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# The Molecular Characterization of a Predicted Glycosyltransferase from *Lycopersicon esculentum*

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APPROVAL PAGE

HONORS THESIS

**Molecular characterization of a predicted glycosyltransferase  
from *Lycopersicon esculentum***

Presented by

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2005

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## Abstract

The synthesis of the plant cell wall is very complex, and understanding how this process occurs will lead to many benefits for future research and industries dependent upon cell walls for their products. The recent discovery of the functions of AtMUR3 and AtGT18 in *Arabidopsis thaliana* as xyloglucan galactosyltransferases has led to the identification of many more putative glycosyltransferases in the *Arabidopsis* genome. Due to the structural differences between the xyloglucans of *Arabidopsis* and solanaceous plants, we decided to search for putative arabinosyltransferases in the Solanaceae. Solanaceous xyloglucan is substituted by one to two arabinosyl residues at the second xylose position, and sometimes contains an arabinose at the first xylose position. In contrast, *Arabidopsis* xyloglucan does not contain arabinose, and is substituted by galactose at the second and third xylose position. Furthermore, the second galactose residue in *Arabidopsis* xyloglucan is usually fucosylated, a modification not found in solanaceous plants. Searching the database of expressed sequence tags (dbEST), we identified many likely glycosyltransferases in solanaceous plants, including tomato (*Lycopersicon esculentum*). AtMUR3 and AtGT18 search queries resulted in the identification of three putative glycosyltransferases in *L. esculentum*, which were tentatively designated *LeGT1*, *Le1GT18*, and *Le2GT18*. Based on phylogenetic considerations, *Le2GT18* was thought to be a putative arabinosyltransferase. The gene was transformed into *atmur3-3* and *atgt18* mutant plants, and the resulting plants will be screened for homozygous plants with the inserted gene. The homozygous T2 plants can then be screened for changes in the composition of their cell walls. Because *Le2GT18* is

thought to be an arabinosyltransferase, the levels of arabinose may be increased in the xyloglucan fraction of the cell wall. If so, further testing can be performed to reveal the true function of *Le2GT18*.

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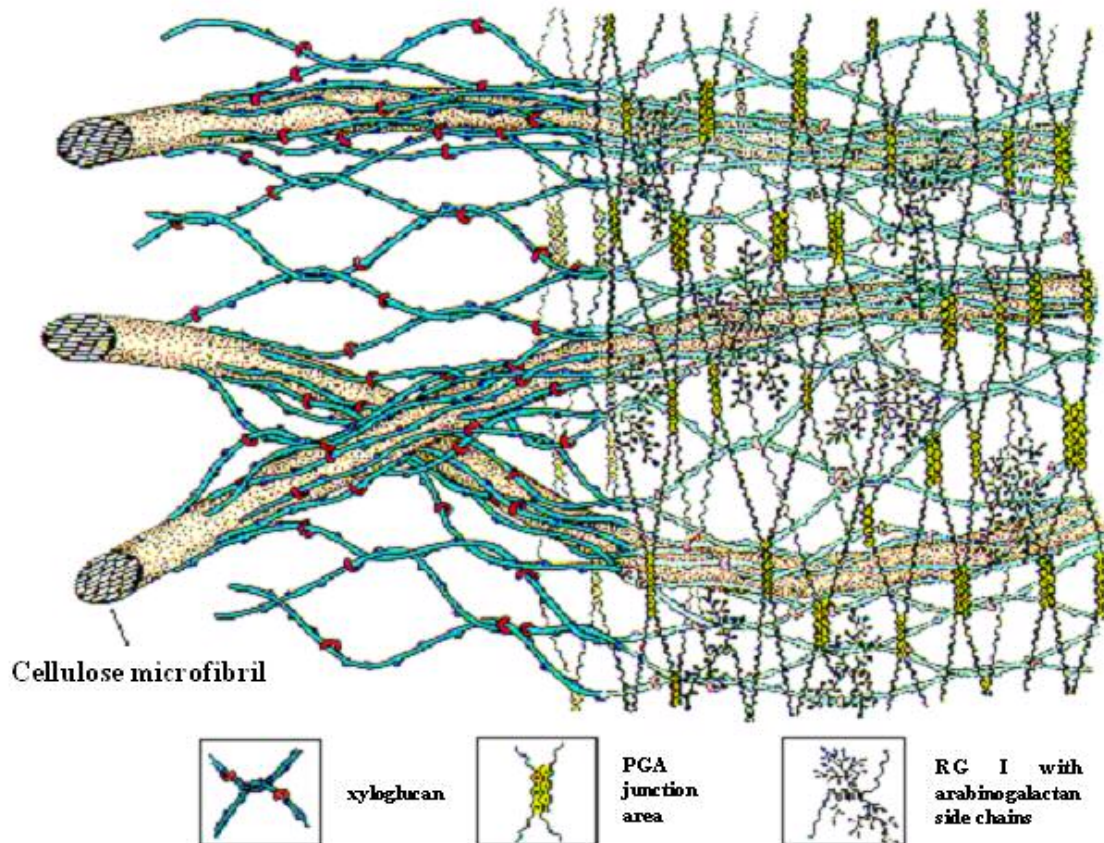
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## I. Introduction

Plant cell walls play an important part in our daily lives. Paper, lumber, textiles, agricultural items, and many other products are made of cell wall material. Studying cell walls may ultimately lead to increases in the economic productivity of industries that use cell wall materials. Some studies have focused on modifying pectin cross-linking or cell-cell adhesion to increase the shelf life of fruits and vegetables, enhancing dietary fiber content of cereals, improving yields and quality of fibers, and the relative distribution of carbon to wall biomass for use as biofuels (Minorsky, 2002). Cellulose is the world's most abundant renewable resource and a major component of cotton fibers and wood, which makes knowledge of its synthesis desirable to increase economic efficiency. One important topic in applied cell wall research is the digestibility of cell wall material by microorganisms. For instance, cellulose can be enzymatically hydrolyzed to glucose, which can then be fermented to ethanol to yield an excellent source of fuel. Unfortunately, naturally occurring cellulose is very difficult to degrade, so modifications of its structure may improve its usefulness as a renewable source of energy. Similarly, the microflora in the stomach of ruminants cannot completely degrade cell wall material because of its highly complex structure. Genetically engineered modifications of cell wall polysaccharides may enable ruminants to make more efficient use of their plant-based diet.

The components of the plant cell wall and how they are put together require further investigation. Plant polysaccharides comprise 90 percent of the primary cell wall.

The remaining 10% is made up of proteins (Reiter, 1998). Primary cell walls are produced during growth, but once growth stops secondary cell walls are produced in several cell types. The secondary plant cell wall is much more rigid than the primary cell wall due to increased cellulose content, the absence of pectin and glycoproteins, and the addition of lignin. The highly cross-linked structure of lignin provides rigidity to the secondary cell wall (Reiter, 1994). The cell wall is made up of three components as shown in Figure 1: cellulose, hemicellulose, and pectin (McNeill, et al., 1984; Carpita and Gibeaut, 1993). Cellulose forms semicrystalline microfibrils that provide strength to the cell wall. The microfibrils are embedded in a matrix of hemicelluloses and pectic polysaccharides. Hemicelluloses are composed of a carbohydrate backbone similar to cellulose with sugar side chains. Pectic polysaccharides are highly negatively charged, and form an interweaving network encompassing the hemicellulose and cellulose matrices (Carpita and Gibeaut, 1993).



**Figure 1:** The type I primary cell wall of most flowering plants. Cellulose microfibrils are interlaced with xyloglucans, and this structure is embedded in a pectic matrix composed of polygalacturonic acid and rhamnogalacturonans (Carpita and Gibeaut, 1993).

Recent research into the synthesis of these polysaccharides focuses on glycosyltransferases (GTs). These enzymes synthesize specific cell wall structures by catalyzing the formation of glycosidic bonds, attaching a sugar moiety to a specific acceptor substrate (Keegstra and Raikhel, 2001; Scheible and Pauly, 2004). GTs are necessary in synthesizing the cell wall; therefore, a mutation in a gene encoding any single GT might have adverse effects. Further research on GTs is necessary to reveal the synthesis and functions of the different components of the cell wall.

## **A. The Plant Cell Wall**

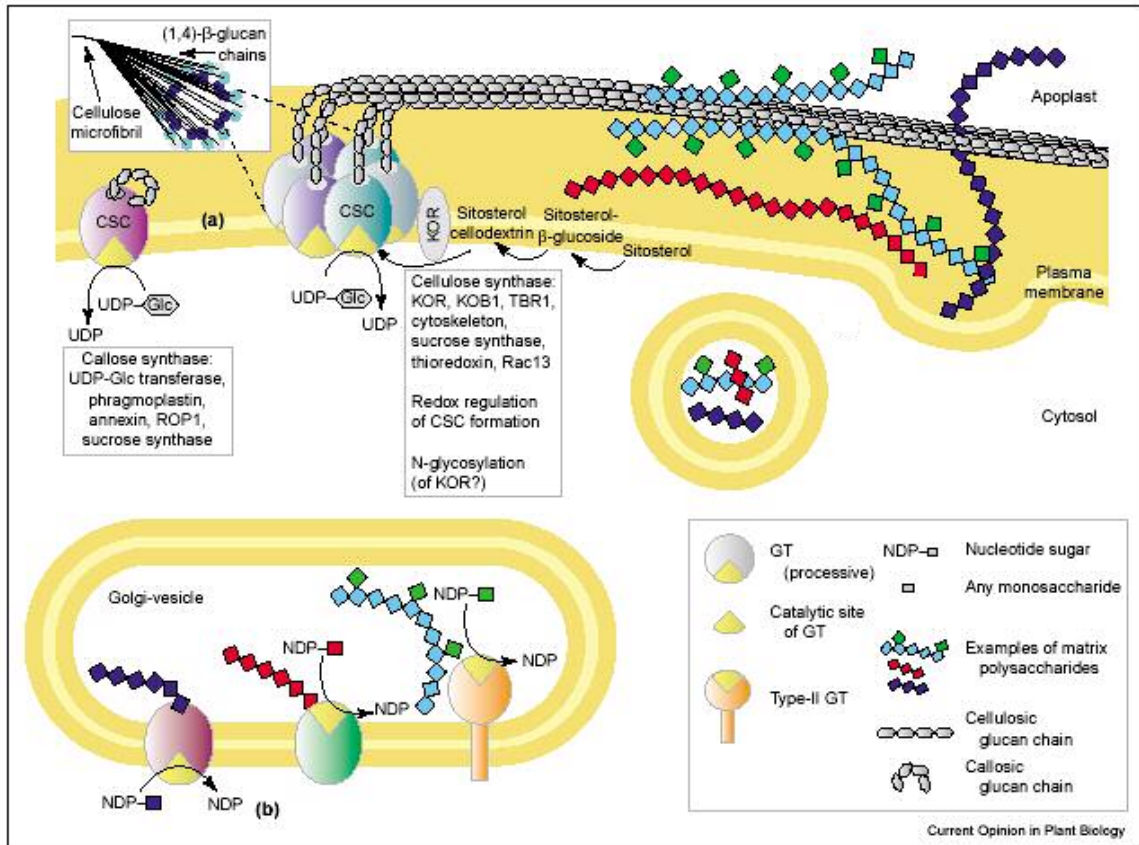
### **1) Functions**

Every individual plant cell is surrounded by a “wall” that serves multiple purposes. Cell walls must serve structural, protective, and regulatory functions throughout the lifetime of the plant. Although the cell walls form a matrix strong enough to support the structural integrity of the plant, including resisting the internal turgor pressure of the cell, they must also be flexible enough to allow growth of the cell and plant (Bacic et al., 1988; Carpita and Gibeaut, 1993). Some cell wall proteins strengthen the cell wall by forming cross-links with each other. This is suggested by removal of the cell wall polysaccharides, leaving a network of hydroxyproline-rich glycoproteins (Mort and Lamport, 1977). Another group of proteins, the expansins, allow extension of the cell wall by weakening the adhesion between cellulose and other polysaccharides. This allows the microfibrils to slide within the cell wall matrix (Cosgrove, 1997). Cell walls are also important in regulating molecules entering the cell and in allowing communication with neighboring cells. Pectin interactions and cell wall proteins are important in cell-cell communications. The cell walls are permeated by symplastic continuations named plasmodesmata, which allow cells to directly communicate with each other (Robards and Lucas, 1990). Defending against invaders is another important function of the cell wall. For example, cell wall signaling due to insect predation induces the production of defense molecules (Ryan, 1990). In fact, plants produce rapid bursts of extracellular hydrogen peroxide, which acts to stiffen the cell wall and to destroy invaders (Levine et al., 1994; Cosgrove, 1997). Another instance of cell walls defending

a plant is when protein and lignin shells are produced due to invasion of fungal and bacterial pathogens (Bowles, 1990). Defensive changes, regulation, signaling, and allowing growth illustrate how the cell wall is much more than a rigid box with no other purpose than structural support.

## 2) Cellulose

Cellulose provides the framework for the plant cell wall. It is made up of linear  $\beta(1\rightarrow4)$ -linked D-glucan chains assembled into water-insoluble, crystalline microfibrils by hydrogen bonding (Reiter, 1994; Gibeaut and Carpita, 1994; Scheible and Pauly, 2004). Microfibrils are about 5 – 12 nm in diameter, and are covered by hemicelluloses (Roberts, 1990). Cellulose is one of the two (the other being callose) polysaccharides known to be synthesized at the plasma membrane (Gibeaut and Carpita, 1994) (Fig. 2a). Cellulose synthase of plants contains several subunits from a group of proteins belonging to the GT2 family. The *Arabidopsis* genome contains ten coding regions for GT2 proteins, six of which are known to be part of the cellulose synthase catalytic subunit (CESA) (Pear et al., 1996; Scheible and Pauly, 2004). The strong cellulose microfibrils are the major load-bearing portion of the plant cell wall (Reiter, 1994).

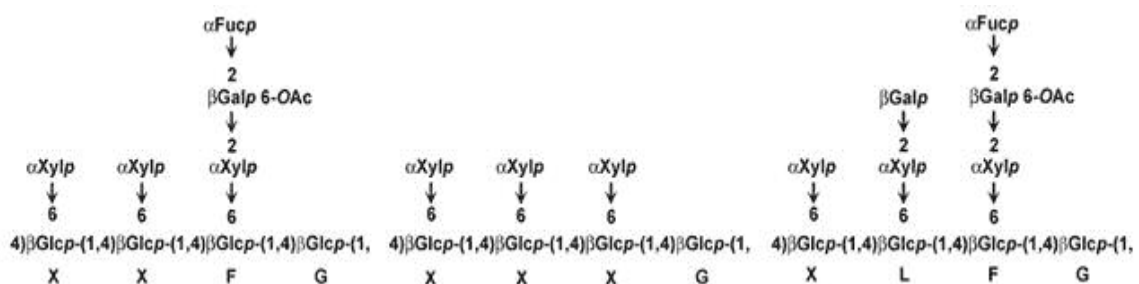


**Figure 2:** Synthesis of cell wall polysaccharides. **(a)** Cellulose and callose are synthesized at the plasma membrane. The CESA proteins combine to form a “rosette” known as the Cellulose Synthase Complex (CSC), which produces the cellulose microfibrils. **(b)** Matrix polysaccharides are produced in the Golgi apparatus by the action of GTs. The polysaccharide chains are then secreted to the apoplast by exocytosis, where they form networks with the cellulose microfibrils (Scheible and Pauly, 2004).

### 3) Hemicellulose

Hemicelluloses are interwoven throughout the cell wall, and are capable of tightly linking with the cellulose microfibrils by hydrogen bonding (Reiter, 1994). Xyloglucans and xylans are the two major types of hemicellulose. Xyloglucans are the predominant hemicelluloses present in the primary wall of dicots, while xylans pre-dominate in the

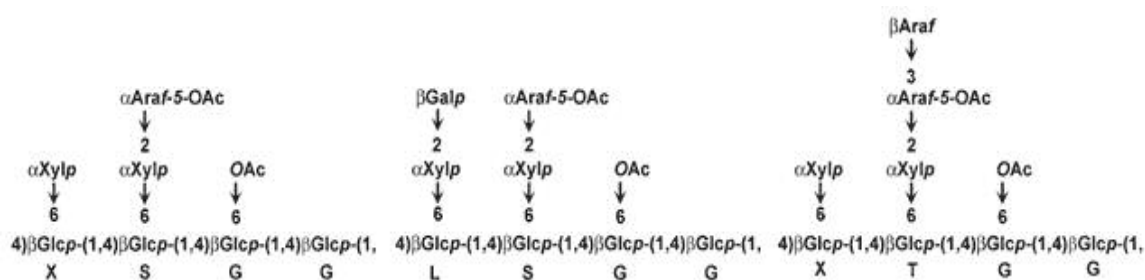
primary wall of grasses and in the secondary wall of higher plants (Bacic et al., 1988). Xyloglucans are composed of a  $\beta(1\rightarrow4)$ -linked D-glucose backbone (like cellulose) that is modified by attaching  $\alpha(1\rightarrow6)$ -linked D-xylosyl residues about 50 – 75 percent of the time. The xylosyl residues are further modified by attachment of  $\beta(1\rightarrow2)$ -linked D-galactosyl residues at specific positions that depend on the plant species.  $\alpha(1\rightarrow2)$ -linked D-fucosyl residues are attached to some of the galactosyl residues. The xyloglucan of *Arabidopsis thaliana* (Fig. 3) includes xylosyl residues on three glucose molecules in a row followed by an unsubstituted glucose. Galactosyl residues are found at the second and third xylose residues, and a terminal fucose is often found on the second galactose position.



**Figure 3:** The typical xyloglucan subunit structure in many dicots, including *Arabidopsis thaliana* (<http://www.ccruc.uga.edu/>).

Solanaceous plants have a different xyloglucan structure (Fig. 4). Solanaceous xyloglucan contains arabinosyl residues and does not include fucose. The arabinosyl residue is attached to the second xylose position, while galactose may attach to the first xylose position. In some cases, a second arabinose may be attached to the first arabinosyl residue. The third and fourth glucosyl residues do not have any sugars attached to them.





**Figure 4:** The xyloglucan structure found in solanaceous plants, including *Lycopersicon esculentum* (<http://www.ccrcc.uga.edu/>).

Xyloglucans have important functions in the plant cell wall in addition to contributing to its mechanical strength. Xyloglucans do not bind strongly to each other; however, the strong hydrogen bonding of xyloglucans with cellulose microfibrils allows the xyloglucan to act as a coating for the cellulose, preventing aggregation of the cellulose microfibrils (Hayashi et al., 1987). Another important function of the xyloglucan is to permit cell wall expansion. The xyloglucan is capable of undergoing hydrolysis through endoglycolytic cleavages during auxin-stimulated cell growth (Taiz, 1984). Expansins allow the cell wall to become more flexible by interfering with the hydrogen bonding between the xyloglucan and cellulose fibers, permitting the extension of the cell and its wall (Cosgrove, 1997). A third proposed function of the xyloglucan is an involvement in signaling events. Degradation of the xyloglucan produces oligosaccharides, which may behave as second messengers that influence cell growth (Fry et al., 1993). These messengers are believed to act in a negative-feedback loop to slow cell expansion.

The major hemicellulose of graminaceous plants, xylan, contains arabinosyl or 4-*O*-methyl-glucuronosyl residues attached to a  $\beta(1\rightarrow4)$ -linked D-xylose backbone (Darvill

et al., 1980; Gibeaut and Carpita, 1994). Like xyloglucan, xylan is capable of hydrogen bonding to cellulose or to other xylan chains when it is unbranched; however, increased branching decreases the ability of xylan to bind to other molecules due to steric hindrance. The side groups also make xylan water-soluble; therefore, highly branched xylans are common in dividing and growing cells, while more unbranched xylans are found after growth is completed (Carpita and Gibeaut, 1993).

Glycosyltransferases (GTs) are required to produce cellulose, xyloglucans, xylans, and other polysaccharides. GTs are located in the Golgi apparatus, where the xyloglucan and pectin are produced (Driouich et al., 1993) (Fig. 2b). The Golgi has been shown to be the site of synthesis and export of polysaccharides by autoradiography (Gibeaut and Carpita, 1994). The backbone sugars can be donated from either the cytosolic or luminal areas of the Golgi, but the branched sugars tend to come from the luminal side (Gibeaut and Carpita, 1994). The CAZy database (<http://afmb.cnrs-mrs.fr/CAZY>) lists 448 GT genes in *Arabidopsis*, but many more are likely to be present.

#### **4) Pectin**

Pectic polysaccharides, or pectin, forms a three-dimensional network intertwined with the cellulose and xyloglucan. Pectin is composed of three types of complex polysaccharides in dicots: homogalacturonans and rhamnogalacturonans I and II (RG-I and RG-II) (Darvill et al., 1980; Carpita and Gibeaut, 1993). Pectin molecules are generally assumed to form a gel-like matrix, in which the cellulose and hemicellulose are embedded; however, recent studies have shown that pectin may actually form a fibrillar

network (McCann et al., 1990) that determines wall porosity (Baron-Epel et al., 1988). Furthermore, suspension cultures of tomato cells grown in a medium containing dichlorobenzonitrile (a herbicide that inhibits cellulose synthesis) adapt by tightly cross-linking the pectin molecules forming a “pectin cell wall”. Therefore, pectin on its own can be used to make an effective cell wall (Shedletzky et al, 1990).

One component of pectin, the homogalacturonans, may account for up to 60 percent of the pectin matrix (<http://www.crc.uga.edu/>). Homogalacturonans are made up of polygalacturonic acid (PGA), which is a helical polymer of  $\alpha(1\rightarrow4)$ -linked D-galacturonosyl residues (Gibeaut and Carpita, 1994). PGA is methyl esterified when it is produced in the Golgi, but this is then partially de-esterified in the cell wall by the enzyme pectin methylesterase (Carpita and Gibeaut, 1993), leading to a high negative charge.  $\text{Ca}^{++}$  can then cross-link the negatively charged PGA molecules to each other, giving PGA the strength to hold the cellulose-hemicellulose network together (Bacic et al., 1988). Homogalacturonan is referred to as pectin if it has a high degree of methyl esterification, and it is known as pectic acid if it has a low degree of methyl esterification (<http://www.crc.uga.edu/>).

RG-I is composed of a backbone of  $\alpha(1\rightarrow4)$ -linked D-galacturonosyl residues along with  $\alpha(1\rightarrow2)$ -linked L-rhamnose. The L-rhamnose residues can be modified with side chains of arabinose, xylose, glucose, fucose, and galactose. RG-II is more complex than RG-I due to the presence of twelve different monosaccharides (Reiter, 1994). RG-II molecules are cross-linked by borate esters at the apiose residues (O'Neill et al., 1996). The borate cross-linking strengthens the cell wall as well as playing a part in the wall's porosity (Fleischer et al., 1999). In addition to giving the wall structural strength and

porosity, pectin also functions in adjusting the pH and ion balance of the cell wall with its charged surfaces.

## **B. *Arabidopsis thaliana***

*Arabidopsis thaliana* is a member of the Brassicaceae (mustard or crucifer) family and is an excellent model for plant biology because it is closely related to several hundred thousand flowering plant species (Somerville and Koornneef, 2002). *Arabidopsis* has a short life cycle of about eight weeks, is self-fertile, produces thousands of seeds, and is very easy to grow in limited space due to its small size (20-40 cm tall). In addition, *Arabidopsis* has many natural variants, produces fertile hybrids, and has only five chromosomes (Somerville and Koornneef, 2002). Many cell wall mutants have been found using multiple strategies including screening for mutants resistant to cell wall synthesis inhibitors, visible phenotypes, immunological approaches, and direct biochemical screening to gain knowledge about the synthesis and function of the plant cell wall and its polysaccharides. In direct biochemical screening, the amount of certain monosaccharides found in the cell wall was measured to search for changes in cell wall composition (Reiter, 1994). The discovery of mutants by screening mutagenized plants for altered monosaccharides is a major step to discover the functions of many putative glycosyltransferases (GT).

The numerous GTs are classified into families based upon their conserved sequence motifs (Li et al., 2004; Henrissat and Davies, 2000). The function of only a few of these GTs is currently known. The GT47 family in *Arabidopsis* was identified by PSI-

BLAST searches of the *Arabidopsis* genome using the known galactosyltransferase *MUR3* to find similar genes within the GT47 family. All of the GT47 members contain the conserved sequence motif pfam03016, which is a part of the  $\beta$ -D-glucuronosyltransferase domain of exostosins, animal enzymes involved in the synthesis of the polysaccharide heparan sulfate. A phylogenetic tree of the family was created using TreeView that was subdivided into smaller, more similar (homologous) groups (Li et al., 2004) (Fig. 5). *MUR3* and 10 closely related genes are in subgroup A of the GT47 family (Fig. 6). Amino acid sequence comparisons of the proteins coded by the genes in subgroup A show between 31% and 73% identity (Li et al., 2004). This similarity suggests that since *MUR3* is a  $\beta(1\rightarrow2)$ -D-galactosyltransferase, then the rest of the group and family members must be glycosyltransferases of some type. As described earlier, examining mutant plants that have the gene of interest knocked out helps in revealing what the function of the protein may be. Mutant plants found with a T-DNA insertion are advantageous compared to other mutagenic methods because the sequence of the insertion is known. This allows the mutated gene to be easily identified using PCR. Also, the insertion contains a marker, such as GFP or an antibiotic resistance gene, that allows for easy screening of mutant plants. By concentrating research on the genome of *Arabidopsis*, information about the genes and their processes in this plant can then be applied to economically important plants.

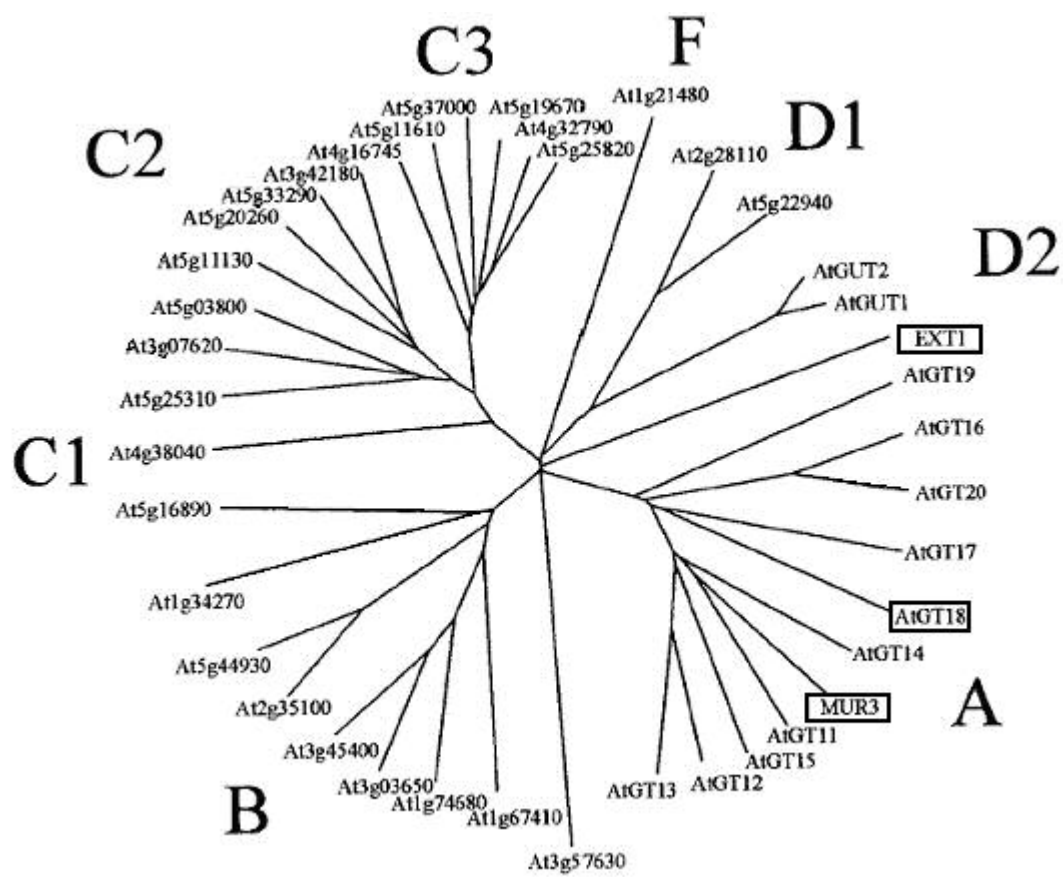
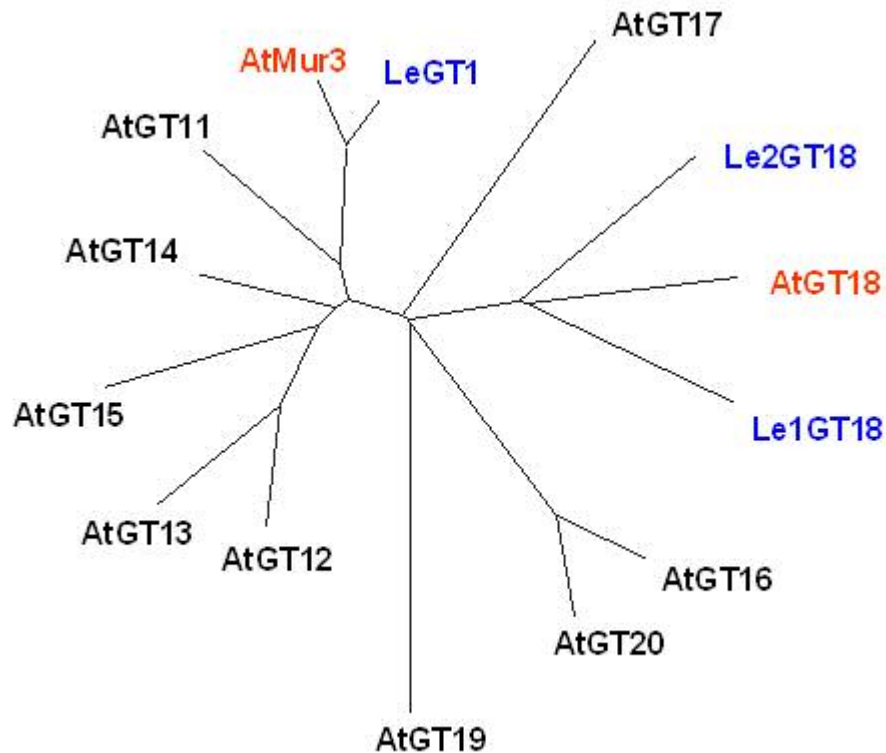


Figure 5: Phylogenetic tree of the GT47 family members of *Arabidopsis thaliana* (Li et al., 2004).



**Figure 6:** Phylogenetic relationship of Subgroup A in the GT47 family of *Arabidopsis* with selected genes from *Lycopersicon esculentum* (blue). The red genes are homologues of the tomato genes in *Arabidopsis thaliana*.

### 1) The *mur3* and *gt18* Mutants of *Arabidopsis*

The *mur3* mutation alters the xyloglucan structure by eliminating the  $\alpha$ -L-fucosyl- $\beta(1\rightarrow2)$ -D-galactosyl side chain that is attached to the third xylose in the wild type xyloglucan of *Arabidopsis* (Madson et al., 2003). The plant compensates for the missing side chain by increasing galactosylation of the second xylosyl residue (Madson et al., 2003). *MUR3* was cloned positionally and found to include a single transmembrane domain near its N-terminus. This is typical of Golgi-localized glycosyltransferases.

Since *MUR3* acts as a galactosyltransferase only at the third xylose position of the xyloglucan in *Arabidopsis*, another galactosyltransferase must attach the galactose at the second xylose position (Scheible and Pauly, 2004). *AtGT18* was a likely candidate to perform this function, so a T-DNA insertion line within this gene was identified at the University of Wisconsin knock out facility, and the mutant showed a 13.5% reduction of galactose in total cell wall material (Li et al., 2004). Further work showed that *AtGT18* acts on the second xylose position.

Searches of the dbEST database for homologues to *MUR3* in plants revealed close matches in important crop plants such as alfalfa, soybean, rice, and tomato. Other than being of interest because it is an important crop plant, the finding of tomato homologues to the *Arabidopsis* GTs is appealing since tomato and other solanaceous plants replace D-galactose with L-arabinose in its xyloglucan (Madson et al, 2003). Searches using *AtGT18* as well as *MUR3* uncovered multiple genes that have a high identity to those in *Lycopersicon esculentum*, the tomato plant. Arabinose is only missing the C-6 hydroxymethyl group in comparison to galactose, suggesting that the tomato homologues to *MUR3* and *AtGT18* may be arabinosyltransferases as opposed to galactosyltransferases.

## **2) Homologues to *MUR3* and *AtGT18* in *Lycopersicon esculentum***

Cloning tomato homologues of *MUR3* and *AtGT18* should allow us to find GTs acting at the same positions as the *Arabidopsis* GTs. The resulting sequences from the EST database (<http://www.ncbi.nlm.nih.gov/>) search did not encompass the entire

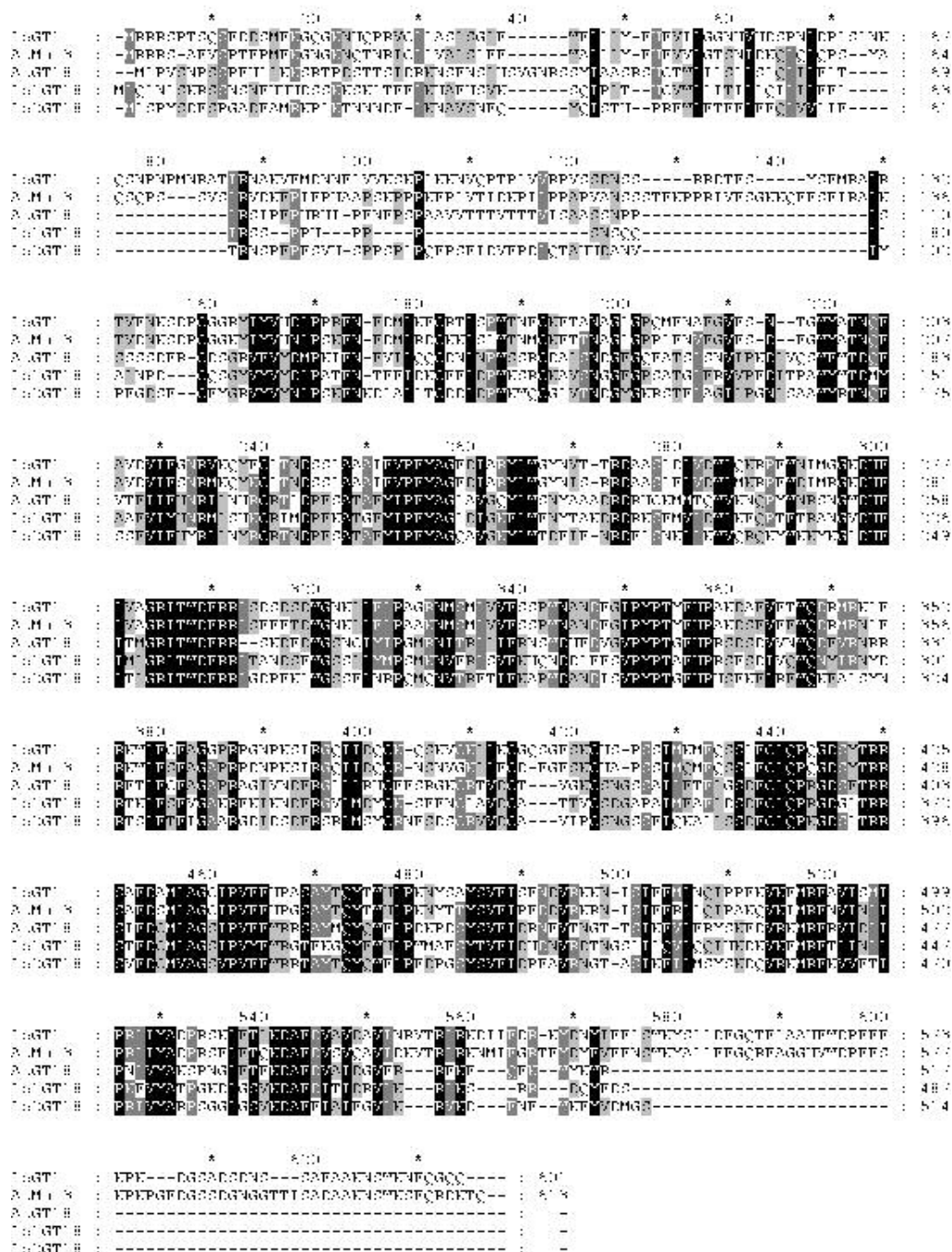


sequence of *MUR3* and *AtGT18*, respectively; therefore, an Inverse Polymerase Chain Reaction (I PCR) was used to find the complete tomato homologues (Fig. 8). In PCR, the template DNA is first denatured; i.e., the double helix is unwound by heating the reaction to a temperature of about 94°C. Next, the primers (Table 1) anneal, or bind, to the complementary bases on the DNA template by lowering the temperature. The third step is the synthesis of the new DNA strand by DNA polymerase at a temperature of 72°C. The reaction is then heated to denature the newly formed double helix.

For the I PCR, total tomato DNA was first digested using restriction enzymes and religated to form ringlets. I PCR was initiated by annealing two primers to the known sequence. The primers were placed at the ends of the gene and faced away from each other; that is, the primers faced each other over the unknown portion of the gene. This set up allows the unknown sequence to be amplified. A nested primer set was then used to clean up the I PCR by using primers even closer to the ends of the known segment. This process was repeated until the entire gene was cloned (when the start and stop codons were reached). Three putative GTs (*LeGT1*, *Le1GT18*, and *Le2GT18*) were cloned using this method. *LeGT1* was most similar to *MUR3* while *Le1GT18* and *Le2GT18* were most similar to *AtGT18*. The amino acid sequence alignment of these proteins in Figure 7 shows their high amount of identity. Adding the genes to the *Arabidopsis* phylogenetic tree also demonstrates how closely related the genes are (Fig. 6). In fact, the three tomato proteins also contain the single transmembrane domain typical of proteins in the Golgi apparatus. In order to test the hypothesis that *Le2GT18* encodes an arabinosyltransferase in *Lycopersicon esculentum*, the gene was transformed into the *Arabidopsis mur3-3* and *atgt18* backgrounds. This will allow us to test if

Le2GT18 can use these mutant xyloglucans as a substrate and to identify the sugar that Le2GT18 transfers.

An *in vitro* approach was also used in which *Le2GT18* was ligated into a protein expression vector (pET41a). The *Le2GT18*/pET41a construct was transformed into *E. coli* that expresses the protein upon induction. A SDS-PAGE protein gel was run to visualize the protein of interest. There was no difference between the control and induced lines, which points to no protein produced by the *E. coli* with a possibility of degradation of any protein produced. A Western blot can also be used to further visualize a protein, especially if it cannot be seen on the SDS-PAGE gel. The isolated protein can then be used to test its function by adding the protein to a preparation of *atg18* and *atmur3-3* xyloglucan along with an appropriate substrate sugar.



**Figure 7:** Amino acid multiple sequence alignment of *Arabidopsis* MUR3 and GT18 with putative glycosyltransferases from *Lycopersicon esculentum*. Dark areas show complete consensus for an amino acid. Gray denotes areas where most of the protein sequences agree (Corpet, 1988; <http://prodes.toulouse.inra.fr/multalin/multalin.html>).

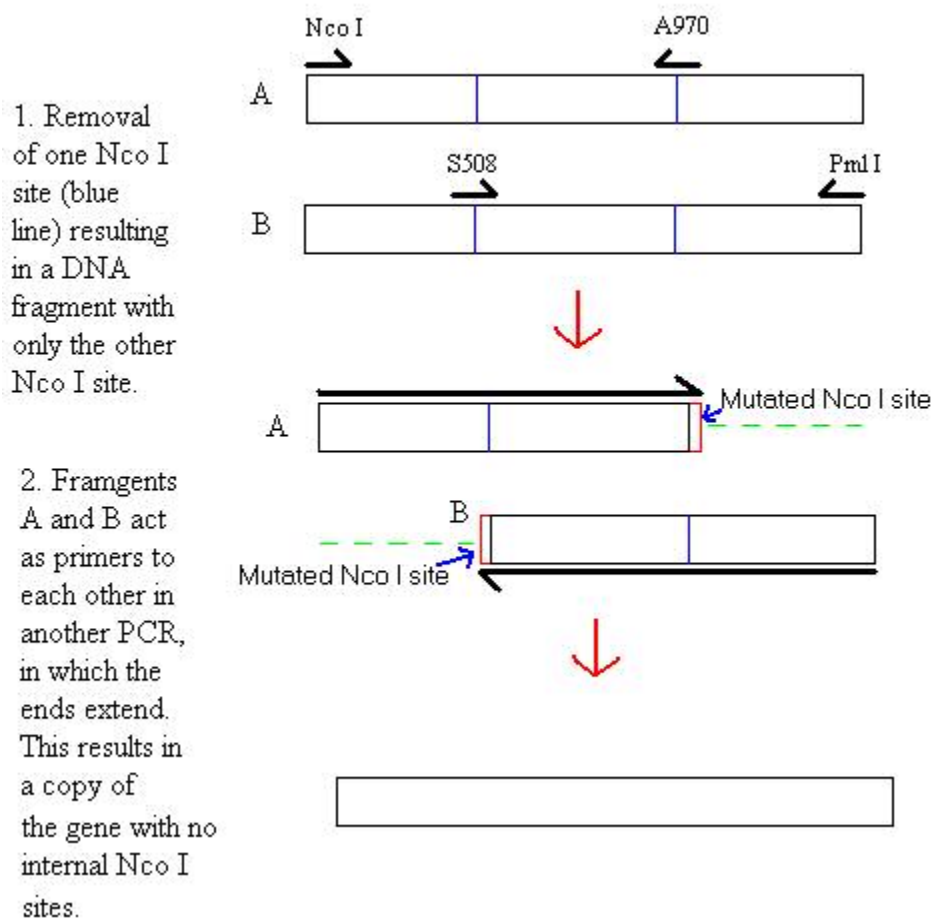
## II. Results and Discussion

Two different alleles of *Le2GT18* were cloned from tomato. One of the alleles was found in the Better Boy tomato variety, while the other was in Marglobe. Comparing the sequences of the two alleles allowed sequencing errors to be easily identified. We inserted the gene into a plant transformation vector so that we could overexpress *Le2GT18* in *Arabidopsis*. We decided to use the pCambia 1302 vector. This vector includes a version of *gfp* (green fluorescent protein) known as *mgfp5\**, which is brighter and red-shifted. At the 5' end of *mgfp5\** is the *CaMV 35S* promoter, which leads to strong transcription of the desired gene. The 3' end of *mgfp5\** includes the *Nos poly-A* terminator, which makes the mRNA message very stable. PCR was used to introduce Nco I and Pml I restriction enzyme sites at the 5' and 3' ends of *Le2GT18*, respectively. We removed *mgfp5\** by a restriction enzyme digest of pCAMBIA 1302 using the enzymes Nco I, which cuts the DNA strand at the 5' end of *mgfp5\**, and Pml I, which cuts at the 3' end. It should have been possible to ligate *Le2GT18* into the place formerly occupied by *mgfp5\**. However, it was a difficult ligation. One reason why the ligation may not have succeeded at first may be that the T4 DNA ligase was inactive or degraded. We tested the activity of a new tube of ligase against that of the old tube and found that the older enzyme was not functioning correctly. A second reason for the unsuccessful ligation may have been that we did not obtain enough DNA when extracting *Le2GT18* out of gels. This may have been because internal Nco I sites would cut most of the DNA,

causing us to lose extractable DNA. We attempted to isolate *Le2GT18* through a partial-digest procedure, but the DNA purified from the gel that was not cut by the internal Nco I sites was not enough. Nonetheless, this technique had been successfully used to clone *Le1GT (LeMur3)*.

To solve the *Le2GT18* /pCAMBIA 1302 ligation problem, we decided to remove the internal Nco I sites using PCR. Primers were made that spanned the Nco I site, but included an uncomplementary base pair to remove the Nco I site upon amplification. The first PCR reaction led to two pieces of DNA, each of which included the other Nco I site. Each piece of DNA was then placed into a second PCR reaction. The two original PCR products acted as primers to each other and amplified into a final piece of DNA that was *Le2GT18* without the Nco I sites. This allowed us to retrieve more insert from subsequent digests. We were then able to successfully ligate *Le2GT18* into the pCAMBIA 1302 vector using the new ligase, and then transformed it into *E. coli*. The sequence of the new vector construct was verified using CEQ sequencing to ensure that it contained a good copy of *Le2GT18*. The construct was next placed into *Agrobacterium tumefaciens*, which is used to effectively transfer genes to plants (Broothaerts et al., 2005). We transformed *mur3-3* and *gt18 Arabidopsis* plants with the *Agrobacterium*, and will soon begin screening the seed for transformants.

## Removal of Internal Nco I sites using PCR



**Figure 8:** Removal of internal Nco I sites from *Le2GT18* using PCR.

The control plants for this experiment are *Atgt18* mutants of *Arabidopsis* that have been transformed with *Agrobacterium* containing the intact pCAMBIA 1302 plasmid with *gfp*. Currently, we have three *Arabidopsis* lines homozygous for the pCAMBIA 1302 plasmid; however, Line 1 is sterile, possibly due to the T-DNA inserting into an important gene. Lines 2 and 3 have been successfully completed with the collection of seeds from the homozygous plants, which makes all of the current seed stock for these lines homozygous. The *mur3* control plants will be supplied by Xuemei Li, and they are

transformed with pCAMBIA 1301 instead of 1302, which contains the *Gus* reporter gene instead of *mgfp5\**.

### A. Protein Expression

We also wanted to try *in vitro* assays of the Le2GT18 protein. Since this protein is membrane anchored and thus difficult to express, we decided to truncate the protein, removing its transmembrane domain. The truncated protein was cloned into the pET41a expression vector. This vector has a *GST* domain in frame upstream of the cloning site to help with protein folding and purification.

The SDS-PAGE protein gels of Le2GT18 did not show high expression of the protein. If the protein was ever present, the Rosetta DE3 cells may have degraded it since the cells were producing a foreign protein. The fusion protein has a predicted molecular weight of about 92 kDa, but no clear band was present at this weight. We did not attempt a Western blot or protein purification. This is an option that we can pursue in the future, but we have decided to focus on the *in vivo* approach.

Since we were unable to clearly demonstrate that we were producing the Le2GT18 enzyme and are unsure of the substrate, we decided to focus on the *in vivo* approach. Xyloglucan from *Lycopersicon esculentum* would be the best choice for the *in vivo* experiment, but we need a tomato plant with the *LeGT18* gene inactivated. This can be done via RNA interference (RNAi), which is expected to decrease expression of the *Le2GT18* gene. Still, tomato plants have a longer generation time than *Arabidopsis*, require more room to grow and are more difficult to transform. For this reason,

*Arabidopsis* is used instead of tomato. Therefore, we decided to focus on ligating *Le2GT18* into pCAMBIA 1302 and transforming the mutant plants, which would be a much easier procedure. *Le2GT18* has been transformed into *atgt18* and *atmur3-3* background plants. T1 seed from the *atmur3-3* plants has been collected and plated on hygromycin (440 µg/mL)/vancomycin (500 µg/mL) plates. Transformants were isolated and transplanted onto soil.

## **B. Putative Function of Le2GT18**

It is impossible to say with any certainty whether *Le2GT18* encodes a galactosyltransferase or an arabinosyltransferase. Even if we knew, it remains to be determined exactly where the protein acts. Recent data from similar experiments with *LeGT1* suggests that LeGT1 acts as a galactosyltransferase when placed into the *atgt18* background. However, no significant changes were observed when the gene is transformed into *mur3-3 Arabidopsis* mutants (Bruce Link, pers. comm.). Therefore, LeGT1 seems to perform a similar role as AtGT18 by acting as a galactosyltransferase. In the solanaceous xyloglucan, a galactosyl residue may be placed on the first xylose of the repeating subunit. LeGT1 probably acts at this position since it appears to be a xylose-dependent galactosyltransferase. If LeGT1 truly has this function, then it is more likely that *Le2GT18* will act as an arabinosyltransferase in the synthesis of tomato xyloglucan. In tomato, it is possible to have a xyloglucan structure where two arabinosyl residues are attached to each other above the second xylose. This may explain the need for two closely related genes such as *Le1GT18* and *Le2GT18*, where one gene would be a



xylose dependent arabinosyltransferase while the other would be an arabinose dependent arabinosyltransferase. Alternatively, both genes could have the same function.

### III. Future Directions

Screening for successfully transformed mutants will begin very soon, and will follow the same procedure as screening for pCAMBIA control plants. Basically, T1 seeds will be plated on hygromycin (440  $\mu\text{g/mL}$ )/vancomycin (500  $\mu\text{g/mL}$ ) plates. Any seedlings that grow are expected to be transformed because of their resistance to hygromycin. After successfully harvesting T2 seeds from hemizygous lines, the T2 plants will be grown in the dark for four days. These plants will be sent to Markus Pauly at the Max Planck Institute in Germany, where the structure of their xyloglucan will be determined by MALDI-TOF mass spectrometry of enzymatically released fragments. Homozygous plants will also be examined for changes in phenotype as a result of the transformation. Another procedure we can follow after finding homozygous lines is to grow a flat of homozygous plants, grind up the plant material, and fractionate the cell wall material. The pectin will be removed first, followed by the xyloglucan, and cellulose. The xyloglucan will then be analyzed by GC to determine its sugar composition. This method will not be as precise as sending the plants to Markus Pauly because arabinose from the pectin will contaminate the sugar composition of the xyloglucan fraction, making the results difficult to interpret. However, we can get some early results in our own lab with GC so that we will have an idea of the protein's function. We may also use RNAi to silence *Le2GT18* expression in *L. esculentum*. We

will then be able to see how the xyloglucan structure of the tomato plant changes. Although it was pointed out in the previous section that using tomato would be difficult, once a possible function of the gene in *Arabidopsis* is found, the function would then be easier to detect in *L. esculentum* since we will know what to look for.

## A. Conclusion

Determining the function of glycosyltransferases will advance our current understanding of the biosynthesis of the plant cell wall. Although the role of only a few GTs has been demonstrated so far, there are many possible GTs that are currently under investigation. The isolation of mutants has led to the discovery of many GTs, and helps researchers find homologues in other plants based on sequence similarities. Future work on GTs will require researchers to develop assays that include the proper substrates, and that show little loss in activity when the protein is extracted or expressed (Scheible and Pauly, 2004). Nonetheless, the continued and growing use of genetic procedures will greatly help in understanding the function of individual cell wall proteins in the composition of the complicated cell wall.

The importance of the cell wall to everyday life makes the understanding of its synthesis even more important. Many benefits will arise from engineering of the cell wall, especially in the economic sector. Cell wall material is used in the manufacturing of a multitude of products. The production of paper, lumber, textiles, agricultural items, alcohol, and renewable energy will greatly benefit from changes in the plant cell wall that improve cell wall utilization.

## IV. Materials and Methods

### A. Plant Material

*Arabidopsis* plants were grown in an environmental growth chamber set to 25°C during a 16-hour light period under fluorescent light ( $150 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) and an 8-hour dark period at 18 °C. The entire day-night cycle occurred at 70% humidity. The plants were either grown on sterile media plates or on potted soil (ProMix BX, Canada). To plant many seeds on soil, the seeds were suspended in 0.1% (w/v) Bacto agar (Difco Laboratories, Detroit, MI) and dripped onto soil. When plating seeds, they were first sterilized in sterile 15 mL disposable tubes by soaking in 30% commercial bleach containing 50  $\mu\text{L}$  Tween 20. The tubes were placed on a rocking table for 15 minutes. The seeds were then rinsed five times with sterile distilled water. The seeds were then placed onto appropriate selective media plates and spaced evenly.

### B. DNA Extraction

Genomic DNA was extracted from *L. esculentum* leaf material using the DNeasy Plant Mini kit (Qiagen, Valencia, CA). Plasmid DNA from *E. coli* cells was extracted from cell pellets of overnight liquid cultures using the Plasmid Mini kit (Qiagen). DNA extractions from gels were done using the Gel Extraction kit (Qiagen).

### C. DNA Sequencing

DNA was sequenced to make sure that there were no errors present or to find the nucleotide sequence when first isolating *Le2GT18*. Sequencing was completed using either Big Dye (ABI prism 377, Applied Biosystems manual) or CEQ (Beckman Coulter manual) instruments at the UConn DNA Biotechnology Facility. The DNA first goes through a cycle sequencing to prepare the samples for the sequencing instruments at the Biotech Facility. For CEQ, 100 ng per kb of DNA was placed into a mixture with 2  $\mu$ L of the appropriate primer at 1.6  $\mu$ M, and 8  $\mu$ L CEQ. This final volume was 20  $\mu$ L. Big Dye requires much more DNA at 500 ng per kb of DNA. This DNA was added to a reaction including 8  $\mu$ L Big Dye reaction mix and 20 pmol primer.

### D. Polymerase Chain Reaction (PCR)

PCR involves the *in vitro* amplification of DNA through simultaneous primer extensions of complementary DNA strands (McPherson et al., 1991). The materials needed for a PCR reaction include the DNA to be amplified, primers, deoxynucleotides (dNTPs), DNA polymerase, and buffer. PCR was used in cloning *Le2GT18*, in verifying ligations and transformations, and in amplifying DNA for various reasons (including removal of the internal NcoI restriction enzyme sites). Three types of DNA polymerase were used for different PCR reactions. Either *Taq* (Eppendorf), *ExTaq* (TaKaRa), or *PfuTurbo* (Stratagene) polymerase were placed into the reaction depending on the type of PCR being done. *ExTaq* is capable of quickly and greatly amplifying the original DNA

template as well as doing some proofreading of its product. *PfuTurbo* has a very high fidelity with its proofreading capabilities, but does not amplify the gene as well. *Taq* is used when verifying that the gene is present in a step of the project (i.e., checking for the presence of the insert *Le2GT18* after ligation into pCAMBIA 1302) since this does not require high fidelity and *Taq* does not cost as much as the other polymerases. After a primer that was made to specifically match a certain sequence of DNA has annealed to the template gene, the DNA polymerase extends the primer using the free precursor dNTPs placed into the mixture. The primers used in this experiment are listed in Table 1.

After the mixtures have been prepared, they are placed into either Perkin Elmer GeneAmp 9600 or GeneAmp 2700 thermocyclers. These instruments use preprogrammed temperature profiles (Fig. 9) that are run in predetermined cycles in order to properly amplify the DNA. The two most used temperature profiles (programmed by Bruce Link) were *gtom* and *gtom-second* (Fig. 9). For the removal of the internal *NcoI* sites, the annealing temperature was decreased to 40°C to ensure that annealing would occur because one of the base pairs in the primers S508 and A970 (Table 1) was made so that it would not be complementary to its position on the internal *Nco I* site. The mutation produced by the primers was chosen to ensure that the same amino acid would be produced in the protein due to the degeneracy of the genetic code.

When attempting to first find the sequence of *Le2GT18*, inverse PCR (I PCR) was used. An I PCR protocol for tomato genomic DNA was used in preparing this reaction as described earlier in the Introduction (Fig. 8). The basic idea behind I PCR is that primers facing outwards at the beginning and end of the known sequence of a gene will prime towards each other on a circular piece of DNA over the unknown sequence. Bruce Link

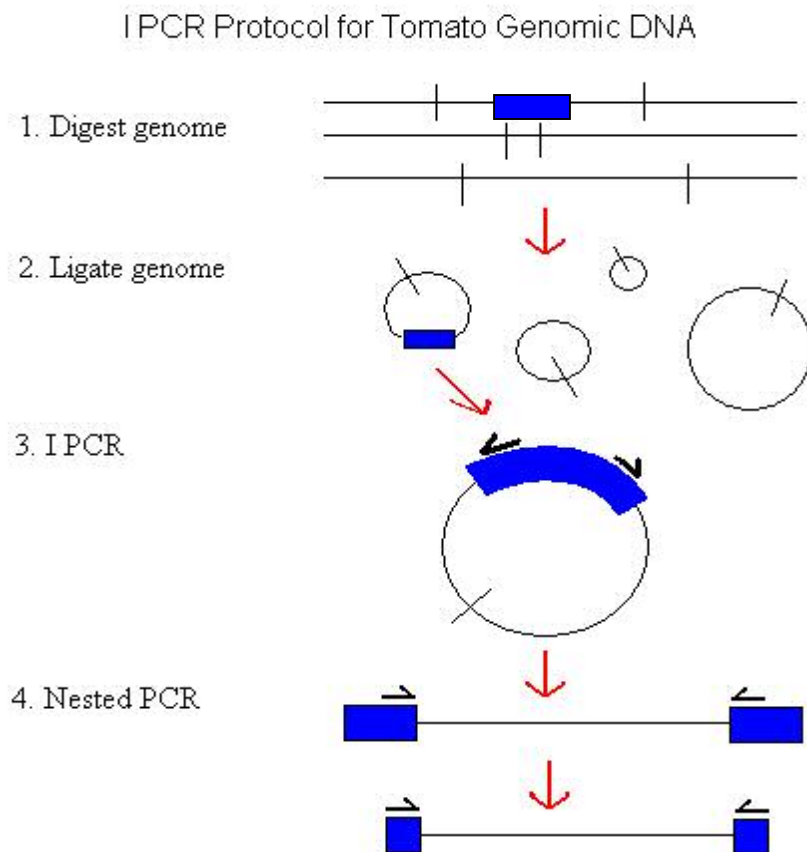
completed the steps in finding the sequence of *Le2GT18*, beginning with I PCR (Link et al., 2001).

Genotype	Primer Name	Direction	Tm	Sequence	
<i>AtGT18</i>					
<i>At5g62220</i>	JL270	anti	57.1	TTTCTCCATATTGACCATCATACTCATTG	
	K8S2	anti	56.6	CCAAAATCAAATTCTACTTCTCCTACCTA	
	K8R1	sense	58.4	ATAAACTATGCTTCCTGTCTCAAATCCTT	
	K8R2	sense	57.2	GACGGATTCTAATCTCATTTTCAGCTAAT	
<i>Le2GT18</i>	Le2GT18SEQ_S1	sense	59.8	GATTCTGAATGCGAGTATGGTC	
	Le2GT18SEQ_S2	sense	60.8	TTCCGGCGATTAGGTGAC	
	Le2GT18SEQ_S3	sense	61	CAAAGGGGACAGCTTGACTC	
	Le2GT18SEQA1	anti	59.4	AGAATTACCCGTCTGGCTTC	
	Le2GT18SEQA2	anti	60.3	TTGTCTCTACAACCTTTTCCCTCA	
	Le2GT18SEQA3	anti	60.5	TCATTAACCTCGATCTGAAATCAC	
	Le2GT18SEQA4	anti	60.4	GTGTGAGGAAATGGTCCAAAC	
	Le2GT18SEQA5	anti	58.9	CATATCCATCATTGGTCACAAG	
	Le2GT18SEQA6	anti	60.1	GCCAGAAACGAGGATGAGTAG	
	S508	sense	62/69.5	CCCTGGAAGTGGCAGTGTGGTC	
	A970	anti	66.4/67	AGGGAGCCTTCTCGATTGTGAATC	
	5'CAMBIA Nco I	sense	59/70	CCATGGTATCACCTTACTCCGACGAGTC	
	3'CAMBIA Pml I	anti	59/70	CACGTGACTGCCCATATCCACGTATTC	
	3'CAMBIA Pml I	anti	60/74	CACGTGTCAACTGCCCATATCCACGTATTC	
	Le2GT18PET_S1	sense	62/78	GGATCCGAATTCCGCAATTCCTTTCCC	
	Le2GT18PET_A1	anti	60/80	GTGGTGCTCGAGTCCACTGCCCATATCCACGTATTC	
	Le2GT18PET_AT	anti	62/79	GTGGTGCTCGAGTTCAACTGCCCATATCCACG	
	<b>Other primers</b>	M13F (-20)	sense	54.5	GTAAAACGACGGCCAG
		M13R	anti	47.5	CAGGAAACAGCTATGAC

**Table 1:** List of primers used in Polymerase Chain Reactions. Primers M13F (sense) and M13R (antisense) were used for the sequencing of inserts in the TOPO vector. Primer sequences created by Bruce Link.

### **E. PCR Genotyping of Plants**

In order to isolate DNA from plant material for use in PCR genotyping of plants, a small leaf or a cotyledon was placed into a 0.5 mL tube that already contained 40  $\mu$ L of 0.25M NaOH. The tube was boiled for 30 seconds, and immediately placed on ice. 40  $\mu$ L 0.25M HCl was then added to the NaOH, followed by 20  $\mu$ L of a 0.5M Tris/HCl (pH 8.0) and 0.25% Tween 20 mix. This solution was then vortexed and boiled for 2 more minutes. The final step was to centrifuge the tube. In order to get DNA into the PCR reaction, 2  $\mu$ L of the solution was taken up along with a small piece of plant material, and these were placed directly into a PCR tube.



**Figure 9:** Simplified visualization of I PCR protocol used in isolating *Le2GT18*. The nested PCR is used to “clean up” and increase the sensitivity of the I PCR results. The blue box represents the known sequence of *Le2GT18*. Black arrows represent primers to *Le2GT18*.



gtom	96 2:00	4x 94 :30 55 :30 72 3:00			31x 92 :30 55 :30 72 3:00			2 Hld 72 7:00 4 ∞			
gtom-prime	96 2:00	2 Tmp x2 94 :30 72 4:00		2x 94 :30 60 :30 72 4:00		2x 94 :30 58 :30 72 4:00		16x 94 :30 58 :30 72 4:00		2 Hld 72 7:00 4 ∞	
gtom-second	96 2:00	2 Tmp x2 94 :30 72 4:00		2x 94 :30 60 :30 72 4:00		2x 94 :30 58 :30 72 4:00		33x 94 :30 58 :30 72 4:00		2 Hld 72 7:00 4 ∞	
removal of internal Nco I sites	94 2:00	4x 40 :30 72 :30 94 3:00			7x 72 3:00 94 :30		1 Hld 4 ∞		<div style="border: 1px solid black; padding: 5px; width: fit-content; margin: auto;"> Temp. (degrees Celsius)  Time (min:sec) </div>		

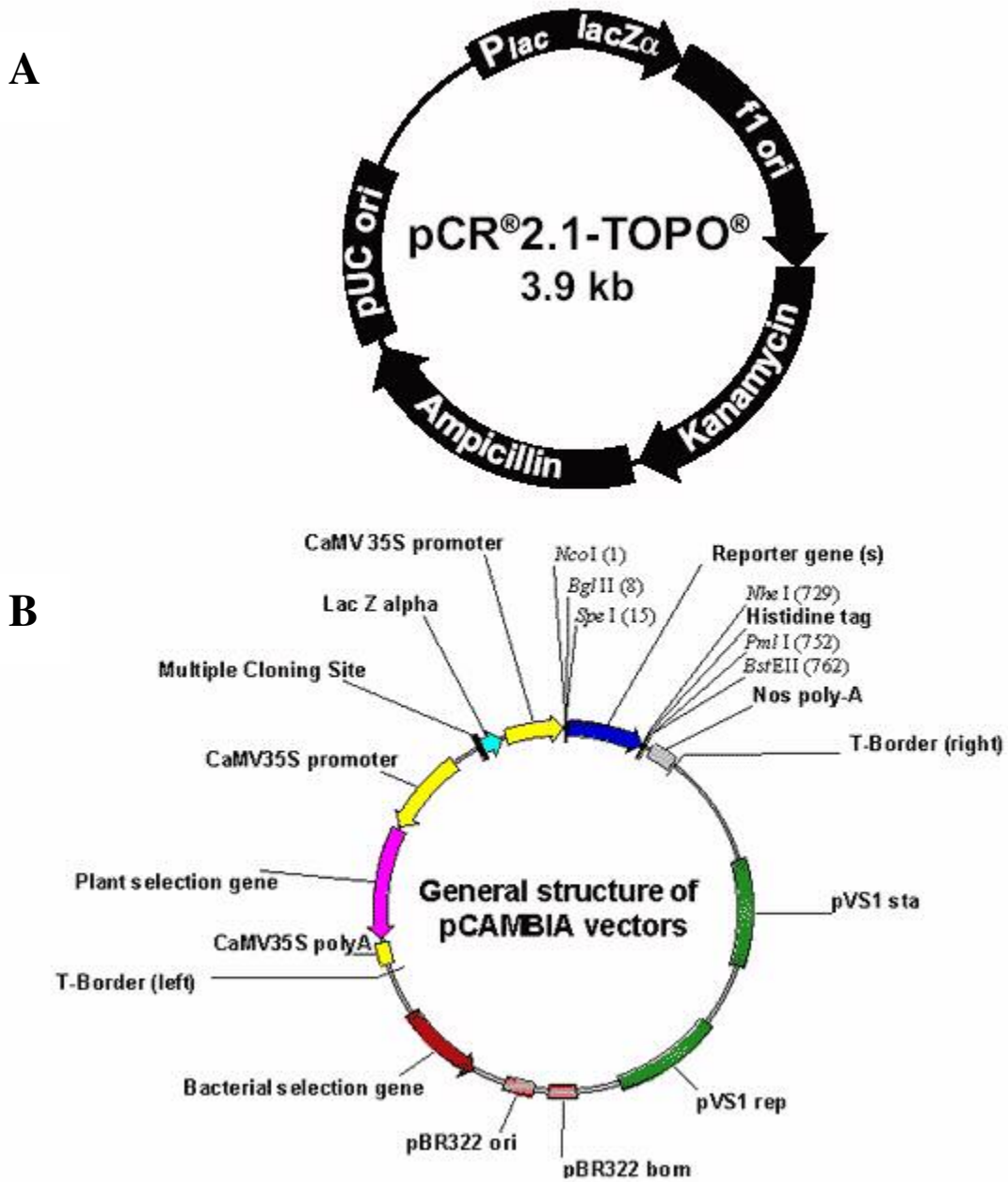
**Figure 10:** PCR temperature profiles used in this experiment. Bruce Link programmed all of the profiles. Gtom and gtom-prime were used for I PCR. Gtom-second was used for most PCR's, including the Nested portion of I PCR.

## F. Vectors and Transformation

Once *Le2GT18* was cloned and sequenced, it was ligated into the pCR2.1-TOPO cloning vector using the TOPO TA cloning kit (Invitrogen). The ligations were then transformed into *E. coli* (TOP10F' genotype) and spread onto plates containing kanamycin (50 µg/mL). The TOPO vector (Fig. 10) contains a resistance marker to kanamycin, so only *E. coli* that have picked up the vector will survive. The plasmid was digested with Nco I and Pml I to confirm the presence of an insert. Clones were sequenced using M13F, M13R, SEQ\_S1, SEQ\_S2, SEQ\_S3, and SEQA1 through SEQA6 primers to verify that the gene did not have any mutations from PCR amplification. The gene was then subcloned into the plant transformation vector

pCAMBIA 1302 and transformed into TOP10F' cells. Once positive colonies were identified, the *Le2GT18* gene was resequenced.

The pCAMBIA 1302/*Le2GT18* construct was then transformed into *Agrobacterium tumefaciens* via electroporation. The *Agrobacteria* were grown on kanamycin (50 µg/mL) plates to select for colonies that included the pCAMBIA construct. Individual colonies were isolated and five colonies were grown overnight in 5 mL LB with kanamycin (50 µg/mL) and rifampicin (100 µg/mL). The next day, all five cultures were added to a liter of LB/kanamycin (50 µg/mL)/rifampicin (100 µg/mL) and grown for another 24 hours. The culture was centrifuged at 8000 g for 5 minutes to collect a pellet of *Agrobacteria*. The pellet was resuspended in a 50% MS and 5% sucrose solution to an OD<sub>600</sub> of 0.8. *Atgt18* and *mur3-3* plants were dipped into the solution for one minute and then drip-dried (Clough and Bent, 1998). Seed were collected after two to three weeks. The seed were sterilized as described in the “Plant Materials” section and plated onto hygromycin (440 µg/mL) /vancomycin (500 µg/mL). Surviving T1 transformants were transplanted to soil.



**Figure 11:** Vectors used in cloning *Le2GT18*. **A)** pCR2.1-TOPO used for cloning *Le2GT18* and verifying its sequence (Invitrogen). **B)** pCambia, Binary plant transformation vector used for plant transformations (Cambia).

## G. Transformed Mutant and Control Plants

The genotype of *atgt18* background plants was verified by PCR using *WT* primers and *gt18* T-DNA primers in two separate reactions (*gt18* primer set: JL270 and K8S2; *WT GT18* primer set: K8R1 and K8R2). True mutants would give a negative result with *WT* primers and a positive result with *gt18* primers. *Mur3-3* mutant plants were used from lab stocks and are easily identified by their short inflorescence phenotype. *Atgt18* plants (T0) were transformed with *Agrobacteria* containing empty pCAMBIA 1302 vector (*gfp* instead of *Le2GT18*). Seeds were collected from the T0 plants. These seeds (T1) were placed onto plates containing hygromycin (440 µg/mL) and vancomycin (500 µg/mL). All surviving plants were transformants and were transplanted into soil and grown for seeds. Seed (T2) were collected for each individual transformant. T2 seed were plated on the same antibiotics. Individuals were transplanted and grown for seed as before. Seeds (T3) from any individual T2 plant that all grew on the plates were homozygous for the mutation, and were transplanted into soil once again to be grown for seeds (T4). These T4 seeds formed stocks for the pCAMBIA 1302 control plants.

## H. Protein Expression

The ends of the *Le2GT18* coding region were modified by PCR to introduce EcoR I and Xho I sites for cloning into pET41a. The TM domain of *Le2GT18* was removed by designating the 5' primer (Le2GT18PET\_S1) with an EcoR I restriction

enzyme site after the TM domain. A 3' Xho I primer (Le2GT18PET\_A1, which we used due to higher amplification, or Le2GT18PET\_AT) was used at the end of the coding sequence. These primers amplified only the luminal portion of the Le2GT18 protein and allowed it to be cloned into the EcoR I/Xho I sites of the pET41A vector. Individual transformants (colonies) were tested for protein expression by growing them overnight in 2 mL of LB with chloramphenicol (34  $\mu\text{g/mL}$ ) and kanamycin (50  $\mu\text{g/mL}$ ). The following morning, 60  $\mu\text{L}$  of liquid culture was added to 2 mL of LB with the same antibiotics, and grown at 37°C for 2 hours, at which point a zero time point was taken (30  $\mu\text{L}$  of culture collected). Adding IPTG at .05, .1, and .15 mM induced the protein expression by derepressing the *lac* operon in pET41A. Time points of 30  $\mu\text{L}$  of culture were taken each hour for three hours after induction. Cells were collected by centrifuging the 30  $\mu\text{L}$  cultures at 12000  $g$  for 2 minutes. The cell pellets were boiled in 20  $\mu\text{L}$  of 6x SDS Loading Dye and run out on 12% SDS-PAGE gels. Gels were stained with Coomassie Blue to visualize the protein and check for expression.

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