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The *MUR1* gene of *Arabidopsis thaliana* encodes an isoform of GDP-D-mannose-4,6-dehydratase, catalyzing the first step in the *de novo* synthesis of GDP-L-fucose

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ABSTRACT **GDP-L-fucose is the activated nucleotide sugar form of L-fucose, which is a constituent of many structural polysaccharides and glycoproteins in various organisms. The *de novo* synthesis of GDP-L-fucose from GDP-D-mannose encompasses three catalytic steps, a 4,6-dehydration, a 3,5-epimerization, and a 4-reduction. The *mur1* mutant of *Arabidopsis* is deficient in L-fucose in the shoot and is rescued by growth in the presence of exogenously supplied L-fucose. Biochemical assays of the *de novo* pathway for the synthesis of GDP-L-fucose indicated that *mur1* was blocked in the first nucleotide sugar interconversion step, a GDP-D-mannose-4,6-dehydratase. An expressed sequence tag was identified that showed significant sequence similarity to proposed bacterial GDP-D-mannose-4,6-dehydratases and was tightly linked to the *mur1* locus. A full-length clone was isolated from a cDNA library, and its coding region was expressed in *Escherichia coli*. The recombinant protein exhibited GDP-D-mannose-4,6-dehydratase activity *in vitro* and was able to complement *mur1* extracts *in vitro* to complete the pathway for the synthesis of GDP-L-fucose. All seven *mur1* alleles investigated showed single point mutations in the coding region for the 4,6-dehydratase, confirming that it represents the *MUR1* gene.**

L-Fucose (6-deoxy-L-galactose) is a monosaccharide found in a diverse array of organisms. The sugar is a known component of bacterial lipopolysaccharides, mammalian and plant glycoproteins, and polysaccharides of plant cell walls such as xyloglucan and rhamnogalacturonans I and II. The precise function of L-fucose within these polysaccharides is not clear, but it may stabilize conformations of xyloglucan, which can efficiently bind to cellulose microfibrils (1), possibly aiding in cell wall integrity. Furthermore, xyloglucan fucosylation is essential for the biological activity of some xyloglucan-derived oligosaccharides (2). The pathway for the synthesis of L-fucose has been studied biochemically, but genes for the corresponding enzymes have not been cloned from any eukaryote.

GDP-L-fucose (guanosine-diphospho-L-fucose) is the activated form of this sugar, synthesized *de novo* from GDP-D-mannose via a three-step mechanism or through a salvage pathway involving phosphorylation of free L-fucose and subsequent nucleoside-diphosphate attachment (3–5). The *de novo* pathway for GDP-L-fucose production is shown in Fig. 1. The first step is catalyzed by GDP-D-mannose-4,6-dehydratase and involves the formation of the intermediate GDP-4-keto-6-deoxy-D-mannose, which is then used in the second and third steps of the pathway by 3,5-epimerase and 4-reductase activities to yield GDP-L-fucose. The pathway was initially elucidated in bacteria but has since been characterized in mammalian and plant systems (6–11).

Recently an L-fucose-deficient cell wall mutant, *mur1*, was isolated from *Arabidopsis thaliana* and characterized phenotypically (12). Eight recessive *mur1* alleles were obtained from this screen, most of which exhibit 50- to 200-fold reductions in L-fucose in the cell walls of shoot organs, while the L-fucose content in roots is only reduced by 40%. The *mur1* mutation causes reduced tensile strength of elongating stem segments and slight dwarfism. When *mur1* plants are grown in the presence of exogenous L-fucose, the L-fucose content is restored to wild type and all other mutant phenotypes are rescued. This suggests that the salvage pathway is intact and that the lack of L-fucose observed in the *mur1* mutant may be due to a defect in the *de novo* synthesis of GDP-L-fucose.

Our knowledge of plant genes encoding enzymes involved in nucleotide sugar interconversions is limited with only two genes isolated so far, a UDP-D-glucose-4-epimerase from *Arabidopsis* (13) and a UDP-D-glucose dehydrogenase from soybean and *Arabidopsis* (14). Bacterial pathways have been studied in more detail and genes for putative GDP-D-mannose-4,6-dehydratases have been identified and are believed to play a role in the synthesis of GDP-L-fucose and/or GDP-colitose (GDP-3,6-dideoxy-L-galactose; refs. 15 and 16). However, no biochemical evidence for the postulated function of these enzymes has been described. Here, we report on the cloning of the *MUR1* gene of *A. thaliana* and demonstrate that it encodes an isoform of GDP-D-mannose-4,6-dehydratase. This represents the first example of a gene in plant nucleotide sugar interconversions for which a mutant has been obtained.

MATERIALS AND METHODS

Plant Material. All plants were grown in environmental chambers at 23°C and 60–70% humidity under continuous fluorescent light (60–70 μmol·m^{-2}·s^{-1}). Wild-type plants of the Columbia ecotype and mutant plants carrying the *mur1-2* allele (12) were used for all biochemical assays.

Enzyme Extraction and Assay. Crude extracts containing GDP-D-mannose-4,6-dehydratase and GDP-4-keto-6-deoxy-D-mannose-3,5-epimerase-4-reductase activities were obtained from freshly harvested leaf tissue from 14-day-old seedlings homogenized with a mortar and pestle in two volumes of buffer 1 (100 mM Tris·HCl buffer, pH 7.6/2 mM DTT) containing 1% (wt/vol) polyvinylpolypyrrolidone. The homogenate was strained through Miracloth (Calbiochem) and centrifuged at 5000 rpm for 5 min in Sorvall rotor SS34, and pelleted cell debris was discarded. Protein was precipitated from the soluble extract by $(NH_4)_2SO_4$ fractionation, and the

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Abbreviation: EST, expressed sequence tag.
Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. U81805).
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protein fraction precipitated between 25% and 75% saturation was collected by centrifugation at 15,000 rpm for 30 min in a Sorvall SS34 rotor. This material was then resuspended in a minimum volume of buffer 2 (20 mM Tris·HCl buffer, pH 7.6/0.5 mM DTT) and dialyzed (3500 Da molecular mass cutoff) against the same buffer. All steps were completed at 0–4°C. Both enzyme activities were stable for several months at −20°C.

Crude extracts from 50-ml cultures of *Escherichia coli* were prepared by sonication in 4 volumes of buffer 1 per g of fresh weight. The bacterial extracts were centrifuged at 10,000 rpm for 20 min in Sorvall rotor SS34, and the supernatant was dialyzed against buffer 2. All steps were completed at 0–4°C.

The combined activities of both dehydratase and epimerase-reductase converting GDP-D-mannose to GDP-L-fucose (steps 1–3 in Fig. 1) were measured in a reaction mixture comprising 20 μl of enzyme extract and 10 μl of Tris·HCl buffer (pH 7.6) containing 925 Bq of GDP-D-[U-^{14}C]mannose (specific activity 10.4 GBq·mmol^{-1}; Amersham), 0.15 μmol of NADPH, and disodium EDTA at a final assay concentration of 10 mM. Incubation was for 1 h at 37°C. The reaction was stopped by heat denaturation at 100°C for 3 min. Assay of the 4,6-dehydratase activity alone (step 1 in Fig. 1) was completed in the above reaction mix minus NADPH and the GDP-4-keto-6-deoxy-D-mannose intermediate was chemically reduced by the addition of 1 μmol of $NaBH_4$ to the stopped reaction and a further incubation for 90 min at 37°C. GDP-4-keto-6-deoxy-D-mannose for the assay of the 3,5-epimerase-4-reductase (steps 2 and 3 in Fig. 1) was produced using the same conditions without subsequent chemical reduction, and the assay was completed by the addition of 0.15 μmol of NADPH and 20 μl of protein extract and a further 1-h incubation at 37°C.

Reaction products were analyzed by TLC on Baker-Flex 250-μm cellulose plates (J. T. Baker). GDP-D-mannose and GDP-L-fucose were analyzed directly in ethanol/1 M ammonium acetate, pH 7 (7:3 by volume). Nucleoside-diphospho sugars were hydrolyzed, where indicated, by addition of 100 μl of 2 M trifluoroacetic acid and incubation at 95°C for 20 min to produce free monosaccharides. Trifluoroacetic acid was removed *in vacuo* and monosaccharides analyzed in 1-butanol/acetic acid/water (12:3:5 by volume) followed by ethyl acetate/pyridine/water (8:2:1 by volume) in the same direction. Nucleoside-diphospho sugars were identified by radiolabeled standards, and monosaccharides by authentic sugars stained with aniline-hydrogen phthalate (17). Developed chromatograms were analyzed using the Bio-Rad Molecular Analyst phophorimaging system and software.

Isolation and Analysis of *Arabidopsis* and Plasmid DNA. *Arabidopsis* DNA was isolated from 3-week-old plants (18) and further purified using anion exchange columns according to the manufacturer's protocol (Qiagen, Chatsworth, CA). Restriction enzyme digests were performed using the manufacturer's specifications (New England Biolabs). For Southern blots, ≈1 μg of digested genomic DNA was loaded per lane and separated by electrophoresis through 0.8% agarose gels. Radiolabeled probes were created by excision of the cDNA insert from pZL1 clones using *Eco*RI and *Bam*HI, purification via the Qiaquick gel extraction kit (Qiagen), and random primer labeling with [α-^{32}P]dCTP (specific activity 111 TBq·mmol^{-1}; Amersham). Nucleic acid hybridizations were done as described (19). Overnight exposure at −80°C using BioMax film with a BioMax intensifying screen was performed according to the manufacturer's specifications (Kodak).

Plasmid DNA from *E. coli* cells was extracted and purified using the Qiaprep spin miniprep or midiprep kit following the protocol suggested by the manufacturer (Qiagen).

Isolation of cDNA Clones. A digoxigenin-labeled probe corresponding to expressed sequence tag (EST) clone 90A12T7 was created via PCR using the T7 and SP6 primers flanking the multiple cloning site of pZL1 (GIBCO/BRL). Labeling conditions were as described in the manufacturer's protocol (Boehringer Mannheim), except that the ratio between dTTP and digoxigenin-dUTP was adjusted to 4:1. The digoxigenin-labeled probe was used to screen a lambda PRL-2 library (20) obtained from the *Arabidopsis* Biological Resource Center (Ohio State University) via a colorimetric detection procedure, as specified by Boehringer Mannheim.

Genetic Mapping. The *mur1-2* mutant (background Columbia) of *A. thaliana* was crossed to wild-type Landsberg *erecta* (L*er*) plants, F_1 plants were obtained, and these were selfed to obtain an F_2 population. Identification of *mur1* plants via gas–liquid chromatography of alditol acetates was done as described previously (12).

Restriction fragment length polymorphisms were identified between Columbia and L*er* DNA by probing Southern blots with ^{32}P-labeled cDNA clones *GMD1* and *GMD2*. Total genomic DNA from *mur1* plants obtained from the F_2 population was digested with *Hpa*I and *Scr*FI, respectively, and probed with their corresponding ^{32}P-labeled cDNA (*GMD1* and *GMD2*).

RNA Isolation and Northern Blots. RNA was isolated from leaves of 3-week-old *Arabidopsis* plants by grinding in liquid nitrogen, phenol extraction, and precipitation with LiCl (21). Approximately 10 μg of RNA was prepared for agarose gel electrophoresis by glyoxylation, run at 3–4 V/cm on an Owl Scientific (Woburn, MA) Buffer Puffer recirculating electrophoresis system, blotted to a positively charged nylon membrane (Hybond N+; Amersham), and hybridized with ^{32}P-labeled *GMD2* via standard procedures (21). To determine the approximate size of the *GMD2* mRNA, bacteriophage lambda DNA digested with *Hin*dIII was heat-denatured, glyoxylated, and probed separately with ^{32}P-labeled lambda DNA after blotting.

PCR Conditions and Cloning. The sequences of the oligonucleotide primers used for subcloning coding regions of the *MUR1* gene into pBluescript (KS+) and the pET11d expression vector (Stratagene) were as follows: *MUR1*/pBlue/pET11d, upper, 5′-ACACTGCAGCCATGGCGTCAGAGAACAACG-3′, and *MUR1*/pBlue/pET11d, lower, 5′-ACAGATATCAAGGTTGCTGCTTAGCATCC-3′, with *Nco*I and *Pst*I sites engineered into the upper primer and an *Eco*RV site engineered into the lower primer. PCR was performed using a model 2400 Gene Amp PCR System and PCR Core Reagents (Perkin–Elmer) with the following conditions: denaturation at 94°C for 10 min, cooling to 20°C, and addition of reaction mix, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 59°C for 1 min, extension at 72°C for 1.5 min, and final extension at 72°C for 10 min. The final concentration of $MgCl_2$ was 2 mM.

Cloning of PCR products into pBluescript KS+ was done as follows: PCR products gel-purified using the Qiaquick gel extraction kit from Qiagen were digested with *Pst*I and *Eco*RV. Following ligation to pBluescript KS+ cleaved with *Pst*I and *Eco*RV, the DNA was used for transformation into *E. coli* XL1 Blue MRF′ supercompetent cells according to the manufacturer's protocol (Stratagene). Seven *mur1* alleles and the wild-type allele were cloned by this procedure.

To clone the *MUR1* coding region into the pET11d expression vector, 3′ recessed ends of *Bam*HI-digested vector were filled in with Klenow enzyme (GIBCO/BRL) using standard methods (21), followed by digestion with *Nco*I. The pBluescript clone containing the wild-type *MUR1* gene was digested with *Nco*I and *Eco*RV. The insert DNA was gel-purified and ligated to the pET11d vector as described above. Transformation into *E. coli* BL21 (DE3) competent cells was performed according to the manufacturer's protocol (Stratagene).

Nucleic Acid Sequence Determination and Analysis. All sequencing reactions were done by the enzymatic method with ≈10 μg of template DNA labeled using the CY5 Auto Read

Labeling kit (Pharmacia) and an automated laser fluorescent sequencer (Pharmacia). Data analysis of nucleic acid and amino acid sequences and hydropathy plots of the derived MUR1 amino acid sequence were carried out with MACVECTOR software (Oxford Molecular Group, Oxford, U.K.).

To determine the DNA sequences from PCR products, cultures from at least six independent clones were pooled before plasmid purification. All DNA sequences were determined from both strands.

SDS/PAGE. Protein from control cultures containing pET11d vector with no insert and pET11d vector containing the *MUR1* coding region were boiled in sample buffer and run on a 10% SDS/polyacrylamide gel (22) for 3 h at 135 V using the Penguin vertical gel system (Owl Scientific).

RESULTS

The *mur1* Mutant Lacks GDP-D-Mannose-4,6-Dehydratase Activity but Exhibits GDP-4-Keto-6-Deoxy-D-Mannose-3,5-Epimerase-4-Reductase Activity *in Vitro*. To determine the biochemical defect in the *mur1* mutant, protein was extracted from leaves of wild-type and *mur1* leaves and assayed for the ability to produce GDP-L-[^{14}C]fucose from GDP-D-[^{14}C]mannose *in vitro*. The expected products from this assay are shown in Fig. 1 and the results displayed in Fig. 2 *A* and *B*, lanes 1 and 2, indicate that protein extracts from wild-type leaves were able to convert GDP-D-[^{14}C]mannose to GDP-L-[^{14}C]fucose, while *mur1* extracts lacked this activity.

Since the *de novo* synthesis of GDP-L-fucose comprises three steps, probably using two separate enzymes (a 4,6-dehydratase and a 3,5-epimerase-4-reductase) in eukaryotes, we wished to determine which step was blocked in the *mur1* mutant. We found that, in agreement with previously published data (6), the final stereospecific reduction of the 4-keto group into the L-*galacto* configuration required exogenous reduced pyridine dinucleotide (NADPH). Omission of NADPH from the assay mixture resulted in the buildup of the GDP-4-keto-6-deoxy-D-mannose intermediate and allowed us to assay the 4,6-dehydratase activity separately. Due to the chemical instability of the 4-keto-6-deoxy intermediate, we reduced the 4-keto group and assayed the resultant diagnostic monosaccharides, 6-deoxy-D-talose, and 6-deoxy-D-mannose (D-rhamnose). Protein from wild-type leaves was able to catalyze the formation of the 4-keto-6-deoxy intermediate (Fig. 2*B*, lane 3), whereas *mur1*-derived extracts were not (Fig. 2*B*, lane 4), indicating that no 4,6-dehydratase activity was present. Small amounts of [^{14}C]rhamnose were formed during these *in vitro* assays even in the absence of chemical reduction, a result which is in agreement with previous reports (23). We confirmed that *mur1* plants possessed functional 3,5-epimerase-4-reductase activity by using wild-type protein without NADPH to generate the 4-keto-6-deoxy intermediate and using this as the substrate for the epimerase-reductase. In the presence of NADPH, both *mur1* and wild-type extracts were able to convert the intermediate into GDP-L-fucose (Fig. 2*B*, lanes 5 and 7).

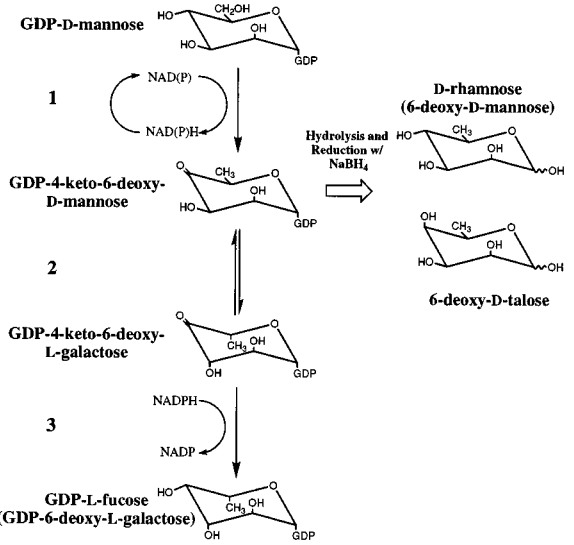

FIG. 1. Reaction scheme for the *de novo* synthesis of GDP-L-fucose from GDP-D-mannose, involving 4,6-dehydratase (step 1), which produces a 4-keto-6-deoxy intermediate, 3,5-epimerization (step 2), and the final NADPH-dependent reduction of the 4-keto group (step 3). Expected products from the chemical reduction and hydrolysis of the 4-keto-6-deoxy intermediate, are also included.

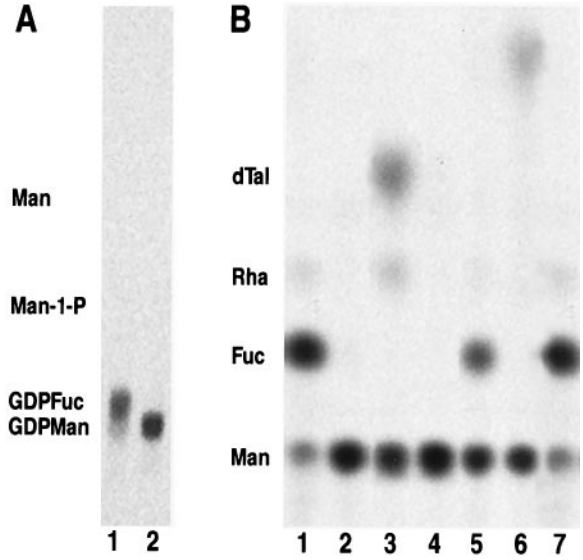

FIG. 2. Assay of enzyme activities in the *de novo* synthesis of GDP-L-fucose. (*A*) Thin layer chromatogram of nucleotide diphospho-sugar products from the *in vitro* assay, following incubation of GDP-D-[^{14}C]mannose with wild-type protein extract (lane 1) or *mur1* protein extract (lane 2). (*B*) Thin layer chromatogram of the monosaccharides from the acid hydrolysis of *in vitro* assay products using GDP-D-[^{14}C]mannose (lanes 1–4) or a mixture of GDP-D-[^{14}C]mannose and GDP-4-keto-6-deoxy-D-[^{14}C]mannose (lanes 5–7) as substrates. Lane 1, wild-type protein extract with exogenous NADPH; lane 2, *mur1* protein extract with exogenous NADPH; lane 3, wild-type protein extract without exogenous NADPH; lane 4, *mur1* protein extract without exogenous NADPH; lane 5, *mur1* protein extract in the presence of NADPH; lane 6, same as lane 5 but using heat-inactivated *mur1* protein; and lane 7, same as lane 5 but using wild-type extract. Samples in lanes 3 and 4 were reduced with $NaBH_4$ before hydrolysis. Positions of authentic standards are shown. Man, mannose; Man-1-P, mannose-1-phosphate; Fuc, fucose; Rha, rhamnose; and dTal, 6-deoxy-talose.

Two Similar cDNAs Were Isolated from a cDNA Library Using an *Arabidopsis* EST with High Sequence Similarity to Putative Bacterial GDP-D-Mannose-4,6-Dehydratases. An EST (clone 90A12T7) that showed high sequence similarity to putative bacterial GDP-D-mannose-4,6-dehydratases was identified in the database of ESTs (dbEST; ref. 20). This clone was provided by the *Arabidopsis* Biological Resource Center, and its entire nucleotide sequence was determined. Sequence comparisons with bacterial clones suggested that the cDNA was truncated at the 5′ end. To obtain a full-length cDNA, a lambda PRL-2 cDNA library (20) was screened using the EST as a probe. Twenty clones were isolated from this screen that could be divided into two separate classes based on restriction mapping of individual isolates. The nucleotide sequences from the longest cDNAs in each class were determined, indicating the identification of two different cDNAs with substantial sequence similarity to each other and to putative bacterial GDP-D-mannose-4,6-dehydratases. The two cDNAs were ten-

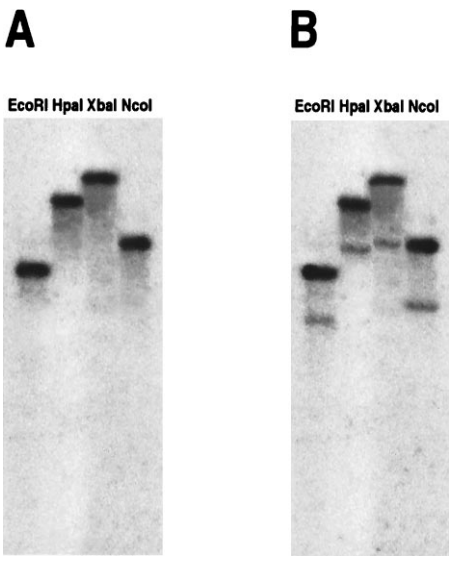

FIG. 3. Southern blot analysis of total *Arabidopsis* DNA at high and low stringency using ^{32}P-labeled *GMD2* as a probe. (*A*) Digested Columbia DNA probed at high stringency (68°C hybridization, 65°C washes). (*B*) Digested Columbia DNA probed at low stringency (58°C hybridization, 55°C washes).

tatively designated *GMD1* and *GMD2* (guanosine-diphospho-D-mannose-4,6-dehydratase).

Southern Blot Analysis Reveals at Least Two Genes for Putative GDP-D-Mannose-4,6-Dehydratases Within the *Arabidopsis* Genome. As shown in Fig. 3, Southern blots probed with GMD2 at high stringency revealed a single hybridizing fragment, while two fragments were observed at reduced stringency, providing further evidence that two genes for putative GDP-D-mannose-4,6-dehydratases were present in the *Arabidopsis* genome. Since both cDNAs represented candidate *MUR1* genes, they were used to determine possible linkage to the *mur1* locus.

***GMD2* Is Tightly Linked to *mur1*.** Restriction fragment length polymorphisms (RFLPs) were identified on Southern blots between *Hpa*I-digested Columbia and Landsberg DNA when *GMD1* was used as a probe and between *Scr*FI-digested Columbia and Landsberg DNA when *GMD2* was used as a probe. These RFLPs were then used to determine the genotype (Columbia or Landsberg) of 50 *mur1* plants obtained from a segregating F_2 population (Landsberg (*MUR1*) × Columbia (*mur1*)). *GMD1* segregated independently from *mur1*, while *GMD2* showed complete cosegregation with the *mur1* locus providing initial evidence that *GMD2* was a *MUR1*-derived cDNA (data not shown).

All *mur1* Alleles Harbor Mutations Within the Coding Region of *GMD2*. After establishing linkage between *GMD2* and *mur1*, we determined that three separate *GMD2* cDNAs contained a 373-aa open reading frame. As shown in Fig. 4, the sequence surrounding the first codon showed a perfect match with the plant initiation codon consensus sequence AACAAUGGC (24). Northern blot analysis indicated a length of ≈1.4 kb for the *GMD2* mRNA, matching the size of the longest cDNAs obtained (data not shown). These results indicate that cDNAs containing the entire *GMD2* coding region had been cloned.

The coding regions of the genes corresponding to the *GMD2* cDNA were isolated via PCR from seven *mur1* alleles and wild-type Columbia. The nucleotide sequences of the PCR products were then determined to identify possible sites of mutations. The sequences of the wild-type PCR product and the *GMD2* cDNA were identical, indicating the absence of introns within the coding region. The PCR products from all seven *mur1* alleles investigated showed single point mutations as indicated in Fig. 4. Based on these data we conclude that *GMD2* represents a *MUR1*-derived cDNA.

The MUR1 Protein Is Similar in Amino Acid Sequence to Putative Bacterial GDP-D-Mannose-4,6-Dehydratases. A comparison between the derived amino acid sequence of the *MUR1* gene product from *Arabidopsis* and putative bacterial GDP-D-mannose-4,6-dehydratases is shown in Fig. 5. The first 25 aa of the MUR1 protein are absent from the bacterial sequences, suggesting a function as a signal sequence. However, hydropathy plots (data not shown) and the presence of GDP-D-mannose-4,6-dehydratase activity in the cytosolic fraction (see *Materials and Methods*) suggest that the MUR1 protein is soluble and cytosolic. Furthermore, the amino acid sequence of the N-terminal extension does not fit the criteria for chloroplast or endoplasmic reticulum transit peptides (25, 26).

MUR1 Protein Expressed in *E. coli* Has GDP-D-Mannose-4,6-Dehydratase Activity That Complements the Biochemical Defect of *mur1* Protein Extracts *in Vitro*. To verify the predicted function of the MUR1 protein, we expressed the *MUR1* coding region in *E. coli*, determined the size of the protein, and

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                                                    MetAlaSerGluAsnAsnGlySerArgSerAspSerGluSerIleThrAlaProLysAlaAspSerThrValValGluProArgLysIleAlaLeuIleThr
1   CACGCGTCCGAGCATTTGTCTCTGAAATCCCAGATCCGAAAACCAACAATGGCGTCAGAGAACAACGGATCCAGATCCGATTCCGAATCCATCACCGCTCCCAAAGCTGATTCCACCGTCGTTGAACCGAGGAAGATAGCTCTGATCACC 150

    GlyIleThrGlyGlnAspGlySerTyrLeuThrGluPheLeuLeuGlyLysGlyTyrGluValHisGlyLeuIleArgArgSerSerAsnPheAsnThrGlnArgIleAsnHisIleTyrIleAspProHisAsnValAsnLysAlaLeu
151 GGAATCACCGGCCAGGACGGATCATACCTGACGGAGTTCCTTCTCGGAAAAGGCTACGAAGTTCATGGTCTGATCCGTCGATCATCGAATTTCAACACCCAGCGAATCAACCATATCTACATCGATCCACACAATGTCAACAAAGCTCTG 300

                                                                Leu(mur1-2)
                                                                |
    MetLysLeuHisTyrAlaAspLeuThrAspAlaSerSerLeuArgArgTrpIleAspValIleLysProAspGluValTyrAsnLeuAlaAlaGlnSerHisValAlaValSerPheGluIleProAspTyrThrAlaAspValValAla
301 ATGAAACTCCACTACGCCGATCTCACCGACGCTTCCTCTCTCCGTCGTTGGATCGATGTGATCAAACCTGACGAAGTTTATAACCTAGCTGCTCAATCTCACGTCGCTGTCTCCTTCGAGATCCCTGATTACACAGCCGATGTAGTCGCA 450
                                                                        T
                Cys(mur1-7)                                     Cys(mur1-5)                     Phe(mur1-1)
                |                                               |                               |
    ThrGlyAlaLeuArgLeuGluAlaValArgSerHisThrIleAspSerGlyArgThrValLysTyrTyrGlnAlaGlySerSerGluMetPheGlySerThrProProProGlnSerGluThrThrProPheHisProArgSerPro
451 ACCGGTGCTCTCCGTCTCCTTGAAGCCGTCAGATCTCACACCATCGACAGTGGCCGTACCGTCAAGTATTACCAAGCCGGATCTTCGGAGATGTTTGGATCAACTCCTCCTCCACAATCGGAGACGACGCCGTTTCACCCCAGATCTCCT 600
                T                                               T                               T

                        Val(mur1-3)                             Val(mur1-6)             Gln(mur1-4)
                        |                                       |                       |
    TyrAlaAlaSerLysCysAlaAlaHisTrpTyrThrValAsnTyrArgGluAlaTyrGlyLeuPheAlaCysAsnGlyIleLeuPheAsnHisGluSerProArgArgGlyGluAsnPheValThrArgLysIleThrArgAlaLeuGly
601 TACGCAGCTTCCAAATGCGCTGCTCATTGGTACACAGTGAATTACAGAGAGGCGTACGGTCTCTTCGCTTGTAACGGAATCTTGTTCAATCACGAGTCACCTCGCCGTGGTGAGAATTTCGTGACGAGGAAGATCACAAGAGCATTGGGA 750
                        T                                       T                       A

    ArgIleLysValGlyLeuGlnThrLysLeuPheLeuGlyAsnLeuGlnAlaSerArgAspTrpGlyPheAlaGlyAspTyrValGluAlaMetTrpLeuMetLeuGlnGlnGluLysProAspAspTyrValValAlaThrGluGluGly
751 AGGATCAAGGTTGGTTTGCAGACGAAGCTATTCCTTGGGAATTTGCAAGCGTCAAGAGATTGGGGATTTGCAGGAGATTATGTGGAAGCAATGTGGTTGATGTTGCAGCAAGAGAAGCCAGATGATTACGTTGTGGCAACAGAGGAAGGA 900

    HisThrValGluGluPheLeuAspValSerPheGlyTyrLeuGlyLeuAsnTrpLysAspTyrValGluIleAspGlnArgTyrPheArgProAlaGluValAspAsnLeuGlnGlyAspAlaSerLysAlaLysGluValLeuGlyTrp
901 CACACAGTGGAAGAGTTTCTTGATGTGTCATTTGGGTATTTGGGACTCAATTGGAAAGATTATGTTGAGATTGACCAGAGGTACTTTAGGCCTGCTGAAGTAGATAACCTTCAAGGAGATGCAAGCAAGGCAAAGGAAGTGTTGGGGTGG 1050

    LysProGlnValGlyPheGluLysLeuValLysMetMetValAspGluAspLeuGluAlaLysArgGluLysValLeuValAspAlaGlyTyrMetAspAlaLysGlnGlnPro *
1051 AAACCACAAGTAGGGTTTGAGAAGCTTGTGAAGATGATGGTTGATGAAGATCTTGAGCTTGCTAAGAGGGAGAAAGTGCTTGTTGATGCTGGATACATGGATGCTAAGCAGCAACCTTGATTTGATCAATTATACAAAGATGGAGTTTCT 1200
1201 TACATTTATCTGCTTTTATAGAGTTTTAAATTTCGTTTTTTTTTCTTTCTCTTTTTGTTGCTTTGGTGGTTGTTTGTTGTCTTTTTATGTTTTAGTTTCTTTGTTGCTTTTTAAGTTATCTAAATTGTTTCCCTCCAATGAGTTGAGGAT 1350
1351 GAAAGCGCAGTGAATTTTGAATGAAGGAGCAAAGAATCATAAAAAAAAAAAAAAAAAAAAAAA                                                                                                1413
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FIG. 4. Nucleotide sequence of the *MUR1* cDNA and derived amino acid sequence of the MUR1 protein. Nucleotide changes and predicted amino acid substitutions in each of seven *mur1* alleles are indicated. The consensus sequence for recognition of the initiation codon and a putative polyadenylylation signal are underlined.

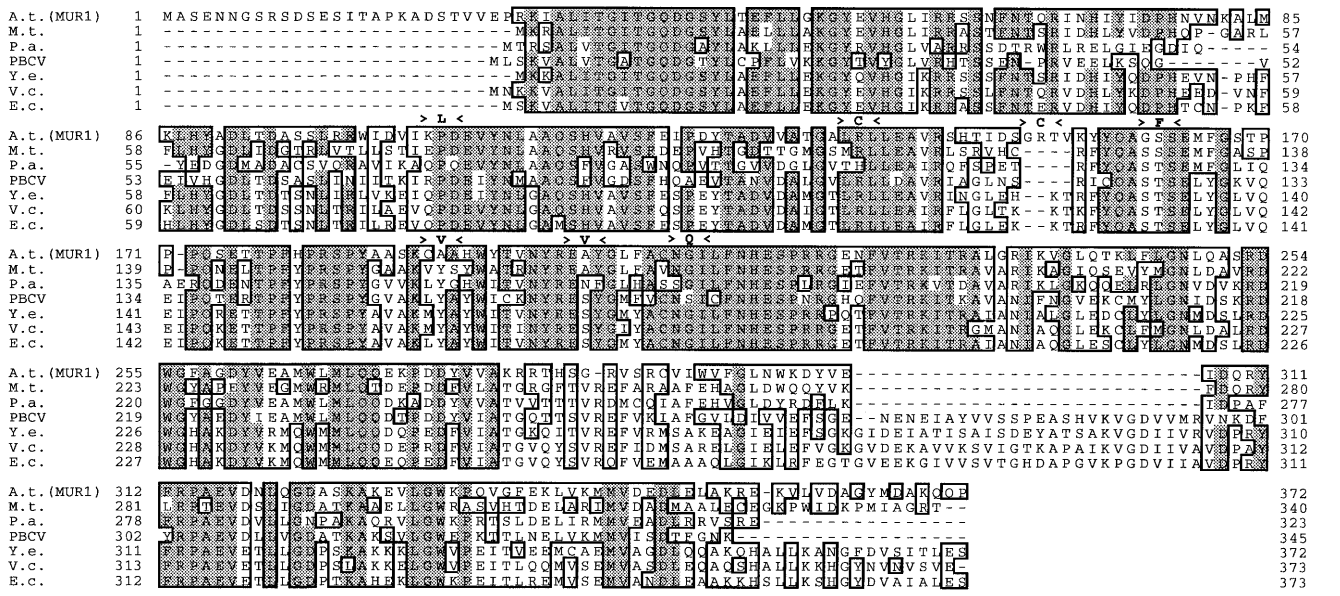

FIG. 5. Alignment between the derived MUR1 amino acid sequence and putative GDP-D-mannose-4,6-dehydratases. Identical residues are shaded in gray and conserved residues are boxed. Positions of the amino acid substitutions from seven *mur1* alleles are indicated above the *Arabidopsis* sequence. A.t., *A. thaliana*; M.t., *Mycobacterium tuberculosis*; P.a., *Pseudomonas aeruginosa*; PBCV, *Paramecium bursaria Chlorella* virus; Y.e., *Yersinia enterocolitica*; V.c., *Vibrio cholerae*; and E.c., *E. coli*. Sequence alignments were done with CLUSTAL W and viewed with SEQVU.

subjected it to the 4,6-dehydratase assay. Fig. 6 shows an SDS/PAGE analysis of protein extracts from induced cells, indicating that the *MUR1* gene encodes a protein ≈42 kDa in size, which is in agreement with the size predicted from the amino acid sequence (41.9 kDa). As shown in Fig. 7, lanes 1 and 2, extracts from the MUR1-expressing cells converted GDP-D-[^{14}C]mannose into GDP-4-keto-6-deoxy-D-[^{14}C]mannose, while extracts from control cells exhibited no 4,6-dehydratase activity. Moreover, when the bacterial extract was combined with a protein extract from *mur1* plants, GDP-L-[^{14}C]fucose was produced (Fig. 7, lane 3). We also noted that the *E. coli* strain possessed an endogenous 4-reductase activity that converted some of the GDP-4-keto-6-deoxy-D-[^{14}C]mannose intermediate to GDP-D-[^{14}C]rhamnose; however, in the absence of the *MUR1* gene, bacterial extracts were unable to convert GDP-D-[^{14}C]mannose into any detectable products.

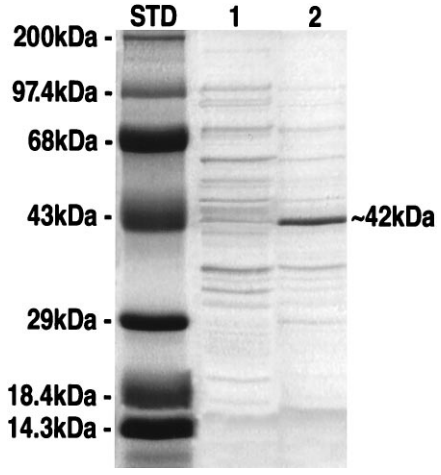

FIG. 6. SDS/PAGE analysis of crude extracts from *E. coli* harboring the pET11d vector with or without the *MUR1* coding region. Lane 1, protein extracted from *E. coli* containing a pET11d vector without an insert; and lane 2, protein extracted from *E. coli* containing a pET11d vector with the *MUR1* coding region. STD, prestained protein molecular weight standards.

DISCUSSION

The *mur1* mutant of *A. thaliana* has allowed us to study the effects of L-fucose deficiency in the cell wall of higher plants and provided us with a means of cloning a plant gene involved in the *de novo* synthesis of an important cell wall component. The work reported in this paper provides biochemical evidence that a cloned gene encodes a functional GDP-D-mannose-4,6-dehydratase.

Enzymatic assays of protein from *mur1* and wild-type plants led us to conclude that the *mur1* mutation eliminated GDP-D-mannose-4,6-dehydratase activity in the mutant plants. A candidate EST was identified through sequence similarity searches and used to isolate two similar yet distinct cDNAs. One of the cDNAs showed complete linkage to the *mur1* locus, and the corresponding gene was sequenced from wild-type as well as seven *mur1* alleles. Nucleotide sequence comparisons indicated sequence identity between the cDNA and the wild-type gene but revealed single point mutations in all seven *mur1* alleles investigated. We then verified the suspected function of the cDNA-encoded protein by expressing it in *E. coli* and assaying for 4,6-dehydratase activity. This analysis indicated that the cDNA encoded a functional GDP-D-mannose-4,6-dehydratase, which was able to restore L-fucose synthesis to *mur1*-derived protein extracts *in vitro*. This combination of genetic and biochemical data indicates that the *MUR1* gene has been cloned.

Even with the information we have gained on the *MUR1* gene, we are still unable to account for the presence of L-fucose in root tissue. The simplest explanation for this observation is the existence of additional gene(s) encoding GDP-D-mannose-4,6-dehydratase activity in the root. *GMD1* may encode another isoform of this enzyme based on its sequence similarity to *MUR1* and putative bacterial GDP-D-mannose-4,6-dehydratases. Further characterization of this cDNA clone will reveal the function of this putative isoform and its role within *A. thaliana*.

Our sequence data of the seven *mur1* alleles indicate that all of the mutations are caused by transitions leading to amino acid substitutions. The mutations are clustered within a region of ≈300 nucleotides, and six out of seven nucleotide changes represent C → T transitions on the coding strand. Some of the

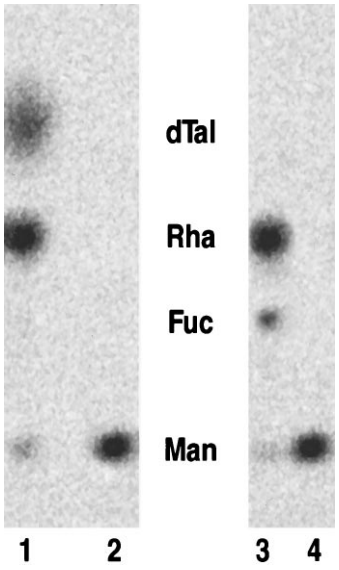

FIG. 7. Assay of the MUR1 protein for GDP-D-mannose-4,6-dehydratase activity and reconstitution of the L-fucose biosynthetic pathway in *mur1*-derived protein extracts. Lane 1, protein from *E. coli* expressing the *MUR1* gene, incubated with GDP-D-[^{14}C]mannose; lane 2, protein from *E. coli* transformed with pET11d vector minus the *MUR1* insert; lane 3, same as lane 1 but with *mur1*-derived protein extract added; and lane 4, same as lane 2 but with *mur1*-derived protein extract added. All final products were hydrolyzed and reduced as described earlier.

substitutions are within conserved regions of the protein as compared with the bacterial sequences, but without any knowledge of the protein structure it is difficult to determine how these substitutions may affect protein folding or function. These point mutations are characteristic of ethyl methanesulfonate mutagenesis, but it is noteworthy that nonsense mutations were not identified.

Another intriguing aspect of the results from the cell wall mutant screen is that all mutations leading to L-fucose deficiency mapped to the *mur1* locus. If *GMD1* does in fact encode an isoform of GDP-D-mannose-4,6-dehydratase with a root-specific expression pattern, mutations in this gene could not have been identified in a mutant screen based on leaf material (12). Since the GDP-4-keto-6-deoxy-D-mannose-3,5-epimerase-4-reductase necessary for the second step in the *de novo* pathway of GDP-L-fucose synthesis must be encoded by a second gene, we wonder why no L-fucose deficient mutants affecting this enzyme were isolated. Mutations eliminating this activity may be lethal. Alternatively, genetic redundancy in this enzymatic step may defeat any attempts to identify mutants.

Changes in cell wall polysaccharides in the *mur1* mutant have been recently investigated, leading to the discovery that L-fucose is partly replaced by L-galactose in mutant-derived xyloglucan (27). This apparent switch from one monosaccharide to another closely related sugar could be explained based on our knowledge of the remaining steps in the pathway for the *de novo* synthesis of GDP-L-fucose. In the absence of a functional GDP-D-mannose-4,6-dehydratase, the 3,5-epimerase-4-reductase normally involved in L-fucose synthesis may be able to use GDP-D-mannose directly forming GDP-L-galactose. If this hypothesis were true, it would be interesting both biochemically and genetically to study organisms such as jojoba (28) and the unicellular green alga, *Chlorella* (29), that produce significant amounts of L-galactose.

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