (33 h and 69 h). The relative expression levels of GMD, GMER, and FUK were measured in triplicate by qRT-PCR (Fig. 3). Ct values obtained after 18.5 h of incubation were chosen as the control values in order to calculate fold change in gene expression over time for each treatment. The GMD, GMER, and FUK genes were transcribed in all growth phases. The expression levels of GMD and GMER in cells collected were changed less than 1 fold in all grows phases of *M. alpina* (Fig. 3). Interestingly, in comparison with the sample collected at 18.5 h when nitrogen was nearly exhausted, the expression of FUK was up-regulated 4.3 folds in cells collected at 22 h when nitrogen was completely exhausted and lipids started to accumulate (Fig. 3C).

Expression and purification of FUK. FUK was expressed as a His-tagged fusion protein in *E. coli* by IPTG induction, and purified to near homogeneity by nickel ion affinity chromatography. The molecular mass was estimated to be approximately 49 kDa by SDS-PAGE (Fig. S1), which corresponds well with the calculated mass. The yield of FUK was 12.15 μg/ml.

Amino acid sequence alignment of *M. alpina* FUK protein and other homologous proteins. The predicted peptide sequence of GFPP does not contain a secretory signal or transmembrane domain, suggesting that FUK is located inside the cell. The sequence of *M. alpina* FUK shares low but significant similarities with FUK from Pig (14%), Homo sapiens (15%), and *Dictyostelium discoideum* (10%) at the amino acid level. Optimal superposition of the three structures and alignment of many sequences in each of the galactokinase, homoserine kinase, mevalonate kinase, and phosphomevalonate kinase (GHMP kinase) family proteins revealed a set of common conserved residues, distributed in five sequence motifs (Phosphate1, Connect1, Phosphate2, Adenosine, and Connect2), which are involved in ATP binding and in a putative inter domain hinge (Inagaki, E., et al. 2006, Kotake, T., et al. 2008a). Multiple sequence alignment of the *M. alpina* FUK and other characterized FUK proteins reveals the presence of certain conserved motifs and residues (Fig. S2), including an ATP-binding motif 188-197 (Pro-Val-Gly-Ser-Met-Gly-Thr-Ser-Ser-Ala) that forms a phosphate binding loop wrapping around the β-phosphate group of ATP in GHMP family proteins (Inagaki, E., et al. 2006, Kotake, T., et al. 2008a), five residues (Glu81, Gly83, Gly84, Val89, and Ile91) involved in the "Phosphate1" motif that form a ATP binding hairpin, three residues (Val171, Gly177, and Glu180) involved in the "Connect1" motif involved in connecting the two lobes of the structure, six residues (Leu282, Tyr284, Gly286, Leu295, Val298, Leu299), involved in the "Adenosine" motif involved in direct contact with ATP, and five residues (Ala379, Gly380, Gly383, Phe384, and Leu385) involved in "Connect2" motif crossing in the opposite direction for the connecting of the two lobes (Bork, P., et al. 1992, Hinderlich, S., et al. 2002, Inagaki, E., et al. 2006, Kotake, T., et al. 2008a). A phylogenetic

tree was generated with FUK sequences and other hexokinases (Fig. S3). *M. alpina* FUK clusters as a distinct group from FUK in human, mouse and *D. discoideum* in the phylogenetic tree.

Activity of FUK. L-fucose was incubated with the purified FUK protein and the reaction products were identified by LC-MS, as shown in Fig. 2. Extracted ion chromatogram (XIC) extraction (243.03 m/z, L-fucose-1-phosphate) of FUK reaction product presented a peak at 18 min (Fig. 2E), corresponding well to the position of the L-fucose-1-phosphate standard (Fig. 2C). In the sample incubated in the absence of the FUK, this peak was not observed (Fig. 2D). These results confirm that the recombinant protein shows FUK activity.

Properties of FUK. FUK was active at each of the temperatures tested (in the range of 4°C–65°C), with the greatest activity detected at 37°C (Fig. 4A). Maximal activity in the FUK reaction (defined as 100%) occurred at 37°C under the aforementioned conditions**.** The optimal temperature for FUK was in agreement with data previously obtained for *Arabidopsis* (Kotake, T., et al. 2008a). FUK retained 95% and 85% of its initial activity after 2 h of exposure at 4°C and 12°C (Fig. 4C), and lost more than 80% of its initial activity after 1.5 h of exposure at 20°C, 28°C and 37°C. FUK showed no activity after 0.5 h of exposure above 50°C (data not show). This indicated that FUK was relatively unstable at high temperature.

FUK showed optimal activity at pH 8.0 in 100 mM Tris-HCl buffer (Fig. 4B), which is consistent with the pH optima observed for recombinant pig and *Arabidopsis* FUK (Kotake, T., et al. 2008a, Park, S.H., et al. 1998). In addition, less than 40% FUK activity was retained at pH 3 and pH 12 after incubation at 4°C for 2 h (Fig. 4D), indicating that FUK was unstable under acidic and alkaline conditions.

FUK displayed null activity in absence of metal ions, and only had catalytic activity when divalent cations were added. In addition, even in the presence of metal ions, the enzyme lost all its activity when EDTA was added as metal ion chelating agent. Therefore, the recombinant FUK is a strictly metal-dependent enzyme, which is consistent with *Vitis vinifera*, human, and pig FUK (Cheng, C., et al. 2014, Kilker, R.D., et al. 1979, Park, S.H., et al. 1998, Richards, W.L. and Serif, G.S. 1977). To investigate the effect of metal ions, the relative activity of FUK was detected in presence of different metal ions. As shown in (Fig. 4E), Mg^{2+} gave the best stimulation, with maximal activity seen at a concentration of 2 mM. Mn^{2+} also stimulated the enzyme to nearly the same degree as Mg^{2+} with optimal activity occurring at about 2 mM. However, when Mg^{2+} was replaced with Co^{2+} or Fe^{2+} at a concentration of 2 mM, the enzyme activity was reduced to less than 5% of that in the presence of Mg^{2+}, respectively. A variety of other metal ions were tested and found to be inactive, including Ca^{2+}, Cu^{2+} , K^{+}, Hg^{2+}, Ni^{2+}, and Zn^{2+}. However, when Co^{2+}, and Fe^{2+} were added at 20 mM concentrations, they completely inhibited FUK activity.

FUK was found to be inhibited by the final product of this salvage pathway, GDP-L-fucose at the concentration of 55 μM in pig (Park, S.H., et al. 1998). The data in Fig. 4F shows the effect of increasing amounts of GDP-L-fucose to FUK reaction mixture. However, when the concentration of GDP-L-fucose was up to 200 μM, more than 70% FUK activity was retained.

The kinetics of the reaction catalyzed by FUK present a good fit with the Lineweaver and Burk model (Fig. S4). The Km for L-fucose was determined to be 1.59 mM, which is in accordance with the data previously obtained for *Arabidopsi*s FUK (Kotake, T., et al. 2008a).

The Vmax and Kcat values of FUK for L-fucose were determined to be 0.035 mM min^{-1} and 23.35 S^{-1} respectively.

Discussion

Chemical synthesis of GDP-L-fucose is a time-consuming process, generally requiring anhydrous chemical techniques, which are often beyond the methodological scope of the researcher who needs to use GDP-L-fucose (Liu, T.W., et al. 2011). Commercial preparations of the commonly occurring nucleotide sugars are available, but tend to be very costly. *M. alpina* could be used as an alternative and efficient source for the production of GDP-L-fucose, as >0.4 mg/L GDP-L-fucose was produced. It should also be noted that no optimization of the culture conditions has been performed so far to increase the yield of GDP-L-fucose. The generation of GDP-L-fucose through the salvage pathway is supposed to be important for the synthesis of L-fucose-containing glycoconjugates under severe growth conditions where GDP-L-fucose cannot be sufficiently obtained through the *de novo* pathway (Kotake, T., et al. 2008a). Since the yield of GDP-L-fucose is relatively higher in lipid accumulation phase, and the transcript level of FUK is up regulated by nitrogen exhaustion when *M. alpina* starts to accumulate lipid, FUK may have considerable importance for GDP-L-fucose biosynthesis under nitrogen starvation.

In higher animals, the salvage pathway of GDP-L-fucose is catalyzed by two separated enzymes: FUK and GFPP (Hinderlich, S., et al. 2002, Niittymaki, J., et al. 2004, Park, S.H., et al. 1998). In plants and bacteria, these two separate salvage reactions are catalyzed by a bifunctional enzyme (FKP) with both FUK and GFPP activities (Cheng, C., et al. 2014,

Kotake, T., et al. 2008a, Liu, T.W., et al. 2011). The *M. alpina* FUK can only convert L-fucose to L-fucose-1-phosphate, indicating that fungi also possess a salvage pathway for free L-fucose similar to that of higher animals. The *M. alpina* FUK has weak but significant similarities to mammalian FUK, bacterial FKP, and plant FKP and contains ATP-binding consensus motifs. These facts suggest that the *M. alpina* FUK gene has evolved from an ancestral gene common to mammalian FUK, bacterial FKP, and plant FKP genes. It is possible that the mammalian and fungal FUK have then lost their GFPP activity in the evolutional process, whereas plant FKP and bacterial FKP preserved both activities. The low sequence similarity among fungal FUK, mammalian FUK, plant FKP, and bacterial FKP suggests that the horizontal gene spread is an old event.

Fucosylation, present in vertebrates, invertebrates, plants, and bacteria, is crucial for many biological processes and the fucosylated carbohydrate structures expressed on the outer membrane of microbes play an important role in bacterial pathogenesis through interacting with the human host (Ma, B., et al. 2006). Despite the significant progress in understanding the various fucosylation processes made in the past decade, many areas related to fucosylation are presently unknown. Because most fungi do not synthesize GDP-L-fucose and lack GDP-L-fucose-dependent fucosyltransferases, the function of GDP-L-fucose in fungi is unclear. To investigate the functions of GDP-L-fucose in *M. alpina*, the genome was searched for the presence of potential GDP-L-fucose-dependent genes; fucosyltransferase 10 (FUT10), fucosyltransferase 11 (FUT11), and O-fucosyltransferase (OFUT) genes were found. Based on our previous transcriptome analysis data (Chen, H., et al. 2015), the expressions of these genes were confirmed and shown in Table S2. In higher organisms, GDP-L-fucose functions

as a biological donor for fucosyltransferases, which are required for the catalysis of L-fucose to various acceptor molecules including oligosaccharides, glycoproteins, and glycolipids (Oriol, R., et al. 1999). Although the same function has never been demonstrated in fungi, it is highly likely that GDP-L-fucose plays a similar role in *M. alpina* based on the identification of the fucosyltransferase homologue. Still, this needs to be confirmed experimentally in future studies.

To the best of our knowledge, this is the first time that a FUK in the GDP- L-fucose salvage pathway has been characterized at the molecular level in a fungus. The role of GDP-L-fucose in *M. alpina* and lipid accumulation are deserving of further investigation. *M. alpina* could be used as an alternative source for the production of GDP-L-fucose.

Materials and methods

Strains and growth conditions.

M. alpina (ATCC 32222) was cultured on potato dextrose agar at 25ºC for 7 days. The cultures were initially cultivated in 200 mL Kendrick medium (Kendrick, A. and Ratledge, C. 1992) and incubated at 25ºC for 36 h. These cultures were used to provide a 20% (v/v) seed to inoculate a 2 L (working volume) fermenter containing modified Kendrick medium, supplemented with ammonium tartrate (2 g/L) and glucose (30 g/L) (Wynn, J.P., et al. 1999). The fermenter contents were incubated at 25ºC and stirred at 500 rpm with aeration at 0.5 v/v per min. The mycelia were collected by filtration through sterile cheesecloth, and frozen immediately in liquid nitrogen for RNA extraction.

Gene searching. Predicted genes in the *M. alpina* (ATCC 32222) genome (GenBank accession number ADAG00000000) were annotated by BLAST (White, S.W., et al. 2005) searches against protein databases with E-value 1E-5: NR (www.ncbi.nlm.nih.gov), KOGs and COGs (Tatusov, R.L., et al. 2003), KEGG (Kanehisa, M., et al. 2004), Swiss-Prot and UniRef100 (Wu, C.H., et al. 2006), BRENDA (Chang, A., et al. 2009), and by InterProScan (Quevillon, E., et al. 2005) against protein domain databases with default parameter settings. Pathway mapping was conducted by associating EC assignment and KO assignment with KEGG metabolic pathways based on BLAST search results.

Extraction of GDP-L-fucose. The GDP-L-fucose in *M. alpina* was extracted using the method of Liu with minor modifications (Liu, T.W., et al. 2011). Cells were homogenized in liquid nitrogen with a pestle and then resuspended in 1 mL of ice-cold 1 M formic acid containing 10% 1-butanol (v/v, a lipid solvent). The samples were pipetted gently and kept on

ice water for 30 min. The supernatant was then cleared of cell debris by centrifugation at 6000 × g for 10 min at 4°C and subjected to LC-MS analysis.

qRT-PCR. Total RNA extraction was performed using Trizol Reagent (Invitrogen) according to the manufacturer's instructions and RNA was subjected to RNase-free DNase I digestion and then purified using an RNease Mini kit (Qiagen). Total RNA was reverse transcribed with the PrimeScript RT reagent kit (Takara Bio, Inc) following the manufacturer's instructions. qPCR was conducted with 2 µl of reverse-transcribed cDNA using a CFX96 Real-Time PCR System (Bio-Rad) and SYBR® Green PCR Master Mix (Bio-Rad) according to the manufacturer's guidelines. The primer pairs used for qPCR are shown in Table I. The PCR cycling conditions were: 10 s at 95°C followed by 30 s at 55°C for a total of 40 cycles. The 18S rDNA gene was used as the reference gene. Fold-changes in gene expression were calculated using the formula $2^{-(\Delta\Delta Ct)}$, where ΔΔCt is ΔCt (treatment) – ΔCt (control), ΔCt is Ct (target gene) – Ct (18S), and Ct is the cycle value at which the fluorescence of the genes of interest crosses threshold levels.

Cloning and plasmid construction. The FUK gene was amplified by PCR from cDNA using the primer pairs shown in Table I. The PCR condition used was as follows: denaturation at 94ºC for 30 s, annealing at 53ºC for 45 s and extension at 68 ºC for 2 min (for 25 cycles in total); the final volume in each well was 50 µl. The amplified product was cloned into $pET28a^{+}$ to construct pw710 (containing FUK), which was confirmed by sequencing using an ABI 3730 Sequencer.

Protein expression and purification. The plasmid construct was transfected into the BL21 (DE3) gold strain of *E. coli*. The cells were grown overnight at 37ºC with shaking in LB

medium containing 50 μg/mL kanamycin. The overnight culture (10 mL) was inoculated into 500 mL of fresh medium and grown until the OD600 reached 0.6. Expression of FUK was induced by the addition of 0.01 mM IPTG at 12ºC for 48 h. After the IPTG induction, cells were harvested by centrifugation, washed with binding buffer (50 mM Tris-HCl, 300 mM NaCl and 10 mM imidazole; pH 7.4), resuspended in the same buffer (supplemented with 1 mM phenylmethanesulfonyl fluoride and 1 mg/mL lysozyme), and then sonicated. Cell debris was removed by centrifugation, and the supernatants (containing soluble proteins) were collected. The His-tagged fusion protein was purified by nickel ion affinity chromatography using a Chelating Sepharose Fast Flow column according to the manufacturer's instructions (GE Healthcare). Unbound proteins were washed with 50 mL of wash buffer (50 mM Tris-HCl, 300 mM NaCl, and 25 mM imidazole; pH 7.4). Fusion proteins were eluted with 5 mL of elution buffer (50 mM Tris-HCl, 300 mM NaCl and 500 mM imidazole; pH 7.4) and dialyzed overnight at 4℃ against 1 M Tris-HCl buffer containing 20% glycerol (pH 7.4). The protein concentration was determined by the Bradford method and proteins were stored at -80℃.

Enzyme activity assays. The FUK activity of the recombinant enzyme was determined by monitoring the formation of L-fucose-1-phosphate in the presence of L-fucose and ATP. The FUK activity was assayed using the method of Kotake (Kotake, T., et al. 2008b) with minor modifications. The reaction mixture for FUK contained 50 mM Tris-HCl (pH 7.4), 2 mM $MgCl_2$, 20 mM L-fucose, 2 mM ATP and 25 nM purified FUK protein in a total volume of 50 μl. After incubation at 37 °C for 30 min, the reaction was terminated by the addition of 50 μl ethanol. This assay method was defined as the standard assay condition for FUK activity.

Samples were prepared for LC-MS after centrifugation.

LC-MS analysis. LC was operated on Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLC systems, including a WPS3000 autosampler, a HPG3400 binary pump, and a TCC3000 column oven. This system was coupled online with the Q Exactive™ Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific Inc., MA, USA) to acquire the MS data. The mass spectrometer (Q-Exactive) was calibrated daily using the commercial calibration mixture (caffeine, n-butylamine, MRFA, sodium dodecyl sulfate, sodium taurocholate and Ultramark™ 1621). A heated electrospray ionization (HESI-II) source was operated at negative mode, with the following parameters: ionization voltage, -2.8 kV; sheath and auxiliary gas, 35 and 10, both were nitrogen and at arbitrary units; probe heat temperature, 250 °C; capillary temperature, 325 °C. Full scan with an *m/z* range of 55-700 and selected ion monitoring (SIM) scan of 588.08 (GDP-L-fucose) and 243.03 (L-fucose-1-phosphate) with an isolation width of 1 *m/z* were performed simultaneously. The resolution and the maximal injection time for both scan modes were 70,000 FWHM and 200 ms. The AGC target (maximal target capacity of the C-trap) was 1×10^6 ions for full scan and 2×10^5 ions for SIM scan. The LC-MS was controlled by Xcalibur 2.2 SP1.48 (Thermo Fisher Scientific Inc., MA, USA). Chromatographic separation was carried out on a zwitterionic ZIC®-HILIC column (100 mm × 2.1 mm, 3.5 μm) (Merck, Darmstadt, Germany) which was maintained at 35 °C. The mobile phase consisted of solvent A (5 mM CH_3COONH_4 in water) and solvent B (Acetonitrile). Gradient elution program was shown as in Table S3. The injection volume was 5 μL. The amount of GDP- L-fucose and L-fucose-1-phosphate was calculated from the peak areas by reference to their external standard samples.

Effects of temperature, pH, metal ions, and GDP-L-fucose on the activity of FUK. In order to determine the optimal temperature, FUK reactions were carried out at 4°C, 12°C, 20°C, 28°C, 37°C, 45°C, 55°C, and 65°C for 30 min at pH 7.4. For determinations of optimal pH, three different buffers were used over a range of pH 3 to 11, including 100 mM sodium acetate-acetic acid (pH 3-6), 100 mM Tris-HCl (pH 7-8), and 100 mM glycine-NaOH (pH 10-12). The thermal stability of the enzyme was studied by incubating the enzyme in Tris-HCl buffer (100 mM, pH 7.4) at various temperatures. At given time intervals, samples were withdrawn and the residual activity was measured under above standard assay conditions. To determine the pH stability, the enzyme was incubated at pH 3–12 at 4°C for up to 2 h, and the remaining enzyme activity was measured at time intervals (0.5 h, 1 h, 1.5 h, and 2 h) under standard assay conditions. A range of metal ion concentrations (0.5 µM-20 mM) was used to test the effect of metal ions on the FUK activity, including KCl, $MgCl_2$, $MnCl_2$, $FeCl_2$, $CuCl_2$, and $CoCl_2$. These reactions were carried out under standard assay conditions and replicated 3 times. A range of GDP-L-fucose concentrations (0-200 µM) was used to test the effect of product concentration on the FUK activity, and these reactions were carried out under standard assay conditions and replicated 3 times.

Measurement of kinetic parameters. To measure the K_m and V_{max} values for FUK using L-fucose as a substrate, reactions were carried out using various concentrations of L-fucose (1-20 mM) in conjunction with a fixed concentration of ATP (2 mM). The FUK reactions were performed in Tris-HCl pH 7.4 at 37°C for 30 min in a final volume of 50 µl. The kinetic parameters were obtained at various concentrations of one substrate at a fixed concentration of the other based on the Lineweaver and Burk equation. To prove that this approach can

measure the initial rate of reaction, the amount of L-fucose-1-phosphate was calculated from the peak areas by reference to L-fucose-1-phosphate standard. This experiment was replicated 3 times.

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Abbreviations

FUK, L-fucokinase; GDP, guanosine 5′-diphosphate; GMD, GDP-D-mannose 4, 6-dehydratas; GFPP, GDP-L-fucose pyrophosphorylase; GMER, GDP-keto-6-deoxymannose 3, 5-epimerase/4-reductase; LC-MS, liquid chromatography and mass spectrometry; PCR, polymerase chain reaction; PUFAs, polyunsaturated fatty acids; qRT-PCR, real-time quantitative PCR; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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Figure Legends

Figure 1. *De novo* (A) and salvage (B) pathways of GDP-L-fucose synthesis.

Figure 2. LC-MS chromatographs: Extracted ion chromatogram (XIC) of GDP-L-fucose standard (m/z 588.08) (A); XIC of GDP-L-fucose in *M. alpina* (m/z 588.08) (B); XIC of L-fucose-1-phosphate standard (m/z 243.03) (C); XIC of blank reaction (m/z 243.03) (D); XIC of FUK reaction product m/z 243.03) (E);

Figure 3. Relative expression levels of GMD (A), GMER (B), and FUK (C) genes during the accumulation of lipids in *M. alpina*. Values are means of three replications ± standard deviation.

Figure 4. Effects of temperature, pH, metal ions, and GDP-L-fucose on the activity of FUK. Panel A and B show the effect of temperature and pH on the activity of FUK. Panel C and D represented the effect of temperature and pH on the stability of FUK. Panel E and F represented the effect of metal ions and GDP-L-fucose on FUK activity. The pH stability was analyzed by pre-incubating the enzyme buffers of different pH values at 4 °C for 2 h and measuring the remaining activity at 37 °C and pH 7.4. Values are means of three replications ± standard deviation.

Tables

Table I. Primers used in this study.

Gene	*Primer sequence*[a]
FUK (forward)	CTAGCTAGCATGCTACTCGGGAGTGCAG
FUK (reverse)	CCCAAGCTTTTACGGACATTCACTCCCG
FUK (qPCR)	GGCGAATCCTACACGACTGAC
FUK (qPCR)	GAGTTCTAGCGAGGTTGCTGTGA
GMD (qPCR)	CGAGAAGGGCTACCAGGTTC
GMD (qPCR)	GAGACCACAGGTACGGATGG
GMER (qPCR)	GAACCGTGCCTACAACCAGC
GMER (qPCR)	CGAAGGGTCCAGATGAAGAGC
18S rDNA	CTATTGGCGGAGGTCTATTCGT
18S rDNA	GCACGCATTCGGATAATTGGT

[a] Restriction cleavage sites are underlined.