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A PLANT CELL WALL BIOSYNTHETIC ENZYME

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XYLOGLUCAN FUCOSYLTRANSFERASE, A PLANT CELL WALL BIOSYNTHETIC ENZYME

by

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ABSTRACT

XYLOGLUCAN FUCOSYLTRANSFERASE, A PLANT CELL WALL BIOSYNTHETIC ENZYME

by

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Cell walls are major determinants of plant growth, morphology, development, and interactions with the environment. Components of the plant cell wall include polysaccharides such as cellulose, pectins, and hemicelluloses as well as structural proteins. Although much is known about the architecture of the plant cell wall, far less is known about the biosynthesis of its structural components. The focus of this project is the biosynthesis of a hemicellulosic polysaccharide component of the plant primary cell wall, xyloglucan. Xyloglucan is comprised of a beta-(1,4)-glucan backbone decorated with side chains consisting of xylose, xylose and galactose, or xylose, galactose and fucose. An enzyme that adds the terminal fucose to galactose on xyloglucan in an alpha-(1,2)-linkage, xyloglucan fucosyltransferase (XyG FUTase), was purified from rapidly elongating segments of etiolated pea epicotyls. Amino acid sequence data from this purified enzyme allowed the identification of an expressed sequence tagged cDNA (EST) encoding an *Arabidopsis* XyG FUTase. A full-length cDNA was obtained for this *Arabidopsis* gene and named *Arabidopsis Fucosyltransferase 1* (*AtFUT1*). The biochemical function of *AtFUT1* was confirmed by 1) using antibodies against the protein encoded by this cDNA to immunoprecipitate XyG FUTase activity from *Arabidopsis* microsomal proteins, and 2) expressing *AtFUT1* in mammalian Cos-7 cells, thereby conferring XyG FUTase activity.

The regulation of XyG fucosylation was studied in *Arabidopsis* as a model cell wall biosynthetic process. The expression of *AtFUT1*, the distribution of XyG FUTase activity, and the fucosylation state of XyG was studied in seven *Arabidopsis* tissues. Gene expression and enzyme activity levels were highest in the uppermost regions of the expanding *Arabidopsis* inflorescence stem and lowest in fully expanded tissue such as mature rosette leaves. *AtFUT1::GUS* transgenic plants were used to determine the spatial regulation of *AtFUT1* expression. Results generally paralleled gene expression data obtained by other techniques. A gradient of reporter gene expression was observed in the elongating inflorescence stem.

35S::AtFUT1 transgenic plants were generated to study the effect of strong constitutive *AtFUT1* expression on XyG structure. These plants show higher expression of *AtFUT1* and XyG FUTase activity compared to wild-type controls. Altered XyG structure is also observed in the transgenic plants in comparison to wild-type controls; however, these alterations consist not of highly elevated levels of fucose content, but of approximately two-fold higher acetylation of galactose.

AtFUT1 is one of a small number of genes encoding a cell wall biosynthetic enzyme with confirmed biochemical function. The sequence information from this gene and enzyme, as well as gene expression data, should assist in the identification and evaluation of other plant cell wall biosynthetic genes.

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I have a confession to make. I always read acknowledgements – in books, in dissertations, in presentations. They allow insight into the author's experiences and emphasize how progress towards any goal is a collective effort. So the first person I'd like to thank is you, the reader, for your audience. The documentation of work such as this would be neither necessary nor meaningful if there were no parties interested in its conclusions.

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INTRODUCTION

Were it not for the plant cell wall, the mechanisms of plant growth and development would be fundamentally different. The plant cell wall acts as a cellular exoskeleton, providing support and positional context to the cytoplasm of each cell. Herbaceous plants adopt an upright form because of the turgor pressure exerted against the rigid cell wall. Turgor pressure is not possible in cells that lack a wall. Ultimately, cell walls are responsible for the morphology characteristic of each organ so familiar in nature. The extensibility of the cell wall delimits cellular elongation, and continued elongation or expansion requires synthesis and deposition of new cell wall material. Thus, growth and development is determined by properties of the cell wall in plants. The mechanism of growth is much different in animals, which lack a cell wall. In short, cell walls provide structure to the cell, leading to formation of forces that sculpt plant form.

In addition to being required for normal growth and development, the cell wall is absolutely required for cell division in plants. Plant cells divide by forming a new cell plate at the phragmoplast, a structure that forms at the point of division, where callose and nascent cell wall materials are deposited to partition the cytoplasm into two daughter cells. This mechanism is distinct from animal cells, which pinch in two to form new cells. However, the plant cell cannot proliferate by any other means. Naked cells or "protoplasts" that lack a cell wall cannot divide.

The wall is also the means by which plants interact with their environment. Pathogens that attack plants must either penetrate the cell wall or grow within the apoplast. Perhaps

arising as a response to the cell wall lesions that can accompany pathogen attack, fragments of cell wall material can serve as signaling compounds or "elicitors" of plant defense responses. Plants perceive abiotic signals through the cell wall, resulting in differential cell wall extensibility (as in gravotropism or phototropism) or cell wall reinforcement (as in responses to touch or wind stress.) Many signals and hormones involved in the perception of and response to environmental conditions must negotiate the apoplastic region of the plant before binding to specific receptors, some of which may have apoplastic domains.

In the first chapter of this thesis, I will describe the structure and function of the plant cell wall in more detail. I will consider polysaccharide biosynthesis, including the enzymes involved in this process, and then will focus on the process of cell wall polysaccharide biosynthesis specifically. I will discuss the regulation of cell wall biosynthesis as it relates to plant growth. The cellular localization of cell wall biosynthetic processes will be considered, and known genes and enzymes involved in these processes will be described. I will also briefly summarize known mechanisms for remodeling of the cell wall during the life of the plant. Throughout this discussion, I will focus in detail upon one polysaccharide component of the primary cell wall, xyloglucan. The second chapter will describe the purification and biochemical characterization of a plant cell wall biosynthetic enzyme, xyloglucan fucosyltransferase, from garden pea. The third chapter will describe the identification of the gene encoding xyloglucan fucosyltransferase in *Arabidopsis thaliana* and the confirmation of its biochemical function. The fourth chapter will describe studies in which the regulation of xyloglucan fucosylation was

assessed in *Arabidopsis* as a model cell wall biosynthetic process. Finally, the fifth chapter will present conclusions and remarks on possible future research directions.

CHAPTER ONE

The Plant Cell Wall: Composition, Synthesis and Biological Roles

1.1 Plant cell wall structure

The plant cell wall can be divided into the primary cell wall, which is associated with growing cells, and the secondary cell wall that forms later in some cell types and is associated with cessation of growth, structural reinforcement or specialized differentiation (as in vascular cells). These two wall types differ in composition, and only the primary cell wall will be considered in detail in this chapter. In general the plant cell wall consists primarily of polysaccharides, though other components include proteins and phenolic compounds. The structure of the cell wall is complex, but ordered. Some polymers in the plant cell wall are among the most intricate found in nature and, for that reason, challenging to analyze structurally. Because of this complexity, it has taken decades of study of cell wall material for a comprehensive picture of wall architecture to emerge. This section will begin with a survey of cell wall polymers, and will then consider models of primary cell wall architecture and techniques used to study cell walls.

1.1.1 Primary Cell Wall Structural Components

Polysaccharides

Cellulose

Cellulose is the most characteristic feature of the plant cell wall and is the most abundant biopolymer on Earth. This polymer is comprised of β-(1,4)-glucose units linked together to form chains that are up to at least 15,000 units long (Brett and Waldron, 1996).

Although the structure of cellulose appears simple at first glance, these polymers are highly ordered into a metastable crystalline form called cellulose I through hydrogen bonding. Coalesced molecules of cellulose form into microfibrils, long structures with a width of about 10 nm in higher plants (though they may be as thin as 2 nm in young cells) (Brett and Waldron, 1996). Solid-state NMR studies showed that native cellulose is composed of two allomorphs, called Iα and Iβ, with the Iβ form being most prominent in higher plants (Atalla and VanderHart, 1984; Delmer, 1999). Subsequent studies determined that individual glucan chains in cellulose are arranged parallel to each other, with all reducing ends at the same end of the microfibril (Delmer, 1999). Additionally, the Iα alloform was shown to have a triclinic unit cell while the Iβ form has a monoclinic unit cell (Delmer, 1999).

Unlike other cell wall polysaccharides, cellulose is synthesized at the plasma membrane by a catalytic complex of proteins visualized in electron microscopy as rosettes (Kimura et al., 1999). In the past five years there has been a great deal of research on cellulose biosynthesis, and a family of enzymes have been identified that are thought to act as the catalytic subunit of the cellulose synthase complex (Arioli et al., 1998; Holland et al., 2000; Pear et al., 1996). More information about cellulose synthesis will be presented later in this chapter.

Hemicelluloses: Xyloglucan

Hemicellulose is a generic term for polymers that interact closely with cellulose microfibrils to form a cellulose-hemicellulose network. Traditionally, these polymers

have been defined as those cell wall polymers that can be extracted with molar concentrations of alkali, no matter what their structures are (McCann and Carpita, 2000). Since these polysaccharides aren't really "half" ("hemi") of a cellulose molecule, a more appropriate term might be "pericellulose", although for historical reasons the term hemicellulose continues to be used. Xyloglucan (XyG) is the major hemicellulose found in dicotyledonous plants and all monocotyledonous plants with the exception of Commelinoids, which include most notably the grasses but also bromeliads, palms, gingers, and cypresses (Hayashi, 1989). These latter species have a type II cell wall (described below) and utilize glucuronoarabinoxylan (GAX) as the major cross-linking hemicellulose. Thus, in the majority of higher plants, xyloglucan is the most abundant polymer that serves to cross-link cellulose microfibrils.

XyG has a backbone structure that is identical to cellulose. Unlike cellulose, however, glucose units are substituted with side chains (Hayashi, 1989). The substitution patterns vary, and the most common side chains consist of Xyl alone, Gal-Xyl disaccharides, or Fuc-Gal-Xyl trisaccharides (see Figure 1). A shorthand nomenclature is used to describe XyG side chain substitutions, shown in Table 1. In most dicots and noncommelinoid monocots, the structural pattern tends to consist of three substituted Glc residues followed by a fourth unsubstituted Glc (McCann and Carpita, 2000). The predominant subunits tend to be XXXG and XXFG, but XLFG is also observed (Guillen et al., 1995). In some species, rare instances of XFFG or XFLG have been observed, but fucosylation at the second position of the subunit is not common (Hisamatsu et al., 1991). Additional modifications may include acetylation of galactose residues and addition of α-L-Ara

(Brett and Waldron, 1996; McCann and Carpita, 2000). Solonaceous species (tomato, tobacco, peppers) and peppermint have arabino-xyloglucan, where fucose is absent but Ara may be added to Xyl. Major subunits in these cases are AXGG, XAGG, or AAGG (York et al., 1996). A very small proportion of the commelinoid monocot primary cell wall may contain XyG, but little substitution has been observed other than irregular Xyl residues (McCann and Carpita, 2000). Some species, notably tamarind (*Tamarindus indica*) and nasturtium (*Tropaeolum majus*), accumulate non-fucosylated XyG in the periplasmic space between the plasma membrane and cell wall of endosperm cells (York et al., 1990). This polysaccharide is broken down during seed germination and is thought to serve as a seed storage carbohydrate. Thus, it may be used as an acceptor substrate for XyG fucosylation assays. Non-fucosylated seed storage XyG is distinct from the endogenous cell wall XyG found in tamarind and nasturtium, which does contain fucose.

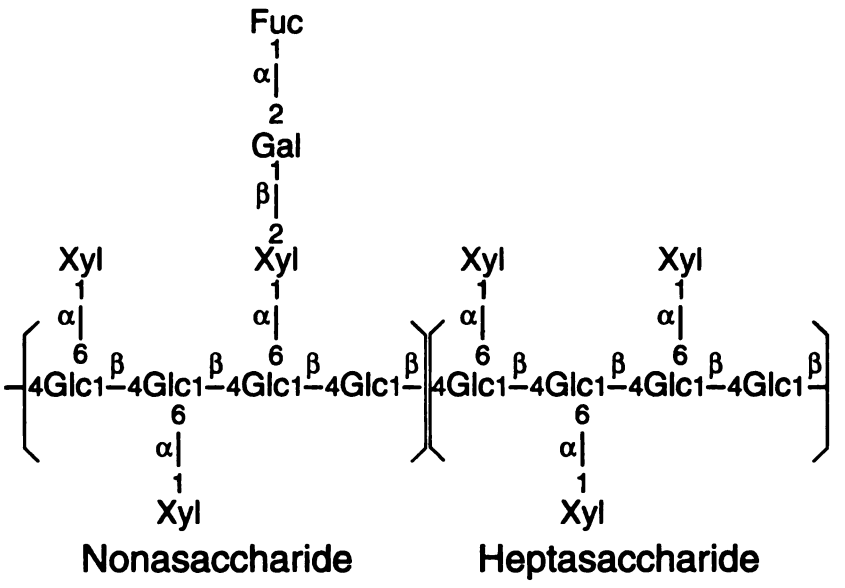

Figure 1. General structure of xyloglucan. Xyloglucan consists of a β (1,4) glucan backbone decorated with side chains of xylose, xylose and galactose, or xylose, galactose and fucose. The shorthand descriptions of these units are: XXFG (nonasaccharide), XXXG (heptasaccharide) (see Table 1.)

Table 1. Xyloglucan nomenclature. An abbreviated nomenclature has been adopted to describe different subunit structures in xyloglucan. The most common structures are in bold. Modified from (Fry et al., 1993b).

Abbreviation	Structure
G	β-D-Glc*p*
X	α-D-Xyl*p*-(1,6)-β-D-Glc*p*
L	β-D-Gal*p*-(1,2)- α-D-Xyl*p*-(1,6)-β-D-Glc*p*
F	α-L-Fuc*p*-(1,2)- β-D-Gal*p*-(1,2)- α-D-Xyl*p*-(1,6)-β-D-Glc*p*
A	α-L-Ara*f*-(1,2)- β-D-Glc*p* | α-D-Xyl*p*-(1,6)
B	β-D-Xyl*p*-(1,2)-)- β-D-Glc*p* | α-D-Xyl*p*-(1,6)
C	α-L-Ara*f*-(1,3)- β-D-Xyl*p*-(1,2)- β-D-Glc*p* | α-D-Xyl*p*-(1,6)
S (found in Solonaceous spp.)	α-L-Ara*f*-(1,3)- β-D-Xyl*p*-(1,6)- β-D-Glc*p*

Hemicelluloses: Glucuronoarabinoxylans

Glucuronoarabinoxylans (GAXs) are the major cross-linking polymers in commelinoid monocots, although cell walls from all angiosperms also have some amount of GAXs (McCann and Carpita, 2000). GAXs have a backbone of β-(1,4)-linked Xyl and are substituted with single-sugar additions of α-L-Ara and α-D-GlcA units (Verbruggen et al., 1998). In commelinoid monocots, the Ara units are most commonly added at the O-2 position; the GlcA residues are attached at the O-2 position of different Xyl units (McCann and Carpita, 2000). The GlcA units may be methylated at the 4-*O* position (Rizk et al., 2000).

Hemicelluloses: (1,3), (1,4)-β-D-glucans

Grasses (Order Poales) also have (1,3), (1,4)-β-D-glucans as an additional hemicellulose. This polymer is also known as mixed-linkage glucan. The structure of this polymer consists of three-Glc (cellotriose) and four-Glc (cellotetraose) units, present in a ratio of about 2:1, connected by β-(1,3)-linkages (Buckeridge et al., 2001). The β-(1,4) regions adopt an extended conformation, while the β-(1,3) linkages introduce kinks to ultimately result in interrupted corkscrew-shaped polymers (Brett and Waldron, 1996; McCann and Carpita, 2000).

Other hemicelluloses

Other cross-linking polymers, usually found to a lesser extent in the cell wall, include glucomannans, galactomannans, mannans, xylans, glucuronomannans, and galactoglucomannans (Brett and Waldron, 1996; McCann and Carpita, 2000; Mohnen,

1999; Sims et al., 1997). Glucomannans are the major hemicellulose in gymnosperms and have an unsubstituted β-(1,4)-linked backbone of irregular alterations of Glc and Man. In other angiosperms, members of this collection of hemicelluloses are present in fairly low abundance.

Pectins

Pectins form a gel-like substance and are abundant in the middle lamella region that is sandwiched between adjacent cell walls. This group of polysaccharides is rich in GalA and may be extracted from the wall by treatment with Ca^{2+} chelating agents such as EDTA, EGTA, ammonium oxalate or cyclohexane diamine tetraacetate (Mohnen, 1999). As might be expected, many pectins interact with Ca^{2+} ions, and the functional implications of this interaction is described in more detail below. Pectins are often used as gelling agents in the food industry.

Pectins: Homogalacturonan and Rhamnogalacturonan I

Homogalacturonan (HGA) is a fundamental pectin structure consisting of α-(1,4)-D-GalA polymers that contain up to 200 units (though polymers as long as 2000 units have been reported) and reach around 100 nm in length (Brett and Waldron, 1996; McCann and Carpita, 2000; Willats et al., 2001). Rhamnogalacturonan I (RG I) consists of repeating (1,2)-α-L-Rha-(1,4)-α-D-GalA units, forming a rod-like polymer (Willats et al., 2001). RG I may be covalently connected to HG, and the GalA units on RG I may be modified by methyl esterification (Brett and Waldron, 1996). Rha units of RG I may also be modified by side chains of variable structure, but principally containing Gal and Ara,

though Fuc has also been reported (An et al., 1994; Brett and Waldron, 1996). Together, HG and RG I are the most abundant pectins in most plant species.

Pectins: Rhamnogalacturonan II

Rhamnogalacturonan II (RG II) is a highly substituted, highly structurally complex modified HGA (Whitcombe et al., 1995). Monosaccharides present in RG II include GalA, Rha, Ara and Gal (present in a ratio of 10:7:5:5) with smaller amounts of unusual monosaccharides such as apiose, aceric acid, 2-*O*-methyl-Fuc, KDO and DHA (Brett and Waldron, 1996; Herve du Penhoat et al., 1999; McCann and Carpita, 2000; Whitcombe et al., 1995). It is present in smaller amounts in the cell wall, but its intricate structure is conserved throughout the angiosperms (Brett and Waldron, 1996; McCann and Carpita, 2000). RG II may dimerize by forming diester bonds with boron, suggesting that it may serve an important functional role despite its low abundance in the cell wall (O'Neill et al., 1996). Recently, it has been shown that RG-II isolated from a mutant with a lesion in the GDP-Fuc biosynthetic pathway, *mur1*, has a reduced rate of borate cross-linked dimer formation (O'Neill et al., 2001). Thus, it would appear that Fuc or the 2-*O*-methyl-Xyl linked to Fuc in this molecule is required for dimerization. Another recent study described covalent connections between RG II and HG (Ishii and Matsunaga, 2001).

Other Pectins

Arabinan (α-(1,5)-D-Ara chains substituted with α-(1,3)- or α-(1,2)-linked Ara), galactans (β-(1,4)-D-Gal chains) or arabinogalactans (galactan chains substituted with shorter α-(1,5)-D-Ara chains or terminal Ara units) are also found in cell walls (Eriksson

et al., 1996). Type II arabinogalactans ((1,3) and (1,6)-β-D-Gal chains attached by (1,3), (1,6) branch points) are found associated with particular proteins to form arabinogalactan proteins, described in detail below.

Non-polysaccharide components of the cell wall

Cell Wall Proteins

In addition to polysaccharides, the cell wall may also contain a complement of proteins, many of which are thought to have a structural role and impart reinforcement after most growth has occurred by cross-linking to other wall components (Showalter, 1993). Three major classes of cell wall proteins are hydroxyproline-rich glycoproteins (HRGPs), proline-rich proteins (PRPs) and glycine-rich proteins (GRPs).

Proteins: Hydroxyproline-rich Proteins

HRGPs, as the name suggests, contain an abundance of the unusual amino acid hydroxyproline. Extensin is the best-studied HRGP and contains repeating Ser-$(Hyp)_4$ and Tyr-Lys-Tyr sequences, forming a helical structure that appears rodlike in electron microscopy studies (Kieliszewski and Lamport, 1994). Extensin may be glycosylated extensively with Ara chains attached to Hyp; there may also be single Gal substitutions attached to Ser residues. Tyr residues may participate in intra- or intermolecular crosslinking to form isodityrosine linkages. Extensin glycosylation patterns differ between dicots and monocots, with less glycosylation occurring in monocots (McCann and Carpita, 2000).

Proteins: Proline-rich Proteins

Like HRGPs, PRPs form a large multigene family in most species. PRPs described thus far form two classes: those which have tandem repeats of Pro-Pro-Val-X-(Asp/Thr), where X is often Tyr, His, or Glu, or those which have an N-terminal Pro rich domain and a C-terminal domain that is not rich in Pro (Fowler et al., 1999). PRPs are not heavily glycosylated. They are thought to provide structural reinforcement during development and in response to damage from wounding or pathogen infection (Hong et al., 1989).

Proteins: Glycine-rich Proteins

GRPs may contain over 70% Gly and form β-pleated sheets (Sachetto-Martins et al., 2000). These proteins often are present at the interface of the cell wall and the plasma membrane, and have been suggested to be nucleation sites for lignin formation (Sachetto-Martins et al., 2000). Like PRPs, GRPs are not heavily glycosylated.

Phenolic Compounds

Cell walls may also contain phenolic compounds, normally thought of as being constituents of lignin in the secondary cell wall but also present in some primary cell walls. In commelinoid monocots and plants in the Chenopodiaceae family such as sugar beet and spinach, phenolic compounds such as hydroxycinnamic acids are abundant (Wallace and Fry, 1994). Because of these compounds, cell walls of such species will fluoresce under ultraviolet light. Ferulic acid and para-coumeric acid are the most common hydroxycinnamic acids. Cross-links between these units and GAX serve to structurally reinforce grass cell walls (Wallace and Fry, 1994).

1.1.2 Models for the Organization of Primary Cell Wall Components

Some of the first models of cell wall structure were based on data from cell wall fractionation studies and suggested mechanisms for growth hormone-induced wall loosening. One model suggested that auxin-induced extension of the plant cell wall occurred either by a process of transglycosylation of polymers or by irreversible hydrolysis of covalent polysaccharide bonds (Cleland, 1981). In each proposed mechanism, slippage of the cell wall polymers was suggested to occur during "stretching", and new covalent connections between polymers were thought to form before cessation of elongation. This model did not propose specific polymers that would be targets for transglycosylation or hydrolysis, instead putting forth a generalized scheme for events possibly required during elongation growth. An earlier study developed a classic primary cell wall model, proposed by the Albersheim group (Keegstra et al., 1973). In this model, a structure of the primary cell wall was proposed based on covalent interconnections between cell wall components as suggested by extraction and fractionation experiments. Xyloglucans (XyGs) were suggested to interact with cellulose microfibrils via hydrogen bonds, but authors suggested that the XyG polymers covalently connected to pectic polysaccharides, with further covalent connections between pectins and arabinogalactan chains attached to wall proteins through serine residues (Keegstra et *al.*, 1973). In this model, the proposed mechanism for extension of the cell wall was transient dissociation between XyG polymers and cellulose as the hydrogen bonds were broken and then reformed during cell wall slippage. Several subsequent studies questioned the possibility of covalent bonding between XyG and pectin, and this aspect

of the model was generally disputed (Hayashi, 1989; McCann and Carpita, 2000; McNeil et al., 1984). A recent study, however, indicated evidence for covalent linkage between about one third of the XyG component and pectin in suspension-cultured rose cell walls, and suggested that the Keegstra et al. model be re-visited (Thompson and Fry, 2000).

Carpita and Gibeau later proposed another model of plant primary wall structure (Carpita and Gibeaut, 1993). The authors differentiated between the Type I primary cell wall, characteristic of all dicots and nongraminaceous monocots examined to date, from the Type II primary cell wall, characteristic of the grasses. The model is also generalized, as walls of specific tissues or from certain species may show structural variation.

In the Type I cell wall model, there are three components: the cellulose-xyloglucan framework, a gel-like matrix of pectins, and structural proteins. The main structural features of the wall are suggested to form by the interaction of xyloglucan and cellulose polymers by hydrogen bonding. One fraction of XyG is suggested to interact closely with microfibrils, perhaps even integrating into the microfibrils themselves in some cases. Other XyG polymers are suggested to interact less directly with cellulose, serving instead to bridge the distance between adjacent microfibrils and thus serve as a network of hemicelluloses throughout the wall, with several XyG strands coalescing together in some regions. Evidence for the two fractions of XyG is provided by extraction experiments, in which both easily extracted and tightly associated portions of XyG are derived from cell wall preparations (Pauly et al., 1999). Microscopic studies have also

allowed visualization of cell walls from which pectins have been removed, showing lacy interconnections of polymers between cellulose microfibrils (McCann et al., 1990).

The second domain of the cell wall consists of pectins that form a gel-like matrix. The porosity of the cell wall is thought to be controlled in large part by pectins, as the degree of methyl esterification of GalA residues in homogalacturonan (HGA) as well as the concentration of Ca^{2+} present in the wall may influence the formation of "junction zones". These zones are regions of association between antiparallel chains of HGA cross-linked with Ca^{2+} ions. If esterification occurs, interaction of HGA chains with calcium ions, and thus the formation of junction zones, is prevented. Other manipulations of pectin structure may also influence physical characteristics. For example, pore size limiting unrestricted diffusion in the primary cell wall was shown to be about 4 nm in diameter (Carpita et al., 1979), but enzymatic digestion of pectins may increase the pore size to over 10 nm (Baron-Epel et al., 1988). This effectively means proteins over 100 kDa might be able to move through these 10 nm pores via unrestricted diffusion.

The third domain of the Type I primary cell wall in this model consists of structural proteins, many of which are rich in glycine or hydroxyproline and have characteristic repeating units (Carpita and Gibeaut, 1993). The protein domain is thought to lock the primary cell wall structure into place, and may serve an important role in reinforcement of the wall after elongation.

One analysis of cell wall structure in *Arabidopsis* has been published (Zablackis et al., 1995). This study utilized leaf material from four-week-old Col-0 *Arabidopsis thaliana* plants to determine the content and structures of major cell wall components. It should be noted that Zeblackis *et al.* only determined cell wall structure for one type of tissue, rosette leaves, and that the leaves of four-week-old *Arabidopsis* plants might contain both primary cell wall material and secondary cell wall material (from vascular tissue). Leaf cell walls of *Arabidopsis* contain a high proportion (34% of the total wall material) of phosphate buffer-soluble pectins relative to other plant cell walls that have been analyzed, indicating that pectins are both fairly abundant and fairly weakly held within the wall in this species. The composition of the *Arabidopsis* leaf cell wall is shown in Figure 2. Pectins include rhamnogalacturonan I (RG I), rhamnogalacturonan II (RG II), and homogalacturonan (HGA). Xyloglucan is the primary hemicellulose, though a small amount of glucuronoarabinoxylan (GAX) is also present. Cellulose comprises 14% of the cell wall. Structural proteins also occur in *Arabidopsis* leaf cell walls in relatively high abundance, and the amino acid composition of these proteins differs somewhat from that observed in other species (Zablackis et al., 1995). With the exception of the protein content of *Arabidopsis* leaf cell walls and the high content of phosphate buffer-soluble pectins, the only other notable difference observed was that *Arabidopsis* leaf RG I is less highly branched than RG I from sycamore suspension cells. The authors concluded that in all other respects, cell walls from *Arabidopsis* leaves resembled cell walls from other species that have been analyzed (Zablackis et al., 1995).

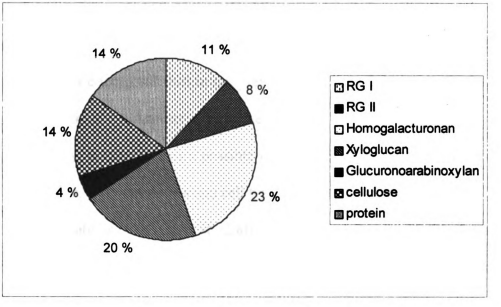

Figure 2. Composition of *Arabidopsis* leaf cell walls. Data from Zeblackis *et al.*, 1995.

Some cell wall models have been necessarily static, seeking to establish a generalized cell wall structure applicable to many types of plant cells. More recent approaches, however, have allowed biologists to appreciate that different types of cell walls exist within the plant, and sometimes even in different regions of a single cell (Roberts, 2001). Antibodies against specific carbohydrate epitopes and other technical advances will continue to allow a conceptual shift in our view of the cell wall from a general structure to a dynamic one. Another technique, Fourier Transform Infrared (FTIR) microspectroscopy, is emerging as a possible means of examining cell wall architecture in growing regions, different tissues and cell types, and plants subjected to environmental or genetic modifications (McCann et al., 2001; McCann and Carpita, 2000). Researchers are seeking to establish a sufficiently robust FTIR spectra database to allow more detailed and more rapid analysis of a variety of different cell wall structures (N. Carpita, personal communication.)

1.1.3 Biological Function of Xyloglucan

Xyloglucan (XyG) is known to associate with cellulose. There are thought to be different fractions of XyG: a portion that interacts intimately with cellulose microfibrils, coating the surface and even penetrating the outer region of the fibrils, and a portion that serves to link adjacent microfibril chains (Hayashi, 1989; Pauly et al., 1999). The existence of the closely-associated portion has been demonstrated by fractionation studies. Analysis of etiolated peas showed that a portion of XyG can be removed with an XyG-specific endoglucanase (XEG), another portion requires treatment with concentrated alkali for removal, and a third portion can only be extricated from cellulose by treating the XEG-

and KOH-treated cell walls with cellulase (Pauly et al., 1999). XyG chains that span the distance between microfibrils have been directly visualized by microscopy studies (Fujino et al., 2000; Itoh and Ogawa, 1993; McCann et al., 1990). Rapid freezing-deep etching (RFDE) microscopy involves cryofixation of a cell wall sample, removal of ice crystals under a vacuum, covering the surface of the sample with platinum and carbon layers, dissolving away the biological material, and visualization under the electron microscope. Crosslinking strands can be seen as narrow interconnections between the thicker cellulose microfibrils using the RFDE technique. Fujino et al. have shown that these strands are resistant to treatment with protein denaturing agents or weak alkali solution, but disappear completely upon treatment with XyG-digesting endo-(1,4)-β-glucanase (Fujino et al., 2000). Anti-XyG antibodies also labeled the crosslinking structures, providing further evidence that they are composed of xyloglucan (Fujino et al., 2000). Attempts to determine the smallest unit of XyG that will bind to cellulose have concluded that oligosaccharides with at least four glucose backbone units are necessary (Hayashi et al., 1994b; Vincken et al., 1995). Another study also indicated that the extent of *in vitro* binding of XyG oligosaccharides to cellulose was greater than that observed for cellulose oligosaccharides of equivalent backbone length, indicating that the presence of side chains on a β-(1,4)-glucan molecule does impart specific binding properties (Hayashi et al., 1994b).

The biological function of XyG, therefore, clearly involves a structural role. Because of the nature of this thesis, the relevance of the fucose residue to the function of XyG is of interest. In that regard, some contradiction is found within the literature. When efforts

were made to predict the conformation of xyloglucan structures by computer modeling, calculations predicted a distinct effect of side-chain presence and composition on the conformation assumed by the XyG polymer (Levy et al., 1997; Levy et al., 1991). Metropolis Monte Carlo simulations of conformational forms of XyG structures indicated that lowest-energy states could be achieved when fucosylated trisaccharide side-chains were present and folded back to interact with one face of the polymer, causing the backbone of the molecule to adopt a flattened shape in this region (Levy et al., 1997; Levy et al., 1991). This in turn was suggested to facilitate binding of XyG to cellulose (Levy et al., 1997; Levy et al., 1991). When tested, researchers found that there was a twofold higher rate of *in vitro* binding between fucosylated pea XyG and cellulose (avicel) compared to nonfucosylated tamarind seed XyG, lending biochemical support to the theory that the presence of fucose on side-chains has structural consequences (Levy et al., 1997). Hayashi et al. also found that fucosylated XyG had a higher adsorption constant for cellulose than tamarind XyG when polymers of equal backbone chain length were compared (Hayashi et al., 1994a). Some speculations about the role of terminal Fuc in *Zinnia* have also been made, as XyG in tissues that require high tensile strength contain Fuc while XyG in tissues that do not are nonfucosylated (McCann and Roberts, 1994). In isolated *Zinnia* mesophyll cells, fucosylated XyG is not produced until elongation has halted, indicating a specialized function for this structure (McCann and Roberts, 1994).

However, another group using a different experimental system has speculated that the presence of fucosylated side-chains bears little impact on the function of XyG (Whitney

et al., 1995; Whitney et al., 1999). The bacterium *Acetobacter aceti* ssp. *xylinum* produces cellulose to form extracellular pellicles during fermentation. Researchers have added exogenous tamarind XyG and other polymers to the fermentation media and examined the resulting products using microscopic and materials science analytical methods (Whitney et al., 1995; Whitney et al., 1999). In the presence of tamarind XyG, a material was formed that contained cellulose microfibrils cross-linked with interconnecting strands. Authors concluded that terminal fucose residues are not necessary for formation of cellulose-XyG cross-links, and questioned the hypothesis posed by Levy et al. (Whitney et al., 1995). Subsequent studies examined the uniaxial tensile strength, small deformation oscillatory rheology, and action of cell wall modifying proteins on this material (Whitney et al., 2000; Whitney et al., 1999). Compared to cellulose synthesized in absence of tamarind XyG, the material was found to have greater extensibility during uniaxial tension tests and extended in response to a cell wall modifying protein, indicating that there are some structural differences imparted by the presence of XyG (Whitney et al., 2000; Whitney et al., 1999). However, no data were presented in these later studies that compared composites generated using fucosylated forms of XyG to composites generated using tamarind XyG. It is possible that the presence of Fuc on XyG affects properties that would be of consequence *in planta* but not detectable in the *Acetobacter* system. For example, the presence or absence of Fuc on XyG may affect the suitability of XyG as a substrate for a wall modifying enzyme(s) not investigated in these experiments; some evidence for this has already been described (Steele and Fry, 2000; Steele et al., 2001). It is also possible that the structure of XyG side chains affects the interaction of XyG with other polymers. A recent study detected

binding between radiolabeled glucuronoarabinoxylan and pectin to XyG (Rizk et al., 2000). This binding was greatly reduced when nonfucosylated XyG was used in assays or when terminal Fuc was removed from pea XyG by mild acid treatment (Rizk et al., 2000).

In addition to its proposed structural roles, XyG has also been suggested to serve a role as a signaling molecule within the plant. In 1984, York et al. digested XyG isolated from sycamore cell suspension cultures with *Trichoderma* endocellulase, a hydrolytic enzyme that cleaves XyG at unsubstituted Glc residues to generate XyG oligosaccharides (York et al., 1984). These oligosaccharides were purified, and surprisingly some of them showed an ability to inhibit auxin-induced elongation of pea epicotyl segments at nanomolar concentrations (York et al., 1984). Thus, fragments of XyG were proposed to act as "oligosaccharins", or small units of polysaccharides that elicit specific biological responses when applied at very low concentrations (Fry et al., 1990). Other instances of carbohydrates acting as signaling molecules have been reported, such as fragments of plant or fungal cell walls serving to elicit plant defense responses against pathogens (Fry et al., 1993a; John et al., 1997). Specific structural requirements for biological activity of XyG fragments have been established, and further studies have shown that differing responses occur at different levels of XyG oligosaccharide application (Aldington et al., 1991; Fry et al., 1993a; Fry et al., 1990; McDougall and Fry, 1989). At low levels (1-10 nM), a fucose-containing nonasaccharide (subunit structure XXFG) inhibits both auxin-induced and acid-induced growth in pea (Fry et al., 1990; York et al., 1984). The Fuc residue is absolutely required for this effect, since oligosaccharides that lack Fuc cannot

inhibit auxin-induced elongation. However, an oligosaccharide containing 2-*O*-methyl-Fuc did not act as an anti-auxin agent, indicating that Fuc is necessary but not sufficient for activity (McDougall and Fry, 1989). At higher levels (1 μM), XyG oligosaccharides promote growth in pea, presumably by acting as substrates for a wall-modifying enzyme called xyloglucan endotransglycosylase (XET) that can incorporate new fragments of XyG into the existing cell wall (Fry et al., 1990). Because the concentration of fragments needed to provoke the biological response is much higher than that required for the anti-auxin response, fragments in this case are not thought to be acting as signaling molecules *per se.* The structural requirements for this activity differ, with the most active XyG oligosaccharides actually lacking Fuc (Fry et al., 1990). The order of effectiveness of XyG fragments matches the order of substrate preference of XEG, supporting the theory that XET-assisted incorporation of fragments into existing polymers leads to extension (Fry et al., 1993a).

Despite this foundation of evidence indicating that XyG oligosaccharides may act as signaling molecules in garden pea, very little additional work has been published that clarifies the nature of this event. No XyG nonasaccharide receptor has been reported, and the significance of these observations remains unclear. Interestingly, a cell wall-localized α-fucosidase has been purified from pea and characterized biochemically (Augur et al., 1993). This enzyme was shown to be localized to the primary cell wall and is developmentally regulated (Augur et al., 1993). It acts only on XyG oligosaccharides, lacking the ability to cleave Fuc from large polysaccharides (Augur et al., 1993). The gene encoding this pea enzyme has been cloned (Augur et al., 1995). However, no

further work describing this gene and enzyme and their possible roles in generation of XyG oligosaccharins has been reported.

It should be noted that the activity of XyG oligosaccharides as signaling molecules has only been studied in pea. Members of the legume family may be predisposed to the use of carbohydrate signaling molecules, as carbohydrate structures play an important role in the lipochitooligosaccharide Nod signaling factors necessary for nodulation by nitrogen-fixing bacteria. It is possible that carbohydrate signaling factors may not play a significant role in other plant species, such as *Arabidopsis*.

1.2 The Cell Wall and Growth

1.2.1 Cell Division

It is impossible to consider plant cell division in absence of the cell wall. Early during cell division, a cytoskeletal structure called the preprophase band forms and determines the future site of attachment of the cell plate to existing parental cell walls. The emerging cell plate forms as polysaccharides are deposited at the division plane after biosynthesis in the Golgi apparatus (for reviews, see Assaad, 2001; Smith, 1999; Sylvester, 2000). These polysaccharides are deposited at the phragmoplast, an equatorial cytoskeletal array, via secretory vesicles. The nascent cell plate contains cell wall materials such as xyloglucan, synthesized in the Golgi apparatus, and an abundance of callose (beta 1,3-glucan), synthesized at the cell plate. The presence of callose causes the immature cell plate to be fluid, a property necessary for later events (Assaad, 2001). The cell plate expands towards the periphery of the cell and contacts existing walls at the point

previously determined by the preprophase band. This process is achieved in part by action of microtubules and is regulated by a signal transduction pathway that involves a MAP kinase cascade (Assaad, 2001). Recent research has begun to identify some of the molecular mechanisms for cell plate formation and positional fixation. For example, a high molecular weight dynamin-like protein called phragmoplastin (or ADL1 in *Arabidopsis*) may be involved in vesicle-tubule-vesicle fusion events in the cell plate (Gu and Verma, 1996; Kang et al., 2001; Zhang et al., 2000). Vesicle trafficking elements required to shuttle cell wall materials to the phragmoplast include a syntaxin called Knolle (Lukowitz et al., 1996) and a Sec1-like protein called Keule (Assaad et al., 1996). Upon maturation of the cell plate, callose is replaced by cellulose (beta 1,4-glucan) and pectin (Assaad, 2001). After the cell plate matures it eventually becomes the site of the middle lamella, a pectin-filled structure shared by adjoining cell walls. Two enzymes that appear to be essential for the process of cell plate maturation are Cyt1, a Man-1-phosphate guanylyltransferase required for N-glycosylation (Lukowitz et al., 2001; Nickle and Meinke, 1998), and Korrigan, an endo-1,4-beta-glucanase (Zuo et al., 2000). Additional primary cell wall material is deposited next, forming a layer that is distinguishable in ultrastructural studies (for example, see McCann et al., 1990). In certain cells, secondary cell wall polysaccharides may be deposited at a later point.

It is clear that the cell wall is essential for cytokinesis in plants, because cells lacking a wall cannot divide (Cooper et al., 1994; Meyer and Abel, 1975; Suzuki et al., 1998; Wojtaszek, 2000). If the cell wall is digested using polysaccharide hydrolytic enzymes, the resulting naked cell is called a protoplast. Cultured protoplasts will not divide

without first forming a cell wall (Meyer and Abel, 1975). In one case, the application of 3,4-dehydroxyproline, which inhibits activity of prolyl 4-hydroxylase and prevents hydroxylation and thus glycosylation of hydroxylproline-containing cell wall proteins, prevents execution of mitosis (Cooper et al., 1994). This indicates that these cell wall components are essential for wall formation and cell division. In contrast, application of 2,6-dichlorobenzonitrile (DCB), a cellulose biosynthesis inhibitor, did not prevent monocot or dicot cells in suspension culture from dividing (Shedletzky et al., 1990; Shedletzky et al., 1992). Instead, these cells, which lacked a cellulose-hemicellulose network, developed greatly altered cell wall composition. Dicot (tomato and tobacco) cell walls were reinforced with pectin linked with phenolic structures, while graminaceous monocot (barley) cell walls were reinforced with increased levels of hemicelluloses, also linked to phenolic compounds (Shedletzky et al., 1990; Shedletzky et al., 1992). Experiments such as these indicate that plant cells can tolerate remarkable alterations in cell wall structure and show a great deal of flexibility in adaptation.

1.2.2 Cell Elongation and Expansion

In addition to being essential for cell division, the cell wall is of fundamental importance for growth processes. During elongation, the cell wall both permits growth and delimits it. If sufficient water is available, water will move into the cytoplasm, where osmotically active solutes are present. Water tends to move from a region of less negative to more negative water potential (reviewed in Hsiao and Bradford, 1983). This eventually causes internal pressure as the cell contents abut against the defined volume dictated by the rigid cell wall. If the cell wall remains inflexible, turgor pressure is maintained and there is no

net movement of water. If the cell wall becomes extensible, however, additional water may move into the cell, which will then begin to increase in volume. The direction of increase can be controlled by the orientation of cell wall components and the localization of cell wall softening. Cell walls in which cellulose microfibrils, a major component of the wall, are not directionally organized will experience an increase in all directions, termed isodiametric growth. Cell walls in which microfibrils have been deposited in a directional manner will elongate along the axis perpendicular to the direction of microfibril alignment, resulting in anisodiametric growth. It is also theoretically possible for localized cell wall softening to dictate the dimensions of cell expansion. Plant cell expansion, therefore, occurs through an interplay between the amount of water available to the cell, the amount of osmotically active solutes contained within the cell, and the extensibility of the cell wall. The cell cannot continue to expand and be viable, however, without at some point synthesizing and depositing new cell wall material. If no new material was added the wall would thin to the point of breakage, as occurs in isolated cell walls undergoing extension *in vitro* (Cosgrove, 2000a).

The force exerted on the cell wall by a turgid cell is not insignificant. Carpita and Gibeaut (Carpita and Gibeaut, 1993) estimated that a turgid, spherical cell with a radius of 50 μm and a cell wall thickness of 0.1 μm exerts a pressure of 2500 bars upon the cell wall. If the cell is of equivalent volume but is cylindrical rather than spherical, it will develop 5000 bars of tension in the tangential axis but the longitudinal tension will remain 2500 bars. Clearly, the cell wall must maintain structural integrity in face of great force.

Plant cell expansion and cell wall biosynthesis are generally well coordinated, with increased rates of cell wall deposition occurring in regions of expansion (Cosgrove, 1997a). A stimulation of arabinan synthetase has been observed in *Phaseolus vulgaris* during extension growth (Bolwell and Northcote, 1981) and subcultured bean suspension cell cultures (Bolwell and Northcote, 1983). Labeling experiments showed a positive correlation between the rate of deposition of radiolabeled uronides in the cell wall, which was taken as a measure of cell wall deposition, and the growth rate in regions of maize root tips (Silk et al., 1984).

The relationship between biosynthesis and elongation can be complex, particularly in the case of auxin-induced elongation. For example, Bret-Harte *et al.* noted that application of auxin to stem segments promoted cell wall deposition when glucose levels were sufficient, but auxin-induced elongation during limiting glucose conditions was not correlated with deposition and instead resulted in cell wall thinning (Bret-Harte et al., 1991). The authors suggested that auxin promotes cell wall synthesis and cell wall loosening by different mechanisms (Bret-Harte et al., 1991), which is not inconsistent with later work on extension of cell walls by action of expansins (discussion below). In an earlier study, it was observed that auxin or fusicoccin treatments promoted glucose uptake, but not incorporation, into the cell wall under conditions in which elongation occurred; however, synthesis was enhanced under conditions that limited elongation (Brummell and Hall, 1983). Thus, the effect of auxin on cell wall synthesis may depend upon the conditions occurring at the time of exposure to the growth hormone.

Several possible outcomes exist as a result of cell wall deposition. If deposition rates are sufficiently high and elongation rates are low, the wall will thicken; if deposition is insufficient during elongation, the wall will thin (Brummell and Hall, 1983; Cosgrove, 1997a). Alternatively, deposition of new material and cell expansion may reach an equilibrium such that the thickness of the wall is maintained despite an increase in cell volume (Brummell and Hall, 1983). In short, the synthesis and deposition rates of new cell wall material may be correlated with growth, particularly with the longer-term maintenance of growth, but are not by themselves sufficient to cause growth.

1.3 The Cell Wall and Environmental Interactions

As sessile organisms, plants face a particular challenge. They must respond to environmental factors such as biotic and abiotic stress without being able to evade them through relocation. Since the cell wall is the first part of the plant body that interacts with the environment, it is frequently involved in responses to the plant's surroundings. Several examples of cell wall participation in reactions to the environment will be described.

Plant responses to abiotic signals frequently require differential growth to occur. For example, phototropism involves the perception of a light signal and growth towards a light source. Similarly, gravitropism allows plants to perceive gravitational forces and orient growth patterns such that shoot tissue grows upwards and root tissue grows downwards. In both cases, growth curvature may occur. While components of the signal

transduction pathway differ between phototropism and gravitropism, auxin is thought to be involved in both responses. A gradient of auxin in a plant organ such as an elongating shoot may result in differential expansion of cells on one side versus the other (Salisbury and Ross, 1992). This ultimately results in curvature of the organ. Alternatively, cells on one side of the organ may be more sensitive to auxin than those on the other side, also resulting in curvature (Salisbury and Ross, 1992). Since auxin affects the extensibility of the cell wall, the capability of the plant to respond effectively to the signal depends not only on the ability to perceive the signal and transduce it, but for its cell walls to undergo sufficient reorganization.

Another differential growth response to an abiotic signal occurs when plants face water shortages. Under limiting water conditions, growth rates in shoot tissues are sharply curtailed while growth rates in root systems are not inhibited. This is an adaptive advantage to the plant, which can continue to mine the soil for water. Recently, mechanisms for this differential growth occurrence were proposed based on events occurring in the cell wall (Wu and Cosgrove, 2000; Wu et al., 1996). Maize roots grown at low water potential produce more expansin, a cell wall protein that causes extensibility (discussed in more detail below) compared to roots grown at normal water potential (Wu and Cosgrove, 2000; Wu et al., 1996). Cell walls of apical elongation zone regions of low water potential roots were also more responsive to expansin, indicating that structural changes had taken place in the wall (Wu and Cosgrove, 2000; Wu et al., 1996). This was in contrast to the basal elongation zone region of the root, which showed less responsiveness to expansin and in which structural modifications had occurred that

caused wall "stiffening". The exact nature of the structural modifications that occur in the apical and basal regions is not known, though stiffening has been proposed to arise by oxidative cross-linking through the action of apoplastic peroxidase (Wu and Cosgrove, 2000).

Some types of environmental responses may involve differential cell wall reinforcement rather than cell wall extensibility. One such example is the modification of the cell wall in response to wind stress or other forms of mechanical stress. Plants exposed to wind or mechanical manipulation may become shorter, stockier, and stronger, which confers an adaptive advantage to further or increased stress (Braam et al., 1997; Salisbury and Ross, 1992). Certainly wind stress can be of significant consequence in agriculture, where plants too fragile to withstand strong winds may be flattened. The morphological pattern associated with wind or mechanical responses is called thigmomorphogenesis. Investigations into the molecular control of thigmomorphogenesis began when a group of differentially expressed *Arabidopsis* genes were identified as being expressed in response to slight mechanical stimulation such as touch, wind, and water spray (Braam and Davis, 1990). This subset of genes was called *TCH* (Touch) genes, and several of the cDNAs were sequenced. Multiple cDNAs encoded calmodulin isoforms, indicating that calcium acts as a second messenger during mechanical stress signal transduction (Braam and Davis, 1990; Braam et al., 1997). Another *TCH* gene, *TCH4*, encoded a xyloglucan endotransglycosylase (XET), an enzyme that cleaves xyloglucan chains and ligates them to adjacent XyG chains (Xu et al., 1995). XETs have been proposed to play a role in incorporation of nascent XyG into the existing cell wall, possibly affecting cell wall

structural properties (Thompson et al., 1997). Thus, it was not perhaps surprising that mechanical stimulation induced a wall-modifying enzyme. The localization of Tch4 protein in stem tissues of wind-treated plants was consistent with a role in morphological changes occurring after mechanostimulation (Antosiewicz et al., 1997).

A second type of cell wall reinforcement response occurs in woody plants. Reaction wood is a type of xylem-rich tissue that is formed in response to mechanical strain (Salisbury and Ross, 1992). Softwood trees form reaction wood (called compression wood) on the underside of branches, causing the branch to be propped upright, while hardwood trees form reaction wood (called tension wood) on the upper side of branches, pulling the branch towards the tree body (Salisbury and Ross, 1992). Reaction wood may also be formed when a tree or branch is subjected to strain, such as the artificial strain imposed by tying a sapling into a U-shaped form or the natural strain that occurs when tree limbs bear the weight of heavy snow or ice. In all cases, the reaction wood involves in part alterations to cell wall structure. The alterations differ among the species, but frequently secondary cell wall thickening is observed, and increased lignification may occur (Salisbury and Ross, 1992).

A final example of cell wall structural modification in response to the environment occurs when a plant mounts a defense against attacking pathogens. In response to invasion by pathogens, plants may alter cell wall structures in an attempt to prevent spread of the pathogen. Such structural modifications may include deposition of callose (thought to be a wounding response), lignification, cross-linking of aromatic compounds

such as ferulate, and increased incorporation of other phenolic compounds (Hardham and Mitchell, 1998; Wojtaszek, 2000).

1.4 Cell Wall Events During the Life of a Cell

The cell wall experiences a natural progression during the life of the cell. At appropriate times, the cell wall is 1) biosynthesized, 2) deposited and assembled, 3) remodeled in response to endogenous or exogenous conditions, and 4) disassembled during senescence or cell death. The biosynthesis of the cell wall will be considered in detail below. Little is actually known about the assembly processes that occur to establish the structure of the cell wall, and this process will not be considered in detail for this reason. Remodeling of the wall may occur during growth, and two major proteins that are capable of cell wall modifications will be reviewed. Because of the nature of this thesis, the process hydrolysis and degradation of cell wall components will not be reviewed.

1.4.1 Plant Cell Wall Polysaccharide Biosynthesis

Polysaccharide biosynthesis in general relies on the specificities of enzymes that link precursor units into polymers. Thus, polysaccharide structure is not determined as a primary outcome of gene activity, but as a secondary outcome through action of carbohydrate-active enzymes (Perrin et al., 2001; Wojtaszek, 2000). Synthesis may be affected by the presence of biosynthetic enzymes, the order in which these enzymes are encountered, the affinity and specificities of these enzymes, and the availability of

substrate molecules such as sugar nucleotide donors (Perrin et al., 2001). These enzymes tend to be integral membrane proteins.

Several aspects of plant cell wall polysaccharide biosynthesis are notable. There are two main sites of synthesis within the cell. Cellulose (β-(1,4)-glucan) and callose (β-(1,3)-glucan) are synthesized at the plasma membrane with extrusion of the product into the apoplast, while all other cell wall polysaccharides are synthesized within the Golgi apparatus and transferred to the apoplast by secretory vesicles. Despite a detailed knowledge of cell wall structural features, there is a relatively small body of knowledge about the enzymes that make cell wall polysaccharides, and an even smaller body of knowledge about the genes that encode these enzymes. In part, this is because these families of enzymes share higher-order structural features, but may not share extensive sequence identity. Thus, it has not been particularly fruitful to attempt to identify plant-specific polysaccharide biosynthesis enzymes by homology to identified enzymes from animals or microbes. I will define the two main types of cell wall biosynthetic enzymes and give an overview of existing examples of plant enzymes for each type.

Glycan synthases

Glycan synthases are processive enzymes devoted to constructing the backbone of polysaccharides (Perrin et al., 2001). For example, cellulose synthase, callose synthase, and the glucan synthase that makes the backbone of XyG are all examples of glycan synthases.

Within the past five years, genes encoding the cellulose synthase catalytic subunit have been identified. This has not been a trivial effort. The lability of rosettes, cellulose-synthesizing enzyme complexes located in the plasma membrane, has caused difficulty for biochemical approaches to studying this enzyme. Identification of cellulose synthase at the molecular level first occurred in the cellulose-producing bacteria *Acetobacter xylinum* and *Agrobacterium tumefaciens*. The use of a product entrapment technique first allowed the purification of a cellulose synthase catalytic subunit enzyme from *A. xylinum*, followed by identification of the corresponding gene (reviewed in Delmer, 1999). A similar gene was subsequently isolated from *A. tumefaciens*. Though it was hoped that these bacterial genes might be used as hybridization probes to identify corresponding plant genes, several attempts to do so were not fruitful (Delmer, 1999). However, a careful study of the bacterial genes using hydrophobic cluster analysis identified some highly conserved regions. Within these regions are three conserved aspartate residues followed by a QXXRW motif (Delmer and Amor, 1995; Saxena et al., 1995). These features seemed characteristic of cellulose synthases, and more a recent structural study on a glycosyltransferase from *Bacillus subtilus* indicated that portions of such regions may be involved in binding to UDP-glucose (Charnock and Davies, 1999). Candidates for plant cellulose synthase genes were derived by random sequencing of cDNA libraries from developing cotton fibers, collected at a developmental stage during which a massive amount of cellulose is synthesized. Two candidate genes were identified that had similarity to the bacterial cellulose synthases and contained the D,D,D,QXXRW motif (Pear et al., 1996). However, the plant homologs contained inserted regions that were not present in the bacterial enzymes, explaining why attempts to utilize the bacterial genes as

probes had failed. Six transmembrane domains were predicted for the cotton enzymes, two at the amino terminus and four near the carboxy terminus. The central soluble region was capable of binding UDP-glucose in a Mg^{2+}-dependent manner, consistent with previous biochemical studies (Pear et al., 1996). Originally named *CelA-1* and *CelA-2*, these genes have recently been designated *GhCesA-1* and *GhCesA-2* (Delmer, 1999).

In vivo support that *CesA* genes do indeed constitute the cellulose synthase catalytic subunit rests largely upon genetic evidence. In the first example, the *rsw1* mutant described above was shown to have a point mutation in a gene homologous to the cotton *CesA* genes, causing cell wall abnormalities throughout the plant when grown at elevated temperatures (Arioli et al., 1998). In the second example, one *Arabidopsis* mutant identified on the basis of collapsed vascular (xylem) cell phenotype was found also to have a mutation in a *CesA* homolog (Taylor et al., 1999). The *irx3* mutant lacks sufficient cellulose deposition in vascular tissues to withstand the negative pressure involved in water conductance, and thus its vasculature collapses. Immunological evidence that CesA is present in rosettes was shown in an elegant microscopy study that utilized a freeze fracture replica labelling technique, applied to plants for the first time, to demonstrate that antibodies directed against a cotton CesA could label rosettes of adzuki bean, a vascular plant (Kimura et al., 1999).

With the sequencing of the *Arabidopsis* genome now complete, it has become clear that that there is much genomic redundancy. Many genes appear to be present as members of families rather than as single copies. This has also been the case for cellulose synthase

genes, but to an extreme. There appear to be approximately 40 genes in Arabidopsis that bear similarity to the original *CesA* genes, comprising a large superfamily. This superfamily has been divided into one family of ten "true" *CesA* genes and six families of cellulose synthase-like (*Csl*) genes that are less closely related (Richmond and Somerville, 2000). A web site is maintained by Richmond and Somerville to document sequence data for cellulose synthase and cellulose synthase-like genes (http://cellwall.stanford.edu). It is possible that some of the *Csl* genes may be involved in synthesis of non-cellulosic glucan polymers, such as the backbone of XyG. Recently, evidence has been generated to suggest that multiple isoforms of cellulose synthase are needed in the same cell for the formation of functional dimeric complexes, indicating why there might be a need for so many *CesA* isoforms in plants (Perrin, 2001).

Glycosyltransferases

Unlike glycan synthases, glycosyltransferases are non-processive enzymes. Glycosyltransferases add single sugar units to polysaccharides, resulting in side-chain structures (Perrin et al., 2001). Typically, these enzymes are type II integral membrane proteins with an N-terminal cytoplasmic tail, a signal-anchor transmembrane domain that spans the Golgi apparatus membrane, and a stem region and C-terminal catalytic domain located in the lumen of the Golgi (Kleene and Berger, 1993). Two plant glycosyltransferases involved in synthesis of cell wall polysaccharides have been identified and will be described.

Galactosyltransferases

Legume seeds utilize galactomannans as seed storage polysaccharides. Galactomannan (GM) consist of a backbone of β-D-(1,4)-Man with substitutions of α-(1,6)-Gal. Using endosperm of hand-dissected germinating fenugreek seeds, Edwards et al. solubilized GM galactosyltransferase (GalT) and demonstrated its activity in an *in vitro* assay using either mannan oligosaccharides with a degree of polymerization of five or more units or GM with a low level of Gal substitution as exogenous acceptor substrates (Edwards et al., 1999). Non-denaturing isoelectric focusing combined with an in-gel assay, followed by SDS-PAGE analysis, indicated that enzyme activity was correlated with a protein of approximately 51 kD with a pI of 6.5 (Edwards et al., 1999). Amino acid sequence data was obtained from the 51 kD protein and used to design degenerate oligonucleotide primers for isolation of the corresponding cDNA by RT-PCR (Edwards et al., 1999). Confirmation of the cDNA as encoding a functional GM galactosyltransferase was achieved by expressing the cDNA in the methylotropic yeast *Pichia pastoris* as a truncated, secreted protein. This protein was purified from the yeast growth media and used in the assay, showing high galactosyltransferase activity (Edwards et al., 1999).

Fucosyltransferases

Previous studies had established certain facts about XyG fucosyltransferase (FUT) and the FUT gene family as a whole. It had been shown by immuno-electron microscopy using anti-α-(1,2)-Fuc-Gal antibodies that fucosylated XyG first appears in the lumen of the trans-Golgi and trans Golgi network before vesicle-mediated secretion to the cell wall (Moore et al., 1991; Puhlmann et al., 1994). This activity appeared to be spatially distinct

from galactosyl- and xylosyltransferase activity (Brummell et al., 1990; Camirand et al., 1987). The Golgi localization of activity agreed with similar studies of fucosyltransferases in mammals (e.g Borsig et al., 1996; Kimura et al., 1995; Narimatsu et al., 1996). FUTs received early attention from the medical community because of their role in synthesis of glycoprotein blood group antigens (such as the ABO blood group) and glycolipids (Field and Wainwright, 1995; Mollicone et al., 1995). Some Fuc-containing antigens and FUT activities also appeared to be correlated with tumor formation, serving as clinical indicators of certain types of cancer (Bolscher et al., 1989; Shah Reddy et al., 1982; Staudacher et al., 1999; Weiser and Wilson, 1981). A rash of cloning and characterization studies in mammalian systems in the early to mid-1990s identified a number of FUTs critical to glycobiological processes. Expression-based cloning proved to be a valuable technique to identify candidate genes in many cases (Ernst et al., 1989; Fukuda et al., 1996). Mammalian studies also established the structure of FUTs, which matches the general structure of glycosyltransferases described above. The critical nature of the C-terminus in catalysis was illustrated by deletion studies showing that removal of as little as one amino acid from the C-terminal end could completely abolish all enzymatic activity, while deletion of as much as one third of the N-terminus had little effect (Xu et al., 1996). With time, FUTs were also identified in parasitic organisms such as ulcer-causing bacteria, apparently serving to synthesize mammalian-type antigens so the pathogen can evade detection by the host (Ge et al., 1997; Martin et al., 1997). A FUT involved in synthesis of Nod factors in nitrogen-fixing bacteria was also identified (Quesada et al., 1997; Quinto et al., 1997). None showed high homology to any plant genomic or cDNA sequences in public databases.

Early studies of plant XyG FUT used rapidly elongating regions of etiolated pea epicotyls as a source of enzyme for partial purification (Hanna et al., 1991). Later studies utilized solubilized, carbonate-washed pea microsomal membrane proteins followed by GDP affinity chromatography and size exclusion chromatography (Faik et al., 2000; Perrin et al., 1999). Chapters two through five of this thesis will describe the purification of XyG FUTase from pea, identification of a gene encoding XyG FUTase from *Arabidopsis* and confirmation of its biochemical function, and analysis of the fucosylation of XyG as a model cell wall biosynthetic process.

1.4.2 Organization and Remodeling of the Cell Wall

After biosynthesis and deposition of the plant cell wall, remodeling may take place to affect wall extensibility and thus growth. Two classes of cell wall proteins that are involved in alterations of wall structure will be described briefly: expansins and xyloglucan endotransglycosylases (XETs).

Expansins

It has long been observed that plant cell walls can undergo extension upon exposure to either auxin or acidic solutions, but the mechanism of such extension was not fully understood (Cleland, 1981). It had been noted that isolated cell walls were capable of undergoing acid-induced elongation, but lost this property if the walls were subjected to protein denaturing conditions (Cosgrove, 1998). Thus, it appeared that acid-induced growth was mediated by a proteinaceous component. McQueen-Mason et al. (1992) used

crude protein preparations from cucumber cell walls to determine whether acid-induced extensibility could be restored to boiled cell wall strips, and identified two proteins that showed activity in the assay. Neither protein caused detectable hydrolysis of wall polymers (McQueen-Mason et al., 1992). These proteins were named "expansins".

Cucumber hypocotyl expansin can promote separation of the pure cellulose microfibrils found in filter paper, indicating that expansins can disrupt hydrogen bonds between glucan chains (McQueen-Mason and Cosgrove, 1994). Based on binding studies indicating that expansin acts at the interface of cellulose and one or more hemicellulose, it was suggested that the point of action of expansins dicots is the cellulose-xyloglucan interface, although this speculation has not been confirmed structurally (McQueen-Mason and Cosgrove, 1995). Since expansins cause mechanical alterations in the interaction between polymers but do not exhibit hydrolytic properties, they are not thought to be enzymes in the sense of being able to break or make covalent bonds, but rather are proteins that alter structural properties of biological components to speed up a biological activity (extension) by lowering activation energy of the process (McQueen-Mason, 1995).

Expansins comprise a large gene family in plants and have been divided into two major groups: the α-expansins, which was the first group identified, and the β-expansins, which are more distantly related proteins first observed as major pollen allergens in grass species (Cosgrove et al., 1997). Beta-expansins do have acid growth-promoting activity on monocot cell walls but not dicot cell walls, and α-expansins show higher activity on

dicot cell walls, indicating that each class of expansin may act on different polymers (Cosgrove, 1998; Cosgrove et al., 1997). Recently, even more distantly related proteins have been identified as expansin-related genes and expansin-like genes (Cosgrove, personal communication.) There are 30-40 α- and β–expansin, expansin-like, and expansin-related genes in *Arabidopsis* (http://www.bio.psu.edu/expansins/). Expression analysis has shown that a variety of expression patterns are seen amongst members of the gene family, and some show very specific developmental or tissue-specific patterns of expression (Cho and Cosgrove, 2000). Expansins appear to play a role not only in elongation growth, but also abscission, fruit ripening, leaf initiation, pollen tube growth, and function of specific cell types such as guard cells (Cosgrove, 1997b; Reinhardt et al., 1998). Expansins are plant-specific genes and have been identified throughout the plant kingdom in organisms ranging from ferns to gymnosperms to monocots and dicots (Cho and Kende, 1997; Cosgrove, 2000b; Kim et al., 2000; Shcherban et al., 1995).

Xyloglucan endotransglycosylases (XETs)

XETs are enzymes that cleave XyG chains internally and transfer them to other XyG chains (Fry et al., 1992). The accepting chain may be either a polysaccharide or an oligosaccharide. XETs are specific for XyG and do not cleave other glucans, and thus are distinct from endocellulases (Fry et al., 1992). Because the products of this reaction are the same as the reactants, tracking the activity of XETs has been challenging, although several assays have been developed to do so (Fry et al., 1992; Sulova et al., 1995). Some XET isoforms have been reported to be induced by auxin treatment, and a physiological role for XET has been proposed during fruit ripening and responses to wind

stress (Catala et al., 1997; Purugganan et al., 1997; Schroder et al., 1998). A maize XET has also been reported to be induced by flooding and ethylene, and may be involved in formation of root aerenchyma (Saab and Sachs, 1996). A localized increase in XET activity has also been detected at the sites of root hair formation in *Arabidopsis* (Vissenberg et al., 2001).

XETs have been proposed to play a fundamental role in modulating cell wall extensibility, but application of XET to boiled cell wall preparations showed that it was insufficient to promote elongation under acidic conditions (McQueen-Mason et al., 1993). Therefore, it seems more likely that XETs serve to integrate newly formed XyG into existing chains in the cell wall, perhaps serving to modulate wall properties in the process. Rather extensive biochemical characterization of XETs has been conducted. The enzyme forms a fairly stable glycosyl-enzyme intermediate with polymeric XyG that may be disrupted upon addition of XyG oligosaccharides, an event which has allowed development of simple enzyme purification strategies (Steele and Fry, 1999; Sulova et al., 1998). Like expansins, XETs form large gene families in plant genomes. Substrate preference and catalytic properties have been evaluated for different XET isomers, and it has been suggested that different family members serve different physiological roles (Steele and Fry, 2000; Steele et al., 2001).

1.5 Approaches to Identify and Evaluate Cell Wall Biosynthetic Enzymes and Candidate Genes

It is important to note that among the full complement of polysaccharides present in the plant cell wall, there are hundreds of different linkages. In general, each linkage is thought to be directed by a specific enzyme. Although there have been occasional examples of multifunctional enzymes that can catalyze two different linkages, they are the exception rather than the rule (e.g. Lee and Spicer, 2000; Nguyen et al., 1998). Thus, there are expected to be hundreds of genes in plant genomes that are devoted to the synthesis of the plant cell wall (Varner and Lin, 1989). One recent review estimated that there are over 1,000 genes in the *Arabidopsis* genome that are involved in making, assembling, and remodeling the cell wall and conferring specific properties to it (Roberts, 2001). A database that catalogs known and predicted carbohydrate-active enzymes (http://afmb.cnrs-mrs.fr/~pedro/CAZY/db.html) shows that Arabidopsis has at least 140 Arabidopsis ORFs within families predicted to be involved in polysaccharide and glycoprotein biosynthesis; this estimation does not include the many enzymes that conjugate sugar units to small metabolites such as hormones (Perrin et al., 2001). However, while it is clear that much of the plant genome is devoted to synthesis of the cell wall, it has been challenging to identify these genes and corresponding enzymes with precision and confidence in their functional roles.

Several different approaches have been or may be taken to identify cell wall biosynthetic genes and enzymes. One approach is to purify a cell wall biosynthetic enzyme to relative homogeneity, obtain amino acid sequence from the purified protein, and use this

information to identify the corresponding gene. Ideally, the role of the enzyme encoded by the gene will be confirmed by a functional test. The major advantage of this approach is that, in general, the results can be definitive. However, biochemical purification projects are inherently low-throughput, as each problem must often be approached anew. In addition, cell wall polysaccharide biosynthetic enzymes are frequently labile and lose activity upon manipulation (Perrin et al., 2001). This approach will also not be amenable to enzymes that require multiple interacting partners for activity, unless the entire complex can be co-purified. A suitable enzymatic assay is needed in order to track activity of the enzyme of interest during the purification procedure, and this may be a limiting factor when working with cell wall enzymes. The complicated structures of cell wall components may make it difficult to obtain suitable acceptor substrates of defined structure, and in some cases the sugar nucleotide donor may not be available commercially (Perrin et al., 2001). Despite these limitations, two cell wall biosynthetic genes have been identified using this approach; one is the subject of this thesis. These projects were described in the proceeding section.

A second approach towards identifying cell wall biosynthetic genes is "forward" genetics, so termed because a mutation characterizing a phenotype is identified first and then the gene responsible for the phenotype is identified by positional cloning (that is, the researcher moves from phenotype forward to gene identification.) This approach has been successful for the identification of genes encoding enzymes that are members of functional complexes (e.g. Arioli et al., 1998). Forward genetics approaches require a feasible screening strategy in which mutant phenotypes are identified. Screens are

usually designed to be as labor-free as possible in order to screen large populations of mutagenized plants quickly. However, mutations in essential genes will result in lethal phenotypes, and it may be expected that certain members of the cell wall biosynthetic machinery may be essential. This requires the identification of conditional alleles. Despite these limitations, several screens designed to identify cell wall biosynthetic genes have been conducted, and a number of genes have cloned as a result (Arioli et al., 1998; Reiter et al., 1997; Turner and Somerville, 1997).

Tools have begun to emerge for an additional approach. Functional genomics strategies identify candidate genes as possibly encoding cell wall biosynthetic enzymes. Candidates are identified using bioinformatics and expression profiling techniques, making use of nucleotide and protein databases and information from expressed sequenced tagged (EST) sequencing projects or microarray experiments. Candidate genes are then further studied, often by analyzing the effects of mutations in these genes (in an approach called "reverse genetics", because the researcher moves from gene towards gene function) or by expressing the gene in a heterologous system and assaying the recombinant enzyme for biochemical function. The functional genomics approach requires a baseline of knowledge for execution, and in the case of cell wall biosynthetic genes this foundation is still being established. Knowledge of similar enzymes is helpful, as is an understanding of motifs and protein structural features that are necessary for activity. Likewise, knowledge of expression patterns of related genes is helpful. The advantage of the functional genomics method is that, unlike the other two methods described above, there is potential for high-throughput or moderately-high throughput

approaches. However, there are limitations. In the area of cell wall biosynthesis, the baseline of knowledge is still relatively small, which can make selection of gene candidates more challenging. Reverse genetic approaches can be of limited use if there is insufficient information as to the role of the gene. For example, a candidate gene may be identified as having a UDP-arabinose binding motif, and a mutant is obtained in hopes that the gene will affect synthesis of a particular arabinogalactan. If the mutant shows no alteration in structure for this arabinogalactan, though, the researcher is then faced with having to systematically analyze all structures containing arabinose – and there is the possibility that, although the candidate protein appeared to have a UDP-Ara binding motif, it actually binds to another sugar nucleotide altogether. Additionally, the redundancy that has been observed in several plant genomes (including *Arabidopsis*) can hamper reverse genetic analysis, as mutations affected in all members of the gene family may have to be obtained in order to observe a phenotypic effect. Finally, even the functional genomics approach may require an enzymatic assay for definitive evaluation of a candidate protein, bringing up the limitations associated with assays mentioned above. It is clear that biochemical techniques will continue to be relevant in the "post-genomic" era. Despite these drawbacks, however, the functional genomics approach may be a feasible method for identifying genes devoted to cell wall synthesis – particularly as the foundation of knowledge about these genes and enzymes continues to become more robust.

1.6 Conclusions

Plant protoplasts are encased in cell walls that are indispensable for normal growth, development, and interactions within the plant itself and the rest of its surroundings. The wall provides positional context to the plant body, permits and delimits growth, and mediates communication both within the plant and between the plant and its environment. Plant cell walls are composed of a complement of polysaccharide molecules, structural proteins that may themselves be glycosylated, and phenolic compounds. Cell wall polysaccharide synthesis occurs by action of enzymes such as glycan synthases and glycosyltransferases. A major goal in the field of plant cell wall biology is to identify all genes encoding enzymes devoted to synthesis of cell wall polysaccharides, but thus far only a few have been identified unequivocally. This thesis is focused upon the identification of an enzyme involved in the synthesis of a plant cell wall polysaccharide, xyloglucan fucosyltransferase.

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CHAPTER TWO

Purification and Biochemical Characterization of Pea Xyloglucan Fucosyltransferase

Credits for experiments described in this chapter are as follows:

- Drs. Amy DeRocher and Maor Bar-Peled developed techniques for the purification of pea xyloglucan fucosyltransferase. I analyzed samples that were purified using these techniques by an in-gel assay and two-dimensional SDS-PAGE.
- I assisted Drs. DeRocher and Bar-Peled with scaling up the purification technique to obtain enough protein for amino acid sequencing. Dr. DeRocher and I prepared the protein sample used for amino acid sequence determination. The amino acid sequencing was performed as a paid service by Harvard Microchemistry (Cambridge, MA).
- Methylation linkage analysis of product generated from an assay performed by Dr. DeRocher was performed as a paid service by the Complex Carbohydrate Research Center (Athens, GA).
- I used protein samples prepared by Dr. Bar-Peled to characterize essential amino acid residue types using group-specific modifying agents.

2.1 Introduction

Polysaccharides are a major structural feature of plant cell walls, imparting specific mechanical properties that ultimately affect the ability of the wall to extend or maintain structural integrity. These polysaccharides are often separated into different components thought to represent domains within the wall: matrix polysaccharides, which cross-link to cellulose, and pectins, which form a viscous gel.

Matrix polysaccharides include hemicelluloses that bind tightly but non-covalently to cellulose microfibrils, crosslinking them into a complex network. The hemicellulose xyloglucan (XyG) comprises approximately 20% of the total cell wall in dicot and nongraminaceous monocot plants and forms a load-bearing network by associating to the surfaces of surrounding cellulose microfibrils through hydrogen bonds (Hayashi, 1989; Zablackis et al., 1995). Xyloglucan contains a β-1,4-glucan backbone decorated with side chains of xylose alone, xylose and galactose, and xylose, galactose, and fucose (Figure 1, Chapter 1). The presence or absence of the terminal fucose residue may have structural and biological significance. Some models suggest that the presence or absence of this fucose residue will determine whether the xyloglucan conformation is planar and thus better able to bind to cellulose (Levy et al., 1997; Levy et al., 1991), though contradicting evidence has been described (Whitney et al., 1995). XyG networks may be modified by xyloglucan endotransglycosylase (XET), an enzyme which cleaves and rejoins adjacent XyG chains. A recombinant XET demonstrated different activity rates for fucosylated versus nonfucosylated XyG oligosaccharide acceptors, indicating that fucosylation state may affect XET modification of the cell wall (Purugganan et al., 1997).

In addition, oligosaccharides consisting of an XyG nonasaccharide prevent auxin-promoted elongation of pea stems if these oligosaccharides contain fucose, but not if they lack fucose (Creelman and Mullet, 1997; Fry et al., 1993; McDougall and Fry, 1989; York et al., 1984). Thus, it is possible that XG fragments act as signalling molecules *in vivo*.

Most matrix polysaccharides are branched molecules modified by various sugars. These modifications are important because they allow heterogeneity in the shape of matrix polysaccharides and patterns of cross-links, resulting in a dynamic and porous cell wall. These polysaccharide modifications occur via glycosyltransferase reactions, many of which occur in the Golgi (Colley, 1997; Driouich et al., 1993; Moore et al., 1991; Puhlmann et al., 1994). Attempts to clone plant glycosyltransferases using sequences derived from bacterial or mammalian glycosyltransferases have been unsuccessful (AED, NVR, KK, data not shown.) This is not entirely unexpected, for although Golgi glycosyltransferases often have similar general structural features, they rarely share extensive sequence similarity (Kleene and Berger, 1993).

This chapter describes the biochemical purification of a xyloglucan fucosyltransferase from garden pea (*Pisum sativum*) and analysis of the corresponding protein by an in-gel assay and two-dimensional electrophoresis. Carbohydrate analysis data on the product of the enzyme are presented. Partial amino acid sequence was determined from the purified enzyme, and amino acid modifying agents were used to analyze residue types necessary for biochemical function.

2.2 Materials and Methods

2.2.1 Purification of Pea Xyloglucan Fucosyltransferase

2-cm segments of etiolated *Pisum sativum*, cv Alaska, excised just below the apical hook, were collected and homogenized in 1.5 volumes buffer (50 mM Hepes pH 7.5, 1 mM EDTA pH 8.0, 0.4 M sucrose, 1 mM DTT, 0.1 mM PMSF, 1 μg/mL each aprotinin, leupeptin, and pepstatin). The homogenate was filtered and centrifuged at 2,000 *g* for 15 minutes, and the supernatant was centrifuged at 100,000 *g* for 1 hour. The resulting pellets were washed and homogenized in the presence of 0.1 M Na_2CO_3 to strip away peripheral membrane proteins (Fujiki et al., 1982). The suspension was centrifuged at 100,000 *g* for 1 hour and the resulting pellets were washed and resuspended in buffer (50 mM Pipes-KOH pH 6.2, 20% glycerol, 1 mM EDTA, 1 mM DTT, 0.1 M PMSF, 1 μg/mL each aprotinin, leupeptin, and pepstatin). The suspension was homogenized, Triton X-100 was added to a final volume of 0.8%, and the sample was stirred for 1-2 hours to solubilize membrane proteins before centrifugation a final time at 100,000 *g* for 1 hour. Supernatant was collected and saved. Pea carbonate-washed supernatants were pooled and separated on a GDP-HA agarose affinity chromatography column and GDP-binding proteins were eluted using excess free GDP. Protein levels were monitored by A_{280}. The protein samples were desalted on a Sephadex G-25 column, concentrated, and further separated on a Phenomenex SEC 4000 size exclusion column. Some samples were further purified using a Poros QE or Resource Q anion exchange column and subsequently separated by SDS-PAGE electrophoresis.

2.2.2. Activity Assays

Samples were incubated at room temperature for the specified time (30 min in most cases) with 25 mM Pipes-KOH pH 6.2, 0.5 mg/mL tamarind xyloglucan, and GDP-[^{3}H]-fucose to a final concentration of 3.3 nM (573 GBq/mmol, NEN, Boston, MA). Most assays also contained 50 μM non-radiolabeled GDP-fucose. Reactions were precipitated using 70% ethanol and ^{3}H incorporation was measured by scintillation counting. The amount of fucose incorporated into the product was used to calculate activity in nanokats (nKat = nMoles substrate incorporated into product per second).

2.2.3 In-gel Assay and Two-Dimensional Electrophoresis

Anion exchange purified samples were loaded into 2 lanes of a nondenaturing polyacrylamide gel that had 0.1 mg / ml tamarind xyloglucan and 0.1% decylmaltopyranoside incorporated into the gel matrix. The gel was run at 4°C. The two lanes were excised from the gel. One was equilibrated in 25 mM Pipes KOH, pH 6.2 and incubated overnight in 25 mM Pipes KOH, pH 6.2 with 0.1% ^{14}C GDP fucose (NEN, 10.5 GBq/mMole, 0.37 MBq/ml). Unincorporated ^{14}C was washed out of the gel, leaving only the ^{14}C fucose that had been incorporated into the xyloglucan. The gel was then fixed, enhanced, and fluorographed. The other gel strip was equilibrated in 2% SDS, 60 mM Tris, pH 8.0, and 2% beta mercaptoethanol, overlaid on a 10 % acrylamide SDS gel, and proteins in the gel were separated by electrophoresis. Protein in the gel was visualized by silver staining. Proteins that co-migrated with the fucosyltransferase activity were identified by aligning the axes of the fluorograph and the stained two-dimensional gel and normalizing for gel shrinkage and expansion.

2.2.4 Carbohydrate Methylation Linkage Analysis

Tamarind seed xyloglucan was fucosylated by purified pea FUTase equal to 33 pKat total activity (assay conditions: 1 mg/mL tamarind XyG, 1.5 mM GDP-fucose, 50 mM Pipes-KOH pH 6.2). XyG product was precipitated with ethanol, re-suspended in water, re-precipitated, and sent to the Complex Carbohydrate Research Center (Athens, GA) for linkage analysis. An equal amount of tamarind xyloglucan was also submitted for linkage analysis.

2.2.5 Amino Acid Sequencing

Proteins in size exclusion column eluate fractions containing peak amounts of fucosyltransferase (FUTase) activity were concentrated using a Millipore 4-mL 10-kDa concentrator and separated by electrophoresis. After brief staining with Coomassie Brilliant Blue and destaining, the separated proteins were excised, rinsed in 50% acetonitrile, stored at –80° C and sent to Harvard Microchemistry (Cambridge, MA) for tryptic peptide sequencing.

2.2.6 Amino Acid Modification

Purified enzyme was subjected to amino acid group-specific modification. The following reagents were used: N-ethylmaleimide (NEM), diethylpyrocarbonate (DEPC), pyridoxal 5'-phosphate (PLP), and N-bromosuccinimide (NBS). Modifying reagents were used according to the methods described by Britten and Bird (Britten and Bird, 1997). Standard assays were conducted as described above in the presence of up to 5 mM of each

modifying reagent using GDP-[^{3}H]Fuc. The reactions were halted by adding 67% ethanol and washed as described above before assessing label incorporation. Controls were conducted in the absence of these modifying reagents.

2.3 Results

2.3.1 Purification of Pea Xyloglucan Fucosyltransferase

The terminal fucosyl residue on XyG side chains is added by a fucosyltransferase (FUTase). We purified enough of this FUTase from pea epicotyls to determine partial amino acid sequences from the enzyme. Microsomes were prepared from the pea epicotyls, carbonate-washed to enrich for membrane proteins, and solubilized with nonionic detergent such as Triton X-100. A specific assay for this enzyme had previously been developed using tamarind seed storage xyloglucan, which lacks fucosyl residues, as an acceptor molecule and radiolabeled GDP-fucose as a donor (Figure 3) (Camirand et al., 1987; Farkas and Maclachlan, 1988). GDP-agarose affinity chromatography, size exclusion chromatography, and anion exchange chromatography were used in conjunction with FUTase activity assays to purify and detect the enzyme (Figure 4). The fucosyltransferase activity co-eluted with 60-kDa and 65-kDa proteins (Figure 4B). Other proteins are also present in various fractions, but their elution profile did not match the profile of the enzyme activity. It was possible to purify XyG FUTase 1400-fold after size-exclusion chromatography, resulting in a total of 50 μg protein containing 70 nKat XyG FUTase activity. Analysis of the purification data indicates that

XyG FUTase is present at very low levels in the cells (less than 0.01% of total protein) (Faik et al., 2000).

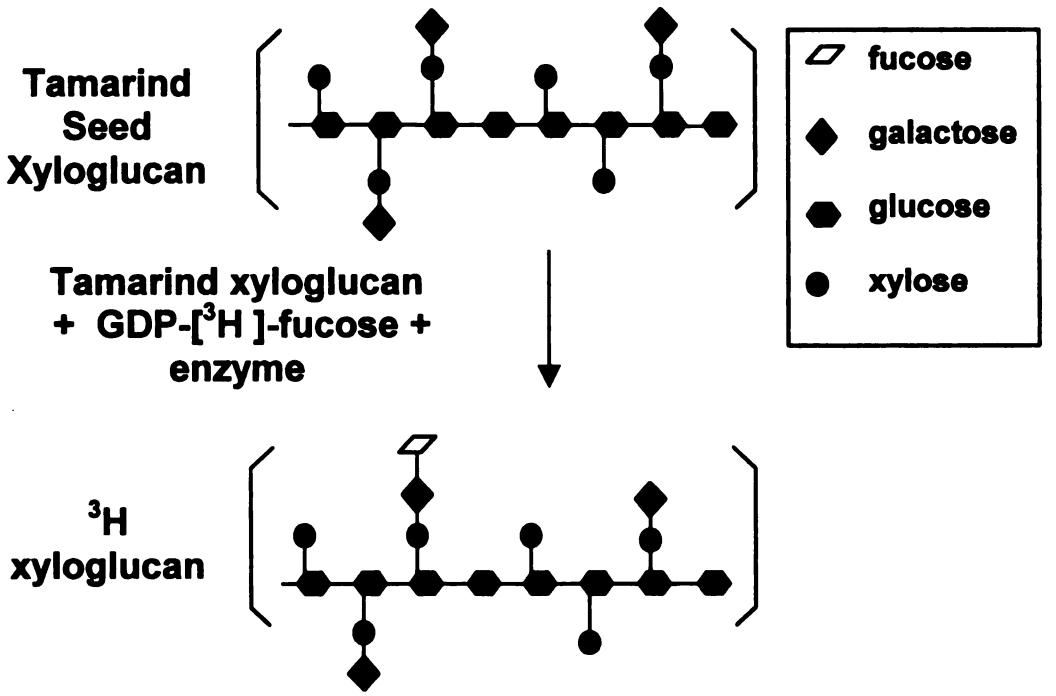

Figure 3. Xyloglucan fucosyltransferase activity assay. Nonfucosylated forms of XyG accumulate as seed storage polysaccharides in a few species, notably tamarind and nasturtium. Tamarind seed storage XyG (top) is available commercially, and is incubated with GDP-[^{3}H]-fucose and enzyme during a XyG FUTase activity assay. The product of the reaction is precipitated with 66% ethanol (bottom) and the amount of radiolabel incorporated is quantified by scintillation counting (Camirand et al., 1987; Farkas and Maclachlan, 1988; Perrin et al., 1999).

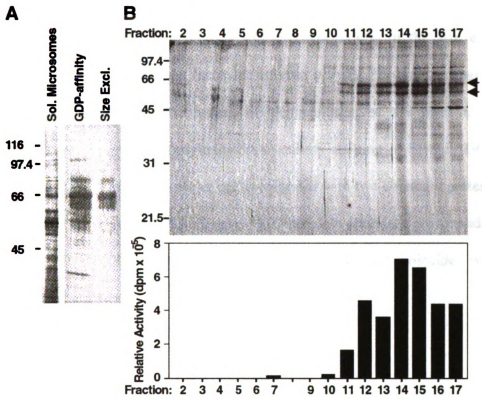

Figure 4. Biochemical purification of XyG FUTase from pea. A. Silver-stained SDS-PAGE gels showing protein profiles from 1) carbonate-washed, detergent-solubilized microsomes from pea epicotyls (Sol. Microsomes), or the fractions containing peaks of FUTase activity from 2) a GDP-agarose affinity column (GDP-affinity) and 3) a size exclusion column (Size Excl.). Arrows indicate 60 and 65 kDa polypetides (see text).**B.** Top: Silver-stained SDS-PAGE gel showing the protein profile from several fractions of an anion-exchange column. Bottom: Total XyG FUTase activity in dpm x 10^{-5} for each fraction of the anion exchange column eluate. Note that bars align with the corresponding SDS-PAGE profile above.

2.3.2 Analysis of Purified Pea Xyloglucan Fucosyltransferase Activity

A second method was used to determine which polypeptide(s) in the size exclusion column eluate co-migrated with the fucosyltransferase activity. For two-dimensional gel analysis, nondenaturing gel electrophoresis of identical samples was used in the first dimension followed by either fucosyltransferase activity staining or SDS-PAGE in the second dimension. Activity in the native gel co-migrated with two abundant proteins with molecular masses of 60 and 65 kDa (Figure 5). Other, less abundant polypeptides were also detected on the silver-stained gel, but they did not coincide with the fucosyltransferase activity.

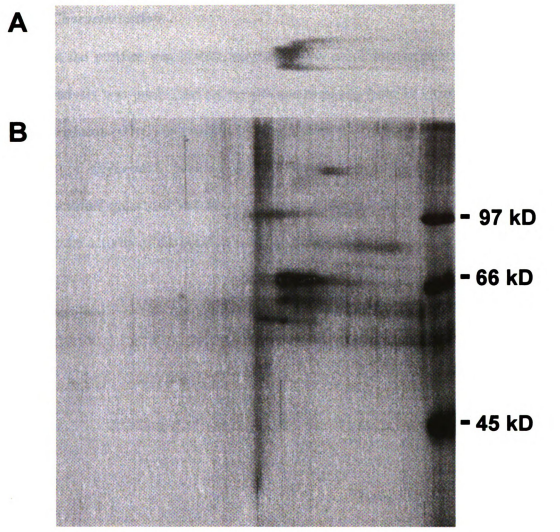

Figure 5. Identification of proteins that co-migrate with fucosyltransferase activity. Duplicate samples of anion exchange-purified sample were separated by native page with tamarind xyloglucan incorporated into the gel matrix. Fucosyltransferase activity in one lane was visualized by incorporation of ^{14}C labeled fucose into the xyloglucan, followed by fluorography (A). The protein profile of the other lane was analyzed by 2-D gel analysis using SDS PAGE and silver staining (B). The native gel dimensions of panels A and B were aligned to identify proteins that co-migrate with fucosyltransferase activity.

2.3.3 Product Characterization

To confirm that the purified pea protein synthesizes an α-1,2 fucose:galactose linkage, carbohydrate analysis was performed on the product resulting from in vitro fucosylation of tamarind xyloglucan (tXyG) by purified FUTase (Table 2). Linkage analysis indicated that incubation of tXyG with purified FUTase resulted in a decrease in the mole percentage of terminal galactose and the appearance of 2-galactose and terminal fucose, thus confirming the activity of the purified enzyme (Table 2).

Table 2. Product methylation linkage analysis. Carbohydrate linkage analysis of tamarind xyloglucan before (tamarind XyG) and after (fucosylated XyG) incubation with purified pea FUTase.

Sugar Residue	% tamarind XyG	% fucosylated XyG
4-Glucose	16.4	17.5
4,6-Glucose	37.0	31.5
terminal Xylose	19.0	13.5
2-Xylose	15.0	14.3
terminal Galactose	12.6	5.5
2-Galactose	-	9.0
terminal Fucose	-	8.7

2.3.4 Determination of Peptide Amino Acid Sequence

After biochemical purification and subsequent analysis, two polypeptides of approximately 65 kD and 60 kD in size were observed to co-purify with XyG FUTase activity (Figure 4). Limited peptide sequence was obtained from both proteins. The 65-kDa peptide was identified as a homolog of BiP, a molecular chaperone usually localized in the ER. It remains unclear whether copurification of BiP with FUTase represents a significant interaction. Six peptide sequences were obtained for the approximately 60-kDa protein: VFGFLGR, YLLHPTNNVWGLVVR, AVLITSLSSGYFEK, YYDAYLAK, LLGGLLADGFDEK, and ESILPDVNR. These peptides were not highly homologous to any proteins of known function in public databases, but an *Arabidopsis thaliana* expressed sequence tag (EST) entry was identified that encodes a homolog of the purified pea protein (described in chapter 3.)

2.3.5 Characterization of Amino Acid Residue Types Essential for Activity

To gain some insight into the structure-function relationship in XyG-FUTase, amino acid residue types essential for catalytic activity were identified. NEM had relatively little effect on the XyG-FUTase activity, with 5 mM giving ~40% inhibition of activity (Table 3). However, *N*-bromosuccinimide (NBS), DEPC, and PLP reagents (which react with tryptophan, histidine and lysine residues, respectively) decreased the activity by about 90-99% when applied at levels of up to 5 mM (Table 3). Preincubation of the XyG-FUTase with GDP-Fuc did not prevent inactivation by NBS treatment (data not shown).

Table 3. Effect of group-specific reagents on purified pea XyG-FUTase activity. The purified enzyme (~0.2 μg) was pre-incubated with the indicated modifying reagent for 20 min and assayed for [^{3}H]-fucose transfer to tamarind XyG under standard assay conditions. Values are expressed as % inactivation. Shown are results from one experiment that was repeated three times.

		Inactivation (%)		
Reagent	Acts on	0.5 mM	2.5 mM	5 mM
NEM	Cysteine	5.9	34.4	41.5
PLP	Lysine	51.4	81.0	87.9
DEPC	Histidine	63.5	88.5	95.1
NBS	Tryptophan	96.0	96.3	96.4

2.4 Discussion

Elucidation of plant cell wall biosynthesis has been a slow process. Despite decades of work by organic chemists which have helped describe the polysaccharide and protein components of the cell wall, and despite the fundamental importance of plant cell walls in agriculture, nutrition, materials science, textiles, and as mediators of plant growth in general, only one gene involved in cell wall biosynthesis (*CesA* , the catalytic subunit of cellulose synthase) had been cloned at the time that this study was initiated (Arioli et al., 1998; Pear et al., 1996). None of the glycosyltransferases that synthesize non-cellulosic cell wall polysaccharides or any of the carbohydrate moieties of plant cell wall glycoproteins had been identified at the molecular level (Brett and Waldron, 1996). In part, this was because these integral membrane proteins have been recalcitrant to purification; many efforts to purify them have resulted in loss of activity such that it became impossible to follow their purification through subsequent steps (Reiter, 1998). Genetic approaches have been attempted, but have both advantages and limitations for identification of cell wall biosynthetic enzymes (for a review, see Chapter 1.) The growing number of glycosyltransferase sequences from other organisms involved in protein glycosylation, bacterial Nod factor synthesis, or bacterial lipopolysaccharide synthesis available in the databases at the time had not yet assisted plant researchers in identifying plant glycosyltransferases by homology alone. This indicated that plant glycosyltransferases might have primary structures rather unique from glycosyltransferases in other organisms.

Although most attempts to purify glycosyltransferases and solubilize them have resulted in a loss of activity, this was not the case with a XyG FUTase. Maclachlan's group had shown that it was possible to purify this enzyme from pea epicotyls and assay activity of solubilized microsomes using tamarind or nasturtium seed xyloglucan (Farkas and Maclachlan, 1988; Hanna et al., 1991; Maclachlan et al., 1992). In seeds of some species such as tamarind or nasturtium, xyloglucan is used as a storage polysaccharide. Storage XyG lacks the terminal fucose residue and may be used in an assay as an acceptor along with radiolabeled GDP-fucose as a sugar nucleotide donor. Purification techniques and the assay were used to obtain enough enzyme to determine limited amino acid sequence data. The purification scheme involved the preparation of detergent-solubilized microsomes from pea epicotyls (enriched in integral membrane proteins during a sodium carbonate wash step), GDP agarose affinity chromatography, and size exclusion chromatography. Anion exchange chromatography was also used as a further purification step in some experiments, though it was not necessary for obtaining sufficiently pure protein for amino acid analysis. By analyzing the xyloglucan-specific fucosyltransferase activity of the chromatographic fractions, we observed two polypeptides that were found consistently in the most active fractions (Figure 4). Sequencing tryptic peptides from these proteins indicated that the larger polypeptide, approximately 65 kDa in size, is highly homologous to BiP, the ER-localized molecular chaperone in the hsp70 family. Whether this was co-purified due to artifactual or physiological reasons is unknown, though it is interesting to consider that the enzyme was subjected to harsh treatment during the purification procedure (for example, exposure to a pH of 11 for over an hour during the carbonate wash) and association with

a chaperone protein could have prevented denaturation and thus loss of activity. Peptides derived from the second (~ 60 kDa) polypeptide were not homologous to any known proteins in the databases but did allow us to identify an Arabidopsis Expressed Sequence Tagged (EST) clone which contained these peptide sequences with 63%-86% conservation of identity (described in Chapters 3 and 4). Gel filtration and SDS-PAGE data indicated that the native form of purified pea XyG-FUTase exists as an oligomer of approximately 250 kDa.

Isopycnic centrifugation had previously provided evidence that the XyG-FUTase is present in both a dense Golgi fraction and in vesicles from the *trans*-Golgi network (Brummell et al., 1990). Later studies using antibodies to the fucosylated region of XyG determined that the fucose-containing product is observed in the *trans*-Golgi cisternae and in the *trans*-Golgi network (Zhang and Staehelin, 1992). Taken together, these studies provide evidence that fucosylation occurs after XyG backbone biosynthesis has been completed. Thus, the purified pea XyG FUTase would be expected to be an integral membrane protein localized to the Golgi. *In vitro* transcription and translation of the *Arabidopsis* XyG FUTase in the presence of canine pancreatic microsomes indicated that it is an integral membrane protein (R. Perrin, data presented in Chapter 3) and localization of this enzyme to the Golgi has been confirmed (R. Sarria, unpublished data.)

Several structure-function studies on FUTase enzymes in other systems have been initiated by first examining the effects of various amino acid modifying reagents on enzyme activity and substrate binding, later followed by point mutagenesis, deletion, or

domain swapping experiments (for a review, see de Vries et al., 2001). As a first approach towards identifying amino acid residue types that are critically important for catalytic activity, activity assays with purified pea XyG FUTase were conducted in the presence of a series of amino acid modifying reagents (Table 3). The sulfhydryl reagent NEM, a cysteine-specific modifying agent, has been widely used in discriminating between glycoprotein fucosyltransferase isotypes (de Vries et al., 1995). Pea XyG FUTase is not NEM-sensitive, indicating that Cys residues may not be essential for substrate binding or catalysis. Similar studies on human α-(1,3) fucosyltransferases coupled with site-directed mutagenesis and amino acid sequence alignments of various isoforms have led other researchers to conclude that FUTases that are NEM-insensitive contain either a Ser or Thr residue in place of conserved Cys residues in the GDP-Fuc binding domain (de Vries et al., 2001; Holmes et al., 1995).

Purified pea XyG FUTase was extremely sensitive to modification of Trp residues (Table 3). There are eight Trp residues present in pea XyG FUTase, six of which (75% of the total) are found in the C-terminal half of the protein. In fact, five Trp residues (62.5% of the total) are within the most C-terminal quarter of the protein. The general structure of glycosyltransferases, including fucosyltransferases, consists of a variable N-terminal region (a short cytoplasmic fragment, the transmembrane domain, and a extended stem region located in the lumen of the Golgi), followed by a more conserved globular catalytic region at the carboxy-terminal portion of the protein (Breton et al., 1998). The catalytic region is also within the lumen of the Golgi. It appears that the distribution of Trp residues in pea XyG FUTase shows bias towards the region of the protein expected

to constitute the catalytic domain. A similar distribution of Trp residues is found in the *Arabidopsis* XyG FUTase sequence, though the *Arabidopsis* protein has an additional Trp in the middle region. Because incubation of pea XyG FUTase with GDP-Fuc before exposure to NBS does not prevent inhibition of activity (data not shown), it is likely that the critical NBS-sensitive Trp residue(s) is involved in binding to XyG or, alternatively, is required to assume a catalytically competent structural conformation. It is interesting to note that Trp residues have previously been noted to be critical for determining acceptor substrate specificity in other FUTases (Dupuy et al., 1999; Elmgren et al., 1997). In one study, a conserved Trp residue in the variable stem region of vertebrate FUTases determined acceptor specificity (Dupuy et al., 1999). Changing this Trp to an Arg in the enzyme encoded by the *FUT3* gene was sufficient to shift acceptor specificity from Lewis H-type 1 to Lewis H-type 2 acceptors (Dupuy et al., 1999).

In addition, pea XyG FUTase was moderately sensitive to chemical modification of Lys or His residues (Table 3). There are 14 His residues in this protein, and the distribution is biased towards the middle and C-terminal regions of the protein. By contrast, the 33 Lys residues are distributed evenly throughout the protein. More studies using site-directed mutagenesis are needed to determine the locations and the roles of all of these essential residues.

Other biochemical characterization of the purified pea XyG FUTase has been conducted and published (Faik et al., 2000). This characterization included kinetic analysis of enzymatic activity, determination of pH optimum, requirement for divalent cations and

inhibition of activity by the byproduct GDP (data generated by M. Bar-Peled and A. Faik.) Substrate specificity assessment indicated that the enzyme is specific for XyG and does not transfer fucose to other fucose-containing cell wall or glycoprotein components (data generated by A. Faik.) This study also reported the identification of a pea cDNA sequence encoding this enzyme (data generated by W. Zeng.)

The purification of this enzyme allowed a detailed study of the biochemical characteristics of XyG fucosylation. It also allowed the identification of both the gene encoding pea XyG FUTase (W. Zeng) and the gene encoding *Arabidopsis* XyG FUTase (R. Perrin, described in Chapters 3 and 4.) This availability of the purified pea enzyme and the pea and *Arabidopsis* genes potentially enables the identification of other plant-specific glycosyltransferases. Hundreds to thousands of different genes are needed to synthesize the various polysaccharides that compose the cell wall. Substrate acceptors and assays remain unavailable for many of these enzymes. Identification of other carbohydrate transferases, perhaps by sequence similarity, could lead to tailored *in vitro* production of carbohydrates, as well as an understanding of how the complex plant cell wall is biosynthesized.

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CHAPTER THREE

Identification and Functional Analysis of an *Arabidopsis thaliana* Gene Encoding a Xyloglucan Fucosyltransferase

Credits for the work presented in this chapter are as follows:

- The pea xyloglucan fucosyltransferase peptide sequence that allowed the identification of an *Arabidopsis* EST was determined by joint efforts of Drs. Amy DeRocher, Maor Bar-Peled, and myself as described in Chapter 2 of this thesis.
- I used this *Arabidopsis* EST as a probe for RNA gel analysis.
- I obtained a full-length cDNA clone for *AtFUT1* by library screening.
- I performed *in vitro* transcription and translation experiments with this cDNA clone to demonstrate that it encodes an integral membrane protein.
- Weiqing Zeng prepared antibodies that recognize a His_6-tagged recombinant version of AtFUT1. I used these antibodies to immunoprecipitate xyloglucan fucosyltransferase activity from solubilized *Arabidopsis* microsomes.
- Dr. Ariel Orellena and Lorena Norambuena used the *AtFUT1* cDNA clone for heterologous expression experiments in Cos-7 cells.

3.1 Introduction

The previous chapter described the purification of a pea xyloglucan fucosyltransferase (XyG FUTase) and identification of limited amino acid sequences from this protein. Although pea is an advantageous model system for biochemical analyses because of the biomass that can be generated in a relatively short period of time, *Arabidopsis* is the organism of choice for plant genetic and molecular biological analysis. The complete sequence of the *Arabidopsis* genome was determined during the course of this research project (Arabidopsis Genome Initiative, 2000), and over 113,000 expressed sequence tagged (ESTs) have been deposited in public databases as of September, 2001. These ESTs are a valuable resource for identification of expressed genes, and the number of ESTs now available from various tissues and plant treatments also allows researchers to determine gene expression patterns "in silico".

The amino acid sequence data described in the previous chapter from purified pea XyG FUTase allowed us to identify an *Arabidopsis* EST that encoded peptides with a high degree of similarity to the pea protein. This, in turn, allowed the identification of a full-length clone of the *Arabidopsis* gene by cDNA library screening. Sequence analysis of this clone, originally named *AtFT1* and later re-named *AtFUT1*, indicated that it contained features expected of a Golgi-localized glycosyltransferase. Attempts to use this clone to produce active enzyme by *in vitro* transcription and translation were not successful, but translation in the presence of canine pancreatic microsomes indicated that the translation product is an integral membrane protein. Two methods were used to confirm the function of AtFUT1 as a xyloglucan fucosyltransferase. In the first method,

antibodies to His_6-AtFUT1 were used to immunoprecipitate XyG FUTase activity from solubilized *Arabidopsis* microsomal proteins. In the second case, *AtFUT1* cDNA was expressed in mammalian Cos-7 cells, conferring XyG FUTase activity to these cells.

3.2 Materials and Methods

3.2.1 RNA Gel Blot Analysis

Northern analysis was performed using standard techniques (Sambrook et al., 1989). The 858-nt insert of EST 191A6T6 was labeled with ^{32}P-dATP in a random prime labeling reaction (Sambrook et al., 1989). Twenty micrograms of total RNA isolated from *Arabidopsis* 7-day-old seedlings, organs of mature plants (flowers, leaves, roots, stems), and cell culture was subjected to electrophoresis in a 1% agarose formaldehyde gel. RNA samples were transferred to Hybond N+ membrane (Amersham Pharmacia Biotech, Pisscataway, NJ) using capillary transfer in 10X SSC (sodium chloride/sodium citrate) buffer. RNA was cross-linked to the blot by UV illumination. The blot was hybridized at 50° C for nine hours. The probe was denatured by adding 1/10 volume 3 M NaOH and was added to the blot. Hybridization occurred at 50° C for 13 hours. The blot was washed twice with 2X SSC/0.1% SDS (50° C, 20 minutes each), once with 2X SSC/0.1% SDS (45° C, 30 minutes), once with 0.1X SSC/0.1% SDS (45° C, 30 minutes), and a final time with 0.1X SSC/0.1% SDS (50° C, 30 minutes.) Autoradiography film was exposed to the blot for 40.5 h.

3.2.2 Library Screening

191A6T7 was labeled with digoxigenin using a DNA labeling and detection kit (C/N 1093657, Boehringer Mannheim, Basel, Switzerland) and used as a probe to screen the CD4-15 portion of a size-fractionated Arabidopsis cDNA library (Kieber et al., 1993) at high stringency. The library was titred and subjected to primary, secondary and tertiary screening. *In vivo* excision was performed to isolate positive clones.

3.2.3 In Vitro Transcription and Translation

In vitro transcription was performed using a full-length cDNA AtFUT1 clone in pBluescript KS^- vector. Commercial reagents were obtained from Promega (Madison, WI.) The transcription reaction included two micrograms of linearized AtFUT1 cDNA, 1X *in vitro* transcription buffer, 0.5 mM rNTP (final concentration, minus rGTP), 1/10 volume methylated cap, 10 mM DTT, 0.05 mg/ml BSA, 100 units RNasin and 25-50 units T3 polymerase. The reaction was incubated for 10 minutes at 37° C, GTP was added to a concentration of 0.5 mM, and the reaction continued for another 90 minutes at 37° C. An aliquot of the reaction was analyzed on an agarose gel stained with ethidium bromide. *In vitro* translation reaction compositions were as recommended by the manufacturer (Promega, Madison, WI) and included rabbit reticulocyte lysate, RNasin, canine pancreatic microsomes, amino acid mix minus methionine, and ^{35}S-Met. In addition to AtFUT1, beta-lactamase mRNA (provided by manufacturer) was used as a positive control for translation and a reaction with DEPC-treated ddH_2O was used as a negative control to determine background levels of translation in this system. The reactions were incubated at 30° C for 1 hour. After incubation, translation reactions were

recovered by centrifugation through a 1 M sucrose cushion in 50 mM Pipes-KOH pH 6.2. AtFUT1 samples were divided into an aliquot that received no further treatment ("total") and an aliquot that was washed with 0.1 M sodium carbonate on ice for thirty minutes with homogenization. The carbonate-washed AtFUT1 sample was centrifuged at 100,000 g for 1 hour. The protein was precipitated with TCA from the supernatent. The pellet was analyzed directly. All samples were subjected to electrophoresis on a 10% SDS-PAGE gel.

3.2.4 Activity Assays

Activity assays were conducted as described previously (see Chapter 2.)

3.2.5 Antibody Preparation

The portion of *AtFT1* encoding amino acids 73 to 566 was PCR-amplified using appropriate primers and cloned into the pET28a expression vector (Novagen, Madison WI). The resulting insoluble fusion protein was purified by washing inclusion bodies four times with 1% Triton X-100, 50 mM Hepes-KOH pH 7.6, 10 mM $MgCl_2$ and one time with 25 mM Hepes-KOH pH 7.0, 8 M urea. The pellet was resuspended in 6 M guanidine-HCl and protein was precipitated from the supernatant with 10% TCA. The protein was emulsified with Titermax adjuvent (CytRx Corporation, Norcross, GA) and injected into a rabbit (Cocalico Biologicals, Reamstown, PA).

3.2.6 Immunoprecipitation

For immunoprecipitation reactions, solid NaCl was added to carbonate-washed solubilized Arabidopsis protein to a final concentration of 200 mM. The Arabidopsis protein was pre-cleared by incubation with 1/10 volume of 50% slurry of protein A-Sepharose beads (Pharmacia) in buffer A (25 mM Pipes-KOH pH 7.5, 50 mM NaCl, 2 mM EDTA pH 8.0). The resulting supernatants were incubated with 50 µl of immune or pre-immune anti-AtFT1 serum for 1 hour. 1/5 volume of protein A-Sepharose slurry was added to precipitate the antigen-antibody complexes and the samples were incubated for an additional 3 hours with rocking at 4° C. Samples were then centrifuged, washed five times in buffer A containing 1% Triton X-100 and two times in buffer A without detergent. The pellets were resuspended in buffer A to a final volume of 120 µl and assayed for AtFUTase activity.

3.2.7 Heterologous Expression

Cos-7 cells were grown on 100-mm plates in DMEM-10% fetal bovine serum. Cells were transfected with different plasmids using Lipofectamine™ reagent (Life Technologies) following the manufacturer's instructions using 9 µg of DNA and 72 µg of Lipofectamine. Cells were incubated for 24 hours in the medium containing DNA-Lipofectamine without fetal bovine serum. The medium was changed to DMEM-10% fetal bovine serum and incubated for another 48 hours. The cells were scraped off the dish in 0.25 M sucrose, 10 mM Tris-HCl pH 7.5 and 0.4% CHAPS. XyG-FUTase activity was measured using 50 µg of protein in the absence (-XG) or presence (+XG) of 100 µg tamarind xyloglucan. The incubation was carried out in a volume of 0.1 mL in

the presence of 1 μM GDP-Fuc (93,000 dpm), 10 mM $MnCl_2$, 20 mM Hepes pH 7.0, 0.05% Triton X-100 at 25°C for 90 min. The reaction was halted by addition of ethanol to a final concentration of 70%. Samples were incubated at 4°C and filtered through 1.5-μm glass fiber filters. The filters were washed with 70% ethanol containing 1 mM EDTA. The filters were dried and radioactivity determined by liquid scintillation. A biological control using pea Golgi vesicles was carried out in parallel.

3.3 Results

3.3.1 Cloning and Analysis of Arabidopsis Xyloglucan Fucosyltransferase

The previous chapter of this thesis described the purification of XyG FUTase from pea and the sequence of several peptides from this purified protein. Peptides derived from this approximately 60 kDa pea polypeptide were not homologous to any known proteins in the databases but did allow us to identify an Arabidopsis Expressed Sequence Tagged (EST) clone which contained these peptide sequences with 63%-86% conservation of identity (Table 4). A RNA gel blot performed with the EST 191A6T7 as a probe (858 bp in length) indicated a transcript approximately 2.0 kb long, from which we concluded that the EST was not a full-length cDNA clone (Figure 6).

Table 4. Comparison of pea xyloglucan fucosytransferase peptides and corresponding *Arabidopsis* peptides.

Pea Peptides	Arabidopsis Peptide	% Identity
VFGFLGR	VFHHLGR	86%
YLLHPTNNVWGLVVR	YLFHPTNQVWGLVTR	80%
AVLITSLSSGYFEK	AVLVTSLNAGYAEN	64%
YYDAYLAK	YYEAYLSH	63%
LLGGLLADGFDEK	LLGGLLASGFDED	85%

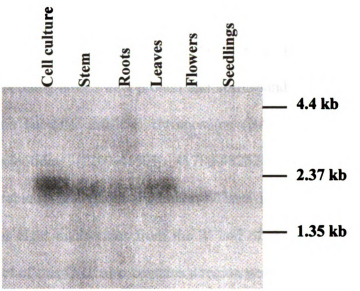

Figure 6. RNA gel blot using an EST for *AtFUT1* as a probe. RNA from *Arabidopsis* cell suspension culture, stem, roots, leaves, flowers, or 7-day old seedlings was used for Northern blot analysis with radiolabeled *AtFUT1* as a probe.

The EST was used as a probe to screen an Arabidopsis cDNA library. Two cDNA clones were isolated, the longest containing a 1768-bp insert. Both lacked 13 nucleotides of the 3' UTR and the poly-A tail found in 191A6T7. The lack of the poly-A tail and part of the 3' UTR was not surprising, as inserts from the library used for screening had been described as beginning 8-10 nucleotides from the polyA-tail due to Klenow 3'-5' exonuclease activity during library construction. The cDNA clones contain a 1677-nt open reading frame encoding a 63.5 kDa protein and correspond to a region of the fully sequenced Arabidopsis bacterial artificial chromosome (BAC) T18E12 derived from chromosome II (nucleotides 41209-41503, 41780-43252), which has been fully sequenced by the Arabidopsis Genome Initiative. There is an AATAAA consensus polyadenylation signal eight nucleotides from the 3' end of the library-derived clones. The translation product of this ORF also contains a region near the N-terminus similar to a fifth pea peptide (see Chapter 2).

The cDNA and corresponding genomic clone were originally designated *AtFT1*; however, the name was changed to *AtFUT1* in July, 2001, to avoid consfusion with an *Arabidopsis* gene named *Flowering Locus T* (*FT*) or *Arabidopsis* farnesyltransferases (also abbreviated FT) (Kardailsky et al., 1999; Yalovsky et al., 2000). Analysis of the BAC indicates that there may be a second glycosyltransferase approximately 300 bp downstream from *AtFT1* that is 63% similar to *AtFT1* at the amino acid level. In addition to this gene, named *AtFUT2*, eight other *AtFUT1*-like genes have been identified (described in Sarria et al., 2001.) Thus, *Arabidopsis* may have a family of FUTases,

each differentially regulated by such factors as environmental stress, tissue localization, or developmental stage, or specific to different acceptors (Sarria et al., 2001, in press).

The protein encoded by *AtFUT1* is not significantly homologous to other proteins in the databases, though low (40-60% over short regions) similarity to NodZ is observed. NodZ is a bacterial alpha 1,6-fucosyltransferase involved in the synthesis of nodulation factors in *Rhizobium* (Quesada Vincens et al., 1997). A motif which is found in all alpha-1,2 and alpha-1,6 fucosyltransferases described to date is also present in the putative *Arabidopsis* fucosyltransferase (Table 5) (Breton et al., 1998). Subsequently, comparison to other FUTases demonstrated that AtFUT1 contains a motif that was previously thought to discriminate between alpha-1,2 and alpha-1,6 FUTases (analysis conducted by C. Wilkerson and A. Faik) (Faik et al., 2000). A Kyte-Doolittle hydrophobicity plot of AtFUT1 indicated that this protein contains a short hydrophilic extreme N-terminus followed by a hydrophobic region and a largely hydrophilic C-terminus (Figure 7).

Table 5. Comparison of motifs found in alpha-1,2 and alpha-1,6 fucosyltransferases. (Motif described in Breton et al., 1998).

Protein	**Motif Sequence**
Pig α–(1,6)–FucT	IGVHVRRTD
Hum α–(1,6)-FucT	IGVHVRRTD
Azorhizobium caulinodans NodZ (α-1,6)	IGVHIRFGN
Bradyrhizobium japonicum NodZ (α-1,6)	IGIHVRHGN
Yersinia enterocolitica α–(1,2) FucT	VGIHIRRGD
Rabbit Se-III (α–(1,2) FucT)	VGVHVRRGD
Rabbit Se-II (α–(1,2) FucT)	VGVHVRRGD
Mouse Sec1 (α–(1,2) FucT)	VGVHVRRGD
Pig H (α–(1,2) FucT)	VGVHVRRGD
Mouse H (α–(1,2) FucT)	VGVHVRRGD
Human Se (α–(1,2) FucT)	VGVHVRRGD
Rabbit H (α–(1,2) FucT)	VGVHVRRGD
Human H (α–(1,2) FucT)	VGVHVRRGD
Consensus	(V/I)G(V/I)H(V/I)Rr(G/T)(D/N)
AtFUT1	**IGIQVRVFD**

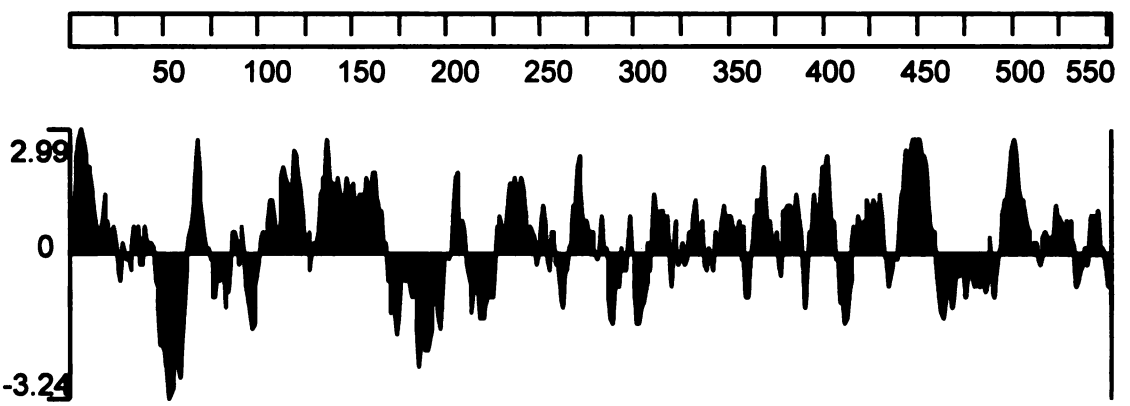

Figure 7. Kyte-Doolittle hydrophobicity plot of AtFUT1. Plot was generated using the Protean component of the DNA* software package. Positive values are hydrophilic, negative values are hydrophobic.

3.3.2 In vitro Transcription and Translation

The *AtFUT1* cDNA clone was used for *in vitro* transcription and translation experiments. Translation reactions using ^{35}S-methionine produced several major products, presumably corresponding to translational initiation at various N-terminal Met residues. Translation was conducted in the presence of canine pancreatic microsomes. Such microsomes have been used to promote *in vitro* translation of integral membrane proteins by allowing association with the membranes (for example, see Bayle et al., 1995). Following translation, a portion of the reaction was subjected to treatment with 0.1 M sodium carbonate, which strips away proteins that are not integrally inserted into membranes (Fujiki et al., 1982). The sodium carbonate-washed sample was separated by centrifugation into supernatent and pellet components and analyzed by electrophoresis and autoradiography (Figure 8.) The largest molecular weight polypeptide from the translation product, representing the full-length protein, is only present in the pellet sample after treatment with sodium carbonate.

Figure 8. Sodium carbonate treatment of *in vitro*-transcribed and translated AtFUT1. *In vitro* transcription and translation followed by sodium carbonate treatment indicates the highest molecular-weight translation product (arrow) is a membrane protein. Translation reactions were conducted in the presence of canine pancreatic microsomes. After translation, an aliquot was removed ("T" for Total), and the remainder was homogenized in the presence of 0.1 M sodium carbonate on ice for 30 min. The sodium carbonate-treated sample was centrifuged at 100,000 *g* for 1 h to separate soluble ("S") and pellet ("P") samples. "L", β-lactamase - positive control (precursor, 31.5 kD; after processing, 28.9 kD), "dH_2O", water negative control.

3.3.3 Immunoprecipitation of XyG FUTase Activity

To confirm the identity of *AtFUT1* as encoding a fucosyltransferase using XyG as an acceptor, we prepared polyclonal antibodies directed against *AtFUT1* overexpressed in *E. coli* and used them to immunoprecipitate proteins from carbonate-washed, detergent-solubilized Arabidopsis proteins. These antibodies were shown to recognize a protein of the expected molecular mass in a sample of *Arabidopsis* microsomal proteins by immunoblotting (analysis by Weiqing Zeng) (Perrin et al., 1999). The immunoprecipitated proteins were then assayed for XyG FUTase activity. FUTase activity found in pellets derived from immunoprecipitation reactions was 2.6-fold higher when immune antiserum was used compared to preimmune serum (Figure 9).

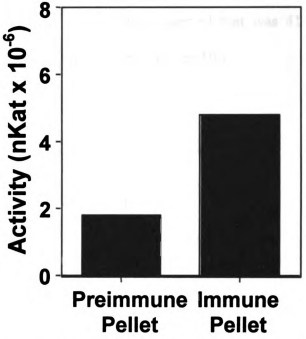

Figure 9. Immunopreciptation of XyG FUTase activity from *Arabidopsis* microsomal proteins. Polyclonal antibodies raised against AtFUT1 were used in immunopreciptation reactions with carbonate-washed *Arabidopsis* microsomal proteins. The resulting pellets were washed extensively and assayed for XyG FUTase activity. This is an example similar to results seen in seven different replicates.

3.3.4 Expression of AtFUT1 *in Mammalian Cells*

As a second means of demonstrating the function of AtFUT1, a cDNA encoding this protein was expressed in the Cos-7 mammalian cell line. This line has no endogenous fucosyltransferase activity towards XyG. Upon expression of *AtFUT1* in this system, substrate-dependent FUTase activity was observed that was 41 times higher than in control lines transfected with empty vector (Figure 10.)

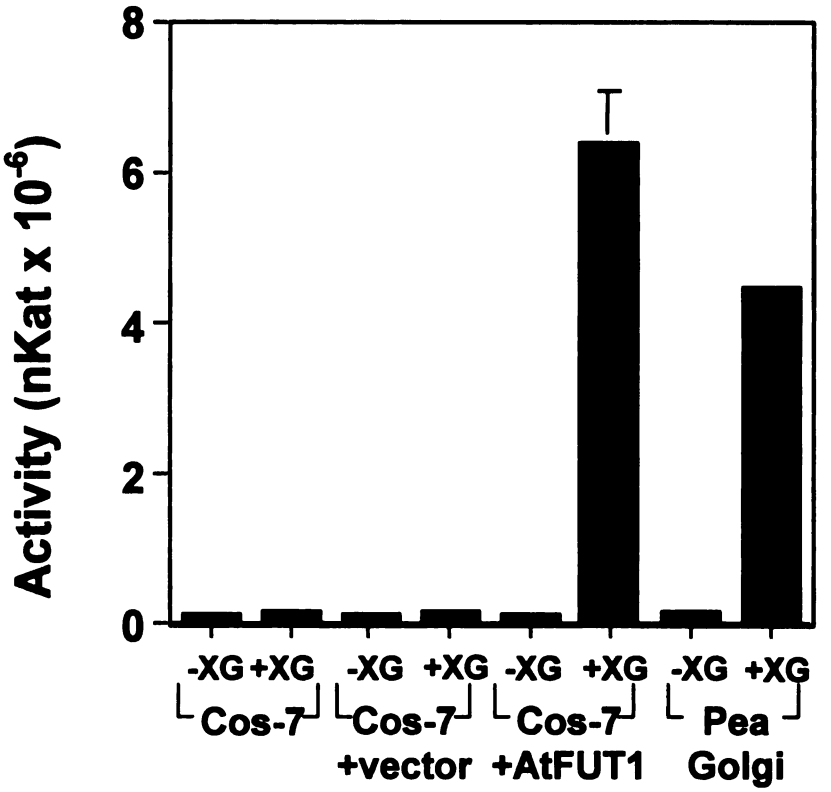

Figure 10. ***AtFUT1*** **expressed in a COS cell line shows XG-specific FUTase activity.** Activity in nKat is shown in the presence or absence of tamarind XG for untransformed Cos-7 cells, cells transformed with vector DNA (Cos-7 vector), cells transformed with vector containing AtFT1 (Cos-7 vector *AtFT1*), or solubilized pea Golgi vesicles (Pea Golgi). For graphs, error bars show $\pm$ 1 standard deviation; if no error bars are visible, standard deviations are contained within the width of the plot element.

3.4 Discussion

The identification of an *Arabidopsis* homolog of the purified pea XyG FUTase allowed molecular and genetic approaches to the study of XyG biosynthesis. The resources available to *Arabidopsis* researchers include a fully sequenced genome, publically available EST databases, collections of T-DNA and transposon mutants, expression analysis using DNA microarrays or DNA chips, an assortment of service facilities, and countless available protocols for genetic, molecular, physiological, cell and biochemical analysis. One of the most valuable resources, though, is an active community of researchers working on this model organism.

Despite the tools that are available, however, few cell wall biosynthetic genes had been identified in *Arabidopsis* at the time that this project was initiated. Even at the point of conclusion of this thesis work, *AtFUT1* joins only a handful of other identified cell wall biosynthetic genes, many of which have been identified by homology to other genes (such as the collections of cellulose synthase and cellulose synthase-like genes).

Identification of a full-length version of *AtFUT1* began by performing a Northern blot to determine how much of the coding region was covered by the existing EST. At that time, it was not known how many other fucosyltransferase-like genes existed in the *Arabidopsis* genome. The Northern blot indicated a transcript approximately 2 kb long in various tissues, which indicated that the EST was not full-length. However, it should be noted that a genome-wide survey for *AtFUT1*-like genes has demonstrated the presence of nine other family members (Sarria et al., 2001). Because the entire EST was used as a

hybridization probe for the Northern blot shown in Figure 6, this blot most likely demonstrates a composite expression profile for multiple family members – a situation which is very likely considering the heterogenous transcript sizes observed between samples. Other expression data will be shown in Chapter 4. However, it is true that EST 191A6T7 does not encode a full-length cDNA for *AtFUT1*.

The library used to identify cDNA clones for *AtFUT1* was originally constructed using RNA from 3-day old *Arabidopsis* seedling hypocotyls (Kieber et al., 1993). cDNAs derived from this RNA had been size-fractionated, and the library subset containing 2-3 kb cDNA inserts was used for screening. Two clones were identified encoding AtFUT1. Sequence analysis of the encoded protein was promising, in that a motif characteristic of α-(1,2) and α-(1,6) FUTases is present (Table 5). The gross structural features were also characteristic of a fucosyltransferase; for example, a hydrophobic region is present at the N-terminus that could act as a signal-anchor domain (Figure 7). *In vitro* transcription and translation supported the idea that AtFUT1 is an integral membrane protein (Figure 8), and other studies have shown that this protein is Golgi-localized (Sarria et al., 2001). The absence of the highest molecular weight product from the soluble fraction indicates that the full-length protein has an integral transmembrane domain and cannot be disassociated from the canine pancreatic membranes by sodium carbonate treatment. However, protein arising from *in vitro* translation experiments did not possess XyG FUTase activity (data not shown), possibly indicating that the protein could not assume a conformation necessary for catalytic activity or that other interacting proteins were required.

Two approaches were taken to determine the biochemical function of AtFUT1. Immunoprecipitation experiments showed that polyclonal antibodies directed against a recombinant, tagged version of AtFUT1 could precipitate XyG FUTase activity from solubilized membrane proteins, indirectly showing that the cDNA clone did encode a functional XyG FUTase enzyme (Figure 9). Heterologus expression experiments also directly demonstrated that the *AtFUT1* cDNA can confer XyG FUTase activity to a mammalian cell line which is normally incapable of transferring fucose to XyG (Figure 10). Subsequent studies have also used tobacco cell cultures and *Pichia pastoris* as heterologous expression systems for *AtFUT1* (A. Faik, T. Wagner). Tobacco, like other Solanaceous species, does not possess fucosylated XyG and no background XyG fucosylation activity is detected in tobacco cell cultures.

Identification of an *Arabidopsis* XyG FUTase allows several areas of study to be undertaken, several of which will be described in Chapter 4. One of the most important consequences is that identification of this cell wall biosynthetic gene should allow other candidate genes to be identified in *Arabidopsis* or other plant systems. Expression profiling should aid in assessing cell wall biosynthetic gene candidates, as it may reasonably be expected that genes involved in primary cell wall synthesis will be coordinately regulated. Analysis of plants affected in this gene will also provide insight into the roles of primary cell biosynthesis in general and XyG fucosylation in particular.

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CHAPTER FOUR

Analysis of Xyloglucan Fucosylation as a Model Cell Wall Biosynthetic Process

Images in this dissertation are presented in color.

I performed all experiments described in this chapter with the exception of xyloglucan analyses performed in collaboration with Drs. William York and Zhonghua Jia (Complex Carbohydrate Research Center, University of Georgia, Athens GA.)

4.1 Introduction

The previous chapter described the identification of *Arabidopsis* xyloglucan (XyG) fucosyltransferase (*AtFUT1*), a cell wall biosynthetic enzyme that adds a terminal fucose residue to galactose units on XyG in an α-(1,2)- linkage. This constitutes one of the last steps of XyG biosynthesis before the polymer is exported from its site of synthesis in the Golgi to the apoplast and incorporated into the growing cell wall.

AtFUT1 encodes one of only two biosynthetic enzymes at the time of this writing for which a suitable assay have been developed, whose carbohydrate product can be readily analyzed, and whose gene has been identified. Only galactomannan galactosyltransferase also falls into this category (Edwards et al., 1999). Few genes encoding cell wall biosynthetic enzymes have been identified at the molecular level, and for some enzyme activities (such as cellulose synthase), no assays have been developed to demonstrate their biochemical function. Several reasons for these limitations have been outlined in previous chapters and include enzyme lability, unavailability of substrates for assays, and a lack of sequence similarity between glycosyltransferases in general that hinders gene identification. Thus, XyG FUTase is a suitable model system to study cell wall biosynthesis at the levels of gene expression, protein activity, and carbohydrate product structure.

There is evidence to support the notion that cell wall biosynthesis is a regulated process. As tissue expands and differentiates, cells comprising that tissue often enlarge, elongate or adapt characteristic shapes. Specific types of cell wall material are required in all of

these cases. Biosynthesis alone is only half of the overall picture during the growth process, because increased deposition of wall material without a corresponding increase in extensibility or means to incorporate new polymers into existing networks would only result in thicker walls, not necessarily increased cell size. However, if the cell were unable to increase biosynthesis of wall material during expansion, it would eventually suffer a loss of wall integrity if the wall thinned to the point of breakage (Cosgrove, 2000).

Determination of expression patterns of *AtFUT1* will also enable identification and evaluation of other candidate genes encoding cell wall biosynthetic enzymes. Co-regulation has been described for other biosynthetic processes in plants. In peppermint, the enzyme activity and gene expression profiles of several members of the monoterpene biosynthesis pathway showed coordinate regulation during the development of oil-producing trichome glands (McConkey et al., 2000). Several genes involved in the biosynthesis of carotenoids show similar expression pattern profiles in various cultivars of marigold, with deeply colored cultivars having up-regulation of multiple pathway members and white cultivars having little expression (Moehs et al., 2001). The completion of the *Arabidopsis* genome sequencing project in combination with global profiling techniques, such as DNA microarray or Affymetrix chip technologies, should further facilitate the identification of co-regulated sets of genes. Careful investigation of the regulation of *AtFUT1* gene expression will allow the identification and evaluation of other gene encoding cell wall biosynthetic enzymes. Although expression profiling by itself is not diagnostic of gene function, cell wall biosynthetic gene candidates that have

expression profiles similar to *AtFUT1*, a known enzyme involved in primary cell wall biosynthesis, may reasonably be considered to be stronger candidates than those that do not.

In this chapter, the regulation of *AtFUT1* expression and enzyme activity are investigated. Sets of tissue from Col-0 *Arabidopsis* were generated and used to study *AtFUT1* expression, XyG FUTase activity, and XyG fucosylation. The genomic region upstream of *AtFUT1* was isolated for preparation of *AtFUT1::GUS-GFP* promoter-reporter constructs. Regulation of *AtFUT1* expression was analyzed in various tissues and in response to various conditions using *AtFUT1::GUS-GFP* transgenic plants. In addition, *35S::AtFUT1* over-expressing transgenic plants were generated and analyzed for *AtFUT1* gene expression, XyG FUTase activity, and XyG structure.

4.2 Materials and Methods

Images in this dissertation are presented in color.

4.2.1 Plant Growth Conditions

Arabidopsis plants were grown under hydroponic conditions as previously described (Gibeaut et al., 1997). Opaque plastic storage boxes (Rubbermaid, Wooster OH) were filled with hydroponic growth medium (1.25 mM KNO_3, 1.5 mM $Ca(NO_3)_2$, 0.75 mM $MgSO_4$, 0.5 mM KH_2PO_4, 50 μM KCl, 50 μM H_3BO_3, 10 μM $MnSO_4$, 2 μM $ZnSO_4$, 1.5 μM $CuSO_4$, 0.075 μM $(NH_4)_6Mo_7O_{24}$, 0.1 mM Na_2O_3Si, 72 μM Fe-diethylenetriamine pentaacetate (Sequestrene 330, Becker Underwood Inc., Ames IA)) and plugs were prepared from Rockwool (GrodanHP, Agro Dynamics, East Brunswick, NJ) with a cork

borer. These plugs were inserted into holes drilled into lids for the plastic containers. Glass Pasteur pipets were used to drill a channel through the middle of the Rockwool plugs. WT Col-0 seeds were suspended in 0.1% agarose and placed onto the center of the plugs. The tanks were placed in growth chambers (16 h days, 22 ° C) and aeration of the nutrient solution was provided using small aquarium pumps. After germination, the plants were thinned to one per Rockwool plug. Plants were allowed to grow until maturity, approximately 8 weeks under these conditions.

Soil-grown plants were grown on greenhouse-prepared soil media suitable for *Arabidopsis* under the same growth chamber conditions. Seeds were suspended in 0.1% agarose, distributed onto soil surface, and subjected to 4° C for three days to promote uniform germination before transferring pots to the growth chamber.

Agarose medium for plant growth (4.3 g/L MS salts, 100 μg myo-inositol, 10 μg thiamine-HCl, 1 μg nicotinic acid, 1 μg pyridoxine-HCl, 1% sucrose, 2.6 mM MES, pH adjusted to 5.7 with KOH, 1.5% phytagar) was used to prepare sterile plates. For selective plates, hygromycin (50 μl/ml) was added after the media had cooled. In some cases, vancomycin (500 mg/L) was added to control contamination. Seeds were sterilized with a series of washes, all 20 minutes long, on a platform rocker: one wash in ethanol solution (80% ethanol, 0.1% Triton X-100) one wash in bleach solution (30% bleach, 0.1% Triton X-100), and three washes in sterile distilled water. After sterilization, seeds were suspended in sterile 0.1% agarose and plated on growth medium.

4.2.2 Collection of Tissue Panels

Hydroponically-grown *Arabidopsis* was harvested at maturity and tissue was collected on ice. Samples harvested were flowers, the uppermost 6 cm of the inflorescence stem, the lowest 6 cm of the inflorescence stem, siliques, cauline leaves, rosette leaves, and roots. Tissue samples were weighed and divided into portions used for Golgi vesicle preparations, RNA preparations, and cell wall preparations. Golgi vesicles were prepared using fresh tissue, while RNA and cell wall samples were prepared using tissue that was frozen in liquid nitrogen and stored at –80° C.

4.2.3 RNA Preparation

RNA was prepared as previously reported in a method designed for use with pine tree samples (Plant Molecular Biology Reporter (1993) 11:113-116) with revisions for *Arabidopsis* (C. Wilkerson and S. Myers, MSU, East Lansing, MI). Extraction buffer (2% hexadecyltrimethylammonium bromide, 2% polyvinylpyrrolidone K 30, 100 mM Tris-Hcl pH 8.0, 25 mM EDTA, 2.0 M NaCl, 0.5 g/L spermidine, 2% β-mercaptoethanol added just before use) was kept at 65° C. Tissue was ground under liquid nitrogen to a fine powder and added to the pre-warmed extraction buffer (5 ml buffer per gram of tissue), then mixed by inverting the tube and vortexing. Samples were extracted twice with an equal volume of chloroform. One-quarter volume of 10 M LiCl was added to the supernatent to precipitate RNA at 4° C for several hours. The samples were centrifuged (7000 rpm, 5800 *g*) at 4° C for 20 minutes and pellets were resuspended in Tris-EDTA solution (0.5% sodium dodecyl sulfate, 10 mM Tris pH 7.5 (DEPC-treated), 1 mM EDTA (DEPC-treated)). An equal volume of phenol/chloroform/isoamyl alcohol

(25:24:1) was added to the samples, which were vortexed and centrifuged at room temperature for 15 minutes (3000 rpm, 1000 *g*). The upper phase was removed and 1/4 volume 10 M LiCl was added. Samples were incubated at 4° C for several hours, then centrifuged (7000 rpm, 5800 *g*) for 20 minutes at 4° C to pellet RNA. The pellets were resuspended in 2 ml DEPC-treated water, and 1/10 volume 3M sodium acetate pH 5.2 and 2.5 volumes ethanol were added. The samples were centrifuged again (7000 rpm, 5800 *g*) for 15 minutes to pellet RNA. Supernatent was removed, an appropriate amount of DEPC-treated water was used to resuspend the pellet, samples were centrifuged briefly to remove any carbohydrate contamination, and an aliquot was removed for quantification.

4.2.4 Expression Analysis by RT-PCR

RNA samples were treated with RNase-free DNase I using the DNA-*free* kit (Ambion, Austin, TX) according to the manufacturer's instructions. RNA (1 μg) samples were used in a reverse transcription reaction with 0.5 μg oligo dT primer (Invitrogen, Carlsbad, CA), 1X first strand buffer, 10 mM DTT, 5 mM dNTPs, and 200 U Superscript II reverse transcriptase (Life Technologies, Rockville, MD). RNA, water and the reverse primer were incubated at 70° C for ten minutes, transferred to ice for 2 minutes, centrifuged, and first strand buffer, DTT and dNTPs were added. The samples were incubated at 42° C for 1 minute and reverse transcriptase was added. The samples were further incubated at 42° C for 50 minutes and then at 70° C for 15 minutes. PCR was performed using an equal volume (0.8 μl) of the original reverse transcription reactions as template in 20 μl PCR reactions with 1X PCR buffer, 150 μM $MgCl_2$, 125 μM each forward and reverse primer,

and 5 U Amplitaq (Roche, Mannheim, Germany). Primers used to amplify AtFUT1 were: Foward primer: 5' GAA GGG CTA CTT GCT TCT GGT TTT 3'; Reverse primer: 5' CCC GAT GAA TGT TTG GTC TCC TT 3'. These primers amplify a 578 bp fragment of *AtFUT1* from nucleotides 571-1149. Thermal cycling parameters for amplification of *AtFUT1* were: 94° C, 1 minute (hot start); 92° C, 30 seconds, 58° C, 1 minute, 72° C, 1 minute 30 seconds for 22 to 24 cycles (which is within the linear range of amplification); 72° C, 5 minutes. Primers used to amplify cytochrome c were: Forward primer: 5' TCG CTT ATT TGA AGG AAG TG 3'; Reverse primer: 5' CTC TTC ACA TCA ATA GCGT AAT 3'. These primers amplify a 212 bp fragment of cytochrome c (AtGI number TC87099), located on chromosome 4. Thermal cycling parameters for the amplification of cytochrome c were: 94° C, 1 minute (hot start); 94° C, 30 seconds, 55° C, 30 seconds, 72° C, 1 minute for 28 cycles (within the linear range of amplification); 4° C. After amplification, samples were analyzed by electrophoresis on a 1% agarose/1X TAE gel.

4.2.5 Golgi Vesicle Preparations

Golgi vesicles were prepared using the method of Muñoz et al., adapted for use in *Arabidopsis* (Muñoz et al., 1996). Tissue was weighed and 1 volume (g/ml) of 0.5 M sucrose solution was added (0.5 M sucrose, 0.1 M KH_2PO_4 pH 6.65, 5 mM $MgCl_2$, 1 mM DTT, and EDTA-free Complete protease inhibitor cocktail tablets (Roche, Mannheim, Germany) according to the manufacturer's instructions). The tissue was chopped by hand with a razor blade, ground with a mortar and pestle, and homogenized with a Pyrex Dounce homogenizer. The homogenate was filtered through Miracloth and centrifuged

at 1000 *g* for 5 minutes. The supernatent was removed and layered on top of a 1.3 M sucrose solution cushion (1.3 M sucrose, 0.1 M KH_2PO_4 pH 6.65, 5 mM $MgCl_2$) and centrifuged at 100,000 *g* for 90 minutes in a swinging bucket rotor. The upper phase was removed without disturbing the interface and 1.1 M sucrose solution (1.1 M sucrose, 0.1 M KH_2PO_4 pH 6.65, 5 mM $MgCl_2$) was layered on top, followed by a layer of 0.25 M sucrose solution (0.25 M sucrose, 0.1 M KH_2PO_4 pH 6.65, 5 mM $MgCl_2$). The samples were centrifuged at 100,000 *g* for 100 minutes in a swinging bucket rotor. The 0.25 M/1.1 M sucrose interface fraction was collected, 1 volume of water was added, and the samples were centrifuged at 100,000 *g* for 50 minutes in a fixed angle rotor. The supernatent was discarded and the pellet was resuspended in STM buffer (0.5 M sucrose, 2 mM $MgCl_2$, 20 mM Tris-HCl pH 7.5, and protease inhibitor cocktail tablets.) Aliquots of the preparations were reserved for quantification by the Bradford assay.

4.2.6 Activity Assays

Activity assays were conducted as described in Chapter 2 of this thesis. All assays of Golgi vesicle preparations included 1% Triton X-100 to permeabilize the vesicles, allowing accessibility of exogenous substrates to the enzyme.

4.2.7 Xyloglucan Analysis

1. Cell Wall Preparations

Alcohol-insoluble residue (AIR) preparations were performed to gather cell wall material for analysis. Tissue was collected, weighed, and frozen in liquid nitrogen. The tissue was ground to a powder under liquid nitrogen using a mortar and pestle, and 80% ethanol

was added (approximately 20 ml per gram of tissue). The tissue was further disrupted using a polytron homogenizer. Cell wall material was collected using nylon mesh and washed with 80% ethanol, then 100% ethanol. The washed material was suspended in methanol: chloroform (1:1) and stirred for one hour at room temperature. The insoluble material was collected on Whatman paper by filtration and washed with acetone.

Further preparation of cell wall material and analysis of XyG structure was performed at the Complex Carbohydrate Research Center (Athens, GA).

2. Endo-polygalacturonase (EPG) and pectin methylesterase (PME) treatment. AIR samples were treated simultaneously with EPG and PME to solubilize pectin oligo- and polysaccharides as follows. AIR samples were suspended in 10 mL of 50 mM NaOAc, pH 5, containing 0.01% thimerosal. EPG (5 units, where one unit releases 1 μM of reducing galacturonate/min at 30°C), purified from *Aspergillus niger* (provided by Dr. Carl Bergmann, CCRC), and PME (5 units) from *A. oryzae* (supplied by Novozymes A/S) were added. The suspension was incubated for 24 h in a shaking incubator at 24°C. The suspensions were filtered and the solid residues were treated a second time with EPG and PME.

3. XG-specific endo-β-1,4-glucanase (XEG) treatment. The residues obtained after EPG and PME treatment were suspended in 10 mL 20 mM NaOAc, pH 5, containing 0.01% thimerosal. XEG (10 units) was added and the suspension was incubated for 24 h in a shaking incubator at 24°C, and then filtered. The filtrate, containing XG

oligosaccharides, was applied to a C-18 cartridge (Supelco Supelcelan LC-18 SPE Tube), which was washed with water (10 mL) to remove salts. The oligosaccharides were then eluted from the cartridge with 25% aq. MeOH (10 mL). The eluant was concentrated under vacuum and lyophilized. The oligosaccharides were further purified on a Superdex 75 column (Pharmacia Biotech) and eluted with 50 mM ammonium formate, pH 5.0, at a flow rate of 0.5 mL/min. A refractive index (RI) detector was used to monitor carbohydrate in the eluant. Fractions containing the oligosaccharides were pooled and lyophilized several times to remove volatile ammonium formate salts, affording pure XEG-released oligosaccharides.

4. 1N KOH and 4N KOH treatment. The residues from XEG digestion were suspended in 10 mL of 1N KOH containing 1% $NaBH_4$ and stirred for 24 h at room temperature. The suspensions were filtered and the insoluble residues were then suspended in 10 mL of 4N KOH containing 1% NaBH4. The suspensions were stirred for 24 h at room temperature and then filtered. The filtrate was neutralized (pH 5) with glacial AcOH and dialyzed (3,500 MWCO tubing; spectrum) against six changes of deionized water over 2 days. The retentates were lyophilized to afford the 4N KOH-solubilized XG fractions, which were digested with XEG (as in step 3) to generate XG oligosaccharides. XG oligosaccharides were isolated from the reaction mixture as in step 3.

5. Spectral Analysis. MALDI-TOF MS and NMR were performed on the oligosaccharides purified from steps 3 and 4. MALDI-TOF MS (Matrix assisted laser desorption time-of-flight MS) was performed with a Hewlett Packard LDI 1700XP

spectrometer, operating at an accelerating voltage of 30 kV, an extractor voltage of 9 kV, and a source pressure of approximately 9 x 10-7 torr. The matrix used was a mixture of 2,5-dihydroxybenzoic acid (DHB, 0.2 M) and 1-hydroxyisoquinoline (HIC, 0.06 M), both in 50% aqueous acetonitrile. Oligosaccharides were dissolved in 0.6 mL of D_2O (99.9%, Cambridge Isotope Laboratories) and NMR spectra were recorded using a Varian Inova 600 (600 MHz for ^1H) at 25°C.

4.2.8 Generation of AtFUT1::GUS-GFP Transgenic Plants

Regions upstream of the *AtFUT1* ORF were amplified from *Arabidopsis* genomic DNA. Three fragments were generated: 1) 1987 bp upstream of the ORF, 2) 3103 bp upstream of the ORF, and 3) a 2934 bp fragment that included a 2 kb region upstream of the ORF, the first exon, and the only intron of *AtFUT1*. The primers used for amplification were: for fragment 1, 5' GGG GGA TCC CTA TAG TGG CTG TCT GCT TGA GGA 3' (primer F2) and 5' GGG CCA TGG ATT GCT CTT GAG GGA 3'(primer KK506); for fragment 2, 5' GGG GGA TCC GTT TCT TGG GTG GAT GTT TGT TTT 3' (primer F1) and primer KK506; for fragment 3, primer F2 and 5' TCG AAA CCC GGT ATT AAC CA 3'. Primers included restriction sites for BamHI and NcoI digestion. Thermal cycling parameters were: 96° C for 2 minutes (hot start); 94° C for 30 seconds, 57° C for 30 seconds, 72° C for 2 minutes for a total of 35 cycles; 4° C. Fragments were cloned in pGemT-Easy vector (Promega, Madison, WI) and then into BamHI and NcoI sites in the pCAMBIA 1303 vector. This caused a fusion of the first ATG codon originating from *AtFUT1* with the gusA-mGFP5 double reporter present in the pCAMBIA 1303 vector. Selection in bacterial hosts and transgenic plants was provided by the kanamycin

resistance marker the hygromycin resistance marker, respectively. The constructs were introduced into *Agrobacterium tumefaciens* by standard transformation methods and transgenic *Arabidopsis* plants were generated using the vacuum infiltration method (Bechtold and Pelletier, 1998).

4.2.9 GUS Staining Conditions

Whole plant seedlings or plant tissues were harvested and placed into cold 90% acetone for 20 minutes on ice to permeabilize tissue. Samples were washed three times for 15 minutes each in working solution (100 mM NaH_2PO_4, 10 mM EDTA, 0.5 mM ferrocyanide, 0.5 mM ferricyanide, 0.1% Triton X-100). Samples were then incubated in staining solution (working solution with 2 mM X-Gluc (Rose Scientific, Edmonton, Canada)) at 37° C for 16-36 hours. Samples were cleared of chlorophyll by several washes in 70% ethanol, then photographed using a Nikon Coolpix 995 digital camera attached to a conventional light microscope or stereoscope. Whole organs of mature plants were photographed without magnification.

4.2.10 Treatments with Hormones or Abiotic Conditions

*AtFUT1::*GUS-GFP plants were treated with various hormones or environmental conditions and stained for GUS as described above. Several treatments involved growth of seedlings on vertical growth media plates for 9 days, then transfer to incubation media (4.3 g MS salts/L, 10 mM Pipes pH 6.0, 50 μg/ml chloramphenicol) with or without hormones (50 μM 1-naphthaleneacetic acid (NAA), 50 μM abscisic acid (ABA), 0.03% BHT (a salicylic acid analog), 100 μM gibberellic acid (GA_3), 1 mM 1-

aminocyclopropane-1-carboxylic acid (ACC) or 2 μM epibrassinolide. Methyl jasmonate (MeJA) treatments were performed by adding 2 μl concentrated MeJA to 100 μl ethanol on a growth plate containing seedlings in an air-tight plexiglass box. All incubations occurred for 24 hours. Cytokinin treatment consisted of adding 6-benzylaminopurine (BAP) to plate growth media to a final concentration of 4 μM. Seeds were plated on this media and allowed to grow for 10 days before staining. Cold treatment involved incubation of 9-day old plants at 4° C under growth lights for 48 hours. Etiolated growth involved growing seeds on vertical growth plates wrapped in aluminum foil for 10 days.

4.2.11 Generation of 35S::AtFUT1 Transgenic Plants

A cDNA fragment encoding the ORF of *AtFUT1* was subcloned into the NcoI/XhoI sites of pET14-b. The *AtFUT1* cDNA was then cloned into the SalI/XbaI sites of pCAMBIA 1300 MCS (a derivative of pCAMBIA with additional sites in the MCS generated by Dr. Anton Sanderfoot, MSU, East Lansing, MI). This generated a construct in which the 35S CaMV strong constitutive promoter controls the expression of *AtFUT1*. The construct was introduced into *Agrobacterium tumefaciens* by standard transformation methods and transgenic *Arabidopsis* plants were generated using the vacuum infiltration method (Bechtold and Pelletier, 1998). Transgenic plants were selected using hygromycin and the presence of the transgene was confirmed by PCR amplification across the *AtFUT1* intron, which results in a 489 bp product for the genomic version and a 212 bp product for the transgene. The primers used for amplification were: forward primer, 5' TCG ACG CCG GAG TTT TC 3'; reverse primer, 5' ATT CAG TAC CCG GAC CAC ATC 3'. The thermal cycling parameters were 95° C for 1 minute (hot start); 95° C for 1

minute, 50° C for 1 minute, 72° C for 1 minute for a total of 30 cycles; 4° C. Products were analyzed on agarose gels. RNA was prepared from T_2 plants using the RNeasy Mini Plant Kit (Qiagen, Germantown, MD) and used to analyze *AtFUT1* expression by RT-PCR as described above. Alcohol-insoluble residue preparations were used to analyze XyG structure as described above.

4.2.12 Quantification of Elongation in Regions of Arabidopsis *Inflorescence Stems*

Growing inflorescence stems of 15 *Arabidopsis* plants were marked at four locations using activated charcoal mixed with water, applied with a paintbrush. At time 0, two marks 0.5 cm apart were made at the top of the inflorescence stem just below the flower bud and two marks 0.5 cm apart were made at the bottom of the stem just above the rosette. The distance between each set of marks was determined 48 hours later. For measuring purposes, distance was recorded between the top of one mark and the top of the second member of the set.

4.3 Results

Images in this dissertation are presented in color.

4.3.1 Gene Expression, Enzyme Activity, and XyG Structural Analyses of Arabidopsis Tissues

Wild-type (Col-0) *Arabidopsis* plants were grown hydroponically and dissected at maturity into various tissue types (Figure 11). These tissue samples were divided into three portions: one for RNA extraction, one for preparation of Golgi vesicles, and one for preparation of cell walls. Tissues collected were flower buds, defined as the cluster of

flowers at the end of an inflorescence stem; the 6 cm of inflorescence stem directly beneath the flower buds; siliques; cauline leaves; the lowermost 6 cm of the inflorescence stem; rosette leaves; and total roots.

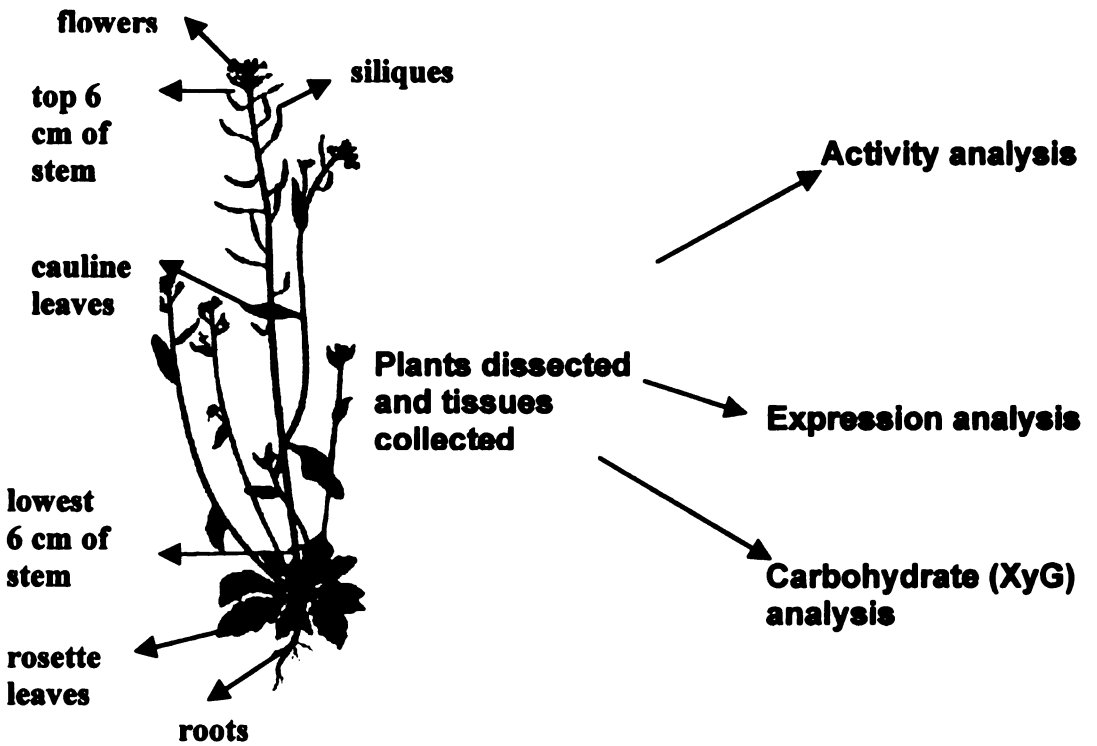

Figure 11. Tissue panel composition. *Arabidopsis* plants were grown in a hydroponic system to maturity and dissected into seven different tissue types, as indicated. These pooled tissues were separated into three different portions for Golgi vesicle preparation and XyG FUTase activity assays, expression analysis by RT-PCR, and XyG structural analysis.

The expression of *AtFUT1* in these tissues was assessed using RT-PCR (reverse transcription-polymerase chain reaction). RNA was prepared from each tissue sample, quantified, and treated with RNase-free DNase to remove any genomic DNA contamination. One microgram of RNA from each tissue was used in a RT reaction with oligo-dT as primer for synthesis of the first-strand cDNA. As a control to detect DNA contamination of the original RNA preparations, one RT reaction contained leaf RNA, but no reverse transcriptase ("No RT"). An equal volume of each RT reaction was used as template for PCR amplification of either *AtFUT1* or cytochrome c. The primers used to amplify *AtFUT1* have been determined to be specific for amplification of this gene (Sarria et al., 2001.) In earlier applications of these primers for *AtFUT1* expression analysis by RT-PCR, amplification was allowed to proceed for thirty cycles (Sarria et al., 2001), which is beyond the linear range of product accumulation (data not shown). Experiments were performed for both the *AtFUT1* primer set and the cytochrome c primer set to determine the optimum cycle number endpoint for qualitative comparisons of starting transcript amount (data not shown). Following PCR, the products were analyzed on an agarose gel and stained with ethidium bromide (Figure 12). The highest level of expression was detected in the uppermost region of the inflorescence stem, followed by roots. It should be noted that previous analyses of gene expression experiments have, at times, shown high levels of expression of *AtFUT1* in RNA derived from flowers.

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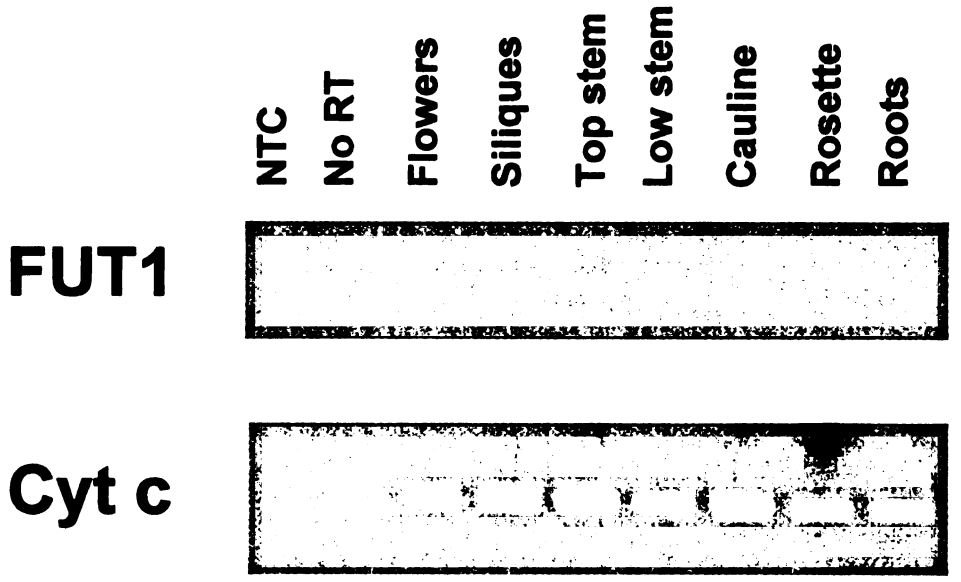

Figure 12. Expression profile of *AtFUT1* by RT-PCR. RNA prepared from *Arabidopsis* tissues and used for reverse transcription (RT) reactions primed with oligo-dT. An equal volume of reverse the RT reactions was used for PCR with primers specific for either *AtFUT1* (top) or cytochrome c as a standard gene control (bottom). PCR was performed for 24 (AtFUT1) or 28 (cytochrome c) cycles, which was within the linear range of amplification as previously determined (data not shown). Multiple technical and biological replicates yielded similar profiles (data not shown).

A second portion of the *Arabidopsis* tissue samples was used to prepare Golgi vesicles. Xyloglucan fucosyltransferase is a Golgi-localized enzyme, and fucosylated XyG accumulates primarily in the trans-Golgi and trans-Golgi network (Brummell et al., 1990). Thus, Golgi vesicles have been used as an enriched source of XyG FUTase enzyme activity (Wulff et al., 2000). The protein content of Golgi vesicle extracts was quantified, and the samples were assayed for XyG FUTase activity in the presence of detergent (1% Triton X-100). The detergent permits accessibility of the nonfucosylated acceptor substrate to the enzyme, which is oriented such that the active site is within the lumen of the Golgi (Wulff et al., 2000). The highest activity levels occur in flowers, the upper portion of the inflorescence stem, and (to a lesser extent) in the roots (Figure 13). Little or no activity could be detected in fully expanded rosette leaves. Over fivefold higher activity was observed in the upper region of the inflorescence stem compared to the lower-most region, which at the time of collection had already lignified.

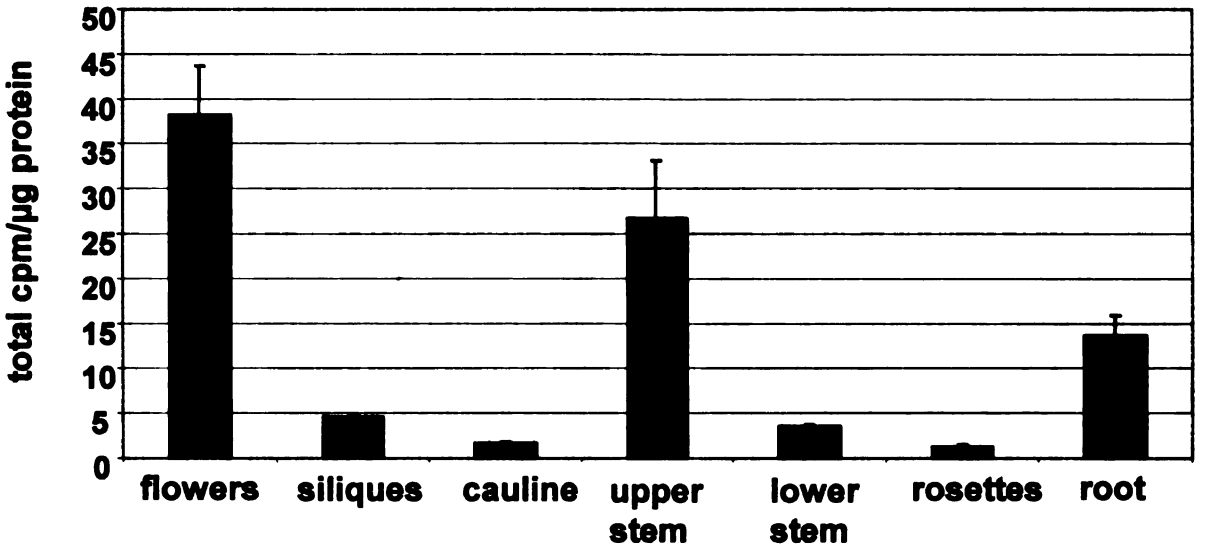

Figure 13. XyG FUTase activity of Golgi vesicle samples. Golgi vesicles were isolated from the indicated tissues using the method of Muñoz et al. (Muñoz et al., 1996). Xyloglucan fucosyltransferase assays were performed as described previously (Chapter 2). Data are averages of three replicates. An independent biological replicate resulted in a nearly identical profile (data not shown). Error bars indicate one standard deviation.

The final portion of tissue samples was used for preparation of cell walls and analysis of XyG fucosylation. Preparations of alcohol-insoluble residues (AIR) were made from the various tissue samples, and xyloglucan was extracted from these preparations with enzymatic and alkali treatment, after enzymatic removal of pectins (Figure 14). Extracted XyG fractions were then digested with a XyG-specific endoglucanase that cleaves XyG at unsubstituted glucan residues, releasing XyG oligosaccharides. NMR and MS analyses were performed on the samples to determine the amount of fucose present per unit of oligosaccharide, using spectral peaks indicative of the presence of Fuc (Figure 15). Two independent biological replicates were performed (both shown in Figure 15). In the first set, the amount of fucose present on XyG oligosaccharides derived from 4 M KOH extraction of rosette leaves was approximately half the amount present in other tissue samples. In the second biological replicate, the amount of Fuc present was approximately constant among all tissue types. Analysis of XyG derived from a series of developing leaves indicated no major differences in the XyG Fuc content of leaves ranging from 1 week to 5 weeks old (data not shown).

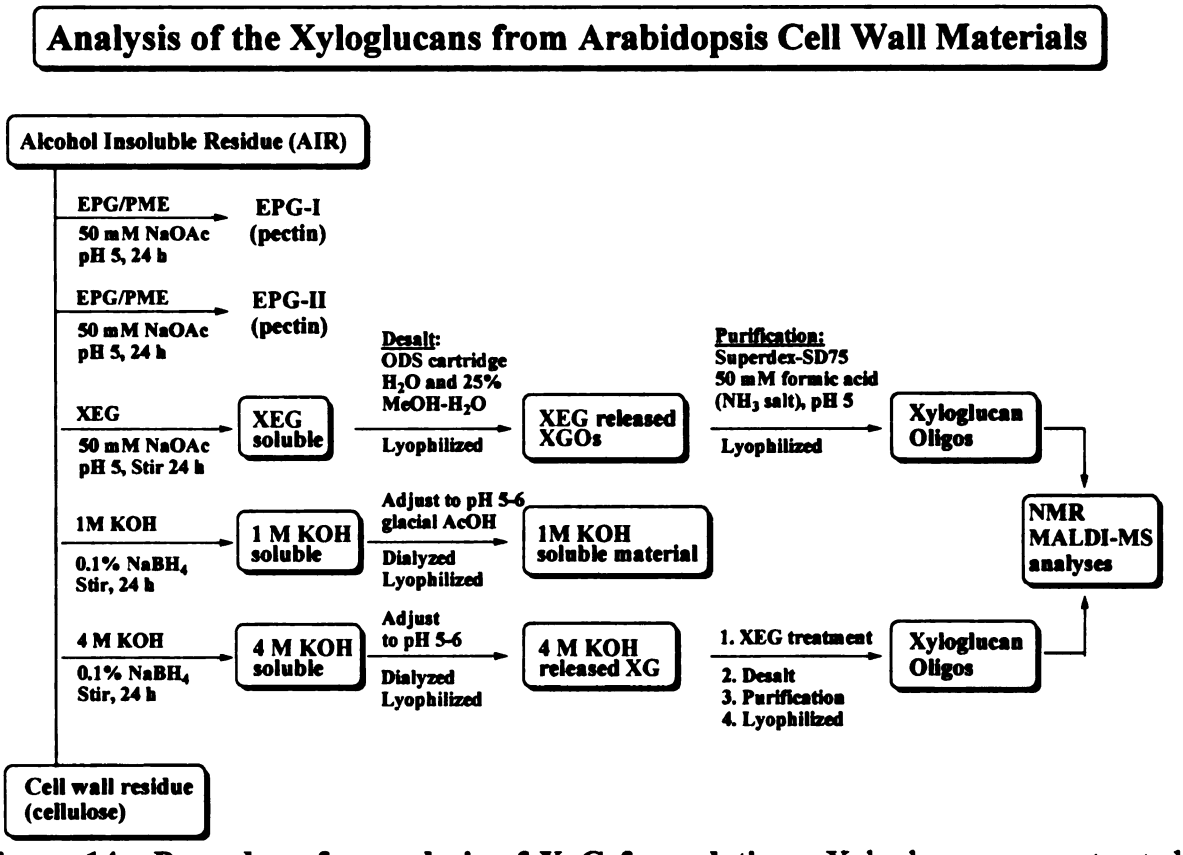

Figure 14. Procedure for analysis of XyG fucosylation. Xyloglucan was extracted from cell walls of *Arabidopsis* tissues and digested into oligosaccharide fragments. The fucose content of xyloglucan oligosaccharide fragments was determined using NMR and MALDI-MS analyses.

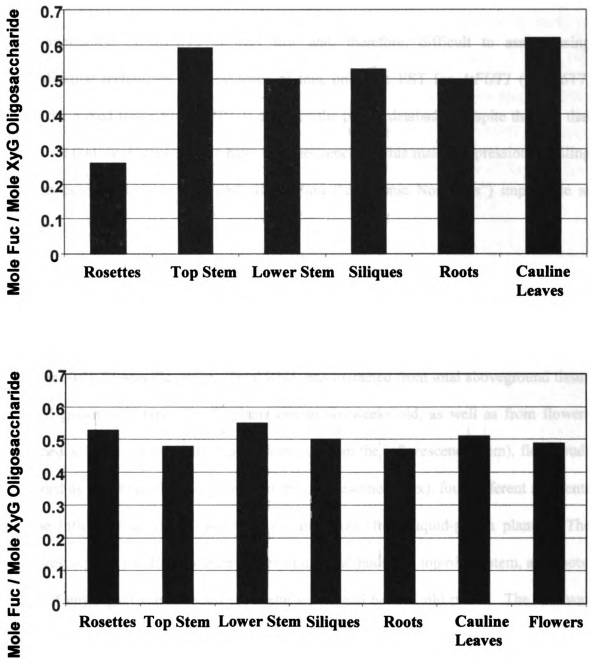

Figure 15. Analysis of XyG fucosylation. Data were obtained by NMR and MALDI-MS techniques that quantify the amount of fucose present per unit of XyG oligosaccharide. Two independent biological replicates were performed (first replicate, top; second replicate, bottom.)

4.3.2 Expression Profiling by RNA Gel Blot and Microarray Analysis

The expression of *AtFUT1* is very low and, therefore, difficult to assess using hybridization techniques. As evidence of this, only one EST for *AtFUT1* (191A6T7, from the mixed tissue library PRL2) exists in the public databases, despite the fact that over 100,000 *Arabidopsis* ESTs have been sequenced. This makes expression profiling of this gene by inventory of EST distribution ("electronic Northerns") impossible at present.

Some hybridization techniques have been applied to detect *AtFUT1* expression, however. Figure 16 shows a RNA gel blot in which 15 μg samples of total RNA were hybridized with an *AtFUT1*-specific probe. Total RNA was extracted from total aboveground tissue of soil-grown wild type (Col-0) plants one to six weeks old, as well as from flowers (defined as fully opened individual flowers cut from the inflorescence stem), floral buds (defined as unopened flowers present on the inflorescence apex), four different segments of the inflorescence stem, rosette leaves, and roots (from liquid-grown plants). The strongest signals for *AtFUT1* were observed in floral buds, the top of the stem, and roots. Little if any signal was detected in rosette leaves and 6-week old plants. The blot was then stripped and re-probed to detect eIF4a, a translation initiation factor, as a loading control.

AtFUT1 expression has also been detected using poly(A)$^+$ RNA. Figure 17 shows the result of hybridizing an *AtFUT1*-specific probe to poly(A)$^+$ RNA derived from 250 μg of total RNA extracted from leaf and root tissue of *Arabidopsis* grown in liquid medium.

Strong signal was detected in the root sample, while very little expression was observed in leaves. Golgi vesicles were prepared from identical tissue (leaves or roots of *Arabidopsis* grown in liquid media) and used for enzyme assays (Figure 17). Approximately fivefold higher activity was associated with Golgi vesicles prepared from root tissue versus leaf tissues. Thus, the steady-state levels of *AtFUT1* transcript determined by RNA gel blot analysis positively correlate with the levels of XyG FUTase enzyme activity for these tissues.

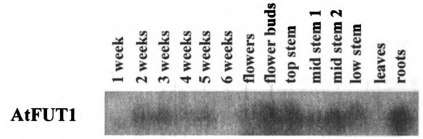

Figure 16. Expression profiling of *AtFUT1* by RNA gel blot analysis. Total RNA

was prepared from total aboveground tissue of soil-grown *Arabidopsis* at ages 1 through 6 weeks, or from organs of mature plants (flowers, flower buds, top 0.5 cm of inflorescence stem (top stem), two successive 1.0-cm segments below this (mid stem 1 and mid stem 2), the remaining stem from the bottom of the mid stem 2 section to the base of the inflorescence (low stem), rosette leaves, and roots). Fifteen micrograms of total RNA was used for RNA gel blot analysis with an *AtFUT1*-specific hybridization probe. The blot was then stripped and hybridized with a probe to detect eIF4a as a loading control.

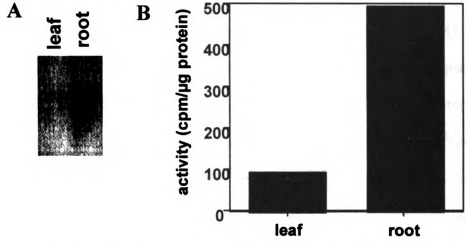

Figure 17. Expression of *AtFUT1* and XyG FUTase activity levels in leaves and roots of *Arabidopsis* grown in liquid culture. Total RNA was prepared from leaf and root tissue of *Arabidopsis* grown in liquid growth medium. Poly(A)$^+$ RNA was prepared from 250 µg of total RNA and used for RNA gel blot analysis with a probe specific for *AtFUT1* (panel A). The same tissue was also used for Golgi vesicle preparations and activity assays (panel B).

4.3.3 Expression Analysis Using Transgenic AtFUT1::*GUS-GFP Plants*

Three different constructs were made for generation of promoter-reporter transgenic plants (Figure 18). These transgenic lines were used for *in planta* analysis of *AtFUT1* gene expression. At the time that the *AtFUT1::GUS-GFP* constructs were made, the nearest predicted ORF upstream of *AtFUT1* was several kilobases away, which did not allow delineation of the *AtFUT1* promoter region with certainty. The constructs generated included 2-kb or 3.1-kb fragments of the genomic region upstream of the *AtFUT1* start codon fused with the GUS-GFP double reporter present in the pCAMBIA 1303 vector. A third construct also included the first exon and the first and only intron of *AtFUT1*, as introns have been reported to contain regulatory regions in rare instances (e.g. Gidekel et al., 1996). It should be noted that the first exon contains the signal anchor region that directs insertion into the Golgi membrane, and thus this could result in Golgi-lumenal localization of the reporter proteins. This would have implications for the accessibility of the X-Gluc substrate to the GUS reporter in transgenic plants containing this construct.

Transgenic *Arabidopsis* plants containing these constructs were subsequently analyzed for GUS expression (Figures 19-23.) Only lines with the construct containing the 2-kb promoter fragment was used for systematic analysis of reporter expression (see Discussion). Transgenic plants homozygous for single insertions of the transgene were identified by analysis of resistance ratios for the hygromycin marker. T_3 lines were used for systematic gene expression analysis in seedlings (3 days, 5 days, 10 days, or 14 days

old) and in organs of mature soil-grown plants. GUS expression was first detected in the veins of emerging cotyledons (Figures 19-24). Signal was later detected in emerging true leaf buds and at the base and tips of true leaves (Figures 20 and 21). Some central vein staining was occasionally noted in the hypocotyl. Little, if any, GUS expression was detected in roots (Figure 19). In mature organs, GUS expression was most notable at the top of the inflorescence stem (Figure 24). Although signal was high in the uppermost portion of the stem, it was not detectable in floral tissue itself. In true leaves, staining could be observed in the midvein at the base of the leaf and at the leaf margins, presumably representing hydathodes (Figure 23). Little staining was observed in cauline leaves (Figure 23).

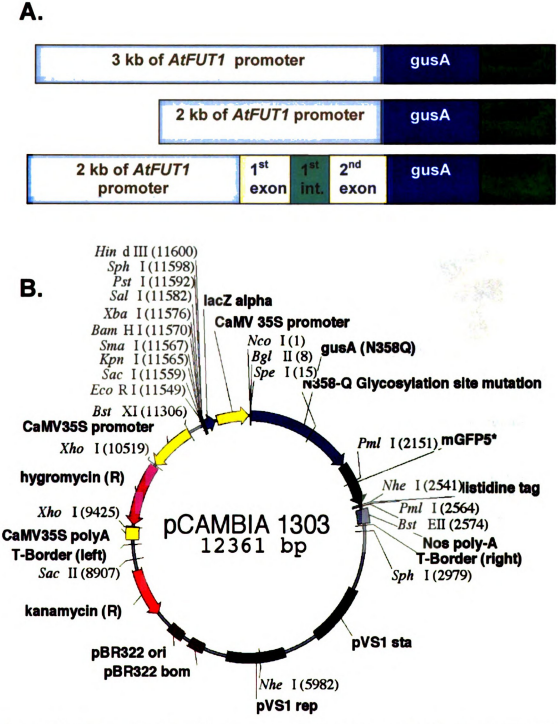

Figure 18. Constructs generated for promoter-reporter analysis. Various fragments of the *AtFUT1* genomic region upstream of the coding region were amplified from genomic DNA (panel A). These fragments were fused to the gusA-mGFP double reporter in pCAMBIA 1303 (panel B).

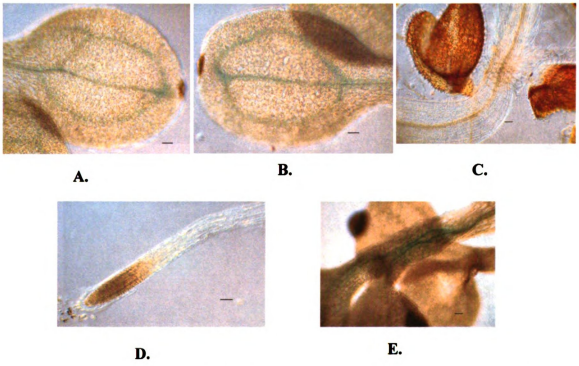

Figure 19. Expression of *AtFUT1* in 3-day-old promoter-GUS transgenic plants. Representative images are shown from staining of four different P2.0 transgenic lines. Staining occurred in the veins of emerging cotyledons (A, B) and was largely absent from the hypocotyl-root junction (C) as well as roots (D). Occasional staining could be seen in veins of the hypocotyl (E). GUS staining was variable, with 10% to 50% of individuals in each homozygous single-insertion line showing visible stain accumulation. Bar = 100 μm.

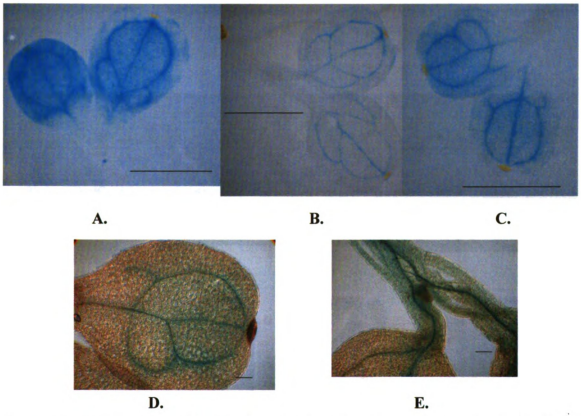

Figure 20. Expression of *AtFUT1* in 5-day-old promoter-GUS transgenic plants. Representative images are shown from staining of four different P2.0 transgenic lines. Staining occurred in veins of cotyledons (A-D) and at base of emerging leaf buds (A, E). GUS staining was variable, with 10% to 50% of individuals in each homozygous single-insertion line showing visible stain accumulation. Bar = 1 mm for A-C, 100 μm for D and E.

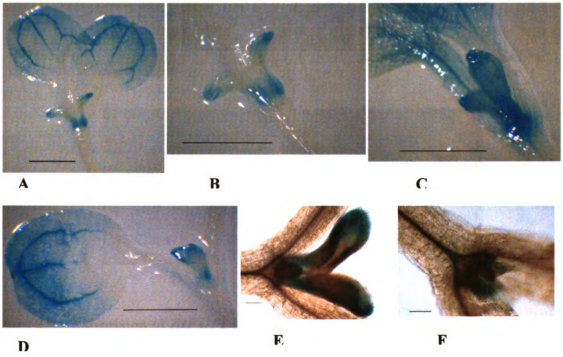

Figure 21. Expression of *AtFUT1* in 10-day-old promoter-GUS transgenic plants. Representative images are shown from staining of four different P2.0 transgenic lines. Staining occurred in veins of cotyledons (A, D) and at base and tips of emerging leaf buds (A-F). GUS staining was variable, with 5% to 66% of individuals in each homozygous single-insertion line showing visible stain accumulation. Bar = 1 mm for A-D, 100 μm for E and F.

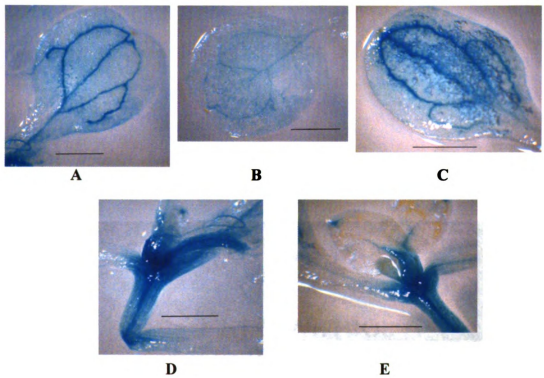

Figure 22. Expression of *AtFUT1* in 14-day-old promoter-GUS transgenic plants. Representative images are shown from staining of four different P2.0 transgenic lines. Staining occurred in veins of cotyledons (A-C) and in the central vein and tips and midveins of true leaves (D, E). GUS staining was variable and generally low, with 0%-10% of individuals in each homozygous single-insertion line showing visible stain accumulation. Bar = 1 mm.

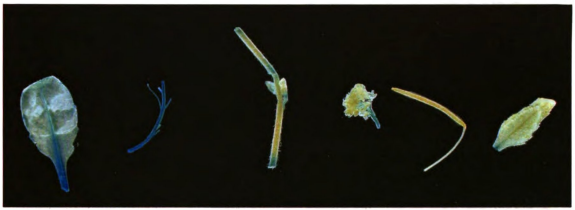

Figure 23. Expression of *AtFUT1* in promoter-GUS transgenic plants and organs. Representative images are shown from staining of four different P2.0 transgenic lines. Plants were grown on soil for 6 weeks and intact organs were stained for GUS activity. Left to right: mature rosette leaf, upper inflorescence stem (just below flower buds), lower inflorescence stem (just above rosette), flowers, silique, cauline leaf.

Figure 24. Expression of *AtFUT1* in intact stems of promoter-GUS transgenic plants. Representative images are shown from staining of four different P2.0 transgenic lines. Plants were grown on soil for 6 weeks and entire inflorescence stems were stained for GUS activity. Left, overview of stem; right, close-up of flowers.

4.3.4 Quantification of Elongation in Inflorescence Stem Regions

Since high expression of the reporter gene was detected in the uppermost region of the inflorescence stem and little expression was observed in the lowermost regions, elongation in these two areas was quantified. Marks were made on inflorescence stems of growing *Arabidopsis* plants at the top of the stem, just below the flowers, and at the bottom of the stem, just above the rosette. The initial distance between the two markings was 5 mm. The distance between these sets of marks was recorded 48 hours later. Figure 25 shows the average distance between each set of markings at 0 and 48 h.

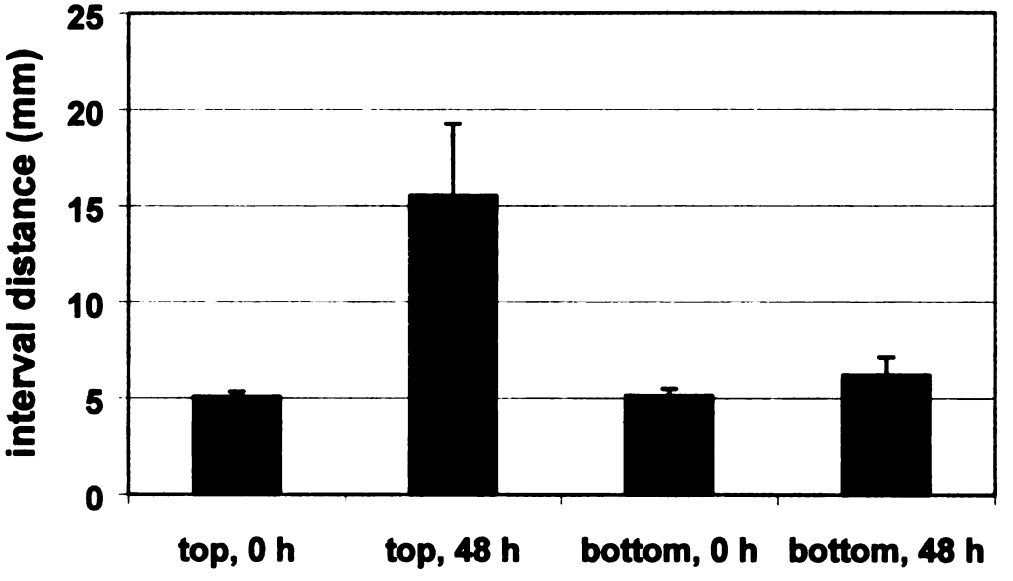

Figure 25. Elongation in upper and lower regions of *Arabidopsis* inflorescence stems. Sets of marks 0.5 cm apart were made in the upper part of growing inflorescence stems just below the flowers and the bottommost part of the stem, respectively, on 15 *Arabidopsis* plants. After 48 hours, the distance between each set of marks was recorded and used to calculate the elongation rates of each region in mm/h. Error bars indicate standard deviation.

4.3.5 Analysis of Cis-acting Promoter Element Motifs in the AtFUT1 *Promoter Region*

The genomic sequence 1060 nt upstream of the *AtFUT1* ORF (see Discussion) was used to search for cis-acting regulatory element motifs in two plant databases, PLACE (http://www.dna.affrc.go.jp/htdocs/PLACE/) and PlantCARE (http://sphinx.rug.ac.be:8080/PlantCARE/cgi/index.html). Promoter element motifs were noted, compiled and annotated (Table 6). Several major categories of motifs were observed, including motifs related to regulation by auxin, light, seed-specific expression, pollen-specific expression, or other types of regulation including regulation by salicylic acid (SA), elicitors, cold temperature, jasmonate or wounding. A number of consensus sequences for transcription factor binding sites were also noted.

To investigate the regulation of *AtFUT1* in response to various hormones and growth conditions, promoter-reporter seedlings were grown on plates for 9 days and then transferred to incubation medium with or without various growth hormones (abscisic acid, auxin (1-naphthaleneacetic acid (NAA)), BHT (a salicylic acid analog), gibberellic acid (GA_3), epibrassinolide, or methyl jasmonate (MeJA) (Figure 26)), or were grown in darkness (Figure 26), subjected to cold treatment (data not shown), or wounded (data not shown). Plants were treated with MeJA by adding 2 µl concentrated MeJA in 100 µl ethanol to the surface of an agar plate containing 9-day old seedlings, and allowing the MeJA to volatilize within an air-tight plexiglass box. All hormone-treated plants with the exception of cytokinin-treated plants were incubated for 24 hours. Exposure to cytokinin was performed by germinating seeds on plates containing 4 µM 6-benzylaminopurine (BAP) and collecting seedlings after 10 days. Etiolated plants were grown on plates

wrapped with aluminum foil for 10 days. Plants were subjected to cold treatment by transferring 9-day old seedlings to 4° C for 48 hours. Plant were wounded by cutting mature leaf tissue with scissors. After treatments, all plants were stained for GUS activity. The results (Figure 26) indicate that cytokinin and, to a lesser extent, the SA analog BHT appear to up-regulate expression of the reporter, while MeJA may repress its expression. Auxin treatment resulted in a different spatial pattern of reporter, with increased staining apparent in the stem, but did not appear to enhance the overall amount of staining. All other treatments resulted in GUS staining patterns that resembled those observed in control plants, i.e., variable staining concentrated primarily in the cotyledon veins and central vein. Data for cold and wounding treatments are not shown, but no induction of expression was observed. It should be noted that the increased GUS activity elicited by cytokinin was not variable, with 100% of the individuals in three different promoter-reporter transgenic lines showing increased reporter activity. Likewise, the repression of GUS activity by MeJA was also not variable, with all plants showing an absence of staining.

Table 6. Promoter cis-elements of *AtFUT1*. 1060 nt upstream of the *AtFUT1* ORF was used to search two plant promoter element databases, PLACE (http://www.dna.affrc.go.jp/htdocs/PLACE/) and PlantCARE (http://sphinx.rug.ac.be:8080/PlantCARE/cgi/index.html). Cis elements were grouped into auxin elements (yellow), light responsiveness (green), transcription factor (TF) binding sites (orange), pollen expression (pink) or other (blue).

cis element	function	number of repetitions
ARFAT	auxin responsiveness	1
AS1 motif	auxin/SA responsiveness	1
NTBBF1Arrolb	auxin responsiveness	2
AuxRR-core	auxin responsiveness	1
TGA-1	auxin responsiveness	2
CCA1AtLhcb1	phytochrome regulation	1
EveningAT	circadian control	1
GATA box	light regulation	4
GT1 consensus	light regulation	6
GT1 core	light regulation, req. for GT1 binding	1
I-box core	light regulation	1
RealPhalGCHCB21	phytochrome regulation	3
T-box AtGAPB	decrease in light-activated expression	1
ACE	light regulation	1
AE-box	light regulation	3
AT1-motif	light regulation	3
ATC-motif	light regulation	2
ATCC-motif	light regulation	1
CATT-motif	light regulation	3
G-box	light regulation	13
GA motif	light regulation	8
GAG-motif	light regulation	4
GT1 motif	light regulation	2
LAMP element	light regulation	2
MRE	myb binding site, light regulation	4
TCCC-motif	light regulation	3
chs-CMA1a	light regulation	1
chs-CMA2a	light regulation	1
Dof core ZM	Dof binding site	20
DPBF core DCDC3	pZIP TF binding site	1
MybPlant	plant myb TF binding site	1
Rav1 AAT	Rav1 TF binding site	1
CANBnNapa	seed storage protein	1
Ebox BnAAPA	seed storage protein	12
Prolamin Box OsGL	prolamine, rice	1
Sef4MotifGM7S	seed storage protein	2
OS2	zein metabolism regulation	2
RY-element	seed-specific regulation, sunflower	1
Skn1-like motif	endosperm expression	1

Table 6, continued.

cis element	function	number of repetitions
GTGANTG10	pollen-specific expression	9
Pollen1Lelat52	pollen-specific expression	7
CGACGOsAmy3	rice amylase, sugar starvation	1
ElReCorePCRP1	elicitor responsive element	1
LtRECoreAtCor15	low temperature responsive element	3
RootMotifTAPox1	RolD promoter (root)	6
CGTCA motif	MeJA responsive element	1
ELI-box 3	elicitor responsive element	1
GC-motif	anoxia-specific inducibility	1
HSE	heat stress	13
P-box	GA regulation	3
TCA-element	SA regulation	3
TGACG-motif	MeJA responsive element	1
WUN-motif	wound responsiveness	9

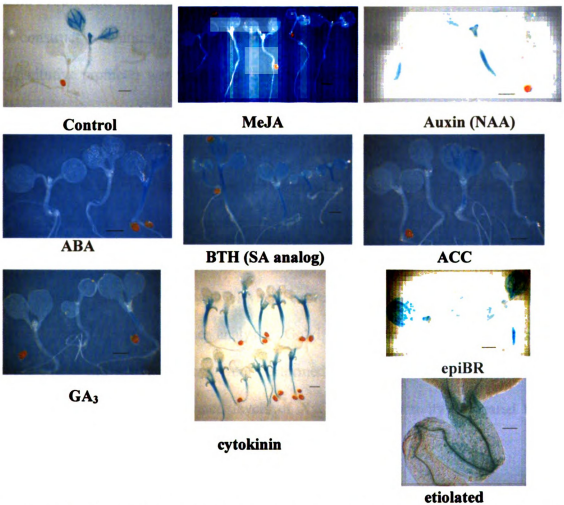

Figure 26. Expression of *AtFUT1* in hormone-treated or etiolated promoter-GUS transgenic plants. Representative images are shown from staining of three different P2.0 transgenic lines. Seedlings were grown for 10 days on growth plates and then transferred to incubation media (control) or treated with methyl jasmonate (MeJA, see methods), 50 μM 1-napthalene acetic acid (auxin), 50 μM abscisic acid (ABA), 0.03% BHT, 100 μM gibberellic acid (GA_3), 1 mM 1-aminocyclopropane-1-carboxylic acid (ACC), 2 μM epibrassinolide (epiBR), or cytokinin. All treatments except that with cytokinin took place for 24 hours. Cytokinin (6-benzylaminopurine, BAP) treatment was performed by growing plants on standard growth plates supplemented with 4 μM BAP for 10 days. Etiolated plants were grown on plates wrapped with aluminum foil. Bar = 100 μm (etiolated), 1 mm (all others).

4.3.6 Production and Analysis of 35S::AtFUT1 Transgenic Plants

A construct containing cDNA encoding *AtFUT1* regulated by the CaMV 35S strong constitutive promoter was used to generate transgenic *Arabidopsis* plants. The presence of the transgene in hygromycin-resistant plants was confirmed by PCR amplification using primers that span the intron of *AtFUT1*, allowing one to distinguish between the transgene and the endogenous genomic version of the gene (data not shown). RNA was prepared from T_2 transgenic plants and used to examine expression of *AtFUT1* by RT-PCR (Figure 27) in leaves, which have very low levels of *AtFUT1* expression in wild-type plants. Multiple lines with elevated expression of *AtFUT1* were identified, and several were chosen for further analysis. Golgi vesicles were prepared from leaves of these lines, and activity assays were performed using this protein (Figure 28). The transgenic lines showed elevated levels of XyG FUTase activity compared to Golgi vesicles derived from wild-type leaves.

Cell wall preparations were made from these lines and analyzed for XyG Fuc content at the Complex Carbohydrate Research Center (Figure 29.) Preliminary analyses indicate that the level of Fuc present in XyG oligosaccharides of the over-expressing transgenic lines is slightly elevated over wild type levels in xyloglucan endoglucanase (XEG)-extracted fractions, but is not elevated in 4M KOH-extracted fractions. There is also an unexpected increase in the amount of XyG subunits containing acetylated Gal, with levels of acetylated Gal units being approximately two-fold higher in the over-expressing transgenic lines as compared to wild type plants.

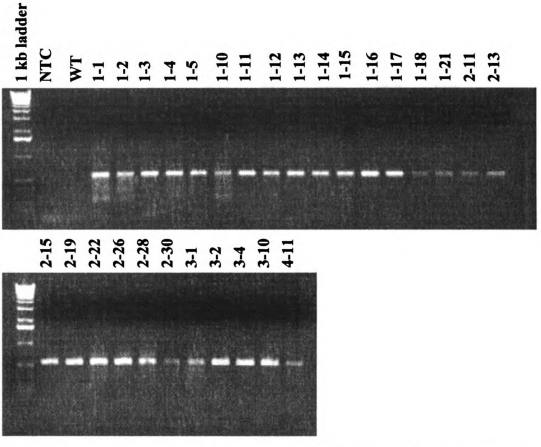

Figure 27. Expression analysis of *AtFUT1* in *35S::AtFUT1* transgenic plants by RT-PCR. RNA was prepared from leaf tissue of wild-type or transgenic lines. One microgram of RNA was used as template in reverse transcription reactions, and an equal volume of RT reaction was used for PCR amplification of *AtFUT1*. Samples were analyzed on an agarose gel stained with ethidium bromide.

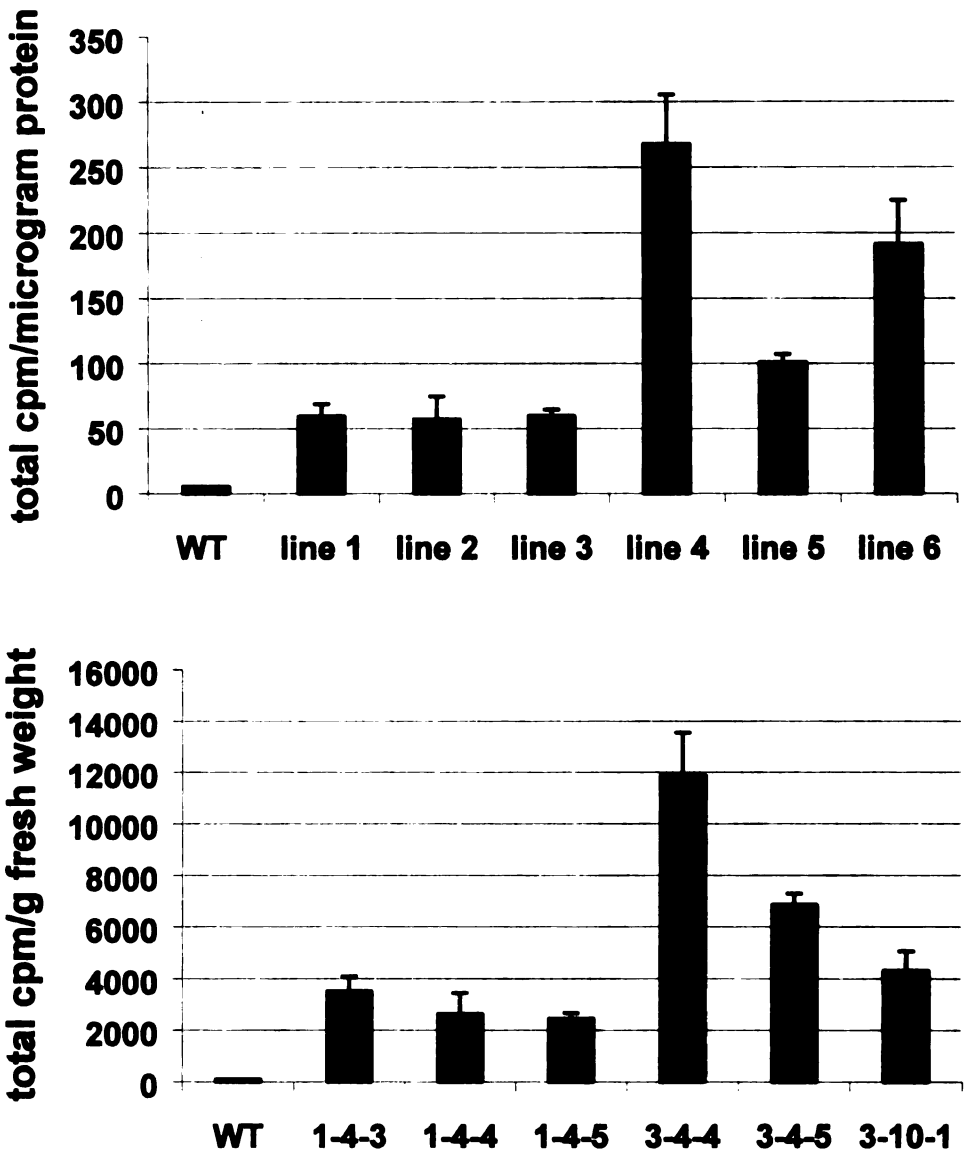

Figure 28. XyG FUTase activity in Golgi vesicles derived from *35S::AtFUT1* transgenic plants. Golgi vesicles were prepared from leaves of transgenic plants and used in XyG FUTase activity assays. Assays were conducted at room temperature for one hour. Duplicate samples were performed. Results are expressed as total cpm/μg protein (top) or total cpm/g fresh weight (bottom). Error bars indicate one standard deviation.

Table 7. Analysis of XyG from 35S::AtFUT1 transgenic plants. XyG was extracted from depectinated cell wall preparations of wild-type and over-expressing transgenic lines. Depectinated cell wall material was first treated with an XyG-specific endoglucanase (XEG fraction), then with 1 M KOH (fraction not analyzed further), and finally with 4 M KOH. XyG polymers from the XEG and 4 M KOH fractions were digested into oligosaccharides and analyzed by ^{1}H-NMR to quantify resonance signals characteristic of specific XyG side chain structures. Data were normalized by setting the total amount of xylose residues per unit of oligosaccharide to 3. Data are expressed as the number of residues or side-chains per oligosaccharide subunit. Fuc, total fucose per XyG oligosaccharide subunit; L, total galactose per XyG oligosaccharide subunit; L(Ac), Gal(Ac)-Xyl side-chains; F, Fuc-Gal-Xyl side-chains (with no acetate substitutions on Gal); F(Ac), Fuc-Gal(Ac)-Xyl side-chains; X, Xyl residues; F(Ac)a, Fuc-Gal(Ac)-Xyl calculated from the area of the acetate methyl signal; F(Ac)b, Fuc-Gal(Ac)-Xyl calculated from the area of the H6/H6' of acetylated Gal in F. Acetate modifications are only detectable in the XEG fraction, because acetate groups are hydrolyzed in 4 M KOH.

XEG fraction

Sample	Fuc	L	L(Ac)	F	F(Ac)	X	F(Ac)a	F(Ac)b
wild-type	0.45	0.44	0.08	0.22	0.23	2.03	0.21	0.18
1-4-2	0.46	0.41	0.11	0.11	0.35	2.02	0.33	0.32
1-4-3	0.47	0.4	0.09	0.09	0.38	2.04	0.36	0.36
1-4-4	0.47	0.4	0.08	0.1	0.37	2.06	0.36	0.35
1-4-5	0.46	0.4	0.1	0.1	0.36	2.04	0.34	0.35
3-4-1	0.47	0.41	0.09	0.12	0.35	2.03	0.33	0.33
3-4-2	0.48	0.39	0.1	0.12	0.36	2.03	0.35	0.32
3-4-3	0.49	0.4	0.06	0.15	0.34	2.04	0.34	0.34
3-4-4	0.48	0.38	0.04	0.16	0.32	2.1	0.32	0.33
3-4-5	0.48	0.4	0.05	0.14	0.34	2.07	0.34	0.34
3-10-1	0.46	0.41	0.09	0.09	0.37	2.04	0.33	0.34

4M KOH fraction

Sample	Fuc	L	F	X
wild-type	0.5	0.47	0.52	2.01
1-4-2	0.49	0.45	0.52	2.03
1-4-3	0.51	0.44	0.52	2.04
1-4-4	0.52	0.46	0.53	2.01
1-4-5	0.51	0.45	0.54	2.01
3-4-1	0.5	0.47	0.52	2
3-4-2	0.5	0.45	0.51	2.04
3-4-3	0.49	0.43	0.53	2.04
3-4-4	0.53	0.45	0.53	2.02
3-4-5	0.52	0.46	0.54	2
3-10-1	0.51	0.46	0.53	2.01

4.4 Discussion

Cell wall biosynthesis is a critical process during plant growth and tissue differentiation. There are, however, limited systems at present that can be used to study cell wall biosynthetic processes at multiple levels. XyG FUTase offers a means of investigating one cell wall biosynthetic event at the level of gene regulation, enzyme activity, and product analysis.

It is important to note that various regions of the mature *Arabidopsis* plant will be undergoing different growth rates at a single point in time. Rosette leaves, for example, go through a period of expansion and growth early during the life of the plant, but cease increasing in size once the plant reaches vegetative maturity and begins to produce a reproductive inflorescence stem. The stem itself grows by division of cells in the shoot apical meristem at the top of the inflorescence stem. These cells elongate and then differentiate. As a result, growth may still be occurring at the top of the stem at the same time that cells at the bottom of the stem have ceased to increase in size. A similar gradient of growth occurs in the roots. Thus, there are inherent differences in growth kinetics among various tissues of mature *Arabidopsis*, and this may be reflected by variations in the rates of cell wall biosynthesis occurring in these tissues. It is also possible, however, that differences exist in the cell wall composition and structure of various tissue and cell types. Such differences have been described in different plant species and are reviewed in Chapter 1. Therefore, it must be determined whether any differences in the regulation of a cell wall biosynthetic process between an assortment of

tissue types are due to variations in growth kinetics or variations in cell wall polymer structure.

Levels of *AtFUT1* gene expression and XyG FUTase activity were assessed in seven different *Arabidopsis* tissues. Distinct differences were observed in gene expression and enzyme activity among the samples. In general, the upper region of the stem showed high gene expression and activity levels, while the lower region of the stem showed less activity. Fully expanded rosette leaves showed a near-absence of gene expression and enzyme activity. Flowers showed high enzyme activity, but RT-PCR analysis of the same sample showed less gene expression, although analysis of other floral samples by RT-PCR and hybridization techniques have shown increased gene expression in flowers.

Although the structure of *Arabidopsis* cell walls has been a subject of previous study (Zablackis et al., 1995), only leaves were used for analysis in their experiments. No systematic comparison of XyG structure from cell walls of different tissues in *Arabidopsis* has yet been published. Although the structure of XyG has been determined for a number of species, it is not known whether there may be subtle variations in the structure of this polymer throughout different regions of the plant. Some small differences have been detected, for example, in the structure of XyG isolated from growing and non-growing regions of etiolated pea epicotyls (Pauly et al., 2001b). Analysis of XyG fucosylation patterns in oligosaccharides derived from various *Arabidopsis* tissues indicated no major differences, with the amount of Fuc present per unit of XyG oligosaccharide remaining at a fairly constant ratio of around 0.5. Although

one rosette leaf sample indicated about half as much Fuc present per XyG unit compared to other tissues, subsequent analysis of independent biological replicates and a leaf developmental series did not show significant differences. It is assumed that biological variability was the source of the initial lower ratio. Thus, the Fuc content of XyG appears to remain fairly constant throughout different tissues of *Arabidopsis*.

One disadvantage of RT-PCR and hybridization-based expression analysis techniques is their limited ability to discern cell-type or tissue-specific patterns of gene regulation. Two methods commonly used to overcome this limitation are *in situ* hybridization and promoter-reporter analysis. Because of the inherently low expression levels of *AtFUT1* and the fact that it is a member of a family containing nine other *AtFUT1*-like genes, promoter-reporter analysis was used to determine spatial patterns of gene expression.

In order to make appropriate promoter-reporter constructs, the delineations of the *AtFUT1* promoter region had to be considered. At the time that the constructs were planned, the nearest predicted ORF upstream of *AtFUT1* was several kilobases away, which did not allow the delineations of the promoter to be determined with any degree of certainty. Between the time that the constructs were generated and the time at which the promoter-reporter transgenic plants were analyzed, however, the genomic region upstream of *AtFUT1* was re-annotated by the Arabidopsis Genome Initiative (Figure 30). There are two predicted ORFs upstream of the *AtFUT1* ORF, the closest of which is currently annotated as a 458 nt-long hypothetical protein (AT2G03230). No ESTs in exist in public databases that correspond to the hypothetical protein, and there is no

evidence that the gene encoding it is transcribed. The predicted hypothetical protein would be 159 aa long protein and approximately 20 kD in size. The next nearest upstream predicted ORF is currently annotated as an unknown protein (AT2G03240). An ORF prediction for the unknown protein did not exist at the time that the promoter-reporter constructs were made, probably because of the complex intron-exon structure of this gene; there are 10 predicted exons and 9 introns over a span of 3924 nt in the unknown protein gene. A BLAST search using the predicted cDNA for this gene returns a partial mRNA sequence for a transcript detected during early response to nematode infection with Z-score of 119 and E value of $1e^{-23}$, indicating that this gene may encode a nematode response protein and is probably transcribed. The end of the nematode response ORF is 1060 bp upstream from start of the *AtFUT1* ORF. Thus, it is expected that the *AtFUT1* promoter region may be 1060 bp long or less, unless regulatory regions exist within the coding region of the nematode response gene. For this reason, only the transgenic plants containing the 2.0 kb *AtFUT1* promoter fragment were used for extensive analysis of gene expression.

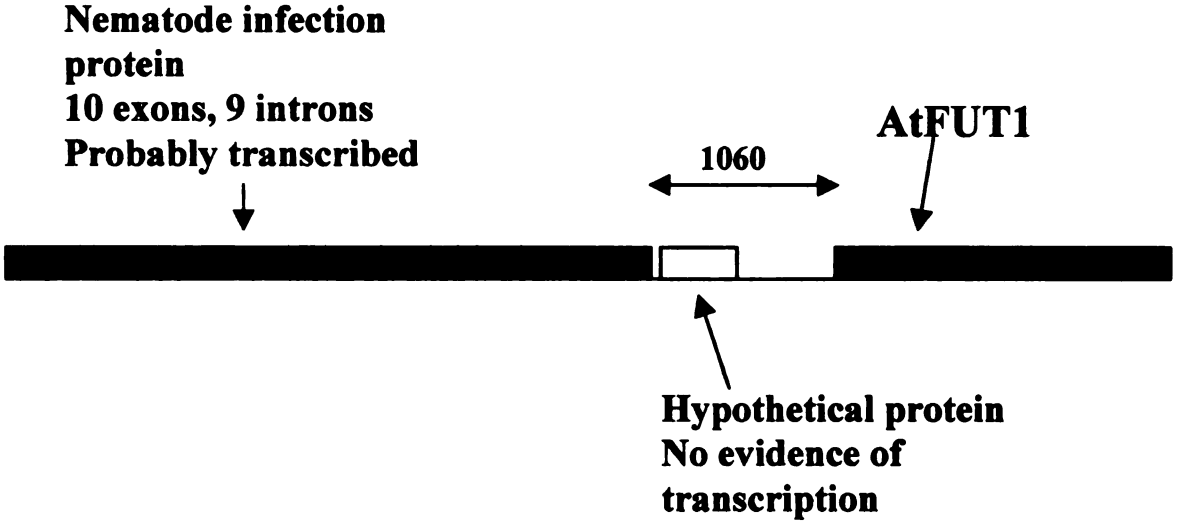

Figure 30. Structure of genomic region upstream of *AtFUT1*. Two ORFs lie upstream of *AtFUT1* on Chromosome 2. Directly upstream is a small predicted ORF (hypothetical protein) that is 458 nt long, shown as a white box. No ESTs corresponding to this hypothetical protein exist in public databases. Further upstream is an ORF encoding a gene that is induced upon infection by nematodes. This gene has a complex intron-exon structure, with 10 exons and nine introns over a total of 3924 nt. There is evidence that the nematode response gene is transcribed. The end of the nematode response gene ORF is 1060 nt upstream of the beginning of the *AtFUT1* ORF, which itself is 2043 nt long. This figure is drawn to scale.

Systematic analysis of transgenic promoter-reporter plants indicates that *AtFUT1* gene expression is regulated. The observations generally agree with gene expression data obtained by RT-PCR and hybridization techniques, with high reporter activity occurring in the top of the inflorescence stem and lower activity occurring in fully expanded rosette leaves (limited mainly to a small portion of the midvein). One unexpected finding was the extent of expression correlated with vascular tissue in cotyledons, regions of the true leaf vasculature, and the central vascular cylinder of the hypocotyl. Fully differentiated vascular tissue is generally thought to be comprised of secondary cell walls, which are not rich in XyG. It is possible that at the early stages of development, the vascular tissue showing increased reporter gene expression had not yet fully progressed into secondary cell wall synthesis. Alternatively, it is possible that the vascular patterns observed were artifacts of the promoter-reporter technique, although ferricyanide and ferrocyanide were included in the staining buffer in an attempt to reduce dispersal of the stain (for example, through the vascular system). However, the overall expression profile does correlate with that observed using other methods, and it is possible that the patterns observed in the transgenic plants are indicative of spatial gene expression regulation *in vivo*.

One important observation is that the expression pattern of *AtFUT1* in the inflorescence stem does correlate very well to the elongation rate in regions of the stem. In other words, high gene expression is observed in the top of the stem, which shows over an eightfold increase in elongation rate compared to the bottom of the stem. The increase in expression in this region of the stem was confirmed by three different methods (RT-PCR,

northern analysis, and promoter-GUS staining). A similar profile was observed for XyG FUTase enzyme activity levels in these regions. This provides strong evidence that XyG fucosylation, a primary cell wall biosynthetic process, is regulated in a manner that is positively correlated with growth in the inflorescence stem.

A similar correlation could not be detected in the other major region of elongation growth in the plant, the root. However, different methods did indicate some instances in which *AtFUT1* expression and XyG FUTase activity were elevated in roots. It is possible that the level of expression in roots of promoter-reporter plants was too low to be detectable by this method. It was not possible to dissect *Arabidopsis* roots, which are thinner than the width of a human hair, very fragile, and prone to entanglement, into elongating and differentiated regions. The variable results in expression and activity levels may be indicative of the ratio of elongating versus differentiated tissue in various biological samples. It should be noted that *AtFUT1* is thought to be the sole gene responsible for fucosylation of XyG, as a T-DNA knockout (Sarria et al., 2001, in press) and the *mur2* mutant affected in *AtFUT1* (Vanzin et al., 2001, submitted) lack Fuc on XyG in all tissues examined, including (in the case of Vanzin et al.) the roots.

XyG FUTase activity profiles showed high activity levels in flowers, but various gene expression techniques did not show strong up-regulation of *AtFUT1* in floral tissue. One possible explanation is that the high activity levels are due not to the presence of floral tissue itself, but the presence of fragments of inflorescence stem collected during tissue harvesting. If, as the promoter-reporter analysis indicates, high gene expression occurs in

the uppermost region of the inflorescence stem, the amount of stem present in a given "floral" tissue sample could have a large effect on the amount of XyG FUTase activity and *AtFUT1* gene expression. Thus, variation between different samples could be due to differences in harvesting technique and exact sites of tissue dissection. With this in mind, it is interesting to note the difference in *AtFUT1* expression between "flowers" and "floral buds" as detected by northern blot (Figure 6). In this case, flowers were defined as individual fully opened flowers cut away from the inflorescence stem (although some amount of floret stem is present, holding the flower together at the base.) Flower buds were defined as the unopened flowers at the top of the inflorescence. Because of the manner in which these samples were harvested, the "floral buds" would contain more inflorescence stem than the "flowers". Greater *AtFUT1* expression is observed in the floral bud sample versus the flower sample (Figure 6). An alternative explanation for the variability in gene expression of flower samples would be that expression could be regulated in a pollen-specific manner, and the extent of detection would be due to how much pollen was present after harvesting, handling, and experimental manipulation. Pollen-specific expression was observed in promoter-reporter analysis of a gene encoding an isoform of GDP-D-mannose 4,6 dehydratase, which synthesizes GDP-Fuc (Bonin et al., in press.) However, no pollen staining has been observed in promoter-reporter analysis of *AtFUT1* gene expression.

Promoter-reporter analysis also allows gene regulation to be assessed easily after exposure to various treatments, such as hormones or different growth treatments. Incubation of promoter-reporter plants with a series of different growth hormones at

levels used in gene regulation experiments reported in the literature indicated that *AtFUT1* may be up-regulated in expression by the cytokinin BAP and, to a lesser extent, by the SA analog BHT. *AtFUT1* expression may also be repressed upon exposure to the volatile signaling molecule MeJA. Cytokinin is a growth hormone involved in cell division and developmental regulation, while SA is thought to be a key signaling molecule for certain plant defense responses. MeJA also participates in defense responses, but seems to play a major role in defense against herbivores rather than fungal, bacterial or viral pathogens. The biological significance of these observations is not clear, but further testing and verification by independent techniques would assist in assessing the regulation of *AtFUT1* in response to cytokinins, SA and MeJA. The expression of *AtFUT1* in various mutants defective in the ability to produce cytokinin, SA and MeJA would also enable further characterization of these results.

In addition to the *AtFUT1* gene expression data described here, I also assessed other publicly available data. During the course of this thesis research, the *Arabidopsis* Functional Genomics Consortium performed microarray experiments for individual researchers wishing to investigate global gene expression under conditions of interest or in various genetic backgrounds. A cDNA encoding *AtFUT1* was present on the arrays used for these experiments, and all experimental data from these efforts are made available to the public after an initial waiting period. These data sets were analyzed for *AtFUT1* expression levels. Table 7 indicates several experiments in which a reciprocal set of microarrays (for example, flowers versus leaves and leaves versus flowers) indicated differential expression of *AtFUT1* between the two samples studied. The

criteria used were that one slide showed a normalized green/red signal for *AtFUT1* greater than two on one slide and the slide for the inverse labeling experiment showed a low, if not exactly reciprocal to the first, normalized green/red signal. In the first study, researchers compared gene expression in flower versus leaf tissues (Jeff Landgraf, personal communication). *AtFUT1* expression results were consistent to other expression data shown above (that is, *AtFUT1* expression was higher in flowers than leaves). In the second study, gene expression profiles were compared between the ABA-insensitive mutant *abi1* to wild type L*er* plants. However, these experiments have been repeated using Affymetrix DNA array chips, and the trend noted in the microarray experiments did not hold (Julian Schroeder and Majid Ghassemian, personal communication). Thus, it must be noted that trends observed in microarray experiments should be confirmed by independent techniques, such as hybridization analysis or RT-PCR, before conclusions can be drawn with certainty. In a third experiment, researchers attempted to identify genes that are targets of the homeobox gene *knotted1* (*kn1*) using transgenic plants expressing *kn1* under the control of the glucocorticoid receptor and induced with dexamethasone to wild-type plants sprayed with dexamethosone (Sarah Hake and Naomi Ori, personal communication). *KNOTTED1* is a transcription factor that is involved in leaf development and meristem identity, and was the first homeodomain-containing protein identified in plants (Vollbrecht et al., 1991). *AtFUT1* expression shows a threefold increase in normalized G/R ratio in the induced *kn1* transgenic plants. These experiments are also being repeated using the Affymetrix system, and expression of *AtFUT1* will be assessed using this technique. Gene expression profile patterns were examined in the fourth study in an attempt to identify genes necessary for adaptation to

low availability of chlorophyll using a leaky magnesium chelatase mutant, *cch1*. This mutant becomes pale green when transferred to moderate light (200 μmol photons m^{-1} sec^{-1}), whereas wild type plants remain green under these conditions (Judy Brusslan, personal communication). Genes expressed in wild type and *cch1* plants under these light conditions were compared, and *AtFUT1* is expressed more highly in wild-type plants than in the *cch1* mutant. It is interesting to note that the promoter region of *AtFUT1* does contain a number of cis-acting elements previously characterized in light-regulated genes (Table 6.) In the sixth experiment, investigators compared gene expression profiles in the *enhanced disease resistance 1* (*edr1*) mutant to those of wild-type plants (Roger Innes and Dingzhong Tang, personal communication). The *edr1* mutant shows enhanced resistance to the fungal pathogen that causes powdery mildew. Germination of asexual spores is inhibited in the mutants, but initial stages of infection are not affected (Frye and Innes, 1998). *AtFUT1* expression is higher in *edr1* plants than in wild type. It is possible that *AtFUT1* expression is altered during response to pathogen infection, though this possibility needs to be investigated by other methods. This might be explained, for example, if pathogen defense includes alteration of cell wall biosynthetic processes. Cell wall structural alterations, such as cross-linking phenolic compounds or localized lignification, have been described previously (Hardham and Mitchell, 1998; Wojtaszek, 2000).

Table 8. Summary of data on *AtFUT1* expression from public database of microarray experiments. Only data with a green channel: red channel normalized (G/R normalized) ratio of >2.0 in one slide and a low G/R signal in an inverse experiment are shown. *, independent Affymetrix chip data are available, and they do not support the trend suggested by the microarray experiments.

Experiment	G/R normalized
Flowers vs. Leaves	2.169
Leaves vs. Flowers	0.346
abi1 slide 1*	6.897
abi1 slide 2*	0.674
downstream genes of kn1 slide 1 (induced kn1_GR green, induced control red)	3.174
downstream genes of kn1 slide 2 (induced control green, induced kn1_GR red)	0.668
genes in chlorophyll starvation slide 1 (WT green, *cch1* red)	2.347
genes in chlorophyll starvation slide 2 (*cch1* green, WT red)	0.265
Pathogen Response 2 - reverse	2.146
Pathogen Response 1	0.654
edr1 Mutant 2 – reverse (*edr1* green, WT red)	2.05
edr1 Mutant 1 (strong spatial bias) (WT green, *edr1* red)	0.584

Ectopic expression is often used to study the effects of gene manipulation *in planta.* Transgenic plants expressing *AtFUT1* under control of a strong constitutive promoter show increased gene expression and XyG FUTase activity compared to wild type controls. Preliminary analysis of XyG structure indicates that Fuc levels are slightly elevated in the transgenic plants. A more surprising observation is that the level of acetylated Gal units (for example, side chains containing Fuc-Gal-Xyl with an acetate group present on Gal) is approximately two-fold higher in the over-expressing plants. Acetylation of Gal has been observed at the O-6, O-4, and O-3 positions in sycamore XyG (York et al., 1988). Mono- or di-acetylation may occur, and in sycamore the predominant form was mono-acetylation at the O-6 position (York et al., 1988). Although it is known that acetyl Co-A is the donor substrate for acetylation (Pauly and Scheller, 2000), the enzyme responsible for the acetylation of XyG or the gene that encodes it has not been identified. The biological role of acetylated Gal units in XyG is not known, although it has been speculated to affect hindrance of polymer breakdown (Pauly and Scheller, 2000). Interestingly, a recent structural analysis of XyG derived from the *mur1* mutant indicated that XyG derived from leaf tissue is less acetylated in the mutant than in wild type (Pauly et al., 2001a). The *mur1* mutant is deficient in an isoform of GDP-D-mannose-4,6-dehydratase and thus partially lacks the ability to synthesize GDP-Fuc. Analysis of *mur2*, a mutant affected in *AtFUT1*, or a mutant with a T-DNA insertion in AtFUT1, also indicates reduced levels of Gal acetylation (W. York, personal communication.) When preliminary data on XyG structure from *35S::*AtFUT1 plants are compared to the *mur1*, *mur2*, and *AtFUT1* knockout studies, it appears that there may be a correlation between the presence of Fuc on XyG and the extent of Gal

acetylation. One explanation for this could be that the presence of Fuc permits a particular structural conformation to be assumed that facilitates acetylation. Alternatively, there could be a process-based relationship between fucosylation and acetylation. For example, if the residence time of the polymer in the Golgi determined the exposure of the polymer to the acetyltransferase, and if over-expression of *AtFUT1* caused an increase in this residence time, an increased level of Gal acetylation could result. Additional studies would have to be conducted in order to evaluate these possibilities, however.

No differences in phenotype are observed for these plants under normal growth conditions. However, it is possible that strong constitutive expression of *AtFUT1* results in phenotypes only apparent under certain conditions, such as exposure to pathogens, herbivores, or abiotic stresses. Further characterization under a series of conditions may allow identification of situations in which alteration of *AtFUT1* expression results in a demonstrable consequence for the plant.

In conclusion, *AtFUT1* expression and XyG FUTase activity were found to be positively correlated with growth in aboveground regions of *Arabidopsis*. Determination of the gene expression profile of *AtFUT1* should assist in the evaluation of other primary cell wall biosynthetic gene candidates, as has indeed been the case within our research group (T. Wagner, C. Wilkerson, S. Meyer, unpublished results.) Various hormone treatments may affect the expression of *AtFUT1*, possibly allowing the plant to regulate this cell wall biosynthetic process in response to these signals. Manipulation of the expression of

AtFUT1 using the strong constitutive 35S promoter results in increased XyG FUTase activity and altered XyG acetylation patterns. It is possible that these changes may have phenotypic consequences under certain conditions, but no phenotypic effects were observed when plants were grown in growth chambers. Overall, this work should lead to a more precise understanding of xyloglucan fucosylation as a model cell wall biosynthetic process.

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CHAPTER FIVE

Conclusions and Future Directions

5.1 Summary of Research Significance

At the beginning of this thesis, no cell wall biosynthetic genes had been identified at the molecular level with certainty. Although there were hints about conserved sequence motifs and plant genes that might be important for cellulose synthesis in 1996 (Pear et al., 1996), it was not until 1998 that genetic evidence was published confirming the necessity of the CesA isoforms for manufacturing cellulose (Arioli et al., 1998). Although there were a number of FUTases identified in various animal systems (for review, see Field and Wainwright, 1995), no plant sequences in public databases showed any similarity to the mammalian proteins.

And yet, a great deal had been determined regarding the structure of the plant cell wall through several decades of investigation, and evidence was beginning to suggest that cell wall biosynthetic glycosyltransferases could be identified. An assay had been developed to detect XyG FUTase activity (Hanna et al., 1991) and preliminary results from this research group indicated that purification of this enzyme was indeed feasible (data from A. DeRocher, M. Bar-Peled, N. V. Raikhel, K. Keegstra.) During the course of this thesis research, XyG FUTase has been purified by a team effort involving several researchers in the Keegstra and Raikhel laboratories, the homologous gene encoding this enzyme has been cloned in Arabidopsis (R. Perrin) and later in pea (W. Zeng), and the regulation of this gene and enzyme system have been studied (R. Perrin). Other research groups have identified additional cell wall biosynthetic genes and enzymes during the

past five years. These include galactomannan galactosyltransferase (Edwards et al., 1999), the cellulose synthase catalytic subunit (Arioli et al., 1998), and cellulose synthase-like genes (Richmond and Somerville, 2001).

This thesis research seeks to contribute some groundwork to the field of plant cell wall biosynthesis, allowing the information on the regulation of *AtFUT1* expression and XyG FUTase enzyme activity to be used to evaluate other candidate genes for cell wall synthesis. The present work has also confirmed that the process of XyG fucosylation is correlated with elongation growth in aboveground regions of *Arabidopsis*, an idea that has long been presumed and is now confirmed at the molecular level. This indicates that XyG fucosylation may be studied as a model primary cell wall biosynthetic event. In addition, it is hoped that this research will begin to provide some insight into the biological significance of XyG fucosylation. Manipulation of the expression of *AtFUT1* by strong constitutive expression causes an increase in XyG FUTase activity, alteration of XyG structure in terms of Gal acetylation (though not, interestingly, in terms of fucosylation), and could have phenotypic consequences under specific circumstances although no major differences are observed under normal growth conditions.

5.2 Future Directions

There are a number of additional experimental lines that may be followed to extend this work, a few of which will be described. One area of study would utilize the *AtFUT1* over-expressing transgenic lines described in this thesis, as well as a T-DNA insertional *AtFUT1* mutant completely lacking Fuc on XyG, to do more physiological studies

comparing the performance of these plants to wild type controls under various conditions. These studies would seek to determine the function of Fuc on XyG. Fuc modification of XyG is a well-conserved structure found throughout all dicot and nongraminaceous monocot plant species (although Solenaceous species replace Fuc modification with other structures such as Ara). The relative regularity of Fuc modification would seem to indicate that there is some sort of selective pressure for this structure in nature. Although both T-DNA knockout and over-expressing plants do not show altered phenotypes under normal growth conditions in greenhouses or growth chambers, it is entirely possible that the presence of Fuc on XyG is advantageous under certain conditions such as challenge with pathogens or herbivores. Alternatively, subtle differences in growth or developmental rates might not be discernable under nonsystematic observation, but could be identified by careful growth kinetic characterization. Such analysis is being done on the T-DNA knockout by Paradigm Genetics and could also be performed using the over-expressing lines as well.

Another line of research would involve confirmation of the cytokinin and SA inducibility and MeJA repression of *AtFUT1* by an independent technique such as RT-PCR. This could be followed by whole plant-level experiments to determine the biological significance of these observations. At present, there are indications that these signaling molecules may regulate the expression of *AtFUT1*, but the consequences of this regulation have not yet been determined.

Although microarray technology has been used during the past five years to investigate global gene expression patterns, Affymetrix arrays are beginning to emerge as the leading technology in these types of experiments. Theoretically, it would be possible to use Affymetrix arrays and the complete *Arabidopsis* genome sequence information to continue to develop a more thorough expression profiling data set for *AtFUT1*, which could be used to identify other co-regulated genes. By comparing promoter sequences of such co-regulated genes, it would be possible to identify cis-acting sequences putatively required for coordinately regulated expression. This could allow another means of identifying and assessing other candidate genes, if they have such regulatory elements in their promoters, and could also help to identify trans-acting factors involved in regulating the expression of cell wall biosynthetic genes.

Finally, existing information about *AtFUT1* and XyG FUTase will continue to assist identification and evaluation of other genes involved in synthesis of XyG and other cell wall polymers. Information on protein structural features and motifs have allowed identification of gene candidates (C. Wilkerson), while expression profiling data is currently providing another means of evaluating several gene candidates of unknown function that may be involved in cell wall biosynthesis (T. Wagner, S. Meyers, C. Wilkerson.) This should provide a complement to other approaches to identify cell wall biosynthetic genes, such as biochemical characterization of candidates expressed in heterologous systems (A. Faik, T. Wagner), suppression of candidate genes or gene families by RNAi or insertional mutagenesis followed by phenotypic characterization (W. Zeng, T. Wagner, S. Hazen, J. Scott-Craig), or identification of quantitative trait loci

associated with cell wall structural features (S. Hazen). Although cell wall biosynthesis research is and will continue to be a challenging area, there are now many different approaches available. It is almost certain that a large number of cell wall biosynthetic genes will be identified and characterized within the next five to ten years.

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