The Plant Journal (2000) **21**(5), 445–454

A bifunctional epimerase-reductase acts downstream of the *MUR1* gene product and completes the *de novo* synthesis of GDP-L-fucose in *Arabidopsis*

Christopher P. Bonin[1] and Wolf-Dieter Reiter[1,2,*]
[1]*University of Connecticut, Department of Molecular and Cell Biology, Storrs, CT 06269, USA, and*
[2]*Institute of Materials Science, Storrs, CT 06269, USA*

Received 3 September 1999; revised 17 January 2000; accepted 20 January 2000.
*For correspondence (fax +860 486 4331; e-mail wdreiter@uconnvm.uconn.edu).

Summary

L-Fucose is a monosaccharide found as a component of glycoproteins and cell wall polysaccharides in higher plants. The *MUR1* gene of *Arabidopsis thaliana* encodes a GDP-D-mannose 4,6-dehydratase catalyzing the first step in the *de novo* synthesis of GDP-L-fucose from GDP-D-mannose (Bonin *et al.*, 1997, *Proc. Natl Acad. Sci. USA*, 94, 2085–2090). Plant genes encoding the subsequent steps in L-fucose synthesis (3,5-epimerization and 4-reduction) have not been described previously. Based on sequence similarities to a bacterial gene involved in capsule synthesis we have cloned a gene from *Arabidopsis*, now designated *GER1*, which encodes a bifunctional 3,5-epimerase-4-reductase in L-fucose synthesis. The combined action of the *MUR1* and *GER1* gene products converts GDP-D-mannose to GDP-L-fucose *in vitro* demonstrating that this entire nucleotide–sugar interconversion pathway could be reconstituted using plant genes expressed in *Escherichia coli*. *In vitro* assays indicated that the GER1 protein does not act as a GDP-D-mannose 3,5-epimerase, an enzymatic activity involved in the *de novo* synthesis of GDP-L-galactose and L-ascorbic acid. Similarly, L-ascorbate levels in *GER1* antisense plants were unchanged indicating that GDP-D-mannose 3,5-epimerase is encoded by a separate gene.

Introduction

L-Fucose (6-deoxy-L-galactose) is an important carbohydrate component of various plant cell wall polysaccharides and glycoproteins. It is found in xyloglucan, a hemicellulose believed to function as a load-bearing polymer in the cell wall by crosslinking cellulose microfibrils, and in the pectins rhamnogalacturonan I and II, whose exact function is not understood (Bacic *et al.*, 1988; Carpita and Gibeaut, 1993). In xyloglucan, L-fucose occupies the ends of trisaccharide side chains which appear to stabilize linear conformations of this molecule, aid in interactions with cellulose and may increase wall strength (Levy *et al.*, 1991; Levy *et al.*, 1997). Indeed, it has been shown for the *mur1* mutant of *Arabidopsis thaliana* that a deficiency of L-fucose leads to decreases in the tensile strength of elongating stem segments (Reiter *et al.*, 1993). L-Fucose is also required for the biological function of xyloglucan-derived oligosaccharides which have been shown to inhibit the auxin-induced elongation of pea stem segments possibly representing a negative feedback loop in controlling extension growth (McDougall and Fry, 1989; York *et al.*, 1984).

The *de novo* synthesis of L-fucose is accomplished through the conversion of a nucleotide-sugar, GDP-D-mannose, into GDP-L-fucose in three catalytic steps; a 4,6-dehydration, a 3,5-epimerization, and a 4-reduction (Figure 1). GDP-L-fucose can also be synthesized via a salvage pathway with a phosphorylation of free L-fucose followed by GMP attachment (for review see Feingold and Avigad, 1980).

To genetically dissect cell wall biosynthetic pathways in higher plants, mutants of *Arabidopsis thaliana* with changes in the monosaccharide composition of their cell wall material have recently been described (Bonin *et al.*, 1997; Burget and Reiter, 1999; Reiter *et al.*, 1997). This screening procedure led to the identification of a single mutant locus, *mur1*, causing a deficiency in L-fucose in the shoot (Reiter *et al.*, 1993; Reiter *et al.*, 1997). Further characterization of the *mur1* mutant has shown that L-galactose is present in about one-third of the positions normally occupied by L-fucose in *Arabidopsis* xyloglucan (Zablackis *et al.*, 1996). The *MUR1* gene has recently been

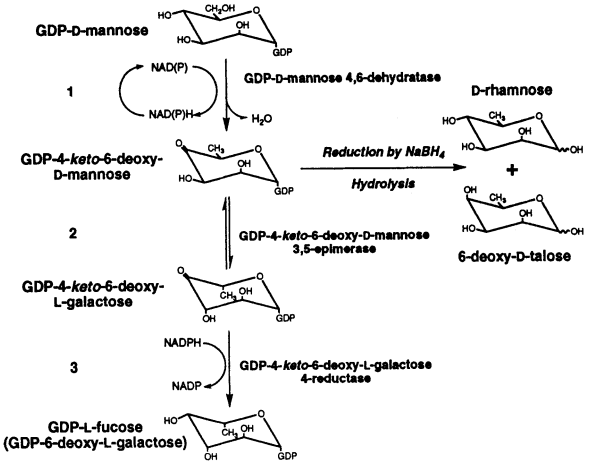

Figure 1. Schematic representation of the *de novo* pathway for the synthesis of GDP-L-fucose.
The procedure for the quantitation of KDM is indicated at the right.

cloned and shown to encode an isoform of GDP-D-mannose 4,6-dehydratase catalyzing the first step in GDP-L-fucose synthesis (Bonin *et al.*, 1997).

Eight independent recessive mutations in the *de novo* synthesis of GDP-L-fucose were identified during the mutant screen, all of them mapping to the *mur1* locus. The absence of known mutations in the remaining steps of the GDP-D-mannose to GDP-L-fucose interconversion pathway suggests that mutations in these steps may be lethal or difficult to identify due to genetic redundancy. To address this issue we decided to use sequence information on genes involved in bacterial capsule synthesis to obtain plant genes acting downstream of the GDP-D-mannose 4,6-dehydratase in the biosynthetic pathway leading to GDP-L-fucose. This work describes the cloning and characterization of a gene encoding a GDP-4-*keto*-6-deoxy-D-mannose 3,5-epimerase-4-reductase catalyzing the last two steps in the *de novo* synthesis of GDP-L-fucose.

Results

Identification of an Arabidopsis *EST with sequence similarity to an* E. coli *gene linked to the synthesis of GDP-L-fucose*

To identify genes from *Arabidopsis* involved in steps 2 and 3 of L-fucose synthesis (Figure 1), we analyzed the genetic data published on the synthesis of capsules and lipopolysaccharides in bacterial systems. The *rfb* (rough B) and *cps* (capsular polysaccharide synthesis) clusters of *E. coli* strains O111 and K-12, respectively, harbor many genes whose products are involved in the synthesis of these surface antigens. Predicted amino acid sequences of a number of genes in these clusters indicated sequence similarity to enzymes involved in the *de novo* synthesis of GDP-D-mannose such as GDP-D-mannose pyrophosphorylase and phosphomannomutase in *Salmonella* (Jiang *et al.*, 1991; Nikaido *et al.*, 1966; Nikaido *et al.*, 1967). Others were assumed to be involved in the *de novo* pathway for the synthesis of GDP-L-fucose and/or GDP-colitose (a 3,6-dideoxy sugar), such as GDP-D-mannose 4,6-dehydratase and GDP-4-*keto*-6-deoxy-D-mannose 3-dehydrase (Aoyama *et al.*, 1994; Bastin and Reeves, 1995). A few remaining open reading frames (ORFs) were also presumed to be involved in these pathways, but no function was assigned to them. We compared their predicted size with other known and putative nucleotide-sugar epimerases and searched for the consensus sequence for an N-terminal pyridine nucleotide co-factor binding site (Rossman *et al.*, 1975). One gene designated ORF 0.9 from the *cps* cluster of *E. coli* strain K-12 (Aoyama *et al.*, 1994), which corresponded to ORF 6.7 from the *rfb* cluster of *E. coli* strain O111 (Bastin and Reeves, 1995), fit our criteria and hence was the best candidate for a gene encoding 3,5-epimerase and/or 4-reductase activities in L-fucose synthesis. With the aid of the NCBI BLAST program (Altschul *et al.*, 1990), an *A. thaliana* EST (clone YAY215) sharing significant sequence similarity with ORF 0.9 was identified from the database of expressed sequence tags (dbEST).

Isolation of the GER1 *gene*

Clone YAY215 was obtained from the *Arabidopsis* Biological Research Center (ABRC, Ohio State University, Columbus, OH, USA) and sequenced. Based on sequence alignments with ORF 0.9 and related bacterial sequences the YAY215 cDNA appeared to be truncated at its 5′ end.

To obtain a genomic clone corresponding to EST YAY215, an EMBL3 *A. thaliana* genomic library was screened using the EST as a probe. From an initial population of approximately 50 000 plaques, four lambda clones hybridized to the probe and were purified through a second round of screening. Southern blotting of *Sal*I-digested phage DNA indicated that two lambda clones contained a 5 kb *Sal*I fragment that hybridized specifically and at high stringency to the ^{32}P-labeled insert of EST YAY215 (data not shown). The hybridizing 5 kb *Sal*I fragment derived from one of the lambda clones was then cloned into pBluescript and sequenced. The gene believed to reside on the cloned fragment was designated *GER1* (GDP-4-*keto*-6-deoxy-D-mannose 3,5-*E*pimerase-4-*R*eductase). The *GER1* gene was mapped to the bottom of chromosome I between markers m453A and m532 using recombinant inbred lines (Lister and Dean, 1993). The chromosomal region encompassing the *GER1* gene has recently been sequenced as part of the

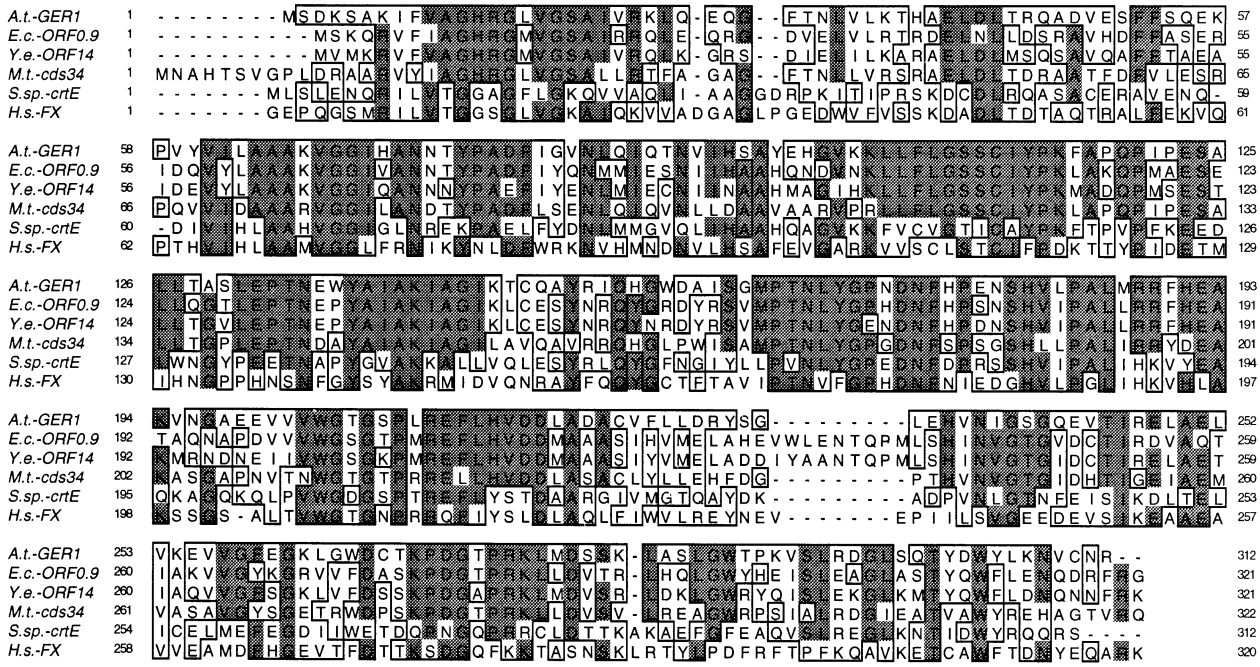

Figure 2. Amino acid alignments between the *A. thaliana* GER1 protein and several homologs.
Shaded residues are identical and boxed residues represent conservative exchanges. All alignments were created with ClustalW and displayed with SeqVu. Abbreviations: *A.t.*, *Arabidopsis thaliana*; *E.c.*, *Escherichia coli*; *Y.e.*, *Yersinia enterocolitica*; *M.t.*, *Mycobacterium tuberculosis*; *S.*sp., *Synechocystis* sp.; *H.s.*, *Homo sapiens*.

Arabidopsis Genome Initiative indicating that the *GER1* coding region is located on BAC T18K17.

The GER1 *coding region is intronless*

Sequencing of the *GER1* genomic clone revealed a continuous open reading frame of 312 amino acids, which suggested that no introns were present. The start codon of the *GER1* coding region was tentatively identified based on alignments with ORF 0.9 and other similar sequences (Figure 2) plus the presence of an in-frame stop codon located upstream of the putative initiation codon. Sequences shared between cDNA clone YAY215 and the genomic DNA were identical, and the absence of significant gaps between the GER1 amino acid sequence and other related sequences (Figure 2) lent support to our hypothesis that no introns were present in the coding region. To test this hypothesis, a PRL-2 cDNA library (Newman *et al.*, 1994) was obtained from the ABRC and amplified. Purified DNA was then used for PCR in parallel with genomic *A. thaliana* DNA employing primers encompassing the entire open reading frame. Gel electrophoresis of the resultant PCR products indicated a single fragment of approximately 0.94 kb in both cases establishing that no introns are present in the coding region of the *GER1* gene (data not shown). Control PCR reactions performed without template DNA did not produce any detectable product.

The GER1 *gene is expressed throughout the plant*

Total RNA was extracted from roots, leaves, stems, flowers and siliques and analyzed by Northern blotting to determine the expression pattern of the *GER1* gene and the size of its mRNA. A single hybridizing RNA species with a size of approximately 1.5 kb was observed in these experiments. The *GER1*-derived transcript was highly abundant in roots and flowers and less abundant in leaves, stems and siliques (Figure 3). The same blot probed with 18S rDNA verified that these differences in hybridization signals were not due to unequal loading of the lanes.

*The GER1 protein exhibits GDP-4-*keto*-6-deoxy-D-mannose 3,5-epimerase-4-reductase activity but lacks GDP-D-mannose 3,5-epimerase activity* in vitro

To determine the function of the *GER1* gene product, the coding region was cloned into the pET28b expression vector, utilizing a C-terminal histidine tag, and transformed into *E. coli* BL21 (DE3). Total protein from crude extracts of *E. coli* in which GER1 was expressed was analyzed using SDS-PAGE before and after purification with Ni-NTA agarose beads (Figure 4). Results from this experiment indicated that histidine-tagged *GER1* is approximately 35 kDa, which is in line with the molecular weight predicted from its amino acid composition (35.5 kDa).

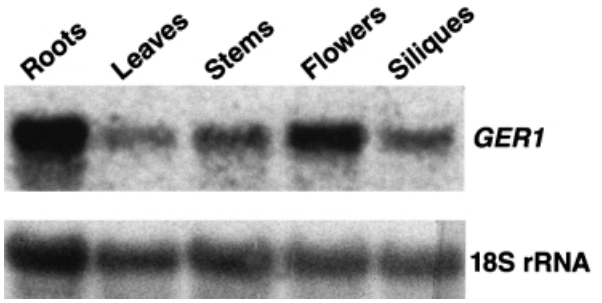

Figure 3. Northern blot analysis of total RNA extracted from roots, leaves, stems, flowers and siliques and probed with a ^{32}P-labeled *GER1* fragment.

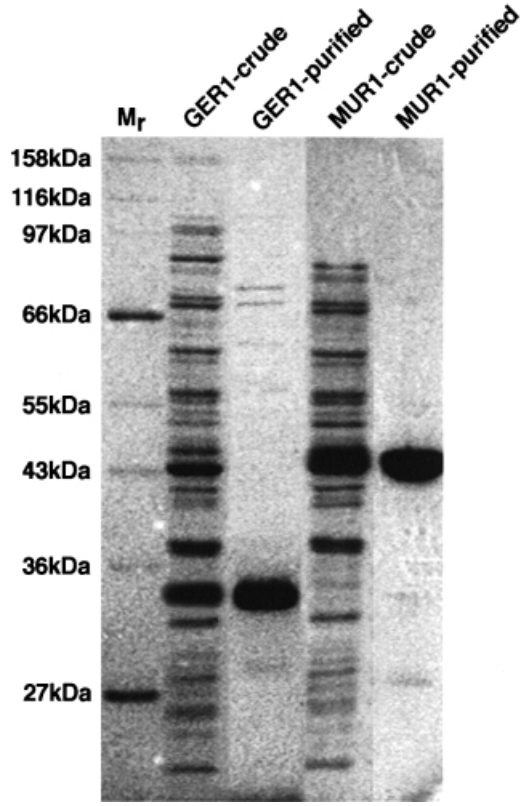

Figure 4. Coomassie blue stained SDS-polyacrylamide gel of crude and Ni-NTA purified GER1 and MUR1 protein from *E. coli*.
M_r denotes a size marker.

To generate pure GDP-4-*keto*-6-deoxy-D-mannose (GDP-KDM) as a substrate for 3,5-epimerase-4-reductase assays, highly purified MUR1 protein was incubated with GDP-D-[^{14}C]mannose until the reaction had gone to completion (see Experimental procedures). Because of the instability of GDP-KDM and the lack of a commercial standard for KDM, reaction products were reduced, hydrolyzed and separated by thin layer chromatography (TLC). Two products, 6-deoxy-D-talose and D-rhamnose, were produced after the reduction and hydrolysis of GDP-KDM (Figure 5a, lane 3; see Figure 1 for explanation). Incubation of purified GER1 protein with GDP-KDM resulted in the complete conversion of GDP-KDM into GDP-L-fucose (Figure 5a, lane 5). The L-fucose produced by this reaction co-migrated with an authentic standard using two separate solvent systems. To verify that this product was L-fucose and not D-fucose, the reaction product was incubated with L-fucose dehydrogenase and separated by TLC. The product of this reaction migrated differently than L-fucose and co-migrated with a standard produced by converting L-[^{14}C]fucose to L-fucono-1,5-lactone using two different solvent systems (data not shown).

A separate assay that involved mixing GDP-D-[^{14}C]mannose, purified MUR1 protein, and purified GER1 protein in the same tube yielded the same results as the first assay (Figure 5a, lane 7). Omission of NADPH from the reaction resulted in a complete lack of 3,5-epimerase-4-reductase activity (Figure 5a, lane 6), supporting a previously reported requirement of NADPH for the reduction of the 4-*keto* group (Bonin *et al.*, 1997). Analysis of the nucleotide-sugars from this reaction indicated that GDP-D-mannose was completely converted to a nucleotide-sugar that co-migrates with GDP-L-fucose (Figure 5b, lane 3). Control cells carrying the pET28 vector without an insert failed to produce any L-fucose (Figure 5a, lane 1), indicating that the *GER1* gene product possesses GDP-4-*keto*-6-deoxy-D-mannose 3,5-epimerase-4-reductase activity *in vitro* (Figure 5a, lanes 5 and 7). Boiled GER1 protein did not exhibit any detectable activity (Figure 5a, lane 4). To verify that MUR1 and GER1 action produced nucleotide-sugars, anion exchange chromatography was used to separate nucleotide-sugars, sugar-phosphates and monosaccharides. Results from this analysis indicated that more than 90% of the radioactivity from the GDP-KDM substrate and the GDP-L-fucose product was found in the nucleotide-sugar fraction (see Experimental procedures).

It has been hypothesized that epimerase–reductase activities in L-fucose synthesis may be capable of catalyzing the 3,5-epimerization of GDP-D-mannose to GDP-L-galactose through a 4-*keto* intermediate (Bonin *et al.*, 1997), thus providing an explanation for the presence of L-galactose in *mur1* xyloglucan (Zablackis *et al.*, 1996). It is now accepted that the conversion of GDP-D-mannose to GDP-L-galactose is one of the first steps in the synthesis of L-ascorbic acid (Wheeler *et al.*, 1998). Incubation of GDP-D-[^{14}C]mannose with GER1 protein (Figure 5a, lane 2) did not produce L-galactose under a variety of conditions, including increases in the concentration of GDP-D-mannose, variations and combinations of pyridine nucleotide co-factors (NAD, NADH, NADP and NADPH) and extended incubation periods.

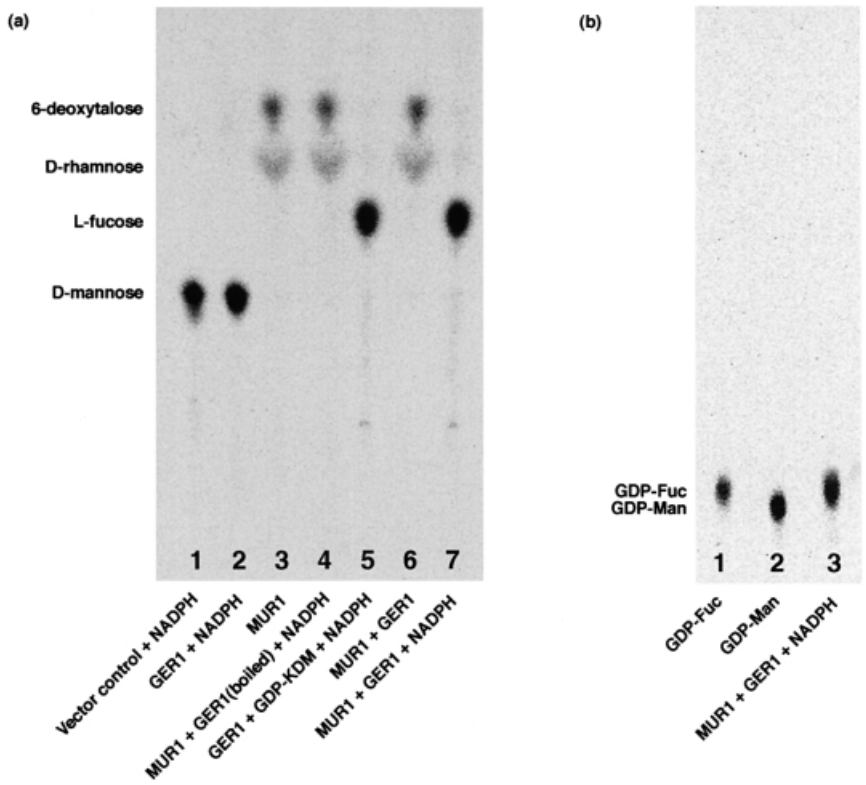

Figure 5. Autoradiograph of ^{14}C-labeled reaction products from assays of 3,5-epimerase-4-reductase activities separated by thin layer chromatography.
(a) Separation of monosaccharides following incubation of enzymes (MUR1, GER1) with GDP-KDM (lane 5) or GDP-Man (all other lanes) in the absence (lanes 3 and 6) or presence (lanes 1, 2, 4, 5 and 7) of NADPH. Nucleotide-sugars were hydrolyzed to monosaccharides before loading. Samples in lanes 3, 4 and 6 were reduced with $NaBH_4$ prior to hydrolysis.
(b) Separation of nucleotide sugars. Lanes 1 and 2: standards. Lane 3: mixture of MUR1 and GER1 proteins incubated with GDP-Man and NADPH.

GER1 *antisense lines exhibit decreased GDP-4-keto-6-deoxy-*D*-mannose 3,5-epimerase-4-reductase activity*

In an attempt to decrease GER1 activity *in planta*, transgenic *GER1* antisense lines were constructed. This was accomplished by cloning the *GER1* gene in the antisense orientation into the pKYLX7 vector (Schardl *et al.*, 1987), which contains the Cauliflower Mosaic Virus (CaMV) 35S promoter upstream of the multiple cloning site, and transforming this construct into plants.

Initial transformants were identified by selection on plates. Antibiotic resistant isolates were further characterized by Northern blot analysis using ^{32}P-labeled *GER1* as a probe. Three separately transformed plants, designated GERA9a, GERA10b and GERA21b, were found to contain high levels of antisense transcript in leaves (data not shown).

Protein extracts were prepared from inflorescences of wild-type, transgenic lines GERA 9a, 10b and 21b and plants containing the vector without an insert. Assays were performed on these extracts to determine if 3,5-epimerase-4-reductase activity was reduced in the transgenic plants. Analysis of the resultant products from these assays indicated that 3,5-epimerase-4-reductase activity was virtually undetectable in all three *GER1* antisense lines *in vitro* (Figure 6b–d, lane 3), while 4,6-dehydratase activity was present (seen as a mixture of 6-deoxy-D-talose and D-rhamnose). L-Fucose comprised less than 1% of total radioactivity using extracts from antisense lines. Extracts from wild-type *Arabidopsis* and transgenic plants containing a vector without an insert converted approximately 50% of the GDP-D-mannose into GDP-L-fucose (Figure 6a, lanes 1 and 4). Moreover, when purified recombinant GER1 protein was added to extracts from *GER1* antisense plants, L-fucose synthesis was restored (Figure 6b–d, lane 4). Boiled control reactions displayed no 3,5-epimerase-4-reductase activity or 4,6-dehydratase activity (Figure 6a, lane 2 and 6b–d, lanes 1).

The approximately 50-fold decrease in 3,5-epimerase-4-reductase activity in the inflorescence of these antisense lines prompted us to determine if there was a reduction in the amount of L-fucose in these tissues. Gas chromatographic analysis of cell wall material from inflorescence stems indicated that wild-type levels of L-fucose were still present, suggesting that GER1 activity does not represent a rate-limiting step in the *de novo* synthesis of GDP-L-fucose in *Arabidopsis* (data not shown).

If the GER1 protein was involved in the synthesis of GDP-L-galactose by acting as a GDP-D-mannose 3,5-epimerase *in vivo*, L-ascorbate levels should be strongly reduced in *GER1* antisense plants. We tested this hypothesis by quantifying L-ascorbate from inflorescence tissue essentially as described by Conklin *et al.* (1996) and found that all of the antisense lines examined contained wild-type levels of L-ascorbate (data not shown).

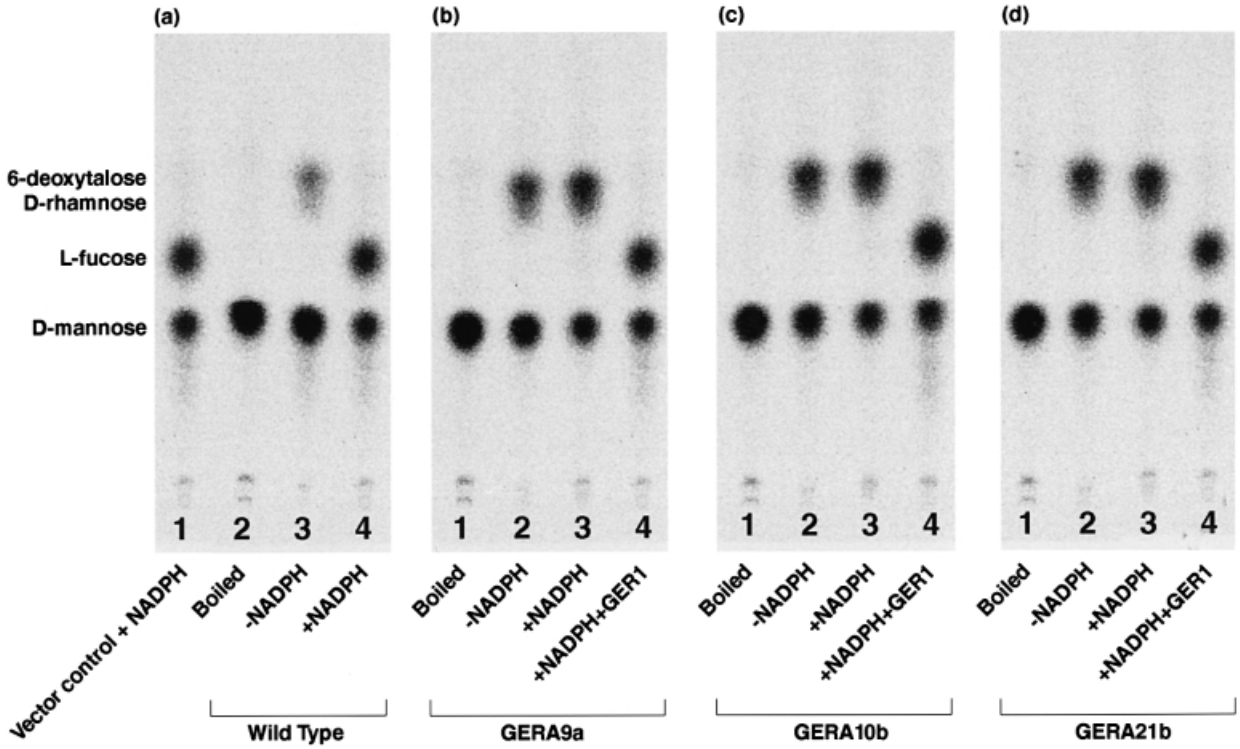

Figure 6. Autoradiograph of ^{14}C-labeled sugars from assays of 4,6-dehydratase and 3,5-epimerase-4-reductase activities in wild-type and *GER1* antisense plants.
(a) Protein from wild-type and vector-transformed plants incubated with GDP-Man in the presence (lanes 1, 2 and 4) or absence (lane 3) of NADPH. (b) Protein from GERA9a antisense plants incubated with GDP-Man in the presence (lanes 1, 3 and 4) or absence (lane 2) of NADPH. Lane 4: purified GER1 protein was added to the reaction mixture. (c,d) Same reactions as in (b) substituting GERA10b (c) or GERA21b (d) protein. Samples in (a) lane 3 and (b–d) lanes 2 and 3 were reduced with $NaBH_4$ prior to hydrolysis.

The Arabidopsis *genome contains a* GER1 *homolog*

Recent database searches revealed the existence of a cDNA clone and a matching BAC end sequence with a high degree of sequence similarity to *GER1*. Based on the limited sequence information available at this time, cDNA clone 701667762 (GenBank accession number AI996642) is 83% identical to GER1 on the nucleotide level between bases 649 and 927 of the *GER1* coding region corresponding to the carboxy terminus of the GER1 protein. The derived amino acid sequences are 84% identical to each other within this region. The cDNA clone is virtually identical in its nucleotide sequence to the T7 end of BAC T3G2, suggesting that the sequence discrepancies between *GER1* and cDNA clone 701667762 are genuine and not caused by sequencing errors. Based on these results we conclude that the *Arabidopsis* genome contains a *GER1* homolog for which the biochemical function remains to be established. The *GER1*-like sequence located at the T7 end of BAC T3G2 has tentatively been designated *GER2*.

Discussion

The *mur1* mutant of *A. thaliana* shows a visible phenotype and decreased wall strength thus indicating that L-fucose is necessary for proper plant growth and cell wall architecture. The *MUR1* gene encodes an isoform of GDP-D-mannose 4,6-dehydratase catalyzing the first step in the *de novo* synthesis of GDP-L-fucose (Bonin *et al.*, 1997). This represents the only plant gene involved in the *de novo* synthesis of GDP-L-fucose for which a mutant has been isolated. The apparent lack of mutants deficient in the remaining steps of the *de novo* pathway may be due to genetic redundancy or lethality of null mutations. These possibilities have prompted us to search for genes encoding the 3,5-epimerase and 4-reductase activities in *Arabidopsis*.

With the aid of BLAST database searches (Altschul *et al.*, 1990) we were able to identify an *Arabidopsis* EST, now designated *GER1*, with similarity to a bacterial gene thought to be involved in the biosynthesis of L-fucose. Other genes involved in the *de novo* synthesis of GDP-L-fucose have recently been cloned from bacteria and humans and were shown to encode GDP-D-mannose 4,6-dehydratase activity (Ohyama *et al.*, 1998; Sturla *et al.*, 1997; Sullivan *et al.*, 1998) or GDP-4-*keto*-6-deoxy-D-mannose 3,5-epimerase-4-reductase activity (Andrianopoulos *et al.*, 1998; Sullivan *et al.*, 1998; Tonetti *et al.*, 1996).

With the aid of purified MUR1 protein, we were able to produce GDP-4-*keto*-6-deoxy-D-mannose to demonstrate that the *GER1* gene product catalyzed the conversion of this GDP-bound intermediate into GDP-L-fucose. This result indicated that the GER1 protein possesses both the 3,5-epimerase and the 4-reductase activities in L-fucose synthesis (steps 2 and 3 in Figure 1). This is in agreement with biochemical data reported on the function of the human and *E. coli* 3,5-epimerase-4-reductases (Andrianopoulos *et al.*, 1998; Sullivan *et al.*, 1998; Tonetti *et al.*, 1996).

Since the *MUR1* gene product represents the only GDP-D-mannose 4,6-dehydratase expressed in the shoot, and the *GER1* gene is expressed in all major plant organs, it can be concluded that *GER1* acts downstream of *MUR1* to complete the *de novo* synthesis of GDP-L-fucose in *Arabidopsis*. The approximately 50-fold reduction in GDP-KDM 3,5-epimerase-4-reductase activity in extracts from *GER1* antisense plants suggests that the *GER1* gene encodes virtually all of the GDP-KDM 3,5-epimerase-4-reductase activity in inflorescence stems. Paralogous genes such as *GER2* may be expressed in specific organs such as the root as evidenced by the isolation of a *GER2* cDNA from a root-specific library. It will be interesting to determine the biochemical activity and expression pattern of the *GER2* gene product to establish possible correlations with the two known isoforms of GDP-D-mannose 4,6-dehydratase, MUR1 and GMD1 (Bonin *et al.*, 1997).

Despite the strong downregulation of GDP-KDM 3,5-epimerase-4-reductase activity in *GER1* antisense plants, the fucose content of cell wall material from inflorescence stems is normal, thus suggesting that *GER1* activity is not rate-limiting *in vivo*. Alternatively, genetic redundancy in L-fucose synthesis may enable roots to provide L-fucose to shoot organs via the transpiration stream.

Arabidopsis plants with high *GER1* antisense expression appear to be slightly less vigorous and to flower earlier than lines containing the vector without insert. This phenotype cannot be explained by a reduction in fucose content but may be related to disturbances in GDP-sugar metabolism caused by the conversion of GDP-KDM into potential dead-end products such as GDP-D-rhamnose (Barber, 1968; Bonin *et al.*, 1997).

It has recently been shown that the synthesis of GDP-L-galactose from GDP-D-mannose via GDP-D-mannose 3,5-epimerase represents an important step in the synthesis of L-ascorbate in higher plants (Wheeler *et al.*, 1998). Since this enzymatic reaction is mechanistically similar to the 3,5-epimerase-4-reductase activity of GER1, we assayed GER1 protein for GDP-D-mannose 3,5-epimerase activity *in vitro* and determined L-ascorbate levels in *GER1* antisense lines. Results from these experiments speak against a role of the *GER1* gene product in the synthesis of GDP-L-galactose, however, the *in vivo* data need to be interpreted with caution since GDP-D-mannose 3,5-epimerase activity may not be rate limiting in the synthesis of L-ascorbate in plant tissues.

Experimental procedures

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}). *Arabidopsis* plants of the Columbia ecotype were used for the isolation of DNA and RNA.

Isolation of the GER1 *gene*

GER1 genomic clones were isolated from an EMBL3 library constructed by Clontech Laboratories Inc. (Palo Alto, CA, USA) using DNA from the Columbia ecotype of *A. thaliana*. Synthesis of a digoxigenin-labeled hybridization probe was accomplished by random primer labeling of the *Eco*RI/*Bam*HI insert fragment of clone YAY215 that was obtained from the ABRC. The EMBL3 library was screened with the digoxigenin-labeled probe using the colorimetric detection system supplied by Boehringer Mannheim (Indianapolis, IN, USA). Lambda clones hybridizing to the DNA probe were grown in liquid media using standard procedures (Sambrook *et al.*, 1989) and DNA was purified using the lambda DNA purification kit from Qiagen Inc. (Chatsworth, CA, USA). A *Sal*I fragment hybridizing to the probe DNA was isolated from one of the lambda clones and ligated to *Sal*I-digested pBluescript KS+ vector. The ligation products were transformed into *E. coli* XL1-Blue supercompetent cells (Stratagene, La Jolla, CA, USA), and transformants containing a *Sal*I insert of the expected size were identified through restriction digestion and analysis.

Genetic mapping was carried out using *Ase*I-cleaved DNA derived from the Lister and Dean RI lines (Lister and Dean, 1993) and the *GER1* gene as a probe for Southern analysis. The hybridization data were evaluated by staff at the Nottingham *Arabidopsis* Stock Centre (Nottingham, UK).

Expression of the MUR1 *and* GER1 *genes in* E. coli

The sequences of oligonucleotide primers used for cloning of the *GER1* coding region into the pET28b expression vector (Novagen, Madison, WI, USA) were as follows: (*GER1*/pET28b-upper), 5′-ACACACCCATGGCTGACAAATCTGCC-3′; (*GER1*/pET28b-lower), 5′-ACCCTCGAGTCGGTTGCAAACATTCTTC-3′, engineering an *Nco*I site into the upper primer and an *Xho*I site into the lower primer. The polymerase chain reaction was performed in a volume of 30 μl with approximately 50 ng of template (wild-type Columbia DNA) using a model 2400 GeneAmp thermocycler and PCR Core Reagents (Perkin Elmer, Foster City, CA, USA) with the following conditions: denaturation of the DNA in a volume of 29.5 μl at 94°C for 5 min, cooling to 4°C, addition of 1 unit of Taq DNA polymerase, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 1 min, extension at 72°C for 1.5 min, and final extension at 72°C for 7 min. 1 μg of each primer was used in the reaction and the final concentration of $MgCl_2$ was 2 mM.

The *GER1* PCR product was cloned into the pET28b expression vector as follows: a PCR fragment of the expected size was purified from an agarose gel using the Qiaquick gel extraction kit (Qiagen), digested with *Nco*I and *Xho*I and purified with the Qiaquick PCR purification kit. The pET28b vector was digested with *Nco*I and *Xho*I and purified using the Qiaquick PCR purification kit. Ligation of vector to insert DNA was followed by transformation of the construct into *E. coli* BL21 (DE3) competent cells according to the manufacturer's protocol (Stratagene).

Conditions for cloning *MUR1* into the pET28b vector were identical except for the primers used which were as follows: (*MUR1*/pET28b-upper), 5′-GTCCATGGCGTCAGAGAACAACGG-3′; (*MUR1*/pET28b-lower), 5′-ACCCTCGAGAGGTTGCTGCTTAGC-3′, engineering an *Nco*I site into the upper primer and an *Xho*I site into the lower primer.

Purification of the recombinant proteins via Ni-NTA columns was carried out according to the manufacturer's protocol (Qiagen) with slight modifications of induction conditions. Culture flasks containing 50 ml of LB and 50 $\mu g\,ml^{-1}$ of kanamycin were inoculated with 1 ml of an overnight culture, grown to an OD_{600} of approximately 0.6 at 37°C in a rotary shaker at 250 r.p.m., IPTG was added to a final concentration of 5–10 μM and cultures were grown for an additional 3 h at an ambient temperature (approximately 25°C).

Isolation of Arabidopsis *and plasmid DNA*

Purification of plasmid DNA and total DNA from plant material was carried out as described previously (Bonin *et al.*, 1997). An *Nco*I/*Xho*I fragment derived from pET28b/*GER1*, which corresponds to the coding region of the gene, was used for random primer ^{32}P-labeling.

Isolation and analysis of Arabidopsis *RNA*

RNA was isolated from different organs of *Arabidopsis* plants as described previously (Bonin *et al.*, 1997). Root RNA was extracted from 2-week-old plants grown vertically under axenic conditions on agar plates containing a nutrient mixture as described previously (Haughn and Somerville, 1986). Leaf RNA was extracted from 2-week-old plants, and RNA from stems, flowers and siliques was extracted from 4-week-old plants. Northern blotting and transcript size determination was performed as described by Bonin *et al.* (1997) using the ^{32}P-labeled *GER1* probe described above. Hybridizations and filter washings were done at 68°C and 65°C, respectively, using the buffer system described by Church and Gilbert (1984). To verify equal loading of the lanes, filters were stripped according to the protocol of the manufacturer (Amersham International plc, Amersham, UK) and reprobed with a ^{32}P-labeled *Eco*RI fragment derived from plasmid CD3–196 (obtained from the ABRC), which contains most of an 18S rDNA gene from *Arabidopsis*.

Nucleic acid sequence determination and analysis

All sequencing reactions were performed by the enzymatic method with approximately 10 μg of template DNA using the Cy5 Auto Read Labeling kit (Pharmacia, Piscataway, NJ, USA) and an automated laser fluorescent sequencer (Pharmacia). Analysis of nucleic acid and derived amino acid sequences of the *GER1* gene was carried out with MacVector software (Oxford Molecular Group, Oxford, UK). All DNA sequences were determined from both strands.

Enzyme assays

All experiments using MUR1 protein were pre-incubated overnight at 25°C with 1 mM NAD in 20 mM Tris–HCl, pH 7.6. GDP-4-*keto*-6-deoxy-D-[^{14}C]mannose was synthesized from GDP-D-[^{14}C]mannose by purified MUR1 protein. Extracts containing approximately 1 μg MUR1 protein were mixed with 926 Bq of GDP-D-[^{14}C]mannose and 1 mM NAD contained in 30 μl of 20 mM Tris–HCl, pH 7.6, and incubated at 25°C overnight. The reactions were terminated by boiling for 3 min, and precipitated protein was removed by centrifugation. Enzymatic activity of the GER1 protein was assayed by adding approximately 1 μg of purified GER1 protein and 10 mM NADPH (final concentration) to the above reaction mixtures and incubation at 25°C overnight. Another assay was performed using identical conditions except that approximately 1 μg of purified MUR1 protein was mixed with purified GER1 protein prior to the addition of GDP-D-[^{14}C]mannose and NADPH. Nucleotide-sugars were separated by TLC on Baker-flex cellulose plates and developed in ethanol/1 M NH_4Ac, pH 7.0 (7 : 3 v/v). Some reactions were boiled, reduced with 1 μmol of $NaBH_4$ at 37°C for 1 h and hydrolyzed by the addition of 100 μl 2 M trifluoroacetic acid and incubation at 95°C for 20 min. Samples were dried under vacuum and resuspended in 20 μl of 70% ethanol. Resultant monosaccharides were separated by TLC either on Baker-flex cellulose plates (J.T. Baker Inc., Phillipsburg, NJ, USA), developed in 1-butanol/acetic acid/water (12 : 3 : 5 v/v/v), dried, and developed further by ethyl acetate/pyridine/water (8 : 2 : 1 v/v/v) in the same dimension or on silica plates in 1-propanol/NH_4OH/H_2O (6 : 2 : 1 v/v/v). Radioactivity was visualized by phosphorimaging (Bio-Rad Laboratories, Hercules, CA, USA) and quantified using Molecular Analyst® Software (Bio-Rad Laboratories). Authentic sugar standards run in parallel were stained with aniline-hydrogen phthalate (Fry, 1988). A control experiment was performed using transformed *E. coli* cells carrying the pET28b vector without an insert. All assays were repeated at least once.

L-Fucose dehydrogenase assays were accomplished by resuspending reaction products, after hydrolysis and drying, in 30 μl of 120 mM Tris–HCl, pH 9.5, 120 mM imidazole, 100 mM NaAc and 0.2 units of L-fucose dehydrogenase (Sigma, St. Louis, MO, USA) and incubating at 37°C for 3 h. To create a standard for detecting the conversion of L-fucose to L-fucono-1,5-lactone, GDP-L-[^{14}C]fucose was hydrolyzed, dried and assayed as described above. Reaction products were separated via TLC using both systems described above.

Anion exchange chromatography

To verify that GER1 was acting on a nucleotide-sugar rather than a monosaccharide or sugar-phosphate, reaction products from the incubation of GDP-D-[^{14}C]mannose with MUR1 protein were analyzed via anion exchange chromatography. To determine the ionic strength required to elute monosaccharides, sugar-phosphates and nucleotide-sugars, three separate Superclean™ LC-SAX solid-phase extraction columns (Supelco, Bellefonte, PA, USA) were loaded with 100 μg of D-mannose, 100 μg of D-mannose-1-phosphate or a mixture of 100 μg of unlabeled GDP-D-mannose and 926 Bq of GDP-D-[^{14}C]mannose, respectively. Monosaccharides and sugar-phosphates were identified by total carbohydrate assays on the eluted fractions (Fry, 1988) and nucleotide-sugars were identified by scintillation counting. D-Mannose eluted at 5 mM KH_2PO_4, pH 6.0 while D-mannose-1-phosphate eluted at 300 mM and GDP-D-mannose eluted at 400 mM of this buffer. After these elution conditions were

established, reaction products derived from the conversion of GDP-D-[^{14}C]mannose by purified MUR1 protein were loaded onto a column, washed with 3 column volumes (3 ml) of 5, 50, 100, 200, 300, 400, 500 and 600 mM KH_2PO_4, pH 6.0 and each fraction was collected. Amounts of radioactivity were determined by liquid scintillation counting. More than 90% of the radioactivity was found in the nucleotide-sugar fraction indicating that the GDP-KDM used for enzyme assays was > 90% pure. This experiment was repeated using reaction products from the conversion of GDP-D-[^{14}C]mannose to GDP-L-[^{14}C]fucose by MUR1 and GER1 with similar results.

Construction of GER1 *antisense plants*

The sequences of oligonucleotide primers used for cloning of antisense *GER1* into the pKYLX7 vector (Schardl *et al.*, 1987) were as follows: (*GER1*Anti-upper), 5′-AACTGCAGAGCTTGAGCGGG-CCGTGAAGTCAAGTG-3′; (*GER1*Anti-lower), 5′-GTGGATCCAA-AATCTTCGTCGCGGGTCATCGTGG-3′, engineering a *Pst*I site into the upper primer and a *Bam*HI site into the lower primer. PCR and cloning was performed as described above except that the PCR product was first cloned into pBluescript and transformed into *E. coli* XL-1 Blue MRF′. The insert was then excised from pBluescript using *Xho*I and *Xba*I and cloned into pKYLX7 cleaved with the same enzymes. The resulting construct (designated pGERA) was purified as described above and transformed into *E. coli* XL1-Blue MR. Approximately 100 ng of pGERA was then used to transform *A. tumefaciens* (GV3101) using a Bio-Rad Genepulser. One out of several kanamycin resistant colonies was then used to transform *A. thaliana* using standard methods (Bechtold *et al.*, 1993). T_1 plants were selected on plates containing MS salts (Murashige and Skoog, 1962) and 50 μg ml^{-1} kanamycin. These were then transferred to soil after 10 days and allowed to self. T_2 plants were again selected on kanamycin, transferred to soil and allowed to self. T_3 plants homozygous for the transgene were used for RNA and protein analysis. Protein was extracted as described previously (Bonin *et al.*, 1997) and quantitated using the dotMETRIC™ protein assay according to the manufacturer's protocol (Geno Technology, Inc., St. Louis, MO, USA). Protein concentrations were normalized in respect to wild-type protein extracts and assayed as described above using approximately 10 μg of protein each.

SDS-PAGE

Protein extracts (10 μl) were heated in 2× sample buffer at 95°C for 5 min. The supernatant was then run on a 10% SDS-polyacrylamide gel (Laemmli, 1970) for 1 h at 8 V cm^{-1} and then 3 h at 15 V cm^{-1} using the Penguin vertical gel system (Owl Scientific Inc., Woburn, MA, USA). A broad range protein molecular weight marker (New England Biolabs, Beverly, MA, USA) was run concurrently and the gel was stained with 0.1% Coomassie Brilliant Blue® R-250 in methanol/acetic acid/water (9 : 2 : 9 v/v/v) overnight at 25°C, and destained for 4 h at 25°C in the same solution without the dye.

Gas chromatography

The monosaccharide composition of cell wall material from 4-week-old inflorescence tissue of WT, GERA9a, GERA10b and GERA21b plants was analyzed by gas chromatography as described previously (Reiter *et al.*, 1993).

Acknowledgements

We would like to thank Gary F. Vanzin for his contribution toward mapping of the *GER1* gene; Ian Potter for his help with biochemical assays; J. Peter Gogarten for the EMBL3 genomic library; the *Arabidopsis* Biological Research Center for providing DNA clones and the PRL-2 library; and the Nottingham *Arabidopsis* Stock Centre for the Lister and Dean RI lines and the evaluation of the mapping data. Supported by grants from the U.S. Department of Energy (no. DE-FG02–95ER20203) and the National Science Foundation (no. MCB-9405735).

References

Andrianopoulos, K., Wang, L. and Reeves, P.R. (1998) Identification of the fucose synthetase gene in the colanic acid gene cluster of *Escherichia coli* K-12. *J. Bacteriol.* **180**, 998–1001.

Aoyama, K., Haase, A.M. and Reeves, P.R. (1994) Evidence for effect of random genetic drift on G + C content after lateral transfer of fucose pathway genes to *Escherichia coli* K-12. *Mol. Biol. Evol.* **11**, 829–838.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990) Basic local alignment search tool. *J. Mol. Biol.* **215**, 403–410.

Bacic, A., Harris, P.J. and Stone, B.A. (1988) Structure and function of plant cell walls. In *The Biochemistry of Plants*, Vol. 14 (Stumpf, P.K. and Conn, E.E., eds). New York: Academic Press, pp. 297–371.

Barber, G.A. (1968) The synthesis of guanosine 5′-diphosphate D-rhamnose by enzymes of a higher plant. *Biochem. Biophys. Acta*, **165**, 68–75.

Bastin, D.A. and Reeves, P.R. (1995) Sequence and analysis of the O antigen gene (*rfb*) cluster of *Escherichia coli* O111. *Gene*, **164**, 17–23.

Bechtold, N., Ellis, J. and Pelletier, G. (1993) *In planta Agrobacterium* mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. *C.R. Acad. Sci. Paris, Life Sci.* **316**, 1194–1199.

Bonin, C.P., Potter, I., Vanzin, G.F. and Reiter, W.-D. (1997) 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. *Proc. Natl Acad. Sci. USA*, **94**, 2085–2090.

Burget, E. and Reiter, W.-D. (1999) The *mur4* mutant of *Arabidopsis thaliana* is partially defective in the *de novo* synthesis of uridine diphospho L-arabinose. *Plant Physiol.* **121**, 383–389.

Carpita, N.C. and Gibeaut, D.M. (1993) Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. *Plant J.* **3**, 1–30.

Conklin, P.L., Williams, E.H. and Last, R.L. (1996) Environmental stress sensitivity of an ascorbic acid-deficient *Arabidopsis* mutant. *Proc. Natl Acad. Sci. USA*, **93**, 9970–9974.

Church, G.M. and Gilbert, W. (1984) Genomic sequencing. *Proc. Natl Acad. Sci. USA*, **81**, 1991–1995.

Feingold, D.S. and Avigad, G. (1980) Sugar nucleotide transformations in plants. In *The Biochemistry of Plants*, Vol. 2 (Stumpf, P.K. and Conn, E.E., eds). New York: Academic Press, pp. 101–170.

Fry, S.C. (1988). *The Growing Plant Cell Wall: Chemical and Metabolic Analysis*. New York: John Wiley and Sons, Inc.

Haughn, G.W. and Somerville, C. (1986) Sulfonylurea-resistant

mutants of *Arabidopsis thaliana*. *Mol. Gen. Genet.* **204**, 430–434.

Jiang, X.-M., Neal, B., Santiago, F., Lee, S.J., Romana, L.K. and Reeves, P.R. (1991) Structure and sequence of the *rfb* (O antigen) gene cluster of *Salmonella* serovar *typhimurium* (strain LT2). *Mol. Microbiol.* **5**, 695–713.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685.

Levy, S., Maclachlan, G. and Staehelin, L.A. (1997) Xyloglucan sidechains modulate binding to cellulose during *in vitro* binding assays as predicted by conformational dynamics simulations. *Plant J.* **11**, 373–386.

Levy, S., York, W.S., Stuike-Prill, R., Meyer, B. and Staehelin, L.A. (1991) Simulations of the static and dynamic molecular conformations of xyloglucan. The role of the fucosylated sidechain in surface-specific sidechain folding. *Plant J.* **1**, 195–215.

Lister, C. and Dean, C. (1993) Recombinant inbred lines for mapping RFLP and phenotypic markers in *Arabidopsis thaliana*. *Plant J.* **4**, 745–750.

McDougall, G.J. and Fry, S.C. (1989) Structure-activity relationships for xyloglucan oligosaccharides with antiauxin activity. *Plant Physiol.* **89**, 883–887.

Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **15**, 473–497.

Newman, T., DeBruijn, F.J., Green, P. *et al.* (1994) Genes galore: a summary of methods for accessing results from large-scale partial sequencing of anonymous *Arabidopsis* cDNA clones. *Plant Physiol.* **106**, 1241–1255.

Nikaido, H., Nikaido, K. and Makela, H. (1966) Genetic determination of enzymes synthesizing specific sugars of *Salmonella* lipopolysaccharide. *J. Bacteriol.* **91**, 1126–1135.

Nikaido, H., Levinthal, M., Nikaido, K. and Nakane, K. (1967) Extended deletions in the histidine-rough-B region of the *Salmonella* chromosome. *Proc. Natl Acad. Sci. USA*, **57**, 1825–1832.

Ohyama, C., Smith, P.L., Angata, K., Fukuda, M.N., Lowe, J.B. and Fukuda, M. (1998) Molecular cloning and expression of GDP-D-mannose-4,6-dehydratase, a key enzyme for fucose metabolism defective in Lec13 cells. *J. Biol. Chem.* **273**, 14582–14587.

Reiter, W.-D., Chapple, C.C.S. and Somerville, C.R. (1993) Altered growth and cell walls in a fucose-deficient mutant of *Arabidopsis*. *Science*, **261**, 1032–1035.

Reiter, W.-D., Chapple, C.C.S. and Somerville, C.R. (1997) Mutants of *Arabidopsis thaliana* with altered cell wall polysaccharide composition. *Plant J.* **12**, 335–345.

Rossman, M.G., Liljas, A., Brenden, C.I. and Banaszak, L.J. (1975) Evolutionary and structural relationships among dehydrogenases. In *The Enzymes* (Boyer, P.D., ed.). New York: Academic Press, pp. 61–102.

Sambrook, J., Fritsch, E.F. and Maniatas, T. (1989) *Molecular Cloning: A Laboratory Manual*. 2nd edn. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Schardl, C.L., Byrd, A.D., Benzion, G., Altschuler, M.A., Hildebrand, D.F. and Hunt, A.G. (1987) Design and construction of a versatile system for the expression of foreign genes in plants. *Gene*, **61**, 1–11.

Sturla, L., Bisso, A., Zanardi, D., Benatti, U., De Flora, A. and Tonetti, M. (1997) Expression, purification and characterization of GDP-D-mannose-4,6-dehydratase from *Escherichia coli*. *FEBS Lett.* **412**, 126–130.

Sullivan, F.X., Kumar, R., Kriz, R., Stahl, M., Xu, G.-Y., Rouse, J., Chang, X.-j., Boodhoo, A., Potvin, B. and Cumming, D.A. (1998) Molecular cloning of human GDP-mannose 4,6-dehydratase and reconstitutions of GDP-fucose biosynthesis *in vitro*. *J. Biol. Chem.* **273**, 8193–8202.

Tonetti, M., Sturla, L., Bisso, A., Benatti, U. and DeFlora, A. (1996) Synthesis of GDP-L-fucose by the human FX protein. *J. Biol. Chem.* **271**, 27274–27279.

Wheeler, G.L., Jones, M.A. and Smirnoff, N. (1998) The biosynthetic pathway of vitamin C in higher plants. *Nature*, **393**, 365–369.

York, W.S., Darvill, A.G. and Albersheim, P. (1984) Inhibition of 2,4-dichlorophenoxyacetic acid-stimulated elongation of pea stem segments by a xyloglucan oligosaccharide. *Plant Physiol.* **75**, 295–297.

Zablackis, E., York, W.S., Pauly, M., Hantus, S., Reiter, W.-D., Chapple, C.C.S., Albersheim, P. and Darvill, A. (1996) Substitution of L-fucose by L-galactose in cell walls of *Arabidopsis mur1*. *Science*, **272**, 1808–1810.

GenBank accession numbers AF045286 and AI996642.