Characterization of the Putative Xyloglucan Glycosyltransferase GT14 in Arabidopsis thaliana

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Honors Scholar Thesis

Characterization of the Putative Xyloglucan Glycosyltransferase GT14 in *Arabidopsis thaliana*

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University of Connecticut
Department of Molecular and Cell Biology
Spring 2010

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Abstract

Plant cell walls largely consist of matrix polysaccharides that are linked to cellulose microfibrils. Xyloglucan, the primary hemicellulose of the cell wall matrix, consists of a repeating glucose tetramer structure with xylose residues attached to the first three units (‘XXXG’). In Arabidopsis thaliana, the core XXXG structure is further modified by enzymatic addition of galactose and fucose residues to the xylose side chains to produce XLXG, XXLG, XLLG and XLFG structures. GT14 is a putative glycosyltransferase in the GT47 gene family. Initial predictions of GT14’s hydrophobic regions, based on its translated amino acid sequence, are almost identical to its Arabidopsis homolog MUR3, which is a xyloglucan galactosyltransferase targeted to the Golgi membrane. This suggests that, like MUR3, GT14 possesses a transmembrane domain and that it is targeted to the Golgi.

The monosaccharide composition of leaves from T-DNA insertion knockouts of GT14 was analyzed by gas-liquid chromatography. The gt14 plants were found to have lower fucose and higher mannose contents than wild type plants. Analysis of cell wall and soluble fractions from gt14 and wild type plants revealed that most of the deficiency in fucose was accounted for in the cell wall, supporting the idea that GT14’s target is xyloglucan. Finally, gt14 and wild type plants were transformed with GT14 for complementation and overexpression analysis. The majority of transformed plants did not show significant changes with regard to monosaccharide composition. This may be because the plants were in the T1 generation and, thus, hemizygous. Analysis of homozygous plants in the T2 generation may reveal noticeable changes.
Further studies on the xyloglucan composition of \textit{gt14} plants are necessary to put the observed reduction in cell wall fucose into a meaningful context.
Acknowledgments

I extend my sincerest thanks to Dr. Wolf-Dieter Reiter for giving me the opportunity to work in his lab and for all his support and guidance of my work. I am also very grateful to Dr. Rashid Ali for his patience in teaching me everything from the ground up, for his assistance, and for sharing his knowledge with me. In addition, I would like to thank the other members of the Reiter lab for fostering a helpful lab environment. Last, but certainly not least, I want to thank my parents, family, and cousins for their continual love and support.
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Introduction

i. The plant cell wall

The plant cell wall is composed of cellulose microfibrils that are interconnected with hemicelluloses in an extracellular matrix of pectic polysaccharides. The cell wall is involved in regulating growth of the plant, as well as providing structural integrity for the cell and plant body (Carpita and Gibeaut, 1993). This serves to provide a framework for a number of other functions vital to the plant’s development.

Plants have two types of cell walls: primary and secondary. The primary cell wall is deposited while the plant is growing; thus, it must confer mechanical strength without sacrificing pliability in order to allow cell expansion (Reiter, 2002). The primary cell wall is principally composed of networks of three distinct polysaccharides: cellulose, hemicelluloses and pectins (McNeil et al., 1984; Bacic et al., 1988; McCann and Roberts, 1991). Cellulose is a 1,4-β-D-glucan synthesized at the plasma membrane (Reiter, 2002). It is organized into a microfibril structure containing 36 parallel, paracrystalline polysaccharide chains (Delmer, 1999). These are bound together by the hemicelluloses and pectins (i.e., matrix polysaccharides), which are synthesized in the Golgi cisternae (Reiter, 2002). Secondary cell walls are, as the name implies, synthesized after the formation of the primary (growing) cell wall. In Arabidopsis thaliana and other plants, some specialized cell types, such as the xylem and interfascicular fibers, possess a secondary cell wall (Turner and Somerville, 1997).
ii. Xyloglucan in the plant cell wall

![Figure 1](image.png)

Figure 1. A representative unit of the hemicellulose xyloglucan. G = glucose, X = xylose.

The most abundant hemicellulose found in the primary cell wall of most higher plants is xyloglucan. Xyloglucan’s structure usually consists of a 1,4-β-D-glucan backbone with 1,6-α-D-xylose moieties linked to the first three glucose residues. This core xyloglucan oligomer is notated ‘XXXG’. A number of glycosyltransferases are responsible for modification of this initial building block. A D-galactose residue may be added to the second and third xylose residues with a β-1,2-linkage, resulting in the possible xyloglucan structures XLXG, XXLG, and XLLG. An L-fucose residue may be added at the second galactose residue in an α-1,2-linkage to form XLFG or XXFG. Several hypotheses for the purpose of the α-L-Fuc-1,2-β-D-Gal-1,2 side chain have been proposed. Computer modeling has suggested that the disaccharide group straightens the D-glucan backbone in order to facilitate the formation of hydrogen bonds with cellulose microfibrils (Levy et al., 1991, 1997). Another hypothesis proposes that xyloglucan oligomers with the fucosylated side chain are involved in auxin-induced growth and expansion (York et al., 1984; Fry, 1994).
iii. Glycosyltransferases involved in the modification of xyloglucan

Characterizing glycosyltransferases involved in cell wall polysaccharide synthesis and modification has been difficult due not only to the large number of genes involved, but also their intricate interrelationships. One estimate suggests that up to 15% of genes in the plant genome may be involved with cell wall synthesis, remodeling and turnover (Carpita et al., 2001). The Arabidopsis thaliana genome itself has over 800 identifiable carbohydrate active enzymes (Brown et al., 2005). In other words, development and maintenance of the plant cell wall represents a significant proportion of the plant’s resources. It is no surprise, then, that the functions of and interaction between genes that encode carbohydrate active enzymes are complex.

Chemically mutagenized Arabidopsis plants that were screened for an abnormal cell wall monosaccharide composition were found to have defects at 11 particular loci. These 11 loci, designated mur1 through mur11, fell into three groups: 1) mutant lines with reductions in the amount of a single monosaccharide, 2) those with absence of a monosaccharide and 3) plants with changes in the relative amounts of multiple monosaccharides. In mur1 plants, fucose was essentially absent (Reiter et al., 1993). mur2 and mur3 suffered a reduction in cell wall fucose content by roughly 50%, mur4 through mur7 showed reductions in arabinose, and mur8 showed a reduction in rhamnose content. mur9 through mur11, which fell into the third category, displayed complex changes in their monosaccharide composition (Reiter et al., 1997).

Among the mutations that reduced the amount of fucose, the mur1 plants were deficient because of missense mutations in the GMD2 gene. The outcome of the defect is the production of nonfunctional isoforms of the enzyme GDP-D-Man 4,6-dehydratase.
GDP-δ-Man 4,6-dehydratase is necessary for the de novo synthesis of the activated form of L-fucose, GDP-L-fucose (Mulichak et al., 2002). The *mur1* plants displayed a dwarfed phenotype (Reiter et al., 1997), though the phenotype can be rescued by growing the plants in the presence of high concentrations of borate (O’Neill et al., 2001). Boron is responsible for cross-linking the pectic polysaccharide rhamnogalacturonan II (Kobayashi, et al., 1996), and structural changes in the fucose-deficient mutants reduce the ability of rhamnogalacturonan II to form cross-links. Therefore, the mutants require a greater boron concentration than the wild type plants to achieve normal cross-linking. It appears that borate-complexed rhamnogalacturonan II, not fucosylated xyloglucan, is primarily responsible for mechanical strength in *Arabidopsis* (Ryden et al., 2003). Regardless, the fucosylation of xyloglucan in *mur1* plants was largely restored by growing the plants in fucose-supplemented media (Pauly et al., 2001), lending credence to the idea that the *mur1* defect is in the de novo synthesis of fucose. The *mur2* line was found to have a loss-of-function mutation in the fucosyltransferase AtFUT1 (Vanzin et al., 2002). Because AtFUT1 is a fucosyltransferase specific to xyloglucan, the *mur2* mutation only affects fucose in xyloglucan and not the entire plant as with *mur1*. In *mur3* plants, galactosylation of the third xylose residue of xyloglucan is absent (Madson et al., 2003). This, of course, precludes the addition of any fucose to xyloglucan. Therefore, the total fucose content of *mur3* plants is roughly half that of their wild type counterparts. The proportion of xyloglucan with a galactosylated central xylose residue (XLXG) is significantly higher in the *mur3* plants, perhaps to compensate for the loss of the α-L-Fuc-1,2-β-D-Gal side chain (Madson et al., 2003).
iv. MUR3 homologs and GT14

The Arabidopsis genome was searched using the PSI-BLAST algorithm (Altschul et al., 1997) with the MUR3 protein as the query sequence. This identified 38 coding regions in the genome with a high degree of sequence similarity to MUR3. These proteins are part of glycosyltransferase family 47 (GT47) as defined by Henrissat and Davies (Henrissat and Davies, 2000; see http://www.cazy.org/). They share the pfam03016 structural motif, which also represents the glucuronosyltransferase domain of animal exostosins. Results of the PSI-BLAST search were used to divide the GT47 family into a number of subgroups. MUR3 and 10 related homologs (GT11 through GT20) were part of subgroup A (Li et al., 2004).

The focus of my honors thesis is the characterization of the MUR3 homolog GT14. Leaves from a mutant line of plants with a T-DNA insertion in the GT14 gene were analyzed for changes in carbohydrate composition. Wild type and knockout plants were also transformed with GT14 for complementation and overexpression analysis. Finally, GT14’s amino acid sequence was used to predict its location and orientation in cellular membranes.
Materials and Methods

Insertion of GT14 into pCAMBIA and pSPDK vectors

A 1.566 kb GT14 cDNA, which was obtained from a T-DNA insertion mutant from the Salk Institute Genome Analysis Laboratory (La Jolla, CA), was amplified via PCR using the following primers:

Forward primer containing a KpnI restriction site:
5’-GCGCGGTACCATGCGACCCAAGAATTATTCTCAGATGGAG-3’

Reverse primer containing a BamHI restriction site:
5’-CCGCGGATCCTTAAGCCAAAACTTGAGGTTCTTCATATGG-3’

The following program was used for PCR amplification:
1) 1 min at 94°C (1 cycle)
2) 30 s at 94°C; ramp down to 55°C for 30 s; ramp up to 68°C for 2 min (25 cycles)
3) 10 min at 68°C; ramp down to and maintain at 4°C

The amplification product was isolated through gel electrophoresis and gel extraction. The gel was cast using TAE buffer, 0.8% agarose and 0.4 µg/ml ethidium bromide, and run at a field strength of approximately 10 V/cm. The PCR product was run alongside a 1 kb DNA ladder. The desired band was excised, and the slice was purified using a QIAquick Gel Extraction Kit following the instructions provided by the manufacturer. The gel slice was placed in a 2.0 ml microfuge tube and incubated at approximately 65°C until melted. 150 µl of isopropanol was added to the tube and the sample was transferred to a QIAquick spin column and centrifuged. Then, 0.5 ml Buffer QG was added and the tube was centrifuged. Next, 750 µl of wash buffer was added and
the sample was again centrifuged. The sample was eluted into a clean tube with 50 µl of water.

*GT14* was inserted into the pCAMBIA1301 vector (Fig. 2), which contains a kanamycin resistance gene for bacterial selection and a hygromycin resistance gene for plant selection. The *GT14* insert and the pCAMBIA vector were treated with the restriction enzymes BamHI and KpnI in a double digestion. Ligation was achieved using T4 DNA ligase by adding the insert and vector in a 3:1 ratio and leaving the mixture overnight at 16°C. The 13.4 kb ligated plasmid was isolated via gel extraction as described above.

**Figure 2.** The pCAMBIA1301 vector and its multiple cloning sites.
GT14 was inserted into the pSPDK1677 vector (kindly provided by Dr. Dinesh-Kumar at Yale University; see Fig. 3B for the vector map and Appendix for the vector sequence) by means of the pENTR 1A dual selection entry vector (Fig. 3A and Fig. 4B), based on Invitrogen’s Gateway cloning method. The method involves inserting the gene of interest (GT14) between the attL sites of the entry vector by performing a digestion and subsequent ligation. The attL sites in the entry vector, which flank the gene of interest, are recombined with attR sites in the destination vector’s gateway cassette. The pSPDK vector contains a spectinomycin resistance gene for bacterial selection and a gentamicin resistance gene for plant selection.

Figure 3. Maps of the pENTR 1A entry vector (A) and the pSPDK1677 destination vector (B).
Primers for inserting GT14 into pENTR 1A were designed as follows:

Forward primer containing a KpnI restriction site:
5’-GCGCGGTACCATGCACCCAAGAATTATTCTCAGATGGAG-3’

Reverse primer containing a NotI restriction site:
5’-CCGCGCGGCCGCGAAGCCAAAACTTGAGGTTCATATGGAGAA-3’

E. coli transformation and selection

100 µl of DH5α chemically competent cells were combined with 4 µl of ligated vector (containing insert GT14) and 8 µl double distilled H2O in 1.5 ml microfuge tubes and left on ice for 30 minutes. The cells were exposed to a temperature of 42°C for 2 minutes and immediately returned to ice. The cells were allowed to grow at 37°C for 1-2 hours in SOC media (2% tryptone, 0.5% yeast extract, 10 mM sodium chloride, 2.5 mM potassium chloride, 10 mM magnesium chloride, 10 mM magnesium sulfate, 20 mM glucose). The mixture was centrifuged and all but 100-150 µl of the SOC media was discarded. The pellet was resuspended, and the resuspended solution was distributed onto a 2% LB agar plate (1% tryptone, 0.5% yeast extract, 1% sodium chloride, 2% agar) and left overnight at 37°C. The LB agar plates contained either 50 µg/ml kanamycin, for cells transformed with pCAMBIA, or 50 µg/ml spectinomycin, for cells transformed with pSPDK.

Uptake of the entire plasmid was verified by performing a PCR colony screen of bacteria that grew on the plate. Liquid LB media was inoculated with positive colonies and incubated overnight at 37°C. Bacterial DNA from these cells was isolated using the QIAGEN Plasmid Maxi Prep kit as per the instructions provided by the manufacturer.
The plasmid DNA was purified using gel electrophoresis. A small amount of this plasmid DNA was double digested with the aforementioned restriction enzymes and run on the gel to confirm the presence of the vector and insert bands (Fig. 4A).

![Figure 4](image_url)

**Figure 4.** (A) Gel electrophoresis of the PCR amplification product of pCAMBIA (11.8 kb) with insert *GT14* (1.6 kb). One sample was treated with restriction enzymes KpnI and BamHI and run alongside an undigested sample. (B) Gel electrophoresis of the PCR amplification products of *GT14* (1.6 kb) and pENTR 1A vector (3.8 kb). The samples were treated with restriction enzymes KpnI and NotI. In the pENTR 1A lanes, the 1.5 kb and 2.3 kb fragments represent the segments of the digested plasmid. The 3.8 kb fragment represents undigested material.

Both sets of samples were run against HindIII-digested λ phage DNA as a size marker on a 0.8% agarose gel.

The DNA was sequenced in a Ready-to-Run reaction. The following internal sequence primers were designed for this purpose:

Forward primer: 5’-CGGTGTTTTGTCTTCAGCCACCGG-3’

Reverse primer: 5’-GGTCTCTACCAGACATTCCGACCCC-3’

Strains of *E. coli* that had been successfully transformed with pCAMBIA and *GT14*-pCAMBIA were stored at -80°C in 15% glycerol.
Agrobacterium tumefaciens GV3101 transformation

Approximately 5 µl of DNA was added to 50-100 µl of chemically competent cells of A. tumefaciens strain GV3101 (pMP90) that were kept on ice. The solution was transferred to an electroporation cuvette (on ice). The cells were electroporated at 2.40 kV for 9.70 msec. Then, approximately 750 µl of SOC media was added to the cells. The cells were resuspended and transferred to culture tubes where they were incubated at 28°C for 2 hours. The cells were then spread onto 2% LB agar plates containing the antibiotics listed below.

LB media for A. tumefaciens transformation was prepared as follows: 1% tryptone, 0.5% yeast extract, 0.5% sodium chloride, buffered with 10 mM MES and adjusted to pH 5.7. Gentamicin (for the A. tumefaciens helper plasmid), rifampicin (for A. tumefaciens), and either kanamycin (for cells transformed with pCAMBIA) or spectinomycin (for cells transformed with pSPDK) were added at concentrations of 100 µg/ml, 50 µg/ml, 50 µg/ml, and 50 µg/ml, respectively.

Plant growth conditions

Plants were grown on soil or on 0.8% MS agar plates with 1.0% sucrose, as described below. Conditions in the growth room were as follows: 18h light/6h darkness (approximately 100 µmol · m⁻² · s⁻¹), a temperature of 23°C during the light phase and 18°C during the dark phase, and 70% humidity. Wild type plants were of the Columbia ecotype.
Transformation of *Arabidopsis* plants with *A. tumefaciens*

*A. tumefaciens* was cultured in LB media (as described above) overnight. The cell culture was diluted to an OD$_{600}$ of 0.8-1.0 and added to plant transformation mix (2.15 g/L MS salt, 5% sucrose, 0.03% Silwet L-77). Plant inflorescences were dipped into the transformation mix for 1-2 minutes, then removed and laid horizontally in trays lined with moist paper towels. The trays were covered with plastic wrap. The plants were left in the growth chamber overnight. The plastic wrap was removed and the plants were returned to a vertical position.

Selection of T1 seeds from T0 plants

Approximately 200 mg of T1 seeds from transformed T0 generation plants were collected and placed in 50 ml Falcon tubes. 10 ml of 75% ethanol was added to the tubes and decanted after 1 minute. Then 10 ml of 30% bleach and 0.05% Tween 20 were added to the tubes. The tubes were placed on a shaker for 15 minutes, during which time the contents of individual tubes were periodically mixed by manually rotating them for 45-60 seconds at a time. Afterwards, the liquid from the tubes was decanted. The seeds were washed 6-8 times with approximately 30 ml of autoclaved, double distilled water. The seeds were then resuspended in roughly 25 ml of 0.2% MS agar that had been autoclaved at 121°C and maintained at 65°C. The resuspended seeds were spread onto 0.8% MS agar plates containing the ingredients listed below. The plates were sealed with Parafilm and transferred to a growth room. Care was taken to ensure a sterile environment for the plates. Seedlings were allowed to sprout for 3-4 weeks before being transplanted to soil.
The 0.8% MS agar plates were prepared as follows: 0.43% MS salt, 1.0% sucrose, 0.05% MES, pH 5.7 (adjusted using KOH), and 0.8% agar. After autoclaving, Gamborg’s vitamin solution was added to a final concentration of 1 ml/L. In addition, vancomycin and either hygromycin (for plants transformed with pCAMBIA vector) or gentamicin (for plants transformed with pSPDK vector) were added at final concentrations of 500 µg/ml, 50 µg/ml and 100 µg/ml, respectively, to select for transformed seedlings. The plates were left overnight at 4°C before use.

**Ethanol extraction of leaf samples for carbohydrate analysis**

For each sample, 2-3 leaves were collected and placed in 1.5 ml microfuge tubes. If storage was necessary at this point, the samples were placed in dry ice at -80°C. Otherwise, preparation of the samples continued as follows. Approximately 1 ml of 70% ethanol was added to the tubes. The tubes were incubated at 70°C for 30 minutes, then the supernatant was decanted. These steps were repeated once (approximately 1 ml of 70% ethanol for 30 minutes). The tubes were left open and allowed to air dry overnight. The samples were stored at room temperature.

**Fractionation of leaf material**

This method is based on the procedure described by Reiter, et al. (1997). Approximately 10 g of leaf material was collected in a Magenta box with liquid nitrogen, then stored at -80°C.

Before beginning the fractionation, a mortar and pestle were stored at -80°C overnight. Upon removal, they were pre-cooled with liquid nitrogen. The leaf material
was placed in the mortar. Then liquid nitrogen was added and the mixture was ground. This was repeated 2-3 times. The powderized mixture was placed in a Magenta box and stored at -80°C. A small amount was resuspended in water and viewed under a microscope to ensure that the leaf material had been sufficiently homogenized.

The pulverized leaf material was resuspended in 50 ml of buffer A (100 mM MOPS, pH 7.0, 1.5% SDS, 5 mM sodium bisulfite that was heated briefly at approximately 50°C to remove turbidity), and stirred in an ice-water bath. The cell wall material was pelleted by centrifugation for 10 minutes at 4°C in a Sorvall SS34 rotor at 15000 rpm. The material was then washed with 25 ml of ice-cold buffer B (100 mM MOPS, pH 7.0, 0.5% SDS, 3 mM sodium bisulfite), and centrifuged as described above. The two supernatants were combined into fraction S1.

The cell wall pellet was washed with 50 ml of buffer B, and the supernatant was discarded after centrifugation. Following 2-3 washes with water, the cell wall material was resuspended in 16 ml of water and stored at -20°C.

A solution of 10 M ammonium acetate was added to fraction S1 to have a final concentration of 1 M. Polymers were precipitated by addition of five volumes of a 1:1 mixture of ethanol and acetone. The mixture was allowed to stand for 24 hours at room temperature. The precipitate was then recovered by centrifugation at 2000 g for 15 minutes. Neither the addition of more acetone nor the addition of more ammonium acetate to the mixture caused turbidity, suggesting that polymers had been efficiently removed. The pellet was washed thoroughly with 80% ethanol, and stored at 4°C as a suspension in 40 ml of 80% ethanol.
Samples were prepared for carbohydrate analysis by properly resuspending them and pipetting an amount of material roughly equal to 1% (approximately 100 mg) of the original mass of the leaves into microfuge tubes. The samples were centrifuged to pellet the insoluble material. The supernatant was removed and the remaining material was dried by centrifugation under vacuum in a SpeedVac apparatus. The samples were stored at room temperature.

**Cell wall carbohydrate analysis**

This method is based on the cell wall analysis method described by Blakeney, et al. (1983).

Each sample of cell wall material was autoclaved for one hour in 250 µl of 2 M TFA with slow exhaust. To each sample, 100 µg of myo-inositol were added for use as an internal standard. For calibration purposes, a mixture of monosaccharides (100 µg each of L-rhamnose, L-fucose, L-arabinose, D-xylose, D-mannose, D-galactose and D-glucose) with 100 µg myo-inositol in 250 µl of 2 M TFA was used. In addition, blank samples (2 M TFA with 100 µg myo-inositol) were autoclaved.

Samples were stored overnight at 4°C and then transferred to large screw cap vials with Teflon-lined lids. 100 µl of 9 M NH₃ was added to each tube and briefly vortexed. The tubes were transferred to a 40°C water bath. 1 ml of 2% NaBH₄ in DMSO was added to each tube, followed by brief vortexing. The tubes were then covered with aluminum foil, and allowed to incubate at 40°C for 90 minutes.

The tubes were removed from the water bath. Following the addition of 250 µl of acetic acid, the tubes were briefly vortexed. 250 µl of 1-methylimidazole and 4 ml of
acetic anhydride were added to each tube. The tubes were vortexed and incubated at room temperature for 10 minutes. Eight milliliters of double distilled water were added to each tube. The tubes were capped, inverted several times to dissolve a precipitate, and allowed to cool slowly to below 30°C. 1.5 ml CH₂Cl₂ was added to each tube, and the alditol acetates were extracted into the organic phase by inverting the tubes at least 30 times. The tubes were allowed to sit at 4°C for 2 hours (or overnight).

The lower (CH₂Cl₂) phases were recovered using long Pasteur pipets and transferred to small (12 x 75 mm) test tubes. 200 µl of CH₂Cl₂ followed by 2 ml of double distilled water were added to each tube. The tubes were vortexed vigorously for over 15 seconds. After the organic phase settled it was transferred, via a P-200 pipettor, to small pre-numbered GC vials. The filled vials were stored at -20°C.

The samples were analyzed by gas-liquid chromatography essentially as described by Reiter, et al. (1997) with minor modifications. The inlet temperature for the analysis was set to 240°C, the detector temperature was set to 250°C, and the total run time was 22 minutes.
Results

Subcellular localization of GT14

The amino acid sequences of GT14 and MUR3 were analyzed by the TMHMM online tool at http://www.cbs.dtu.dk/services/TMHMM/ (Fig. 5). The algorithm uses bioinformatics data to predict the hydrophobicity of each amino acid in the sequence.

Figure 5. The amino acid sequences of GT14 (A) and MUR3 (B) were analyzed by the TMHMM transmembrane prediction algorithm. The resulting plots predict that amino acids 31-53, in GT14, and amino acids 32-54, in MUR3, comprise transmembrane domains.

Hydrophobic regions of peptides are generally embedded in the lipid bilayer. The algorithm determines the likelihood of any given amino acid in the sequence being part of
such an \(\alpha\)-helical transmembrane domain. The tool predicted that, in GT14, amino acids 31 through 53 comprise an \(\alpha\)-helical transmembrane domain (Fig. 5A). In MUR3, amino acids 32 through 54 were predicted to be part of an \(\alpha\)-helical transmembrane domain (Fig. 5B).

The amino acid sequences of GT14 and MUR3 were also analyzed by the SignalP 3.0 online tool (Fig. 6) at [http://www.cbs.dtu.dk/services/SignalP/](http://www.cbs.dtu.dk/services/SignalP/) (Bendtsen et al., 2004; Nielsen et al., 1997).

![Figure 6](image_url)

**Figure 6.** The amino acid sequences of GT14 and MUR3 were analyzed by the SignalP 3.0 algorithm. The resulting plot predicts the presence of a signal anchor sequence \((p=0.947)\) for GT14 (A) as well as the presence of a signal anchor sequence \((p=0.999)\) for MUR3 (B).
The SignalP hidden Markov model (Nielsen and Krogh, 1998) output predicts with a probability of 0.947 that the polypeptide contains a signal anchor sequence. This is based on the presence of an h-region (hydrophobic region), roughly between amino acids 30 and 50, preceded by an n-region. For MUR3, it predicts the presence of a signal anchor with probability 0.999. This is indicated by an h-region, roughly between amino acids 30 and 60, preceded by an n-region. In both cases, the n-region and subsequent h-region suggest the presence of a sequence responsible for targeting the peptide to a cellular membrane. These data correspond to the output obtained from the TMHMM tool. It should be noted that MUR3 is known to be targeted to the Golgi membrane (Tamura et al., 2005).
Carbohydrate composition of gt14 mutants

The relative amounts of monosaccharides in leaf material from gt14 and WT plants were quantified via gas-liquid chromatography (Fig. 7).

Figure 7. Representative image of a chromatogram obtained by gas-liquid chromatography of the derivatized monosaccharides. Rhamnose, fucose, arabinose, xylose, mannose, galactose, glucose and myo-inositol were separated based on differences in their retention times, and quantified based on their respective peak areas.

Total cell wall material from gt14 plants displayed 20.7% and 12.8% decreases in fucose and rhamnose, respectively, as well as 30.6% and 12.5% increases in mannose and arabinose, respectively (Fig. 8 and Table 1). There was no significant difference between the gt14 mutants and WT plants with regard to xylose or galactose.
Figure 8. Cell wall monosaccharide composition in \textit{gt14} and WT plants. The \textit{gt14} mutants displayed significant decreases in fucose and rhamnose, as well as significant increases in mannose and arabinose.

Table 1. Relative amounts of monosaccharides in \textit{gt14} and WT plants.

<table>
<thead>
<tr>
<th>Monosaccharide</th>
<th>\textit{gt14}</th>
<th>Wild type</th>
<th>Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhamnose</td>
<td>12.9%</td>
<td>14.8%</td>
<td>(-12.8%)</td>
</tr>
<tr>
<td>Fucose</td>
<td>4.6%</td>
<td>5.8%</td>
<td>(-20.7%)</td>
</tr>
<tr>
<td>Arabinose</td>
<td>20.7%</td>
<td>18.4%</td>
<td>(+12.5%)</td>
</tr>
<tr>
<td>Xylose</td>
<td>20.6%</td>
<td>21.0%</td>
<td>(-1.9%)</td>
</tr>
<tr>
<td>Mannose</td>
<td>8.1%</td>
<td>6.2%</td>
<td>(+30.6%)</td>
</tr>
<tr>
<td>Galactose</td>
<td>33.0%</td>
<td>33.2%</td>
<td>(-0.6%)</td>
</tr>
</tbody>
</table>

Differences between the mutant and WT plants were also evaluated by constructing histograms of the data for each monosaccharide (Fig. 9). In the \textit{gt14} plants, there was a distinct decrease in the median amount of fucose (4.5\% vs. 6\%). There was also an increase in the median amount of mannose (8.5\% vs. 6\%), a decrease in the median amount of rhamnose (12\% vs. 15\%), and an increase in the median amount of arabinose (20\% vs. 18\%). There was, however, a relatively large amount of overlap between the WT and \textit{gt14} distributions with regard to rhamnose and arabinose, so the differences are less clear than they may initially seem.
Figure 9. Histograms of the carbohydrate analysis of gtl4 and WT plants. There were relatively large shifts in the median for fucose, which decreased, and mannose, which increased. There were slightly smaller shifts in the median for rhamnose, which decreased, and arabinose, which increased.
In addition, a difference is noticeable in xylose content (19% vs. 21%). It can be observed, however, that these distributions are skewed with regard to xylose (gt14 to the right and WT to the left) which, perhaps, makes these differences less significant. There was no observable change in the distribution of galactose between the two groups.

In order to determine whether or not the changes in monosaccharide composition were the result of changes in the cell wall or in the cytosolic material, fractionated portions of cell wall material and soluble polymers were analyzed using gas-liquid chromatography (Fig. 10).

In the gt14 plants, less fucose (Fig. 10A) was present in the cell wall than in the WT (ca. 4% vs. 4.5%) but in relative terms, there was more fucose in the gt14 soluble fraction than in the wild type soluble fraction (ca. 7% vs. 5.5%). The amount of mannose in the gt14 and WT cell wall fractions was approximately the same (Fig. 10B), though there was significantly more in the gt14 soluble fraction than in the WT soluble fraction (ca. 9% vs. 6%). The gt14 cell wall fraction contained more galactose (Fig. 10C) than the WT cell wall fraction (ca. 33% vs. 26%), though the amount of galactose in the soluble fraction was similar in both sets of plants. There was slightly less rhamnose (Fig. 10D) in both fractions for gt14. There was only slightly more arabinose (Fig. 10E) in the gt14 cell wall fraction than in the WT cell wall fraction, and slightly more arabinose in the WT soluble fraction than in the gt14 soluble fraction. There was no significant change in the xylose content (Fig. 10F) of the soluble fraction, but there was slightly more xylose in the WT cell wall fraction than in the gt14 cell wall fraction.

It should be noted that sample sizes in the fractionation analysis were small. This may explain some peculiarities in the histograms, such as the lack of a distinct median or
peak in the gt14 distribution of cell wall rhamnose (Fig. 10D). Thus, certain changes may not necessarily be significant.

**Figure 10.** Leaf material from gt14 and WT plants was fractionated to separate cell wall components from soluble polymers. (A)-(F) present histograms of the distribution of monosaccharides between the two fractions.
Figure 10. (Continued)
Complementation and overexpression analysis

The *GT14* coding region was inserted into pCAMBIA 1301 and pSPDK vectors and then transformed into WT and *gt14* plants. T1 seeds were collected from T0 generation plants. The monosaccharide composition of the T1 transformants was analyzed via gas-liquid chromatography of alditol acetates. Three sets of plants were analyzed: *gt14* plants transformed with the *GT14* pSPDK construct (*gt14/GT14*-pSPDK), WT plants transformed with the *GT14*-pSPDK (WT/*GT14*-pSPDK), and *gt14* plants transformed with the *GT14*-pCAMBIA construct (*gt14/GT14*-pCAMBIA), in addition to their respective controls. A set of WT plants transformed with the *GT14*-pCAMBIA construct is not included because leaf material for these plants was not ready to be harvested at the time of this writing. Histograms of the data (Figs. 13-18) are located in the Appendix.

There was relatively less fucose in the *gt14/GT14*-pSPDK plants than in the control group (Fig. 13A). The same relationship was observed in the WT/*GT14*-pSPDK plants (Fig. 13B). There was no change in the distribution for fucose in the *gt14/GT14*-pCAMBIA groups (Fig. 13C). The *gt14/GT14*-pSPDK plants (Fig. 14A) actually appeared to have somewhat more mannose than the control, as did the WT/*GT14*-pSPDK plants (Fig. 14B). There was no difference in the relative amount of mannose between the *gt14/GT14*-pCAMBIA and control groups (Fig. 14C). The distributions of galactose in the *gt14/GT14*-pSPDK (Fig. 15A) and *gt14/GT14*-pCAMBIA (Fig. 15C) groups revealed no changes either, though the amount of galactose was noticeably lower (approximately 30% vs. 35%) in the WT/*GT14*-pSPDK group (Fig. 15B). None of the transformants
showed considerable differences in rhamnose (Fig. 16), arabinose (Fig. 17) or xylose (Fig. 18).

The age of the plants at the time of leaf material collection was not uniform. Some transformants were older than others. This, combined with small sample sizes, may have contributed to the variability between the samples and the skewed appearance of the distribution data.
Discussion

GT14 appears to be a type II membrane protein most likely targeted to the Golgi apparatus. This cannot be ascertained based on the bioinformatics data alone; however, it is probable because the TMHMM and SignalP results for GT14 (Fig. 5A and Fig. 6A) are nearly identical to those for MUR3 (Fig. 5B and Fig. 6B). MUR3 is known to be a type II membrane protein with a short cytosolic domain and a large lumenal domain. It is targeted to the Golgi apparatus (Tamura et al., 2005). It would not be unreasonable to assume that GT14 occupies a similar position in the Golgi membrane. The $gt14$ mutants showed a reduction in fucose of approximately 20.7% and an increase in mannose of approximately 30.6%. Analysis of cell wall and soluble fractions confirmed that the reduction in fucose could be traced to the cell wall. Considering these changes within the context of phylogenetic relationships may provide clues as to the exact function of GT14.

*GT14, MUR3, and GT18 are all part of subgroup A in the GT47 gene family (Fig. 11).*

![Figure 11](image)

*Figure 11. Subgroup A of the GT47 family. Subgroup A includes GT14 in addition to its homologs MUR3 and GT18, among others (Li et al., 2004).*

MUR3 is a xyloglucan specific galactosyltransferase and, based on genetic data, GT18 is as well. Therefore, it is relatively safe to assume that the target of GT14 is also
xyloglucan. The exact nature of its function, though, remains unclear. If GT14 is a
galactosyltransferase like MUR3 and GT18, the decrease in fucose in \textit{gt14} mutants may
be explained as an indirect consequence of such activity. Since galactosylated xyloglucan
is an acceptor substrate for the fucosyltransferase AtFUT1, a decrease in the
galactosylation of xyloglucan is also manifested as a reduction in fucosylated xyloglucan.
This effect is seen clearly in the \textit{mur3} mutants. Although MUR3 is a
galactosyltransferase, the most pronounced change in \textit{mur3} plants is a 50\% reduction in
fucose content (Madson et al., 2003). The same may be true of GT14. Unlike the \textit{mur3}
and \textit{gt18} mutants, the \textit{gt14} mutants did not display a reduction in cell wall galactose. This
does not rule out the potential role of GT14 as a xyloglucan-specific
galactosyltransferase. It is entirely possible that \textit{gt14} mutants contain less galactosylated
xyloglucan, but that this decrease is masked by galactose in other cell wall components
like RG-I and arabinogalactans.

One way to gain insight into the function of GT14 may be to observe the
structural and mechanical characteristics of \textit{gt14} mutants, and then compare these to the
characteristics of \textit{mur3} mutants. For instance, growing \textit{mur3} mutants were found to be
visually indistinguishable from their wild type counterparts, save for a collapsed
appearance of the trichome papillae (Madson et al., 2003). Moreover, there was no
difference in the amount of force needed to break elongating inflorescences in \textit{mur3}
mutants (Madson et al., 2003). While the \textit{mur3} mutation does not appear to be
detrimental in relatively older plants, there was, on the other hand, a considerable
decrease in the tensile strength of the hypocotyls in 4 day old \textit{mur3} seedlings (Peña et al.,
2004). Given these qualities of \textit{mur3} mutants, studies on the trichome structure and
mechanical strength of \textit{gt14} mutants at different stages of development would be beneficial. If the \textit{gt14} mutants and \textit{mur3} mutants display similar trends, then it would strengthen the hypothesis that GT14 and MUR3 possess a similar function.

Since mutations in both \textit{GT14} and \textit{MUR3} reduce fucose content, it is possible that GT14 and MUR3 (or other proteins in subgroup A) cooperate in the galactosylation of xyloglucan, though it is also possible that they function independently of one another, to some extent. Further experimentation is necessary to determine if there are any significant interactions between the two. It may be useful to develop and analyze \textit{mur3} \textit{gt14} double mutants for this purpose.

The increase in the mannose content in the soluble polymer fraction of the \textit{gt14} mutants was significant as well. This may potentially be explained by the fact that D-mannose is a substrate in the synthesis of L-fucose (Fig. 12). GDP-D-mannose is converted to GDP-L-fucose by means of GDP-D-mannose 4,6-dehydratase (Mulichak et al., 2002; Bonin et al., 2003). It is unlikely that GT14 is involved in this process; however, \textit{gt14} plants demonstrate a marked reduction in fucose. If less GDP-L-fucose is incorporated into xyloglucan in the cell wall, this may mean that, to compensate, less GDP-D-mannose is converted to GDP-L-fucose. This could lead to an increased concentration of GDP-D-mannose within the cell, though the precise nature of this effect would be unclear. Two ways this may be manifested include reductions in the transcription and translation of the \textit{GMD2} gene, which encodes for GDP-D-mannose 4,6-dehydratase. These reductions could be measured by assays that detect changes in transcription of \textit{GMD2} into mRNA (e.g., Northern blot) and translation of the mRNA into GDP-D-mannose 4,6-dehydratase (e.g., Western blot).
Complementation and overexpression analysis was ultimately inconclusive. A number of factors likely contributed to this. The T1 plants from which the leaf material was harvested were older than the *gt14* plants that were originally used in the determination of carbohydrate composition. Fucose and xylose content have been shown to increase with the age of the plant, whereas arabinose and galactose appear to decrease with age (Reiter et al., 1997). This effect may have dampened some of the differences in monosaccharide composition and exaggerated others. In addition, since T1 generation plants were used, the majority of plants were most likely hemizygous for the *GT14*-plasmid constructs, rather than homozygous. T2 generation plants, which should be homozygous, may display more noticeable phenotypic differences, if any.

Further studies are necessary for a better understanding of GT14 and its activity. The composition of different types of xyloglucan in the mutants remains to be analyzed. Knowledge of the relative amounts of the different forms of xyloglucan (XXXG, XIXG, XXLG, XILLG, XXFG, XLFG) in the *gt14* mutants may shed much light on the function of GT14. *In vitro* enzyme assays of GT14 on xyloglucan polysaccharide may also

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**Figure 12.** A proposed mechanism for the catalytic activity of GDP-D-mannose 4,6-dehydratase. GDP-mannose (a) is converted to GDP-4-keto-6-deoxymannose (d) via the 4-keto and 4-keto-5,6-ene (b, c) intermediates (Mulichak et al., 2002).
indicate more precisely which step in the galactosylation of xyloglucan GT14 is involved in. GUS-tagged (as in the pCAMBIA construct) or citrine-tagged (as in the pSPDK construct) GT14 may also be used to ascertain whether or not GT14 is targeted to the Golgi membrane. This could be accomplished by fractionating the cellular organelles, and then using an immunohistochemical assay like Western blotting or ELISA to determine if the tagged protein is present in a particular fraction. GT14 may also possess other functions. For example, it is known that a different defect in the MUR3 gene, kam1, affects cytoskeletal organization (Tamura et al., 2005). In addition, mur3 mutants demonstrated enhanced resistance to H. parasitica in petiole tissue. The mechanism for this pathogenic resistance is not entirely clear. One hypothesis is that proteins for defense against pathogens are activated by perturbations in the cell wall or in the actin cytoskeleton and that the loss of MUR3 conditions these proteins, essentially lowering the threshold at which they are activated. Another alternative is that there is some molecule that activates pathogen defenses, known as an ‘elicitor,’ normally produced by pathogen activity, and that this elicitor is also produced by changes to the cell wall (Tedman-Jones et al., 2008). Though the mechanisms for these hypotheses are unclear, the homology between MUR3 and GT14 suggests that GT14 may also be involved in such processes.

Ultimately, this work has revealed several characteristic features of GT14. Still, many questions regarding its mechanism and function remain to be answered.
References


Appendix

Histograms for complementation analysis

Figure 13. Histograms comparing fucose composition of T1 gt14 and WT plants transformed with GT14 in pCAMBIA and pSPDK vectors. None of the transformants showed significant changes relative to the control groups.
Figure 14. Histograms comparing mannose composition of T1 gt14 and WT plants transformed with *GT14* in pCAMBIA and pSPDK vectors. Some plants in the pSPDK complemented line (A) may have less mannose than the control group.
Figure 15. Histograms comparing galactose composition of T1 $gt14$ and WT plants transformed with $GT14$ in pCAMBIA and pSPDK vectors. No changes were observed in the complemented (A, C) transformants, but the overexpressed (B) transformants had relatively less galactose than the control plants.
Figure 16. Histograms comparing rhamnose composition of T1 gt14 and WT plants transformed with GT14 in pCAMBIA and pSPDK vectors. None of the transformants showed significant changes relative to the control groups.
Figure 17. Histograms comparing arabinose composition of T1 gt14 and WT plants transformed with GT14 in pCAMBIA and pSPDK vectors. None of the transformants showed significant changes relative to the control groups.
Figure 18. Histograms comparing xylose composition of T1 $gt14$ and WT plants transformed with $GT14$ in pCAMBIA and pSPDK vectors. None of the transformants showed significant changes relative to the control groups.
Sequence of the pSPDK1677 destination vector

5'-tcaccatgttatcaatcaactcactgtctgtaaggttggtgcaccatcctttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt
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