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Untersuchungen zur Biosynthese der Zellwand

Dissertation
zur Erlangung des akademischen Grades
"doctor rerum naturalium"
(Dr. rer. nat.)
in der Wissenschaftsdisziplin Biochemie

eingereicht an der
Mathematisch-Naturwissenschaftlichen Fakultät
der Universität Potsdam

von
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Abbreviations

AGP	Arabinogalactan protein
AXS	bifunctional UDP-D-apiose, UDP-D-xylose synthase
bp	Base pairs
BLAST	Basic local alignment search tool
BSA	Bovine serum albumine
CaMV	Cauliflower mosaic virus
cDNA	Complementary DNA
CSBDB	Comprehensive systems biology database
cv	Cultivar
dCTP	Deoxycytididne triphosphate
dest.	distilled
EDTA	Ethylenediamine tetratacetic acid
EtOH	Ethanol
GAE	UDP-D-glucuronic acid epimerase
GC	Gas chromatography
GDP	Guanine diphosphate
GlcA	Glucuronic acid
GME	GDP-D-mannose epimerase
GRP	Glycine-rich protein
HGA	Homogalacturonan
HPAEC	High performance anion exchange chromatography
HRGP	Hydroxyproline-rich protein
MIOX	*Myo* inositol oxygenase
mRNA	Messenger RNA
MALDI	matrix assisted laser desorption ionization
MS	mass spectrometry
NCBI	National Center for Biotechnology Information
NDP	Nucleoside diphosphate
PAD	Pulsed amperometric detection
PCR	Polymerase Chain Reaction
RG I, II	Rhamnogalacturonan I, II
RHM	putative UDP-L-rhamnose synthase
RNA	Ribonucleic acid
RNAi	RNA interference
RT	Room temperature
SD	Standard deviation
SDS	Sodium dodecyl sulphate
TC	Tentative contig
TFA	Trifluoracetic acid
T-DNA	Transfer DNA
TOF	time of flight
UDP	Uridine diphosphate
UGD	UDP-D-glucose dehydrogenase
UGE	UDP-D-glucose epimerase
UXE	UDP-D-xylose epimerase
UV	ultraviolet
WT	Wild-type

1 Introduction

1.1 The plant cell wall

The plant cell wall is one of the most striking features that differentiates a plant from animals. Even though the plant cell wall was already discovered nearly 350 years ago by the british botanist Robert Hooke, it took more than three centuries to establish basic knowledge of its structure.

The plant cell wall has been established as one of the most important structures of a plant as it harbors many vital functions for the plant. Beside its role to give stability to the plant and to counterbalance the internal turgor pressure (Bacic *et al*. 1998) it offers protection from mechanical injury or pathogen attack (Darvill *et al*. 1980). Moreover comparable to the animal extracellular matrix, plant cell walls can control cell adhesion (Cosgrove 1997), determine porosity (Baron-Epel *et al*. 1998) and extracellular ionic composition (Grignon and Sentenac 1991). Futhermore, it poses an important reservoir for biological molecules such as storage polysaccharides (Franco *et al*. 1996, Santos *et al*. 2004). Despite all these regulatory and stability functions the plant cell wall is flexible and plastic enough to allow plant growth or tropic movements.

Due to these very different functions of the cell wall, three substructures of plant cell walls can be distinguished: Middle lamella, primary and secondary wall. The middle lamella is deposited during cell plate formation (Matar and Catesson 1988). Thus it is a structure shared by two neighboring cells. The primary cell wall, which is deposited after cell division is a rather flexible structure to accommodate cell elongation. The secondary cell wall is deposited upon cessation of growth and adds stability due to its high content of crystalline cellulose and water impermeable lignin.

As a natural renewable product cell walls and derived products are of great importance for various industries. For example paper, textiles, filling materials and food additives are derived from cell walls (Lapain and Pricl 1995).

1.2 Structures in the cell wall

According to current models the primary cell wall (Carpita 2000) consists of four major components interacting with each other. These components are the polysaccharides cellulose, hemicellulose, and pectin as well as proteins. Typically the dry matter of growing dicot walls is composed of about 1/3 of each polysaccharide class and 1-5% proteins (Cosgrove 1997). However, it should be noted that walls contain a large amount of water (Cosgrove 1997). Although most plant species contain similar wall compositions significant differences in the cell wall structure and architecture are found in graminaceous monocots (grasses) (Carpita 1996) reflecting that the cell wall composition can be flexibly adapted .
The individual components of the cell wall will be discussed in further detail here.

1.2.1 Cellulose

Cellulose can occur in a crystalline and non-crystalline (amorphous) form of linear polymers of β-1,4 linked D-glucose. Cellulose-chains often aggregate through noncovalent interactions (H-bonds) and form crystalline microfibrils. Due to these interactions crystalline cellulose has a high tensile strength (Wainwright *et al*. 1976), is insoluble in most solvents, and is chemically stable and resistant to weak acids (Cosgrove 1997). In the wall cellulose-microfibrils represents the load-bearing polymer.

1.2.2 Hemicelluloses

Hemicellulose is a practical term used for a heterogeneous group of glycans which are rather tightly bound to the wall and usually require harsh extraction conditions such as the use of alkaline solutions (Hayashi and Maclachlan 1984, Hayashi 1989). These hemicelluloses are thought to cross-link cellulose microfibrils by hydrogen-bonding (Cosgrove 1997) or can be woven into the cellulose microfibrils (Hayashi 1994) explaining the harsh extraction requirements.
In the primary wall of dicotylodoneous and non graminacaeous monocotylodoneous plants the major hemicelluloses are xyloglucans (Cosgrove 1997), which seem to lie preferentially parallel to the cellulose microfibrils (Séné *et al*. 1994). Xyloglucan is mainly composed of the sugars xylose, glucose, fucose and galactose. Considering the well conserved structural scheme in which these

sugars appear a code was introduced (figure 1) (Fry 1993). In addition, individual sugars might be acetylated.

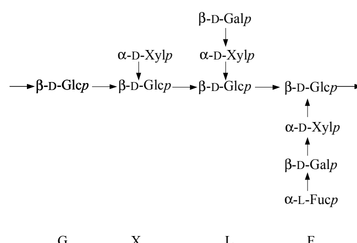


Figure 1 Example of a part of a xyloglucan structure.

The one letter symbolic notation for each of the glucosyl residues is shown below the structure.

Other wall polysaccharides that are classified as hemicelluloses are (arabino-)xylans, mannans and callose (Carpita and Gibeaut 1993).

Xylans are the most abundant hemicelluloses in grasses and hardwoods. They are comprised of β−1,4 linked D-xylosyl residues. Depending on the origin their degree of polymerization varies from 70 to 200 (He *et al*. 1994). Arabinoxylans feature a xylan backbone, decorated with arabinose and glucuronic acid (Darvill *et al*. 1980). Carpita and Gibeaut hypothesized (1993) that arabinoxylans can take over the role of the xyloglucans in grasses, where only small amounts of xyloglucans are present (Labavitch and Ray 1978).

The glucomannans are linear polymers of 1-4-linked mannosyl and glucosyl residues. Three to five percent of the dry weight of most hardwoods consists of glucomannans (Meier 1985).

Callose is an amorphous unbranched β-1,3-D-glucan (reviewed by Verma and Hong 2001). Its presence in the pollen suggests an important role in pollen growth (Sedgley 1979). Furthermore, it is present in the cell plate of dividing cells, and it is deposited upon pathogen attack, where it was implicated in pathogen resistance. However, when a putative callose synthase was downregulated in mutants and transgenic plants, resulting in a lack of wound-induced callose, the affected plants exhibited actually an increased resistance to pathogens (Nishimura *et al*. 2003, Jacons *et al*. 2003).

1.2.3 Pectic polysaccharides

Pectic polysaccharides are characterized by their high content of D-galacturonic acid (D-GalA). They are the most soluble part of the cell wall. They can be extracted with hot water, calcium chelators, or mild alkaline solutions (Cosgrove 1997). In general, pectic polysaccharides are

classified into homogalacturonan (HGA), rhamnogalacturonan I (RGI) and rhamnogalacturonan II (RGII) (Mohnen 1999).

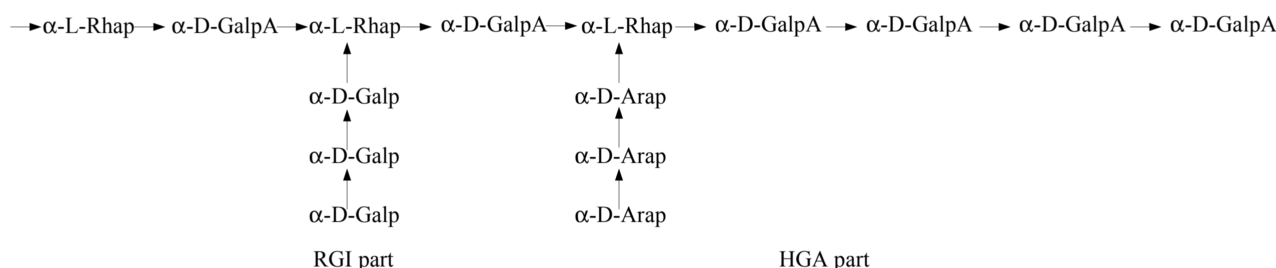


Figure 2 Pectin struture

Left part structure of RGI and right part HGA structure. Note that for clarity reasons HGA has been drawn as a sidechain of RGI her, even though this is still controversial.

HGA is an unbranched chain of approximately 70-100 α-1,4 linked D-GalA units (Thibault *et al*. 1993). It can be methyl-esterified at the C6 carboxylic position of the D-GalA and it can be O-acetylated at the C2 or C3 positions (Ishii 1995, 1997). The degree of methyl-esterfication is an important property of HGA since it modulates polygalacturonase recognition (Dumville and Fry, 2000). Moreover methyl-esterfication neutralizes the otherwise negatively charged, acidic carboxylic group of D-GalA and thus has a major impact on the gelling behavior of pectins. An increased methyl-esterfication leads to decreased binding of calcium (Taitz and Zeiger 1998).

RGI consists of up to hundred blocks of the alternating disaccharide 1,2-α-L-rhamnosyl-1,4-α-D-galacturonic acid (McNeil *et al*. 1980, Visser and Voragen 1996). In contrast to HGA, RGI may contain sidechains in form of arabinans (α-1,5 linked), galactans (β-1,4-linked), or arabinogalactans, which are attached at the C4 position of the rhamnosyl unit (McNeil *et al*. 1980; Lau *et al*. 1985). The proportion of branched rhamnose residues usually is in the range of 0-80 % depending on tissue and plant species (Visser and Voragen 1996, Penfield *et al*. 2001).The sidechains can be further decorated with ferulic and coumaric acid (Jones *et al*. 2003). In smaller amounts fucose, glucose and glucuronic acid might also be present (O'Neill *et al*. 1990). Like HGA in RGI the D-GalA part of the backbone might be acetylated (Komalavilis and Mort 1989) and methyl-esterified (Rihouey 1995).

RGII has a highly complex structure. The backbone of RGII is composed of at least seven α-1,4-linked-D-GalA units to which four different side chains are attached (Vidal *et al*. 2000, Ridley *et al*. 2001). These sidechains feature various sugars and among them some more unusual ones such as 2-keto-3-deoxy-D-manno-octulosonic acid, aceric acid, 2-keto-3-deoxy-D-lyxo-heptulosaric acid

and D-glucuronic acid (O'Neill *et al*. 2001). Thus RGII has the richest diversity of sugars and linkages of any known polysaccharides (Darvill *et al*. 1978).
Pectins do not only occur in the cell wall of plants, but they also account for the major proportion of extractable *Arabidopsis thaliana* and other brassicacae mucilages (Goto 1985, Penfield *et al*. 2001, Usadel and Pauly, unpublished results), where they facilitate seed dispersion and retain water due to their hydrophilic and gelling properties (Fry-Wyssling 1976).
In the plant cell wall, pectins play numerous roles such as mediating fruit ripening possibly through oligosaccharides mediated signaling and/or softening by pectin degradation (Langley *et al*. 1994, Dumville and Fry, 2000). Moreover the strength of the pectic polysaccharide matrix can directly affect the strength of the total cell wall (O'Neill *et al*. 2001, Ryden *et al*. 2003) and determine the porosity of the cell wall (Baron-Epel *et al*. 1988). Furthermore pectins have been implicated in plant growth and cell to cell adhesion (Stephenson and Hawes 1994, Iwai *et al*. 2002). These roles have recently gained further attention after demonstration that the occurrence of pectic epitopes, particularly RGI sidechains, seems to be developmentally regulated (Willats *et al*. 1999, McCartney and Knox 2002, McCartney *et al*. 2003).
In addition, pectins might play a role in pollination by providing an adhesive matrix for the growing pollen tube (Lord 2000, Lenartowska *et al*. 2001, Lord 2003). Due to their hydrophilic water-binding nature, pectins can act as a filling material (Cosgrove 2000) thus pectinaceous gels share some similarity to the hydrophilic components of the extracellular matrix of animals.
Finally, oligogalacturonides released from HGA during pathogen attack or by endogenous plant pectinases (D'Ovidio *et al*. 2004) participate in various plant signal transduction pathways e.g. defense responses (reviewed by Pilling and Höfte 2003).

1.2.4 Wall associated proteins

The wall also contains several kinds of proteins (Showalter 1993), which can be divided into structural proteins and wall modifying enzymes. The group of structural proteins are further subclassifed according to their amino acid composition, e.g. hydroxyproline-rich glycoprotein, glycine-rich protein (Ringli *et al*. 2001) and proline rich protein (Ye *et al*. 1991). Typically these highly glycosylated proteins polymerize by forming diphenylether linkages of protein tyrosines and become almost insoluble (Fry 1986, Brady *et al*. 1996). An exception are arabinogalactan proteins

(AGPs) which are generally soluble (Fincher 1983) and are believed to interact with the pectin network (Cosgrove 1997). AGPs seem to play a role in cell to cell communication (Kreuger and van Holst 1993, Serpe and Nothnagel 1994) and plant development (Pennell and Roberts 1990, Johnson *et al*. 2003). Furthermore they are believed to assist in guidance of pollen tubes in the stylus (Jauh and Lord 1996, Gerster *et al*. 1997) and a removal of AGPs can result in cell death (Gao and Showalter 1999).

The group of the cell wall modifying enzymes encompasses enzymes being able to modify the polysaccharides. Such enzymes (e.g. xyloglucan transhydrolases, expansins, and peroxidases) play a vital role in loosening the wall network during cell-elongation (reviewed by Rose *et al*. 2002) and others (such as endopolygalacturonases and pectin-methylesterases) are involved in cell dehesion and abscission and fruit softening (reviewed by Li *et al*. 2003) .

1.2.5 Lignins

Lignin is a phenylpropanoid based heteropolymer that is mainly present in secondary walls, where it provides rigidity and water impermeability to the cell walls. In addition, lignin deposition may be induced upon wounding and infection to protect plant tissues against invading pathogens. (reviewed by Boerjan *et al*. 2003).

1.3 Cell wall networks

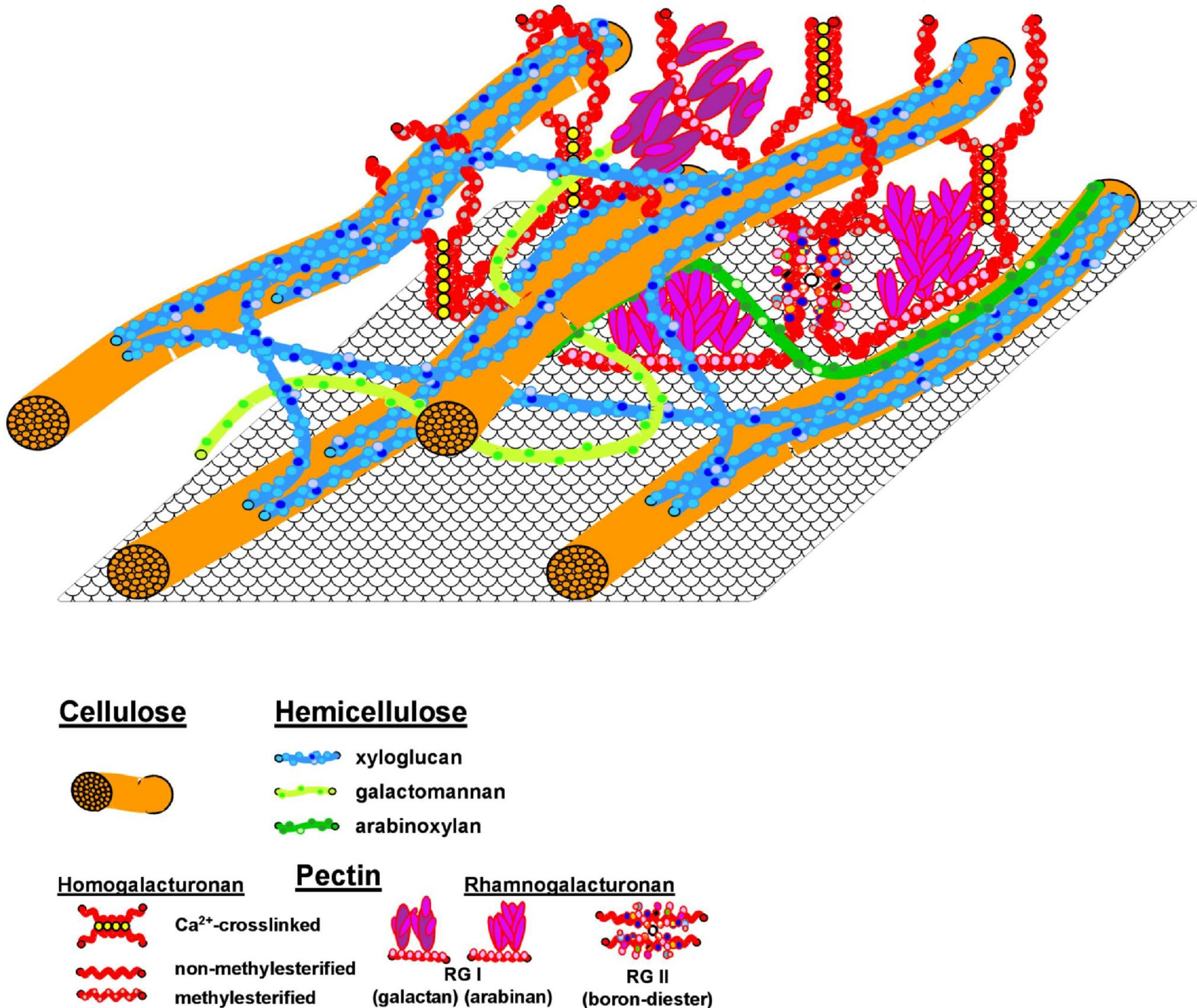


Figure 3 A model of the cell wall

(Modified from Pauly 2003)

The cell wall is involved in a multitude of roles and thus it seems likely that multiple structures need to interact to fulfill these roles. Hence, the cell wall can be described as a liquid crystal where crystalline cellulose is embedded into a water containing matrix consisting of hydrophilic pectins and hemicelluloses (figure 3) (Cosgrove 1997).

In most current models of the cell wall (Cosgrove 2000, Vincken *et al*. 2003) at least three different networks are differentiated. There is a cellulose-hemicellulose network, which is mainly implicated in the load bearing properties of the cell wall (Chanliaud *et al*. 2002). Cellulose microfibrils might act as nuclei to which xyloglucans associate non covalently via H-bonds (Hayashi *et al*. 1994,

Pauly *et al*. 1999). Therefore they might build a cross-linked or an independent network (Talbott and Ray 1992).

The pectin network forms a gel phase which fills the gaps in the cellulose-hemicellulose network and might keep it from collapsing (Cosgrove 2000). It is probably fortified by numerous possible crosslinks between individual pectic polymers. One such crosslink are the so-called "egg-boxes" (Carpita and Gibeaut 1993) which are structures made up of two HGA chains that are cross-linked by Ca^{2+} ions. Moreover, it is speculated that HGA chains can be covalently cross-linked by uronyl esters through the action of pectin methylesterases (Gelineo-Albersheim, CCRC Athens, GA personal communication). Furthermore two molecules of RGII can be crosslinked via borate-diol-esters (Ishii *et al*. 1999, O'Neill *et al*. 2001). RGII might also be a sidechain of HGA (Ishii and Matsunaga 2001). Finally it has been discussed that HGA might be a sidechain of RGI adding further complexity to the pectic network (Vincken *et al.* 2003).

Embedded into these two networks is the third network made of structural proteins, which might be important for expansion (Andeme-Onzighi *et al*. 2002)

1.4 Synthesis of cell wall components

The synthesis of cell wall components occurs in different subcellular locations. Cellulose is synthesized at the plasmamembrane by membrane spanning complexes. Substrates are supplied to the cytoplasmic side of these complexes (Amor *et al*. 1995), whereas the polymer is released on the apoplastic side. In contrast, matrix polysaccharides (hemicelluloses and pectins) are probably synthesized in the golgi-apparatus by golgi membrane spanning enzymes whose catalytic activity seems to be localized most often in the golgi lumen (Driouich *et al*. 1993, Perrin *et al*. 2001). Despite their membrane spanning topology these enzymes often may not have access to the substrates from the cytoplasm (reviewed by Doblin *et al*. 2003). The synthesized polysaccharides are then exported from the golgi to the apoplast by the secretory pathway (Staehlin and Moore 1995).

Despite their different location of synthesis, wall polysaccharides are believed to be synthesized from nucleotide sugars (Scheible and Pauly 2004), which are an activated form of the sugar due to their phosphoric diester (Jencks 1976). The nucleotide sugars are used by glycosyltransferases, which add the sugar moiety of the nucleotide sugar to a nascent polysaccharide chain (reviewed by

Scheible and Pauly 2004). However, due to the high number of possible linkages occurring in the different polysaccharides, numerous transferases are necessary. Bioinformatic analysis has identified 415 putative glycosyltransferases in the *Arabidopsis* genome alone (http://afmb.cnrs-mrs.fr/CAZY/) most of which are thought to be involved in cell wall biosynthesis (Henrissat *et al*. 2001).

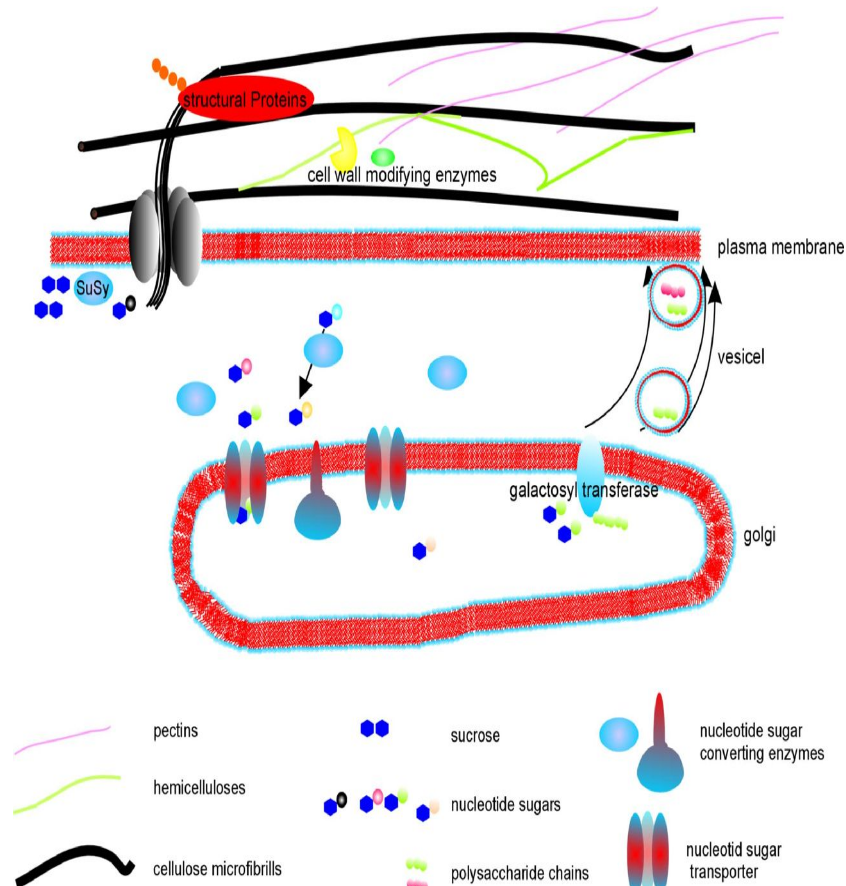


Figure 4 Overview of the cell wall synthesis and modification

The wall polysaccharides contain 14 different monosaccharides and hence 14 different nucleotide sugars are necessary for their synthesis. The plant has therefore established a nucleotide sugar conversion pathway to provide all the substrates for the polysaccharide synthesis.

The major entry point into cell wall synthesis is via UDP-D-glucose, which is synthesized in the cytoplasm (Amor *et al*. 1995). Other nucleotide sugars that seem to be synthesized in the cytosol are UDP-L-rhamnose (Usadel *et al*. 2004a) and UDP-D-galactose (Seifert *et al*. 2002). On the other hand UDP-D-galacturonic acid (Mølhøj *et al*. 2004), UDP-D-xylose (Haper *et al*. 2002) and UDP-

L-arabinose (Burget *et al*. 2003) synthesizing enzymes seem to localize to the lumenal side of the golgi or endomembrane system.

1.4.1 Cellulose synthesis

One of the best studied cell wall polysaccharide synthesis is cellulose. Recently even *in-vitro* production of cellulose has been accomplished (Lai-Kee-Him *et al*. 2002). Cellulose is thought to be synthesized by a membrane spanning cellulose synthase complex (CSC) which is probably similar to a rosette like structure, from which the cellulose microfibrils seem to emerge as judged by freeze-fracture studies (Brown 1996). This cellulose synthase complex is made up of at least three different cellulose synthases which depend on each other for correct rosette complex formation as shown by Taylor and colleagues (2003). These glyosyltransferases use cytosolic UDP-D-glucose as the activated precursor to add to the growing apoplastic microfibrils (Scheible and Pauly 2004). It seems likely that UDP-D-glucose is provided by sucrose synthase which might be membrane associated and in close proximity to the cellulose synthesizing glycosol transferases (Amor *et al*. 1995, Salnikov *et al*. 2001). Interestingly, cellulose synthesis possibly requires an endocellulase, KORRIGAN/IRX2 (Nicol *et al*. 1998, His *et al*. 2001, Szyjanowicz *et al*. 2004). Furthermore in-vitro essays demonstrated that sistosterol- β-glucoside might act as a primer for a cellulose synthase from cotton (Peng *et al*. 2002). However, *Arabidopsis* plants having a more than thirty-fold reduction in sistosterol- β-glucoside did not exhibit a change in cell wall composition or cellulose content (unpublished results of Münster and Scheible, referenced in Scheible and Pauly 2004).

1.4.2 Hemicelluloses synthesis

Recently, some advances have been made in the elucidation of xyloglucan synthesis. A fucosyl transferase has been identified and cloned from peas. It was indeed the first transferase involved in xyloglucan synthesis to be cloned (Perrin *et al*. 1999). Later it could be shown that the reduction in fucose content in the *Arabidopsis mur2* mutants is due to a mutation in an *Arabidopsis* xyloglucan fucosyl transferase gene (Vanzin *et al*. 2002). Similarly, map based cloning of *MUR3* revealed that it encodes a xyloglucan galactosyl transferase (Madson *et al*. 2003). Finally a xylosyltransferase

gene from *Arabidopsis* was cloned. It was shown that the heterologously expressed enzyme transfers xylose residues to cello-oligosaccharides (Faik *et al*. 2002).
Also some light was recently shed on the synthesis of the other hemicelluloses. A transferase gene from fenugreek catalyzing the transfer of UDP-D-galactose to mannan-oligosaccarides and galactomannans was cloned and functionally expressed (Edwards 1999). More recently a mannan synthase gene was cloned from a guar endosperm EST library. Expression and functional assays of the mannan synthase revealed its ability to transfer mannose from a GDP-D-mannose donor molecule to high molecular weight mannan (Dhugga *et al*. 2004).

1.4.3 Pectin synthesis

It has been speculated by Mohnen (1999) that at least 53 different transferases would be necessary for the synthesis of the pectic polysaccharides. However, none of their genes has been cloned and functionally expressed so far.
Recently, some putative glycosyltransferases involved in pectin polysaccharide biosynthesis have been identified with the aid of mutants. In *Nicotiana tabaccum* the lack of the *nolac-H18* gene has been associated with a reduction of glucuronic acid and arabinose contents in RGII (Iwai *et al*. 2002). Furthermore the *Arabidopsis quasimodo* mutant exhibited a reduction in galacturonic acid content which was traced back to a T-DNA insertion in a putative transferase gene (Bouton *et al*. 2002). Although in both cases mutations in putative transferase genes resulted in a change in pectin structure and growth and developmental plant phenotypes were observed, biochemical activity of the proteins encoded by these genes has yet to be demonstrated.

1.4.4 Nucleotide sugar metabolism

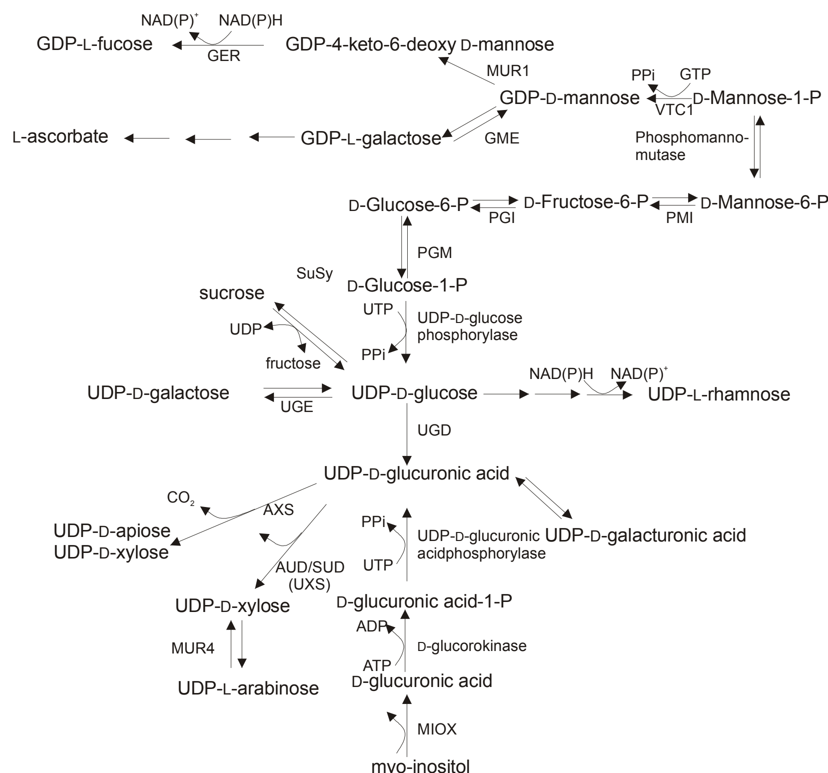


Figure 5 Nucleotide sugar interconversion

Overview of the nucleotide sugar interconversion pathway. For simplicity reasons only the *de novo* synthesis part is shown.

PMI: phosphomannoisomerase, PGM: phosphoglucomutase, PGI: phosphoglucoisomerase

The substrates for the synthesis of the wall polysaccharides are the different nucleoside diphosphate sugars (NDP-sugars). These can either be synthesized *de-novo* (reviewed by Seifert 2004) or they can be recycled by so called salvage pathways (reviewed by Feingold and Avigad 1980).

In the *de-novo* pathway most monosaccharides are generated from fructose-6 phosphate drawn from the calvin cycle (figure 5). Fructose 6-phosphate can then be converted into both glucose-6-phosphate and mannose-6-phosphate, which are the major entry points into the nucleotide sugar metabolism UDP-D-glucose and GDP-D-mannose.

The salvage pathway generally follows a conserved scheme. The free monosaccharide is converted into a phosphate sugar by a C1-kinase using ATP. The phosphate sugar in turn is converted into the corresponding NDP-sugar by action of a NTP-pyrophosphorylase releasing pyrophospate. Due to the high activity of pyrophosphatases in plant tissues (Patra *et al.* 1985) pyrophosphate is readily hydrolyzed into phosphate rendering the initial reaction irreversible. To date it is unknown if salvage pathways exist for all sugars. Pulse chase experiments demonstrated the reutilization of L-arabinose (Neish 1958), D-galactose (Roberts 1967), D-glucuronic-acid (Neish 1958), D-galacturonic acid (Loewus and Dickinson 1982) and L-fucose (Roberts 1968). So far, only one gene for an enzyme involved in the salvage pathway, *AGK1* (Kaplan *et al*. 1997), which is responsible for the phosphorylation of galactose, has been cloned.

1.4.4.1 Synthesis of UDP-D-glucose and UDP-D-galactose

UDP-D-glucose is also produced from sucrose by the action of sucrose-synthase (Amor *et al*. 1993). By converting UDP-D-glucose the nucleotide-sugars UDP-D-galactose, UDP-L-rhamnose and UDP-D-glucuronic acid can be produced in extracts isolated from various plants (see Feingold and Avigad 1980). Indeed an enzyme converting UDP-D-glucose to UDP-D-galactose was the first gene for a nucleotide sugar converting enzyme from *Arabidopsis thaliana* to be cloned and functionally expressed (Dörmann and Benning 1996) and concomitantly named UDP-D-glucose 4-epimerase (UGE1). Under standard conditions *Arabidopsis* UGE-antisense plants did not show any visible phenotype (Dörmann and Benning 1998). However, growing the plants on galactose containing medium resulted in a growth reduction. Recently, four more paralogs (UGE2-UGE5) have been identified by Seifert and colleagues (2002) and in a mutant in one of them (*uge4* also known as *root hair deficient1*) a root-bulging phenotype was shown. The root-bulging phenotype could be reverted by administration of galactose (Seifert *et al*. 2002) or ethylene (Seifert *et al*. 2004). For all members of the UGE family, activity has been shown in a biochemical essay (Seifert *et al*. 2002).

1.4.4.2 Synthesis of UDP-L-rhamnose

At the beginning of this thesis it had been postulated that UDP-L-rhamnose is synthesized from UDP-D-glucose in a three step reaction. This notion was based on experiments with plant extracts from red campion (*Silene dioica*) demonstrating a UDP-4-keto-6-deoxy-glucose intermediate in the

synthesis of UDP-L-rhamnose (Kamsteeg *et al*. 1978, Kamsteeg *et al*. 1979). Furthermore it was based on analogy to bacterial pathways, where three different enzymes, a dehydratase, a reductase and an epimerase, have been characterized in NDP-L-rhamnose synthesis (reviewed by Giraud and Naismith 2000).

1.4.4.3 Synthesis of UDP-D-glucuronic acid

UDP-D-glucuronic acid is synthesized from UDP-D-glucose by the action of a UDP-D-glucose dehydratases (UGDs). By now *UGDs* from many different plant species have been cloned (Tenhaken and Thulke 1996, Turner and Botha 2000, Seitz *et al*. 2000, Hinterberg *et al*. 2002, Johanson *et al*. 2002). In contrast to many other nucleotide sugar converting reactions this reaction seems to be irreversible (Dalessandro and Northcote 1977) thus providing a possible regulation point in nucleotide sugar flux. Indeed, a closer look at the nucleotide sugar conversion pathway (figure 2) reveals that UDP-D-glucuronic acid is a major branching point in nucleotide sugar synthesis. Reiter and Vanzin (2001) identified three additional putative UDP-D-glucose dehydratase genes in the genome of *Arabidopsis* and termed them *UGD2-UGD4*.

In an alternative pathway UDP-D-glucuronic acid can also be synthesized from inositol. This pathway is initiated by an inositol oxygenase (Loewus and Dickinson 1982). This oxygenase oxidizes *myo*-inositol to D-glucuronic acid using molecular oxygen. This intermediate is converted into UDP-D-glucuronic acid via a salvage pathway. Recently it has been shown that UDP-D-glucuronic acid seems to be synthesized by the UGD or inositol oxygenase dependent pathways depending on organ and developmental stage (Seitz *et al*. 2000). However, earlier findings showed that limitation of *myo*-inositol supply did not effect cell wall composition in potatoes (Keller *et al*. 1998). Possibly both pathways can compensate for each other, providing a safety-net for the plant.

1.4.4.4 Synthesis of UDP-D-galacturonic acid, UDP-D-xylose and UDP-D-apiose

UDP-D-glucuronic acid can be converted to UDP-D-galacturonic acid, UDP-D-xylose and UDP-D-apiose. Conversion to UDP-D-galacturonic acid is probably achieved by UDP-D-glucuronic acid epimerase as shown in radish roots (Libjelke *et al*. 1995).

A decarboxylation reaction of UDP-D-glucuronic acid yields UDP-D-xylose. To date six putative UDP-D-xylose synthase genes have been identified in the genome of *Arabidopsis* and termed membrane anchored UDP-D-glucuronic acid decarboxylase (*AUD1* to *AUD3*) and soluble UDP-D-glucuronic acid decarboxylase (*SUD1* to *SUD3*) depending on the presence of a membrane spanning segment (Reiter and Vanzin 2001). At least one member of both classes has been cloned

and UDP-D-xylose synthesizing activity has been shown in recombinantly expressed proteins (Harper *et al*. 2002). Moreover the gene for a bifunctional enzyme (AXS1) synthesizing both UDP-D-xylose and UDP-D-apiose from UDP-D-glucuronic acid has been cloned from *Arabidopsis thaliana* (Mølhøj *et al*. 2003). The unspecific production of UDP-D-apiose and UDP-D-xylose seems to be common in plants (Wellmann and Griesebach 1971, Matern and Griesebach 1977).

1.4.4.5 Synthesis of UDP-L-arabinose

UDP-D-xylose can be converted into UDP-L-arabinose by the action of a UDP-D-xylose epimerase (UXE), which is encoded by the *MUR4* gene (Burget and Reiter 1999, Burget *et al*. 2003). Consequently *Arabidopsis mur4* plants contain only 50 % of wild type levels of cell wall bound arabinose (Burget and Reiter 1999). Mostly arabinose contents in pectin and AGPs were reduced. However, the affected plants did not show a visible phenotype (Reiter *et al*. 1997) possibly due to the residual arabinose. This residual arabinose might be explained by the presence of three putative homologs in the *Arabidopsis* genome (Burget *et al*. 2003). For MUR4, localization to the golgi has been demonstrated by the expression of a chimeric MUR4-green fluorescent protein construct *in planta* (Burget *et al*. 2003).

1.4.4.6 Synthesis of GDP-D-mannose and GDP-L-fucose

Another major entry point for nucleotide sugar metabolism is GDP-D-mannose, which is synthesized by mannose pyrophosphorylase from D-mannose-1-phosphate. Two independent approaches identified mutants affected in the expression of its gene. One mutant was identified by means of a decrease of ascorbate levels in the *vtc1* mutant (Conklin *et al*. 1997). Another approach showed an effect on cellulose and N-terminal glycosylation of proteins and polysaccharides isolated from *cyt1* embryos, which also showed a downregulation of mannose and fucose and an upregulation of rhamnose (Luckowitz *et al*. 2001, Nickle and Meinke 1998). It turned out that both mutants are allelic and are affected in the expression of a GDP-D-mannose pyrophosphroylase gene.

GDP-L-fucose is made from GDP-D-mannose in a three step reaction catalyzed by two proteins (Bonin *et al*. 1997, Bonin and Reiter 2000). The first step is catalyzed by GDP-D-mannose-4,6-dehydratases (GMDs) encoded by the *MUR1* (*GMD2*) and *GMD1* genes. Plants carrying a mutation in *MUR1* have been shown to be deficient in fucose, which can be substituted by L-galactose in xyloglucan and N-glycans (Zablackis *et al*. 1996). Interestingly, MUR1 and GMD1 seem to have a complementary expression pattern as judged by promoter:GUS studies (Bonin *et al*.

2003). Whereas GMD1 is preferentially expressed in the root tip, MUR1 is expressed in all other tissues. Nevertheless an expression of both proteins was detected in the pollen.

The final step in GDP-L-fucose synthesis is catalyzed by a bifunctional GDP-4-keto-6-deoxy-D-mannose 3,5-epimerase-4-reductase termed GER1 (Bonin and Reiter 2000). Even though biochemical activity was demonstrated by expression in *Escherichia coli* (Bonin and Reiter 2000), antisense plants with a reduced *in vivo* GER1 activity showed normal amounts of fucose in their cell wall, possibly indicating that GER1 activity is not rate limiting due to the presence of a similar protein in *Arabidopsis* tentatively termed GER2.

1.4.4.7 Synthesis of GDP-D-rhamnose and GDP-L-galactose

GDP-D-mannose is also the precursor for GDP-D-rhamnose and GDP-L-galactose, but both D-rhamnose and L-galactose are rarely found in cell walls. The enzyme catalyzing the latter reaction, GDP-D-mannose 3,5-epimerase (GME), has been purified and subsequently sequenced by Wolucka and co-workers (2001). The identification of candidate genes for an enzyme for the former reaction is still lacking. GME also forms GDP-L-gulose and is inhibited by GDP-L-fucose, ascorbic acid and L-galactono-1,4-lactone (Wolucka and van Montagu 2003). Since both GDP-L-galactose and GDP-L-gulose are precursors of ascorbate synthesis (Wheeler *et al*. 1998, Smirnoff and Wheeler 2000) GME could be a regulation point for substrate flux into either the cell wall or ascorbate biosynthesis.

1.4.4.8 Transport of nucleotide sugars into the golgi

Since many polysaccharides are synthesized in the golgi but some of the nucleotide sugars are located in the cytosol, nucleotide sugar transporters were postulated to exist. Indeed Neckelmann and Orellana used radioactively labeled substrates (1998) to demonstrate transport of UDP-D-glucose into the golgi lumen. Recently Norambuena and colleagues have been able to clone and functionally express a gene for a UDP-D-glucose/UDP-D-galactose transporter from *Arabidopsis thaliana* which they designated *UTR1* (2002). In addition, nucleotide sugar transporters for GDP-D-mannose and GDP-L-fucose have been recently identified (Wulff *et al*. 2000, Baldwin *et al*. 2001, Norambuena *et al*. 2002).

1.5 Structural analysis of cell walls

Altering the nucleotide sugar fluxes *in planta* might lead to different wall structures. Therefore a number of techniques have been developed allowing a detailed structural analysis of the wall.
First cell wall preparations are usually obtained by preparing alcohol insoluble residues. These residues can then be further subfractionated which can either be done by sequential extractions with calcium chelators (solubilizing pectins) and potassium hydroxide containing solutions (solubilizing hemicelluloses) (Stolle-Smits *et al*. 1999). Alternatively, pectin components can be extracted by solubilization through enzymatic digests with endopolygalacturonase and pectin methylesterase (O'Neill *et al*. 1990) and crystalline cellulose can be purified by removing the matrix polysaccharides with a mixture of acetic and nitric acid (Updegraff 1969).
The complete wall or components thereof are hydrolyzed using acids such as trifluouroacetic acid (Albersheim *et al*. 1967, Mankarios *et al*. 1979) as well as sulfuric acid (Selvendran *et al*. 1979). The resulting monosaccharides can then by qualitatively analyzed by paper chromatography or quantitatively by derivatization to their corresponding alditol acetates (Albersheim *et al*. 1967) or trimethylsylyl methylglycosides (York *et al*. 1985) and subsequent detection by gas chromatography with mass detection. Derivatisation can be avoided when the monosaccharides present in a hydrolyzate are separated by high performance anion exchange chromatography (HPAEC) with coupled pulsed amperometric detection (Vincken *et al*. 1996).
Another important feature of cell walls is their degree of methyl-esterifcation and the presence of O-acetyl-substituents. Both parameters can be investigated by colorimetric assays (Bergmeyer and Möllering 1974) or with HPLC based methods (Voragen *et al*. 1986) Whereas cell wall degrading enzymes have long been used to study cell wall structure (Prade *et al*. 1999), a recent development is the quantitative investigation of polysaccharide fragments released by specific enzymes (Choo *et al*. 2002). A complementary technique is the labeling of these fragments with a fluorofore and gel-electrophoresis of the labeled fragments (Goubet *et al*. 2003).
In addition, infrared (IR) spectroscopy has been used to characterize whole cell walls by clustering spectra obtained from plants with reference spectra from known mutants (Chen *et al*. 1998, McCann *et al*. 2001). Another recent study showed the feasibility of using solid state nuclear magnetic resonance to investigate the wall *in situ* (Lu and Ralph 2003, Lahaye *et al*. 2003).
To investigate cell wall structure at a cellular level, cell wall components staining dyes such as calcoflour (Hughes *et al*. 1975) or ruthenium red (Hanke and Northcote, 1975) can be applied.

Moreover, antibodies recognizing a multitude of specific cell wall epitopes have been generated and characterized indicating the localization of certain wall structures to defined cell wall regions (Willats *et al*. 1998, Willats and Knox 1999, Willats *et al*. 1999, Steffan *et al*. 1995, Williams *et al*. 1996)

1.6 Biotechnology of cell walls

Plant cell walls provide the richest source of biopolymers on earth (Prade *et al*. 1999) thus they represent an important resource for humans. As such plant cell walls have been recognized since ancient times. Besides their obvious applications as for example a source for wood or textiles, it is interesting to note that even more than 6000 years ago Chinese artisans realized that the extracts from the lacquer tree (composed of lignols and apoplastic peroxidases) would solidify upon contact with oxygen and were thus used in artwork (Huttermann *et al*. 2001).

Since these times the products of the wall have found many new applications and developments such as gelling agents in the food and pharmaceutical industries (reviewed by Thakur *et al*. 1997). It is not surprising that in the previous years major efforts have been undertaken to specifically change the plant wall to tailor polysaccharides for new industrial applications and for streamlining processing in various industries. Despite some similar approaches for other polysaccharides which have lead to e.g. altered starches and fructans (Heyer *et al*. 1999) cell wall biotechnology is still in its infancy.

Despite significant efforts spent in a forward-genetics approach of mustering EMS mutagenized *Arabidopsis* pools (Reiter *et al*. 1997, recent advances reviewed by Fagard *et al*. 2000) many questions were left unanswered. Thus new developments target distinct pathways or use heterologous enzymes to modify wall structure *in situ*.

For lignin biosynthesis a successful interference with the biosynthesis pathway using antisense technology was achieved. Thus plants showing changes in their pulping values which would make them eventually more suitable for paper production were obtained (Baucher *et al*. 1996, O'Connell *et al*. 2002).

Burton and coworkers silenced a putative cellulose synthase (2000) and observed a reduced growth in tobacco plants. Interestingly they also observed an increased HGA content in leaves, possibly because of compensatory effects in the plants. However, Park and coworkers obtained a desirable change *in planta* by expressing a poplar cellulases in *Arabidopsis* (2003) and thus obtaining an

increased growth rate. More recently they introduced a fungal xyloglucan specific endoglucanase (2004) in poplar resulting in increased growth and cellulose production.

Since pectins represent an important factor both as a resource for gelling agents but also for the quality of different crop plants (Camara *et al*. 2002, Tajner-Czopek 2003) changing their properties would be desirable. Therefore considerable effort is put into understanding pectin synthesis in *Arabidopsis* and in evaluating *Arabidopsis* pectin mutants for changes in the cell wall. However, at the start of this thesis surprisingly little was known.

Other approaches facilitate modifying or degrading enzymes. The most prominent example probably being the down-regulation of pectin modifying enzymes putatively involved in fruit softening such as polygalacturonases (reviewed by Brummel and Harpster 2001). This approach which most often targeted tomato plants, resulted in fruits being firm longer and even resulted in a short commercial exploitation of these fruits (Langley *et al*. 1994). However, recent experiment might indicate that also apple fruit quality might be affected by polygalacturonase activity (Atkinson *et al*. 2002).

Moreover Sørenson and co-workers expressed an endo-β-1,4 galactanase (2000) and Skjøt and colleagues an endo α-1,5 arabinase (2002) in potatoes (*Solanum tuberosum*). Both approaches led to a change in cell wall structure but only the expression of the arabinase had a major visible phenotype which manifested itself as death of the stolons and discoloring of the leaves. Finally expression of an RGI lyase in potatoes resulted in an unusual fragmentation pattern of RGI but no major phenotype (Oomen *et al*. 2002).

Surprisingly little effort has been put into interfering with the pectin synthesis pathway at the start of the thesis. Only a downregulation of UDP-D-glucose epimerase in *Arabidopsis* which effected growth on galactose containing medium (Dörmann and Benning 1996) was observed but no structural difference in pectins was described.

Due to the recent consumer reservation concerning genetically modified plants an alternative approach where endogenous genes are downregulated or disrupted becomes more important. Here downregulation by means of conventional breeding and mutagenesis can be achieved if the molecular loci of traits would be elucidated. Nevertheless the most important biosynthesis pathways for the important biopolymers such as pectins are not yet understood.

1.7 Aims of the thesis

Following up on the gap in understanding the biosynthesis and function of the various polysaccharides, the reason why polysaccharide sidechains have to be so complex for plant growth and development and interactions with the environment has so far eluded us. One way to gain more insight into the function of cell wall polysaccharides is to generate plants or identify mutants, which contain defined structural alterations. Once these plants are identified their impact on the plant life cycle can be assessed.

The aim of the thesis was to alter wall structure by genetic manipulation of pectin biosynthesis. However, very few genes involved in pectin biosynthesis are known (chapters 1.4.3 and 1.4.4), and therefore the following project plan was developed.

First *Arabidopsis* genes involved in wall biosynthesis were to be identified using an bioinformatics approach. Here particular focus was to be placed on the nucleotide sugars responsible for the synthesis of the pectin backbone (figure 2).

Then the enzymatic activity of these genes and their expression level *in planta* was to be determined. The expression levels *in planta* were then to be altered using insertional mutants and/or RNAi approaches. In these plants the wall structure and the resulting growth and morphological phenotypes were to be characterized. Eventually the gained knowledge was to be extended to crop plants. Novel tools to evaluate these pathways were to be developed to facilitate evaluation of large expression datasets to build hypothesis about cell wall biosynthetic pathway networks.

In addition to the above mentioned goal experiments outlined here will also aid in gaining significant insights in functional genomics of the plant nucleotide sugar pathway.

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals

All standard chemicals were purchased from either Sigma (Taufkirchen, Germany) or Merck (Darmstadt, Germany) in standard purity unless noted otherwise in the text. Standard molecular biology kits and columns were obtained from Machery and Nagel (Düren, Germany).

2.1.2 Plasmids

Plasmid	***Source***
pBluescript KS II+	Novagen, Schwalbach, Germany
pBluescript SK II+	Novagen
pBinAR	MPI Golm, Höfgen and Willmitzer (1990)
pIVEX	Roche Diagnostics, Mannheim, Germany
pCR II -Topo	Invitrogen, Karlsruhe, Germany
pFastBac HTa	Invitrogen
pHANNIBAL	Waterhouse, P. (CSIRO, Canberra, Australia)
pART27	Waterhouse, P.

2.1.3 Primers

RT-PCR

GAE1

5’-CAAGCTTGGCTTTAGTATTGTAACCG-3’, 5’-CATGCCAATATTAGCTCAGCCCG-3’,

GAE2

5’-TCAAGCAGCAGCAGCAACTT TC-3’, 5’- AAGCACGGATTTGCAGACTG-3’;

GAE3

5’-GACCCGTCTGAAGATCGGTGGTG-3’, 5’-TCACCATTCTTGAGAGACTTC-3’;

GAE4

5’-TAGCGGCGGCTTTTTTGTCGC-3’, 5’-ATGCTAATATTAGTTTAGCTCAACG-3’;

GAE5

5’-TCACCAAGAACTCTTCTTCTTCG-3’, 5’-ATGCCAATATCACGTTAGCGCAAGC-3’

GAE6

5’-GGAATCTTCGGCGTGAGAAG-3’, 5’-GCATGCTAATGTGAGTTTAGCG-3’.

RTR

5’-GTTTCAAGCAACACGGTCC-3’, 5’-GGTACATACATTGTCAAATTCTCTCAAG-3’

TDNA Screens

GABI-LB 5'-GGGAATGGCGAAATCAAGGCATCG-3'

GABI8409 5'-ATATTGACCATCATACTCATTGC-3'

GABI2588 5'-CGCCAGGGTTTTCCCAGTCACGACG-3'

RHM2HA 5'-CAGATTTCAAGGATGGATGATAC-3'

RHM2HB 5'-AGAGTAAGGATTCGTCGGTAACAG-3'

2.2 Organisms and cultivation conditions

2.2.1 Escherichia coli

DH10BAC (Invitrogen) F^- mcrA Δ (mrr-hsdRMS-mcrBC) ϕ 80dlacZ Δ M15 lacX74 deoR recA1 endA1 araD139 (ara, leu)7697 galU galK λ^- rpsL nupG /bMon14272 /pMON7124

XL1-Blue (Stratagene, Amsterdam, Netherlands) recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac[F' proAB lacl Z δ M15 Tn10(Tet^r)]

BL21(DE3)pLysE (Novagen, Bad Soden, Germany) F^- ompT hsdS(r_B^- m_B^- gal dcm (DE3) pLysE (Cam^R)

DH5α (Invitrogen) DeoR endA1 gyrA96 hsdR17(r_K^- m_K^-) recA1 relA1 supE44 thi-1 Δ (lacZYA-argFV169) ϕ 80 δ lacZ Δ M15 F^- λ^-

Cultivation was usually performed in LB or YEB as described by Sambrook *et al*. (1989)

2.2.2 Agrobacterium tumefaciens

pGV 3101 pmp90 (Koncz and Schell 1986)

2.2.3 Plant growth

Arabidopsis thaliana

All *Arabidopsis* plants were of the Columbia-0 ecotype and were grown in environmental chambers under standard conditions (120 mmol m^{-2} s^{-1} light, 60% humidity, 20°C) under a 8/16h dark-light regime or in the greenhouse on soil.

2.2.4 Insect cells

Spodoptera frugiperda insect cells (Sf21) (Invitrogen) were cultivated adherently 27°C in "Ultimate Insect" medium (Invitrogen). Every three to four days the insect cells were loosened mechanically and subcultured.

2.2.5 In vitro pollen germination

For pollen germination the basic medium described by Fan and coworkers (2001) was used. In short pollen were smeared onto solid plates containing 5 mM MES, 1 mM KCl, 10 mM $CaCl_2$, 18 mM $MgSO_4$, 1.5 mM boric acid, 16.6 % (w/v) sucrose, 3.65 % (w/v) sorbitol, 1.5 % (w/v) agarose and 10 µg/mL *myo*-inositol. The pH was adjusted to 5.8 with Tris-Base. Pollen germination was performed in a humidified chamber at 25°C under constant light (120 mmol m^{-2} s^{-1}).

2.3 Molecular biology techniques

2.3.1 Standard molecular biology techniques

Standard molecular biology techniques such as cloning, digests with restriction endonucleases, separation of DNA fragments by gel-electrophoresis, the assembly of capillary blot transfers and preparation of common solutions were performed as described in Sambrook *et al*. (1989).

2.3.2 Polymerase Chain Reaction (PCR)

Selective amplification of DNA fragments was preformed using the polymerase chain reaction facilitating a MJ Research PTC 200 thermocycler (Biozym, Oldendorf, Germany). For diagnostic purposes, Taq DNA polymerase (Invitrogen) and for cloning purposes Pfu DNA polymerase was used (Stratagene).

2.3.3 Transformation of bacteria

Chemical transformation of *Escherichia coli* was performed as described by Hanahan *et al*. (1983). *Agrobacterium tumefaciens* was transformed using electroporation facilitating a Gene pulser II (Biorad, Munich, Germany) according to the supplier's instructions.

2.3.4 DNA isolation

DNA was isolated from plants by a modification of the protocol, given by Murray and Thomson (1980). In brief, DNA was extracted by grinding approximately 300 mg of leaf material in a 1.5 mL Eppendorf reaction tube. Afterwards the material was incubated in 300 µL extraction buffer (2% (w/v) cetyl-trimethyl-ammonium bromide, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-Cl pH 8.0) at 65°C for 30 min. The insoluble material was precipitated and the supernatant extracted with 300 µL chloroform. The aqueous phase was then mixed with 300 µL iso-propanol and DNA was precipitated by spinning in a micro centrifuge at 20,000 x g. The DNA was washed once with 70% EtOH and after air-drying re-suspended in 50 µL double-distilled water containing 0.1 µg of RNAseA.

2.3.5 RNA isolation

RNA was isolated form plants using the method described by Chang and colleagues (1993). In short up to 2 g of plant material was snap-frozen in liquid nitrogen and the material was ground to a fine powder. The powder was added to 15 mL of RNA extraction buffer (an aqueous solution containing 2 % (w/v) CTAB 2% (w/v) PVP, 100 mM Tris-HCl pH 8.0, 25 mM EDTA, 2.0 M NaCl and 0.05 % (w/v) spermidin). After incubation for 5 minutes at 65°C, the mixture was extracted twice with one volume of chloroform. The aqueous phase was supplemented with a ¼ volume of 10 M LiCl. After an overnight incubation at 4°C the RNA was precipitated by centrifugation at 10,000 x g for 30 minutes. The pellet was dissolved in 500 µL SSTE buffer (an aqueous solution of 1.0 M NaCl, 0.5% (w/v) SDS, 10 mM Tris-HCl pH 8.0 and 1 mM EDTA) and extracted with chloroform once. Afterwards the RNA was precipitated with two volumes of absolute ethanol for 2 h a -20°C. The precipitate was then dissolved in up to 50 µL diethylpyrocarbonate treated water.

2.3.6 Southern blotting

DNA was isolated form plants according to 2.3.4. Approximately 2-5 µg of whole plant genomic DNA was digested with an appropriate restriction-endonuclease in a 50 µL reaction with 30 U of enzyme for 4 h. After complete digestion the DNA was separated according to size by agarose gel electrophoresis. Afterwards the agarose gel was incubated in a 1 % HCl solution for 20 minutes at room temperature. Then the gel was briefly washed with distilled water and then denatured for 30 minutes in denaturing solution (0.5 M NaOH, 1.5 M NaCl). After denaturation the gel was blotted onto a nylon membrane by capillary transfer (Porablot, Machery-Nagel, Düren, Germany), using 10x SSPE buffer (1.5 M NaCl, 100 mM NaH_2PO_4, 2 mM EDTA, pH 7.4). After transfer the membrane was briefly washed with 2X SSPE to remove salt residues. The DNA was than fixed to the membrane using an UV-crosslinker (Stratagene).

2.3.7 Northern blotting

Arabidopsis RNA was isolated from different tissues according to 2.3.5. Total RNA was separated under denaturing conditions as described by Lehrach and co-workers (1987). Afterwards the RNA was blotted to nylon membranes by capillary transfer using 10X SSC (1.5 M NaCl 0.15 M sodium citrate pH 7.0). After complete transfer the membrane was crosslinked using an UV-crosslinker (Stratagene).

2.3.8 Radioactive probe synthesis

Radioactive labeled probes were synthesized using Promega's (Mannheim, Germany) generalized Prime-a-Gene labeling system following the manufacturer's recommendation. The probe was purified using Amersham (Freiburg, Germany) Micro G-50 Spin-Columns, following the manufacturer's specifications.

2.3.9 Hybridization of radioactive probes

Nylon membranes were pre-hybridized for 1 h in prehybridization solution (5x SSPE, 0.1% (w/v) Ficoll 400, PVP K30, BSA and SDS each and 100 mg/µL salmon sperm DNA) at 65°C. Afterwards the radioactive probe was heat-denatured and added to the pre-hybridization solution and incubated overnight. The next day the membrane was washed twice for 5 min with wash solution I (2X SSC, 0.1% SDS) at ambient temperature and twice for 15 min at 65°C with wash solution II (0.1X SSC, 0.1% SDS). Afterwards the membrane was subjected to autoradiography (Kodak X-Omat AR films, Kodak, Rochester, NY) in an Amersham cassette equipped with intensifying screens at -68° C.

2.3.10 cDNA synthesis

Extracted RNA (2-4 µg) was treated with one unit of RNAse free DNAse (Roche Diagnostics) in digestion buffer (10 mM Tris-HCl, 25 mM $MgCl_2$, 5 mM $CaCl_2$ pH 7.5). After heat-denaturation of the DNAse for 10 minutes at 75 °C first strand cDNA was synthesized with QBiogene's (Heidelberg, Germany) MMLV reverse transcriptase system according to the manufacturer's recommendation using 50 pmoles of dT_{18} primer.

2.3.11 Semiquantitative RT-PCR

The cDNA content of all RT reactions (2.3.10) was normalized by amplifying the APT1 transcript, a constitutively expressed control (Moffatt *et al*. 1994) using previously described primers (Orsel *et al*. 2002). An aliquot (0.5 µL) of this normalized cDNA was used for semiquantitative PCR.

2.3.12 Real time RT-PCR

Real time RT-PCR was performed using 9 µL of a 1/30 (v/v) dilution of the first strand cDNA (2.3.10) reaction and Syber Green master mix (Applied Biosystems, Darmstadt, Germany) in a reaction volume of 20 µL on a GeneAmp 5700 Sequence detection system (PE Applied Biosystems, Darmstadt, Germany). To accurately quantify relative expression levels constitutive control genes were chosen with the help of geNorm application (Vandesompele *et al*. 2002) from the pool of constitutive genes described by Czechowski and co-workers (2004).

2.3.13 Transformation of plants

Arabidopsis thaliana cv. Columbia-0 were transformed using *Agrobacterium tumefaciens* based dipping infiltration as outlined by Clough and Bent (1998). Seeds from plants were harvested and subjected to antibiotic selection on plates.

2.3.14 Molecular analysis of Arabidopsis T-DNA tagged mutant lines.

T-DNA tagged lines were either obtained form the GABI-KAT collection (Rosso *et al*. 2003) (Cologne, Germany) or from the SALK collection (Alonso *et al*. 2003) via the NSBRC.

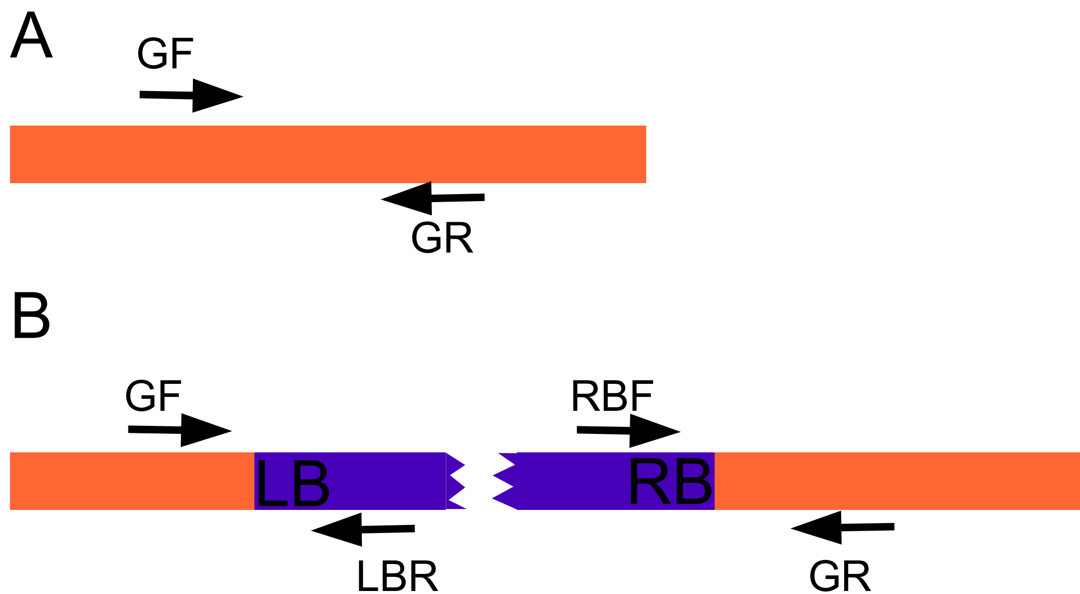


Figure 6 Identification of T-DNA tagged lines

A) schematic representation of a PCR on wild type plants

B) schematic representation of a PCR on plants containing a T-DNA insertion

GF gene specific forward primer, GR gene specific reverse primer; LBR left T-DNA border specific primer and RBF right border T-DNA specific primer.

LB and RB denote the left- and right border-sequences of the inserted T-DNA. The insertion is in blue, the gene in red for clarity reasons the insertion is not drawn to scale.

Homozygous insertional mutant lines were identified by PCR facilitating a gene specific primer (figure 6 GF) and a T-DNA specific primer (assuming the orientation in figure 6 LBR) and two gene specific primers spanning the putative insertion site in a separate reaction (figure 6 GF and GR). Lines showing a product in the former reaction but not in the latter reaction were tentatively termed homozygous insertion plants. For a further characterization these were analyzed by cloning (TOPO TA, Invitrogen) and sequencing (DLMBC, Berlin, Germany) the reaction product of the PCR with the gene and the T-DNA specific primers. Finally a PCR facilitating a RB specific primer (figure 6 RBF) and a gene specific primer (figure 6 GR) was carried out and the resulting product was cloned and sequenced as described above.

2.3.15 Generation of pRRHM

An approximately 700 bp long genomic fragment of *RHM2* encompassing the translation start code was PCR amplified using the primers RHM2HANA 5'-TTAATCTCGAGGGAGCTGCTGGATTTATTG-3' and RHM2HANB 5'-TTAATGGTACCGCCACATCGATCACTCTCC-3' and cloned into the TOPO TA vector

(Invitrogen). Thus a *Xho*I and an *Asp*718 restriction site were introduced. The same fragment was also cloned into TOPO TA with the primers RHM2HAN2A 5'-TTAATGGATCCGGAGCTGCTGGATTTATTG-3' and RHM2HAN2B 5'-TTAATATCGATGCCACATCGATCACTCTCC-3' introducing a *Bam*HI and a *Cla* restriction site respectively. Both fragments were then subcloned into the pHANNIBAL vector (Dr. Perter Waterhouse, CSIRO, Canberra, Australia) cut with either *Bam*HI and *Cla* or *Xho*I and *Asp*718. The resulting vector was digested with *Not*I and the resulting fragment containing the silencing construct was ligated into pART27 (Dr. Peter Waterhouse, CSIRO, Canberra, Australia) cut with the same enzyme.

2.3.16 Generation of pVXGAE6

For in vitro expression the whole open reading frame of *GAE6* was amplified from genomic DNA with PCR using the primers 5'-TATATTGTCGACACGGTCTCTTCTCCGTCCTAG-3' and 5'-TATTACCCGGGTTAAGCGGAATCTTCGGCG-3' introducing a *Sal*I and *Sma*I site respectively. The resulting product was cloned into the TOPO vector. It was mobilized from the TOPO vector using *Sal*I and *Sma*I and the resulting product was cloned into the the pIVEX2.4b vector (Roche) cut with the same enzymes.

2.3.17 Generation of pIXGAE6

For expression in insect cells the whole open reading frame of *GAE6* was amplified with PCR from pVXGAE6 using the primers 5'-GTGCCCCTGTCGGCGACGGCGGATACAA-3' and 5'-TTAAGCGGAATCTTCGGCGTGAGAAGTTT-3' cloned into the TOPO vector. It was mobilized from the TOPO vector using *Eco*RI and the resulting product was cloned into the *Eco*RI site of the pFastBacHTa vector.

2.3.18 Generation of pAXGAE6

The full length open reading frame of *GAE6* was amplified with PCR facilitating the primers NSE35SA 5'-TATATGGTACCATGCCCCTGTCGGCGACGG-3' and NSE35SB

5'-TATATTCTAGATTAAGCGGAATCTTCGGCG-3' introducing an *Asp*718 or *Xba*I restriction site respectively. The resulting product was cloned into TOPO TA to yield the vector pTGAE6. The vector was then cut using *Asp*718 and *Xba*I and the *GAE6* ORF introduced into the pBinAR Vector cut with the same enzymes.

2.3.19 Generation of pRGAE

PTGAE6 was cut *Bam*HI and *Cla* and the resulting approximately 700 bp fragment subcloned into pHANNIBAL digested with the same enzymes yielding pHG1. Approximately the same fragment was amplified from pTGAE6 by PCR with the primers NSE1HANA 5'-TATTAATCTCGAGAAAACGTGCGCATGGAG-3' and NSE1HANB 5'-ATTAATGGTACCAGCCGTGTCTAACGCACC-3' introducing a *Xho*I and *Kpn*I restriction site respectively. The product was cut with these enzymes and cloned into pHG1 cut with the same enzymes. The resulting vector was then digested with *Not*I and the resulting fragment cloned into pART27 to yield pRGAE.

2.4 Biochemical Methods

2.4.1 Protein Expression in insect cells

2.4.1.1 Initial infection

Recombinant bacmid-DNA was generated by transforming pIXGAE6 into DH10BAC *E. coli* cells. Afterwards DNA was harvested according to standard procedures (Sambrook *et al*. 1989) and dissolved in 25 µL double distilled water.

Before reaching confluence insect cells were incubated for 5h with 1 mL infection mixture (0.5 % (v/v) bacmid DNA 0.6 % cellfectin reagent in culture medium) per 25 cm² culture surface and afterwards incubated for two days at 27 °C.

The supernatant containing the virus was obtained by spinning the insect cell medium for 5 minutes at 1000 x g.

2.4.1.2 Reinfection

Insect cells were covered with 1 mL virus containing growth medium per 25 cm² culture surface and incubated for 1 h on a shaker at room temperature. Afterwards 4 mL of Ultimate Insect medium (Invitrogen) per 25 cm² of culture surface was added. After two to three days of incubation at 27°C the cells were harvested for further analysis by centrifugation at 1000 x g for 5 minutes. The supernatant containing the virus was kept for further infections at 4°C. If necessary, the cell pellet was stored at -80°C.

Crude lysate was generated by washing the cells once in 10 mM phosphate buffer (pH 8.0) followed by sonification.

2.4.2 Protein expression in Pichia pastoris

For protein expression in *Pichia pastoris* a clone harboring the desired construct was grown in 25 mL of buffered complex glycerol media supplemented with 100 µg/mL zeocin in a 200 mL flask at 28°C in a shaking incubator (260 rpm) for 24 hours until OD_{600}~2. Cells were harvested by centrifugation (10 min at 20,000 x g) and resuspended in 50 mL of buffered complex methanol media at OD_{600}~1. The cultures were incubated another 48 hours and methanol was added to a final concentration of 0.5 % (v/v) after 24 hours. After centrifugation for 10 min at 20,000 x g, cell pellets from 3 mL were stored at -80°C. Crude cell-free protein extracts were prepared by washing cells once in breaking buffer (50 mM Tris-HCl pH 7.9, 1 mM EDTA, 2 mM DTT, 5% (v/v) glycerol) followed by sequential vortexing for 30 seconds and placing on ice for a total of eight cycles in 200 µL breaking buffer. To the breaking buffer an equal volume of acid-washed 425-600 microns glass beads (Sigma), 4% (w/v) CHAPS (Sigma) and 1×Complete protease inhibitor cocktail (Roche Diagnostics) was added. After centrifugation for 10 min at 20,000 x g, the cleared supernatants were transferred to fresh tubes and assayed.

2.4.3 GAE6 activity essay

Protein activity of recombinantly expressed GAE6 was assessed by incubating 20 µg lysate in either Tris (10 mM, pH 7.9) or phosphate buffer (10 mM pH 8.0) with 50 µg of UDP-D-glucuronic acid in a total reaction volume of 50 µL overnight at RT.

2.4.4 SDS polyacrylamid gel electrophoresis

Proteins were separated by SDS polyacrylamide gel electrophoresis as previously described by Laemmli (1970). Before separation proteins were denatured for by boiling in sample-buffer (100 mM Tris-HCl, pH 6.8, 2 % (w/v) SDS, 10 % (v/v) glycerin, 1 mM EDTA, 2.5% (v/v) β-mercaptoethanol and 0.005% (w/v) bromophenolblue). For visualization the protein gel was stained using a silver staining kit (Biorad).

2.4.5 Western blot analysis

SDS polyacrylamid gels were blotted onto porablot membranes (Machery-Nagel, Düren, Germany) by means of wet Western Blotting facilitating a Biorad Western Transfer apparatus according to the manufacturer's suggestion. Detection of proteins was performed using a Qiagen anti-HIS antibody coupled to horseradish peroxidase (Hilden, Germany) together with a "Super Signal West Pico Chemiluminescence Substrate" (Pierce, Rockford, IL) according to the manufacturers' suggestions.

2.5 Analytical and preparational techniques

2.5.1 Isolation of cell wall material

"Cell Wall Material" was isolated as follows. Fresh plant material was harvested and snap-frozen in liquid nitrogen. The frozen material was ground in methanol using either a retch-mill (Retsch, Haan, Germany) for small amounts (~2 mg scale), or an Ultra-Turrax T25 (IKA-Labortechnik, Staufen, Germany) for larger amounts. Afterwards the material was extracted with a 1:1 (v/v) methanol, chloroform mixture until no more green pigments could be extracted form the plant material. Finally the material was washed once with acetone and left to dry overnight or alternatively subjected to vacuum for 30 min.

2.5.2 Isolation of mucilage material from Arabidopsis thaliana seeds

Mucilage was extracted from seeds (~5 mg) by suspending the seeds in 1 mL of 0.2% (w/v) ammonium oxalate and shaking for 2 h at 30°C. The monosaccharide composition of the dissolved material was determined (2.5.5).

2.5.3 Isolation of pectic fragments from cell wall material

Pectic fractions were isolated from cell wall material by incubating approximately 2 mg of cell wall with 8.5 U Units of endopolygalacturonase (Sigma) and 4.5 U pectin methylesterase (Novozyme) in a total reaction volume of 1 mL 0.1M ammonium formate (pH 4.7) at 37°C overnight.

2.5.4 Gel permeation chromatography of polysaccharidic samples

Gel Permeation Chromatography of polysaccharides was performed on Biorad Biologic Duo-Flow FPLC system with a Superose 12 columns. Polysaccharides were eluted isocratically using 1 M ammonium formate (pH 4.7) at a flowrate of 1 mL/min with coupled refractive index detection.

2.5.5 Monosaccharide composition

2.5.5.1 GC-MS analysis of alditol acetate derivatives

Cell wall material or polysaccharide fractions were hydrolyzed using acids to obtain monosaccharide mixtures. The monosaccharide mixture was derivatised to their corresponding alditol acetates and subsequently separated and quantified using a GC-quadropole MS (GC-MS) analysis was performed as earlier described (Albersheim *et al*. 1967)

Trifluoroacetic acid hydrolysis

Trifluoroacetic acid (2M, 250 µL) were added to approximately 2 mg of a cell wall sample 250 µL of 2 M trifluoroacetic acid was added placed in a borosilicate glass tube. After closure of the tube with a teflon coated lid the mixture was incubated for 1 h at 121°C in a heating block (Labtech-International, Burkhardtsdorf, Germany). Afterwards the TFA was evaporated under a stream of dried air and the residue was washed twice with 300 µL of isopropanol and dried again.

Reduction

The resulting monosaccharides were treated with 250 µL 1 M ammoniumhydroxyde containing 10 mg/ml sodiumborohydride for one hour at room temperature. Superfluous reductant was removed by adding a few drops of glacial acetic acid and evaporation. The residue was washed three times with a acetic acid:methanol (1:9, v:v) mixture followed by methanol washing and evaporation.

Acetylation

Acetic anhydride and pyridine (50 µL each) were added to the sample and incubated for 20 minutes at 121°C. Unreacted reagents and solvents were removed by evaporation and the dried residue was

washed twice with toluene. The resulting alditol acetates were purified by addition of 500 µL water and 500 µL methylenechloride. The mixture was vortexed and after phase separation the organic phase was recovered and placed into an Eppendorf reaction vial. The solvent was evaporated and the dried product was dissolved in 100 to 400 µL of acetone.

Detection

Detection of the alditol acetates was performed facilitating an Agilent 6890N Network GC system (Waldbronn, Germany) using a Supelco 24110-U column. The temperature program was a follows. After an initial temperature of 160°C for two minutes the temperature was raised to 200°C over two minutes and kept for five minutes. Then the temperature was raised to 245°C in two and ¼ minutes and kept for twelve minutes.

2.5.6 Determination of crystalline cellulose content

Determination of cellulose in cell wall material or in TFA insoluble pellets was determined using a modified method according to Updegraff (1969). In brief, 2-5 mg of wall material was incubated for thirty minutes at 100°C in an aqueous solution containing 73 % (v/v) acetic acid and 9 % (v/v) HNO_3. After incubation the supernatant containing hydrolyzed material was discarded and the resulting pellet consisting of crystalline cellulose was washed with water and five times with acetone. Then the pellet was treated with 72 % (v/v) sulfuric acid at RT for 30 min. After hydrolysis the released glucose was quantified using the anthrone assay (Dische 1964).

2.5.7 Uronic acid content

Uronic acids were quantified using a modified protocol of Blumenkranz and Asboe-Hansen (1973). In short, the material was suspended in 100 µL of water and 600 µL concentrated sulfuric acid containing 0.125 M of $Na_2B_4O_7$ was added. The mixture was boiled for 5 minutes and cooled down. Then 20 µL of biphenyl solution (0.15% *m*-hydroxy-biphenyl in 0.5% NaOH) was added. The absorption was recorded at a wavelength of 520 nm using a Spectra Max Plus ELISA reader (Molecular Devices, Munich, Germany) and compared to a standard curve generated of different concentrations of galacturonic acid.

2.5.8 Hexose content

The amount of total sugars present in samples was determined using the anthrone assay (ref!). In short, to one volume of sample two volumes of anthrone reagent (0.2% (w/v) of anthrone dissolved in concentrated sulfuric acid) were added and the mixture was boiled for 5 minutes after thorough vortexing. A portion of the sample (200 µL) was transferred to a 96-well microtiter plate and the absorption at 620 nm was determined. The amount of hexoses was determined by comparison to a glucose standard curve.

2.5.9 Matrix Assisted Laser Desorption Ionization - Time of Flight (MALDI-TOF) mass spectrometry

The molecular mass of nucleotide sugars was determined using a Voyager DE-Pro MALDI TOF instrument (Applied Biosystems, Langen, Germany). Analytes were mixed 1:1 (v/v) with a 10 mg/mL solution of 2,5-dihydroxybenzoic acid. Spectra were recorded in negative reflectron mode at 15 kV with an extraction delay time of 80 ns.

2.5.10 High performance anion exchange chromatography with pulsed amperometric or UV detection (HPAEC-PAD)

High performance Anion Exchange chromatography for detection of nucleotide sugars with pulsed aperometric or UV detection was performed as earlier described by Libjelke *et al*. (1995) facilitating a Dionex DX500 system (Idstein, Germany) equipped with a Dionex PA100 column. Additional standards such as UDP-D-xylose were obtained from the cell wall network (http://xyloglucan.prl.msu.edu/index.html, Michigan, MI, USA) and UDP-D-galacturonic acid from Hazel Crombie (University of Stirling, Stirling, UK).

2.6 Microscopy and Histochemistry

2.6.1 Seed ultrastructure analysis

Whole seed ultrastructure was investigated into by Scanning Electron Microscopy. The scanning electron microscopy was performed by the Fraunhofer Institute for Applied Polymer Research

(Golm, Germany) using an JSM 6330 F (Jeol, Japan) microscope at an acceleration voltage of 5 kV. Thin layers of about 4 nm platinum were deposited by sputtering onto the surfaces of the seed.

2.6.2 Sectioning and bright field microscopy of seeds

Mature seeds were fixed overnight in an aqueous solution of FAA (5% formalin, 5% acetic acid, 67% ethanol) at 4°C. The sample was dehydrated and infiltrated with LR-White (London Resin Co, Berkshire, UK) according to the manufacturer's suggestions. For microscopy 1 µm sections were cut with a glass knife on a Reichert-Jung Ultracut E microtom (Leica, Bensheim, Germany) and mounted on charged glass slides. The sections were stained with 1% (w/v) toluidine blue O in 100 mM sodium phosphate (pH 7.2) for one minute and washed several times with distilled water. Pictures were visualized with a BX41 microscope (Olympus, Hamburg, Germany) connected to a digital SPOT 2.2.1 camera (Visitron Systems, Puchheim, Germany).

2.6.3 Seed staining

Seeds were stained with ruthenium read by placing seeds in a solution of 0.01 % (w/v) ruthenium red and shaking for 10 min. For dextran blue staining seeds were first imbibed in water and then covered with 1 % (w/v) aqueous solution of blue dextran (average MW 2,000 kDa) for 5 min. Seeds were visualized using a MZ FZ III binocular (Leica) fitted with a SPOT 2.2.1 camera (Visitron Systems)

2.7 Bioinformatics

2.7.1 Identification of candidate genes

Proteins potentially involved in in the nucleotide sugar pathway were identified as follows: The Pfam HMMs (release 6.6) (Bateman *et al*. 2002) and the hmmer software package release 2.2g (Eddy 1998) were downloaded and installed locally on a system running SUSE linux 7.2 (Suse, Nürnberg, Germany). The Pfam HMMs were indexed with hmmindex and interesting hmms were retrieved using hmmfetch as suggested by the author of the hmmer software package. These HMMs were searched against a flat file containing all predicted *Arabidopsis thaliana* proteins in the fasta format, which was obtained from TIGR.

2.7.2 Characterization of genes

Bioinformatic characterization was performed using commonly freely available softwares. Detailed descriptions of programs used are presented in the Results section.

2.7.3 Software development

Software to analyze MALDI-TOF spectra and biological sequence data was developed using freely available software. As development platform Eclipse (IBM, Stuttgart, Germany) was chosen. Most programs were written in the PERL scripting language (version 5.6.1 and 5.8.0) where applicable with the help of the BioPerl toolkit (Stajich *et al*. 2002). When the C programming language was necessary modules were compiled using the GNU C Compiler collection. For statistical pre-analysis and validation of models the R statistics package was used. An interactive web-version of AMAS was assembled by making the original PERL program web aware and running it as an CGI script.

3 Results

3.1 Identification of proteins possibly involved in the nucleotide sugar conversion pathway

For the elucidation of candidate enzymes involved in the nucleotide sugar conversion pathway in *Arabidopsis thaliana* a reverse genetics approach was chosen. Since only very few genes encoding for these enzymes have been identified in plants so far (Reiter and Vanzin 2001) initially proteins and their corresponding genes potentially involved in the synthesis and interconversion of nucleotide sugars (figure 5, introduction) had to be identified. Most of these enzymes had already been characterized to some detail in bacteria or other organisms (Tonetti *et al*. 1998, Samuel and Reeves 2003). In addition, earlier studies demonstrated which kind of reactions should occur *in planta* (reviewed by Feingold and Avigad 1980). Therefore, a search for candidate genes was conducted focusing on reactions that often occur in the plant nucleotide sugar interconversion pathways. The keywords "epimerase", "dehydratase" and "dehydrogenase" were used for a keyword search in the Pfam and TIGR profile hidden Markov model (HMM) databases (Bateman *et al*. 2002) and the template HMMs "PF01370", "TIGR_epimerase", and the NDP-sugar dehydrogenase HMMS ("PF00984", "PF03720" and "PF03721") were identified. These templates were then compared to the sequences from all predicted proteins of *Arabidopsis thaliana* using the HMMR software package (Eddy 1998). The candidate protein list was then semi-automatically abridged by blasting each obtained candidate against characterized proteins from various organisms and by identifying other conserved domains using Pfam (Bateman *et al*. 2002), CDD (Marchler-Bauer *et al*. 2003), Interpro (Mulder *et al*. 2003), and SMART (Letunic *et al*. 2004) facilitating the PERL programming language to do bulk annotation. Proteins were removed which probably do not play a role in nucleotide sugar biosynthesis if the nucleotide sugar converting domains were much less likely to be present than other not cell wall related domains. The resulting list presenting the remaining identified candidate genes is shown in table 1.

Table 1 ***Arabidopsis*** **proteins containing dehydratase/epimerase profiles.**

Corresponding AGI accession codes of identified proteins, [a]:description according to the TIGR 3.0 annotation, [b]: name according to Reiter and Vanzin (2001) and later publications mentioned in the introduction # indicates where the annotation "nucleotide sugar epimerase" was an alternative to the given annotation based on automatic annotation results in the MIPS database (http://mips.gsf.de)

AGI Number	*Description*[a]	*Common name*[b]
At1g02000	nucleotide sugar epimerase family	GAE2
At1g08200	unknown protein	
At1g09340	RNA-binding protein -related	#
At1g12780	UDP-glucose 4-epimerase	UGE1
At1g17890	GDP-L-fucose synthetase -related	GER2
At1g19690	expressed protein	
At1g30620	NAD-dependent epimerase/dehydratase family	MUR4
At1g47290	3-beta hydroxysteroid dehydrogenase/isomerase family	
At1g53500	NAD-dependent epimerase/dehydratase family	RHM2
At1g63000	expressed protein	UER1
At1g63180	UDP-glucose 4-epimerase, putative	UGE3
At1g64440	UDP-glucose 4-epimerase, putative	UGE4
At1g73250	GDP-4-keto-6-deoxy-D-mannose-3,5-epimerase-4-reductase	GER1
At1g78570	dTDP-glucose 4,6-dehydratase	RHM1
At2g27860	dTDP-glucose 4-6-dehydratase -related	AXS1
At2g28760	NAD-dependent epimerase/dehydratase family	SUD3
At2g34850	NAD-dependent epimerase/dehydratase family	MUR4 like
At2g45310	nucleotide sugar epimerase family	GAE4
At2g47650	NAD-dependent epimerase/dehydratase family	AUD2/UXS4
At3g14790	NAD-dependent epimerase/dehydratase family	RHM3
At3g23820	NAD-dependent epimerase/dehydratase family	GAE6
At3g46440	NAD-dependent epimerase/dehydratase family	SUD1/UXS5
At3g51160	GDP-D-mannose-4,6-dehydratase	MUR1
At3g53520	NAD-dependent epimerase/dehydratase family	AUD3/UXS1
At3g62830	dTDP-glucose 4-6-dehydratase homolog D18	AUD1/UXS2
At4g00110	nucleotide sugar epimerase family	GAE3
At4g00560	dTDP-6-deoxy-L-mannose-dehydrogenase -related	
At4g10960	UDP-glucose 4-epimerase putative	UGE5
At4g12250	nucleotide sugar epimerase family	GAE5
At4g20460	NAD-dependent epimerase/dehydratase family	MUR4 like
At4g23920	UDP-glucose 4-epimerase, putative	UGE2
At4g30440	nucleotide sugar epimerase family	GAE1
At5g15910	dehydrogenase-related	
At5g28840	NAD-dependent epimerase/dehydratase family	GDP-Man,3,5 epimerase
At5g44480	NAD-dependent epimerase/dehydratase family	MUR4 like
At5g59290	NAD-dependent epimerase/dehydratase family	SUD2/UXS3
At5g66280	GDP-D-mannose 4,6-dehydratase, putative	GMD1

Candidate proteins presented in table 1 were identified using the Pfam domain 1370, which is annotated as being found in epimerases and dehydratases using NAD^+ or $NADP^+$ as a cofactor. A similar set of proteins was identified using the TIGR profile commonly found in UDP-D-glucose epimerases. A non overlapping set of proteins were identified using the profiles commonly found in UDP-D-glucose-dehydrogenases (table 2). However, this list was mainly confined to the *Arabidopsis* UGD proteins (Seitz *et al*. 2000).

Table 2 *Arabidopsis* proteins carrying the UGD profile

Corresponding AGI accession codes of identified proteins, [a]:description according to the TIGR 3.0 annotation, [b]: name according to Reiter and Vanzin (2001) and later publications mentioned in the introduction

AGI Number	*Description[a]*	*Common Name[b]*
At1g26570	UDP-glucose dehydrogenase related protein	UGD4
At2g45630	Oxidoreductase family	
At3g01010	UDP-glucose dehydrogenase related protein	
At3g29360	UDP-glucose dehydrogenase related protein	UGD2
At5g07800	flavin-containing monooxygenase (FMO) family	
At5g15490	UDP-glucose dehydrogenase related protein	UGD3
At5g39320	UDP-glucose dehydrogenase related protein	UGD1

At the time of identification only UGE (Dörman and Benning 1998), UGD (Seitz *et al*. 2000), MUR1 (Bonin *et al*. 1997) and GER1 (Bonin and Reiter 2000) had been described. The other proteins now designated by a name in table 1 and table 2 were subsequently discovered and most of them with the exception of the *MUR4* genes were also found in a study using a conventional BLAST based search (Reiter and Vanzin 2001). Based on their putative function assigned descriptive names were assigned although their function was not demonstrated. The published name of the proteins was adopted here for the remainder of the thesis. Moreover, a five membered family comprising of the previously identified UDP-D-glucose epimerase (UGE1, Dörmann and Benning 1996) was identified (UGE1-5, Seifert *et al*. 2002) and four other previously not described proteins (corresponding AGI codes At1g30620, At2g34850, At4g20460, At5g44480), which showed high sequence similarity to the UGEs thus implicating them in epimerization reactions. Those were later assigned to include MUR4 and its homologs (Burget *et al*. 2003).

Among the candidate proteins is a putative UDP-D-glucuronic acid epimerase family

Among the identified proteins is a gene family comprising six members, which was most similar to proteins implicated in bacterial UDP-D-glucuronic acid 4 epimerization, namely WcaG and Cap1J (Muños *et al*. 1999). Consequently, this gene family was termed GAE (UDP-D-galacturonic acid epimerase) by Reiter and Vanzin (2001). (This gene family will be discussed in further detail in chapter 3.3)

Proteins possibly involved in NDP-rhamnose synthesis were identified

Another three membered protein family designated RHM (UDP-L-rhamnose synthesis) was retrieved. Here, strongest similarity of these proteins was found to bacterial dTDP-glucose dehydratases, one of the enzymes necessary for the synthesis of UDP-L-rhamnose. In fact these proteins were already annotated as dTDP-glucose dehydratases at the time of identification. However, a thorough analysis of these proteins showed that they have a two domain structure. Whereas the first domain was most similar to the mentioned bacterial dehydratases, a second domain was most similar to bacterial epimerases. (This gene family will be discussed in detail in chapter 3.2).

The UGE, GAE and MUR4 families seem to be closely related

A better understanding of the identified proteins and their interrelationship was sought by building a maximum likelihood family tree of all identified proteins (figure 7).

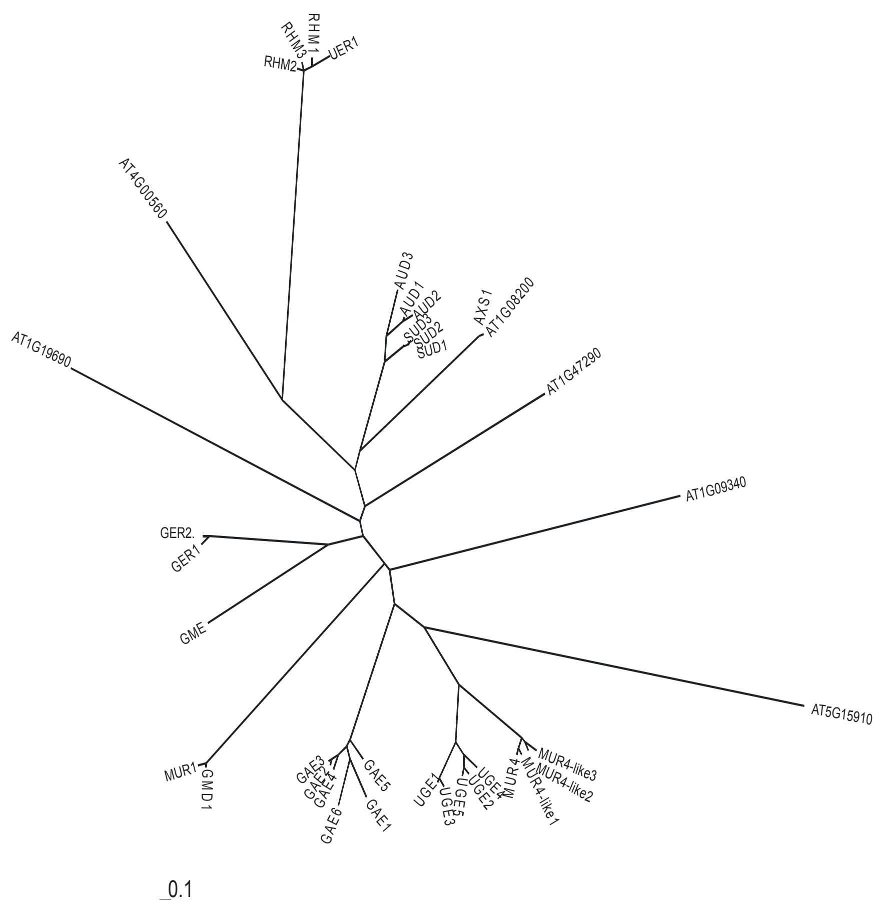


Figure 7 Family tree of proteins from table 1.

Protein sequences of the genes from table 1 and table 2 were aligned using ClustalW (Thompson *et al*. 1994) and a tree was built by maximum likelihood using the PHYLIP package (Felsenstein 2004).

Clearly visible in this tree is a rather close relation of the UGE, GAE and MUR4 families. Indeed, it has also been proposed later that all families seem to encode for sole epimerase activities as discussed by Seifert and colleagues (2002).

Moreover, the family tree indicated that the RHM proteins seem to be related to the AUD/SUD/UXS family possibly due to their reaction mechanism. Moreover the AUD/SUD family seems to be related most closely to the AXS1 and AXS2 proteins, both of which catalyze the synthesis of UDP-D-xylose (Harper *et al*. 2002, Mølhøj *et al*. 2003). A protein encoded by At4g00560 annotated as a putative dehydrogenase seems to be related to the RHM family suggesting that the encoded protein exhibits a similar reaction mechanism.

Interesting candidate proteins were chosen for further analysis

The presence of proteins in the candidate list that have already been shown to be involved in the nucleotide sugar interconversion pathway and previously described protein-signatures found in the candidate list pointed toward the validity of the chosen approach.

From the assembled list (table 1) families that could be involved in pectin synthesis were chosen. The main components of pectin are galacturonic acid (GalA) and rhamnose (see chapter 1.4.4). Hence protein families potentially involved in the generation of the corresponding precursor substrates were chosen for further functional characterization: The RHM family, potentially responsible for the synthesis of NDP-L-rhamnose since this was also identified by Dr. Markus Pauly (Max Planck Institute of Molecular Plant Physiology, Golm, Germany, personal communication) when looking for bacterial paralogs, and the GAE family potentially responsible for the synthesis of UDP-D-GalA as it had been found in a macroarray screen (Gipmans 2001).

The *Arabidopsis* genome encodes putative *myo*-inositol oxygenases

The cyclic alcohol inositol has been shown to be a precursor of UDP-D-glucuronic acid and thus potentially for pectins under some developmental circumstances (Seitz *et al*. 2000). Moreover, it had been demonstrated that *myo*-inositol oxygenase activity, which is one of the enzymes necessary for the utilization of inositol (see figure 5) exist in plants (Loewus and Dickinson 1982). Due to their catalytic mechanism and their substrate specificity it was unlikely that corresponding proteins would be picked up by searching for nucleotide sugar converting enzymes signatures. However, sequence data available from pigs and an EST-contig from *Pinus radiata* described as encoding for a plant protein most similar to the pig *myo*-inositol oxygenase (Arner *et al*. 2001) had been identified. These sequences were searched against the *Arabidopsis* proteom (http://www.tigr.org) using a PBLAST based approach (Altschul *et al*. 1990). Four *Arabidopsis* proteins were identified having similarity to the aforementioned *myo*-inositol oxygenase MIOX (table 3). The nearest neighbors of these proteins were then identified using a BLAST based approach. Again the above mentioned MIOXes were found. Moreover some human renal ESTs exhibiting oxygenase signatures were found which was in good accordance to data showing mammalian *myo*-inositol oxygenase activity in the kidney (Charalampous 1959, Reddy 1998).

Apart from the four identified proteins an additional putative pseudo-gene was identified, localized close to one of the MIOX genes. However, no full length open reading frame seems to be encoded by this locus.

Table 3 MIOX genes in *Arabidopsis*

AGI Number	*Description*	*Assigned Name*
At1g14520	unknown protein	MIOX1
At2g19800	unknown protein	MIOX2
		ψMIOX3
At4g26260	unknown protein	MIOX4
At5g56640	unknown protein	MIOX5

Since the MIOXes would act mechanistically different from the RHM and GAE proteins, the family was also chosen for further characterization.

3.2 Functional characterization of the RHM gene family

3.2.1 Bioinformatic characterization of the RHM protein family

The RHM family was chosen as it was implicated in NDP-L-rhamnose synthesis, a potential precursor for the synthesis of the pectic polysaccharide RGI. In bacteria the rhamnose precursor is generated from dTDP-D-glucose by the action of three different enzymes a dehydratase, an epimerase, and a reductase (Giraud and Naismith 2000, figure 8). The genes encoding for these bacterial enzymes have been cloned and functionally characterized .

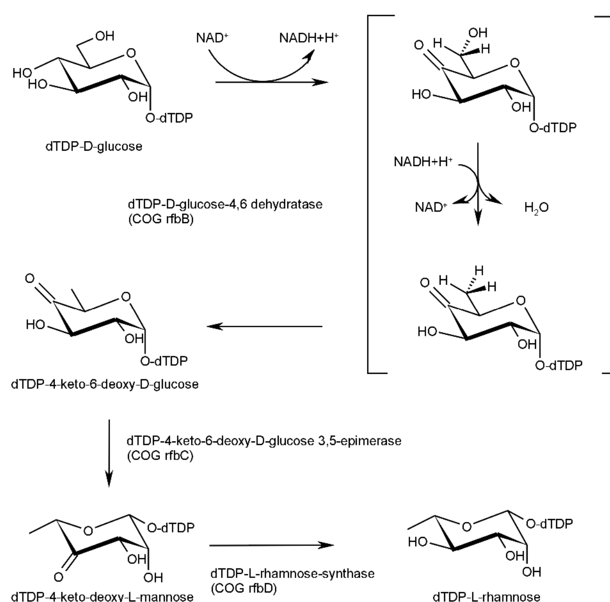


Figure 8 dTDP-L-rhamnose synthesis pathway in bacteria.

(Modified after Giraud and Naismith 2000)

Based on the bacterial data the protein-sequence properties of the identified RHM-proteins were further explored using a bioinformatic approach.

A conserved domain analysis using a NCBI conserved domain search (Marchler-Bauer *et al*. 2003) indicated a two domain structure of the *Arabidopsis* RHM proteins. The N-terminal domain of the proteins showed highest similarity to the bacterial rfbB dehydratase conserved domain (COG1088). This domain was extracted from bacterial proteins involved in the first step of dTDP-L-rhamnose synthesis (figure 8, upper panel). The C-terminal domain of these proteins showed highest similarity to the rfbD domain (COG1091) involved in the third (and last) step of bacterial dTDP-L-rhamnose synthesis (figure 8, lower panel). When the rfbC domain (responsible for the middle step of bacterial dTDP-L-rhamnose synthesis) was extracted from the NCBI conserved domain database

and when an RPS-BLAST (Marchler-Bauer *et al*. 2003) was performed against the *Arabidopsis* proteom no appropriate candidate was found.

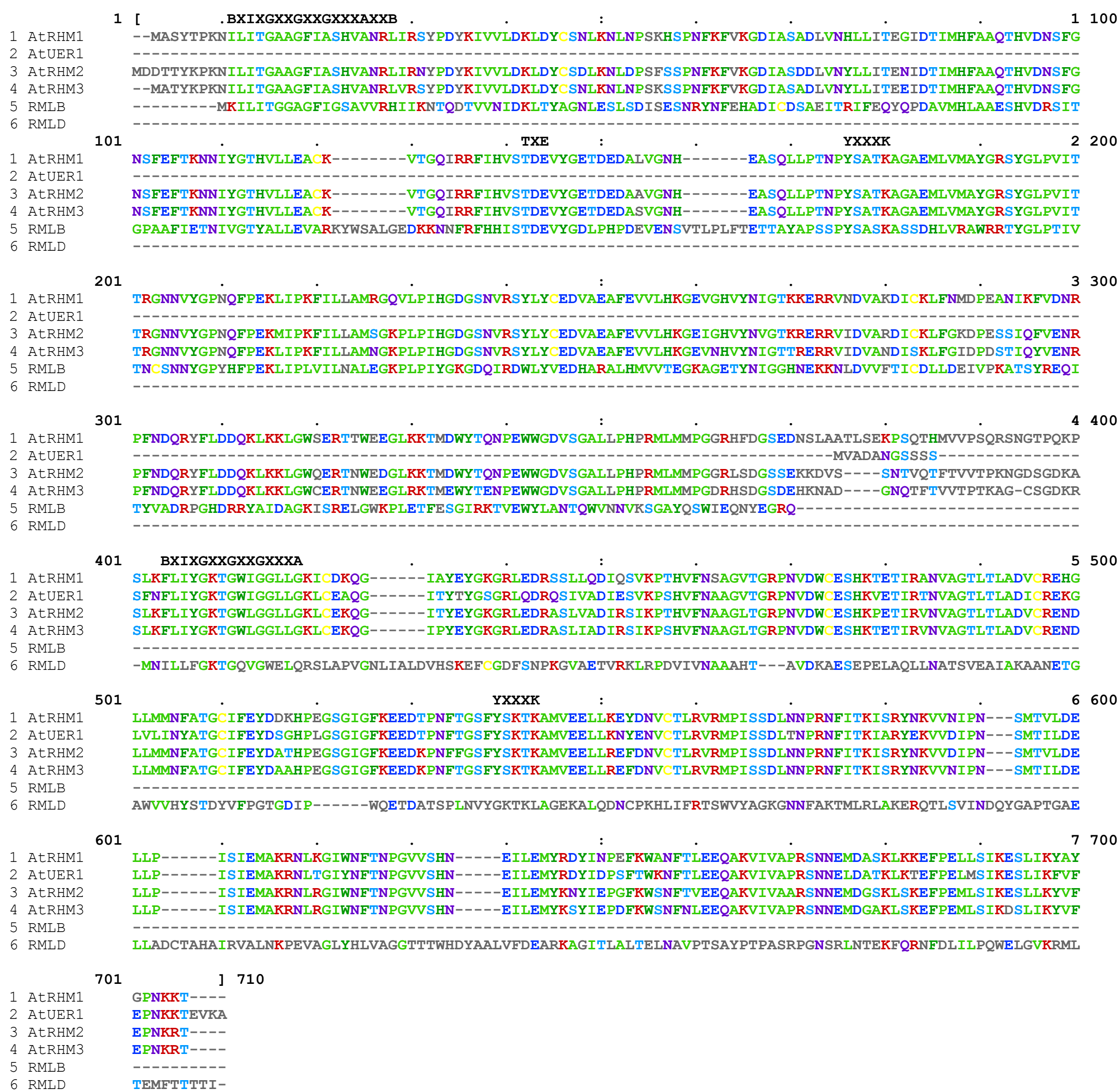


Figure 9 Amino acid sequence alignment between the three putative *Arabidopsis* RHM proteins, the *Arabidopsis* UER1 protein and the bacterial proteins RMLB and RMLD.

Amino acids are colored according to their physiochemical group using MVIEW as detailed in Brown *et al.* (1998) if the similarity between the sequences is above 60%. TXE, YXXK and BXIXGXXGXXGXXXA indicate amino acid signatures implicated in the catalysis in the bacterial enzymes.

This suggests that the second domain of the *Arabidopsis* RHM proteins could be bifunctional and catalyze both the functions of the bacterial rfbC and rfbD domains. Indeed this would not be the first incidence of such a bifunctional enzyme in plants since e.g. GER catalyzes both the reduction and 3,5 epimerization of GDP-4-keto-6-deoxy mannose (Bonin and Reiter 2000).

The three membered RHM protein family and UER1, a protein closely related to the C-terminal domain of the RHM family, were investigated using TMHMM (Krogh *et al*. 2001) to identify potential transmembrane regions. For none of the proteins neither a transmembrane domain nor any targeting signal using the commonly available targeting predictors PsortI (Nakai and Kanehisha 1992), TargetP (Emanuelsson 2002) and Softberry's predictor (www.softberry.com) could be identified suggesting a cytoplasmatic location of all these proteins.

Within the RHM family the similarity on protein level was between 91.5 (RHM1, RHM2) and 95.1% (RHM2, RHM3) as determined by a Needleman Wunsch (1970) alignment. When only the C-terminal domain of the RHM proteins and UER1 was considered a similarity between 85.0 (UER, RHM3) and 96.3 %(RHM2, RHM3) was detected.

When the exon-intron structure of the *RHM* coding region was studied, only a single intron in the region encoding the C-terminal domain of the RHM proteins was found. This intron showed high spatial conservation in all *RHM* genes and *UER1*.

The N-terminal domain of the RHM proteins was searched for signature sequences and the extended Wierenga signature sequence BXV/IXGX$_{1-2}$GXXGXXXG/AXXB” (B denotes a hydrophobic amino acid) found in FAD and NAD(P) binding Rossmann folds (Wierenga *et al*. 1985, Kleiger and Eisenberg 2002) was detected. Moreover the conserved signatures TXE and YXXXK which have been involved in catalysis in the bacterial dTDP-D-glucose dehydratase (Allard *et al*. 2001) could be assigned (figure 9).

The C-terminal domain showed a conserved extended nucleotide cofactor binding site where only the last hydrophobic amino acid is missing, and a conserved YXXXK loop also found in enzymes catalyzing the final step of NDP-L-rhamnose synthesis (Graninger *et al*. 1999) (figure 9).

A three-dimensional model of the N-terminal domain of RHM reveals its similarity to MUR1 a GDP-mannose-dehydratase

Because the similarity of the RHM proteins to known proteins was relatively high a three dimensional homology model of at least one domain of a RHM protein could reveal new insights into the function of the protein or into its probable catalytic reaction. Specifically a spatial conservation of proposed catalytic signature sequences could be indicative of their catalytic function.

Therefore, the N-terminal dehydratase part of RHM2 was used for homology modeling facilitating the SWISS-MODEL framework (Guex and Peitsch 1997). The structures of dTDP- glucose 4,6-dehydratase from *Salmonella enterica* (pdb accession:1keu) (Allard *et al*. 2001) and *Streptococcus*

suis (1ker) (Allard *et al*. 2002) were used as template in manual optimisation mode. After the energy of the model was refined and potential minor side chain clashes visible in the Ramachandran plot (Ramachandran *et al*. 1963) were removed the model was compared to a crystal structure of MUR1 (1n7h) a plant GDP-mannose 4,6 dehydratase (Mulichak *et al*. 2002) which is the only structurally resolved plant nucleotide sugar converting enzyme. Striking similarity was observed between the model of the N-terminal dehydratase domain of RHM2 and the resolved structure of MUR1 (figure 10).

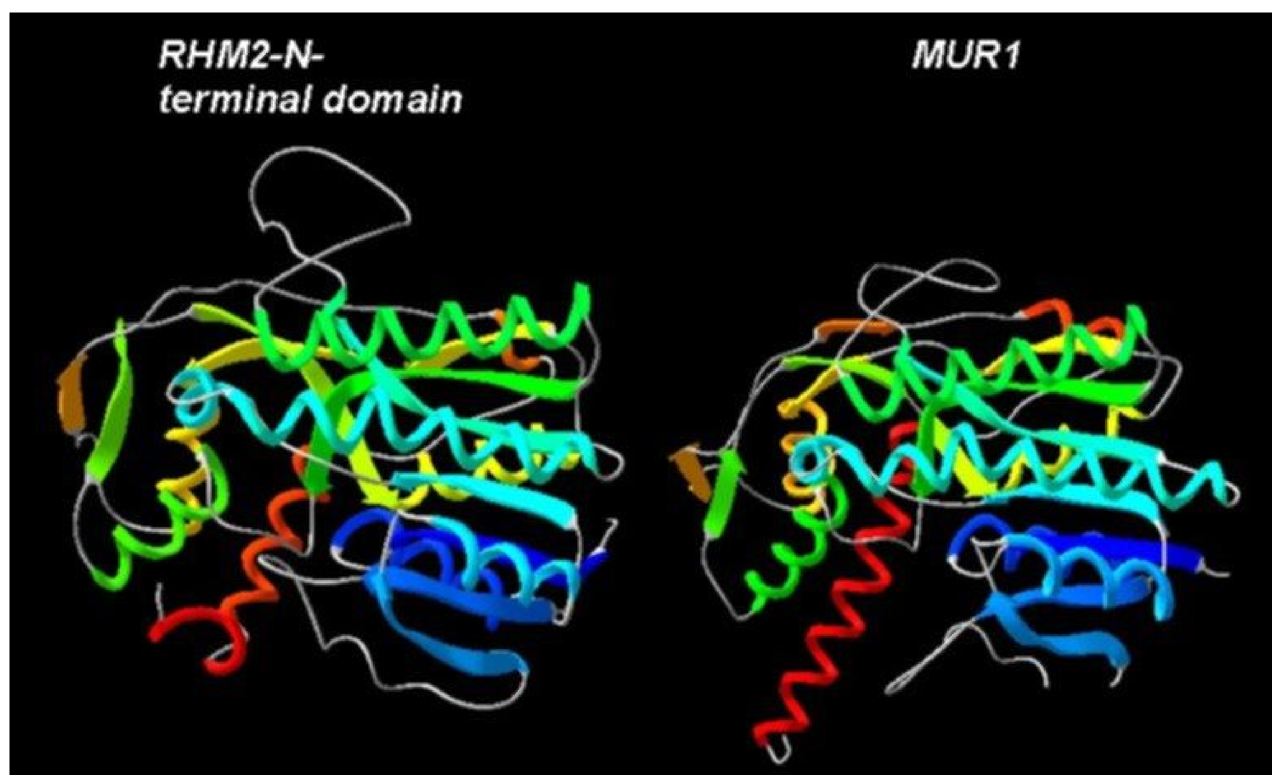


Figure 10 Structure of MUR1 and a model of the N-terminal dehydratase domain of RHM2.

The coordinates of MUR1 (right structure) were obtained from the pdb database and the N-terminal domain of RHM2 (left structure) was homology-modeled using the SWISS-MODEL framework. The resulting structures were viewed as ribbon diagrams using Swiss-pdb Viewer (Guex 1996, Guex and Peitsch 1996) and colored according to secondary structure succession.

A C^{α}-chain alignment between the N-terminal domain of RHM2 and MUR1 using MATRAS 1.1, a tool for structural comparison (Kawabate and Nishikawa 2002), showed a root-mean-square deviation of only 2.22 Å over 305 C^{α} atoms indicative of a good spatial conservation between the backbone of these two structures. This suggests that the N-terminal domain of RHM2 might be similar in functional mechanism to MUR1. This was further explored by highlighting catalytic

residues of MUR1 identified by Mulichak and colleagues (2002) and comparing these to residues found in the proposed catalytic signatures in the N-terminal dehydratase domain of RHM2 (figure 11). Indeed, the previously identified potential catalytic signatures in RHM2 did show good alignment and relative positioning to corresponding amino acid side chains in the MUR1 structure.

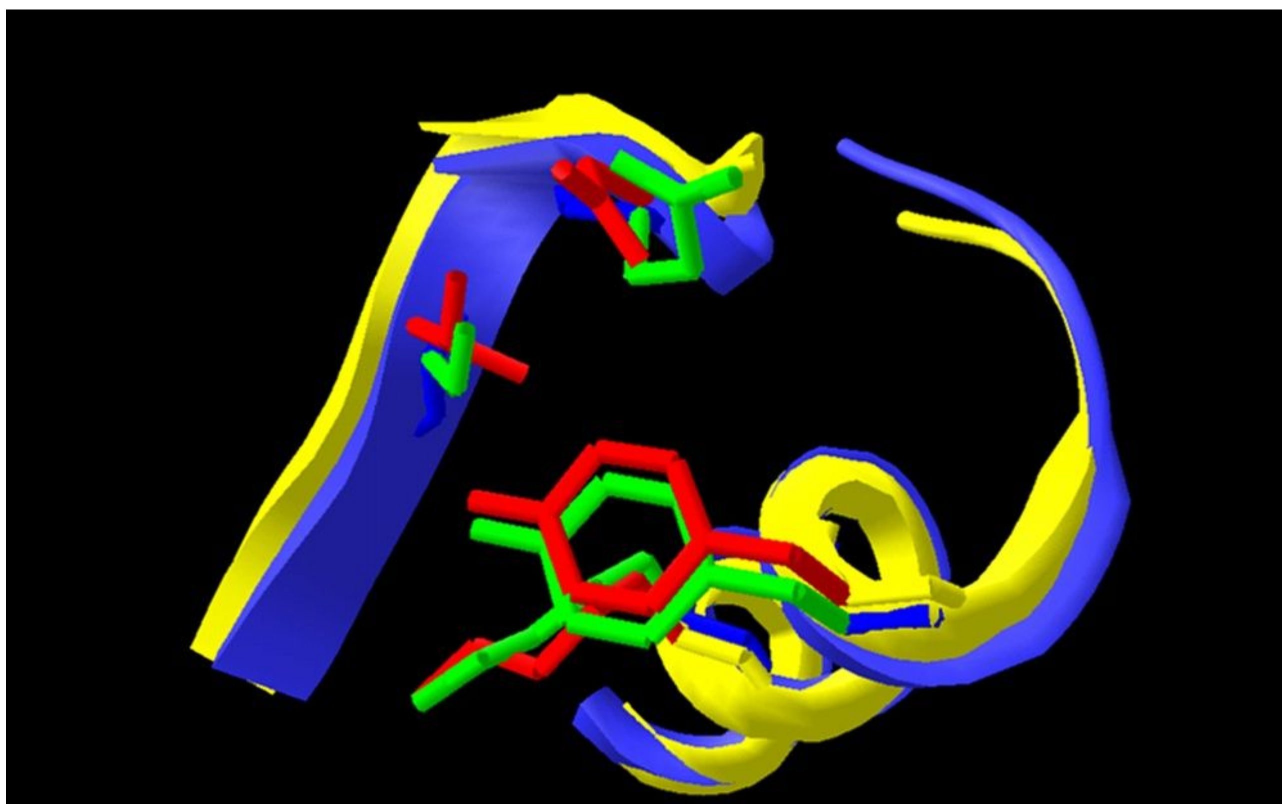


Figure 11 Detailed view of an overlap of the structure of MUR1 and the model of the C-terminal part of RHM2.

The proposed catalytic sidechains from the signature sequences S/TXE and YXXXK are shown for both structures. The RHM2 backbone is displayed in yellow and its sidechains in red. The MUR1 backbone is given in blue with green sidechains.

This added further evidence to the proposed catalytic activity of the N-terminal dehydratase domain of RHM2 being a NDP-sugar 4,6-dehydratase .

3.2.2 RHM transcripts are ubiquitously expressed in Arabidopsis

For a targeted analysis of gene function it is important to identify where and when the gene is expressed to specifically analyze tissues where expression is high.

For an expression analysis of the whole *Arabidopsis RHM* family the MPSS expression database (http://www.dbi.udel.edu) and the publicly available tissue specific microarray data of the

AtGenexpress consortium (ftp.arabidopsis.org) were queried. MPSS data suggested a rather ubiquitous expression of the whole family with preferential expression of *RHM1* in the flower, *RHM2* in the silique, and *RHM3* in the root (table 4). However, due to the higher spatial resolution of the AtGenexpress data a more detailed description is possible. These data suggested a preferential expression of *RHM2* in the siliques, whereas all genes seem to be highly expressed in the pollen (table 5).

Table 4 Expression of the *RHM* gene family in *Arabidopsis thaliana*.

The expression of *RHM* genes was assessed by querying the MPSS expression database with the *RHM* genes and *UER1*. The table shows the normalized tag abundance for each gene.

	root	***shoot***	***flower***	***silique***	***callus***
RHM1	191	147	1024	225	206
RHM2	173	84	137	139	110
RHM3	51	20	28	43	20
UER1	511	124	311	121	144

Table 5 Expression of the *RHM* gene family in *Arabidopsis thaliana based on AtGenexpress data*.

The expression of *RHM* genes was assessed by querying the AtGenexpress data expression database with the *RHM* genes and *UER1*. The table only shows the three tissues showing the highest expression values (more details are given in the appendix).

	Highest expression observed
RHM1	Flowers stage 12, seedling green parts, shoot apex
RHM2	Mature pollen, siliques with seeds stages 4& 5
RHM3	Pollen, flowers stage 12 stamen
UER1	Roots, mature pollen, siliques with seeds stage 4

RHM2 expression was studied in further detail using semiquantitative RT-PCR and a *RHM2*-promoter-β-glucuronidase (GUS) fusion approach.

RT-PCR data showed expression in all examined tissues and was therefore judged to be ubiquitous (figure 12). The promoter::GUS approach was carried out by Anja Kuschinsky described in her Diploma-thesis (2002). Again a rather ubiquitous expression was shown. Moreover strong

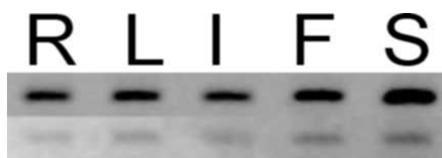


Figure 12 Expression of RHM2

RT-PCR analysis of *RHM2* transcript abundance. Upper lane *RHM2* transcript and lower lane *APT1* control reaction. The letters designate R: root, L: leaf, I: infloresence, F: flower and S: siliques.

expression was detected in sectioned siliques and in the pollen grains (Kuschinsky 2002). Expression was also detected in the pollen tube in pollen growth experiments (appendix, fig I)

Isolation of Arabidopsis thaliana Plants with T-DNA Insertions in *RHM2*

For a functional analysis of the RHM family in *Arabidopsis*, a first *rhm2 Arabidopsis* insertion line was identified in the GABI-KAT collection (http://www.mpiz-koeln.mpg.de/GABI-KAT/), Rosso *et al*. 2003) by screening the GABI-KAT collection with the appropriate identifier (At1g53500). The seeds obtained from GABI-KAT feature a T-DNA encoding sulfadiazine resistance. After establishing seedlings from the obtained seeds, these were screened for the insertion using a PCR based scheme (see material section: 2.3.14). A heterozygous line was picked and progeny of this line were plated on MS plates containing sulfadiazine. A segregation of the T-DNA encoded sulfadiazine resistance of approximately 3:1 was detected (χ^2=0.0054, p=0.941, n=988) indicative of a single full insertion or closely linked multiple insertions. Southern blotting using the full coding part of the sulfadiazine resistance and part of the left-border sequence as a probe also indicated a single full insertion (appendix). Further confirmation of the integration was obtained by PCR amplification and sequencing of the right and left border regions of the inserted T-DNA. Apparently the insertion of the T-DNA resulted in a small approximately 40 bp deletion of the endogenous gene (figure 13). A homozygous insertion line was designated *rhm2-1*.

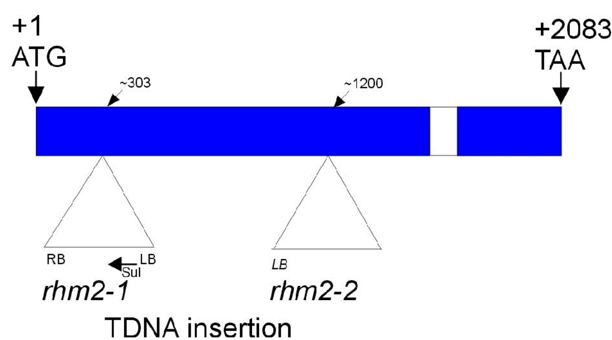


Figure 13 Structure of *rhm2-1* and *rhm2-2*.

The insertion of the T-DNAs into the RHM2 gene is shown. LB and RB designate the left and right border sequence respectively. Blue boxes represent exons and the white box represents the single intron of RHM2.

A second integration allele was obtained from the SALK collection (http://signal.salk.edu) and after isolation of plants homozygous for the insertion, this line was designated *rhm2-2*.

Often an insertion of T-DNA into the genome can lead to complex rearrangements of the genome, sometimes leading to the synthesis of a proper transcript even though the endogenous gene locus is altered. RHM2 transcript in *rhm2-1* and *rhm2-2* plants was investigated by RT-PCR. Primers spanning the insertion sites of the T-DNA in the mRNA were not able to amplify transcript from RNA isolated from either *rhm2-1* or *rhm2-2* but from the control plants, indicative of an alteration of *RHM2* mRNA in the mutants.

3.2.3 Phenotypical characterization of rhm2 lines

Due to its putative role in nucleotide sugar metabolism, RHM2 might play a role in the synthesis of pectic polysaccharides present in the cell wall. Hence cell wall material from different tissues of WT and *rhm2-1* plants was isolated, and its monosaccharide composition was determined to investigate the function of the RHM2 protein in cell wall synthesis *in planta* (sections 2.5.1 and 2.5.5). However, no significant deviation from the wild type wall composition was detected in leaf material from mature greenhouse grown plants or roots from plants grown on Murashige and Skoog (1962) agar plates (data not shown). Also when the pectic components were isolated from the cell wall facilitating an endopolygalacturonase and a pectin methylesterase and separated according to size using gel permeation chromatography no difference was obtained between wild-type and *rhm2-1* plants (data not shown). Taken together, the cell wall structure in the *rhm2-1* leaf did not seem to be altered. Moreover no obvious growth, developmental, or morphological phenotype could

be observed compared to WT plants when *rhm2* plants were grown in growth chambers or in the greenhouse.

Since the pant cell wall is not the only sink for rhamnose a reduction of this compound might also affect other rhamnose sinks. One such sink is the mucilage of *Arabidopsis* seeds (Western *et al*. 2000) which nearly completely consists of a pectinaceous unbranched RGI (Goto 1985, Penfield *et al*. 2001) and thus has a rhamnose content of approximately 40%. Moreover since the mucilage is dispensable under laboratory conditions (Western *et al*. 2001) a reduction in mucilageous rhamnose might not be counterbalanced by subsequent upregulation of other genes leading to rhamnose synthesis.

Therefore mature seeds were stained for seed-mucilage pectin with ruthenium red, a pectin staining dye (Hanke and Northcote 1975) and only little irregularly patterned staining could be observed in both *rhm2-1* and *rhm2-2* (figure 14 B, C). Seeds from wild type plants on the other hand showed intense spherical staining patterns (figure 14 A). The possibility of a compensatory up-regulation of other polysaccharides in the seed mucilage was explored by placing imbibed seeds into an aqueous solution of high molecular weight blue dextran. The blue dextran should not be able to penetrate intact mucilage due to its high molecular weight as has earlier been demonstrated (Windsor *et al*. 2000). Here, wild type seeds showed a large roughly spherical exclusion (figure 14 D) zone whereas the mutant seeds (figure 14 E, F) showed a much smaller patchier exclusion zone indicative of a loss of a penetration barrier (mucilage) in the mutant.

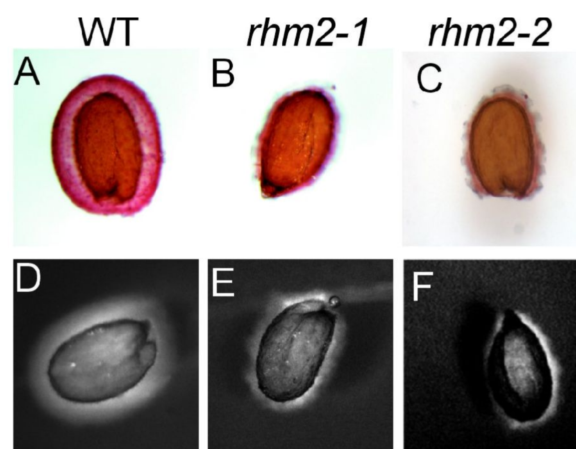


Figure 14 Staining of WT and mutant seeds.

A, B, C staining of seeds with ruthenium red. D, E, F staining with high molecular weight blue dextran

Because structural integrity of the seed coat is affected in some mucilage mutants (Western *et al*. 2001), the structure of the *rhm2* seed coat was investigated by sectioning and subsequent staining with toluidine blue, a polychromatic dye (O'Brien *et al*. 1964). These analyses showed that the columella of *rhm2* seeds were flattened (figure 15) and the general structure of the seed coat was distorted. Moreover, mucilage deposition in the sections of the mutant was less visible than in the wild type seeds (figure 16).

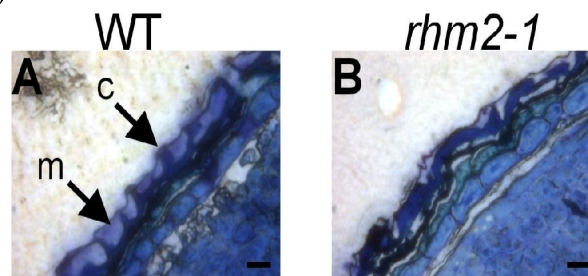


Figure 15 Sections of mature seeds.

Mature seeds were fixed under conditions preserving the mucilage, sectioned and stained with toluidine blue. C indicates a columella and m indicates deposited mucilage. Scale bar is 10 µm

These findings were further corroborated by examining the ultrastructure of whole untreated and imbibed seeds by scanning electron microscopy (SEM). SEM showed that wild type seeds exhibit a regular pattern of elevated columellae within each epidermal cell in accordance to earlier findings by Western and colleagues (2001). In contrast, mutant seeds display a distorted flattened structure, where the columellae are no longer easily distinguishable.

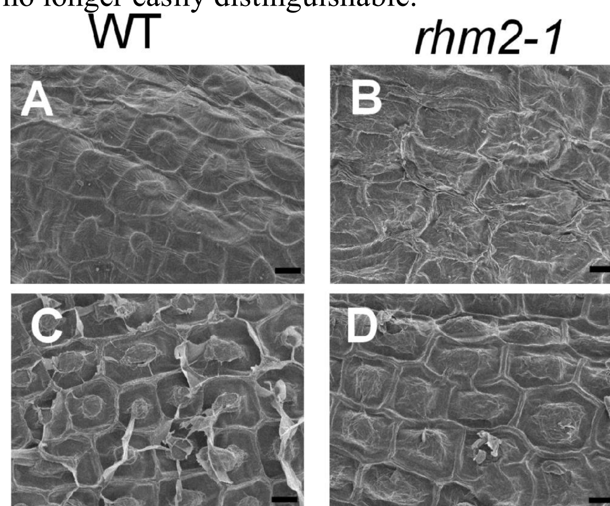


Figure 16 Ultrastructural Investigation of seeds surface

A, B SEM of dry unimbibed seeds; C, D SEM of imbibed seeds after drying. Scale bar represents 10 µm

Upon imbibition cells harboring the mucilage burst and release the mucilage into the surroundings (Western *et al*. 2000). The release of mucilage material was clearly visible in SEM pictures in imbibed WT seeds (figure 16 patches of material in C) and as a result deep valleys around the columella become visible. In contrast, in imbibed *rhm2* seeds the resulting valleys seem to be shallower and the columella seems to be more flattened and less defined (figure 16 D). In essence these findings corroborate the results obtained by staining with ruthenium red and sectioning, where in both cases only a remnant of mucilage could be found.

3.2.3.1 *Rhm2* seed mucilage is drastically reduced

The monosaccharide composition of mutant and WT seeds was studied by extracting the mucilage with ammonium oxalate as previously described (Goto 1985, Western *et al*. 2001). In accordance with the known mucilage composition (Goto 1985, Penfield *et al*. 2001) the most abundant sugars found in both the WT and in *rhm2* extractable mucilage were rhamnose and uronic acids (table 6), representing the backbone sugars of RGI (Visser and Voragen 1996). However, mucilage obtained from *rhm2-1* and *rhm2-2* seeds showed a reduction of approximately 65% and 55% of rhamnose levels compared to the WT respectively. In addition, uronic acids were decreased in approximately the same manner consistent with their presence in the backbone structure of RGI, where each rhamnose unit is linked to one galacturonic acid unit. The absolute amount of the other detected sugars were not changed suggesting that these are not present in any mucilage RGI sidechains (table 6).

Table 6 Sugar content of solubilized mucilage in µg/mg treated seeds.

	Gal	Man	Xyl	Ara	Fuc	Rha**	Glc	UA*
WT	1,6±0,3	0,9±0,1	1,2±0,1	1,1±0,1	1,2±0,1	8,9±2,3	3,9±1,3	9,9±2,9
Rhm2-1	1,4±0,3	0,9±0,1	1,0±0,1	1,0±0,1	1,2±0,1	2,9±0,3	2,9±1,2	4,8±0,5
Rhm2-2	1,5±0,4	0,9±0,1	1,1±0,1	1,1±0,3	1,2±0,2	3,5±0,5	3,1±1,7	5,0±0,9

The values are the average of at least three independent experiments. * indicates significance at 5% level ** indicates significance at 1% level

Other parameters that pertain to the structure of pectic polysaccharides include the degree of methylesterification. However, no difference in degree of methylesterification was observed between *rhm2* and WT, since both samples only contained traces of alkali released methanol.

The molecular weight of the mucilage polysaccharide size was determined by solubilization and subsequent subjection to field flow field fractionation with multi-angle laser light scattering refraction index by Dr. Nora Eckermann (University of Potsdam, Potsdam, Germany) (Wyatt *et al*. 1993). The advantage of this technique compared to gel permeation chromatograph is its high upper working limit and its lack of sensitivity to adsorption (Roessner and Kulicke 1994). The *rhm2-1* mucilage size was determined to have a mass average of 8.8×10^5 g mol^{-1} whereas the WT mucilage showed a molecular mass average of 5.0×10^6 g mol^{-1} . Thus the proposed limiting supply of the rhamnose-precursor in *rhm2* results not only in the production of less mucilage polysaccharides, but also in the production of smaller polysaccharides.

3.2.3.2 Germination rate of *rhm2* seeds is unaltered

Seed mucilage has been implicated in seed-germination under water limiting conditions by Penfield and co-workers (2001). Moreover, *RHM2* was shown to be upregulated upon treatment with abscisic acid which is known to be involved in water stress signaling (Schroeder *et al*. 2001) in a microarray screen (Hoth *et al*. 2002). Therefore the germination rate of WT and *rhm2* seeds was investigated by reducing water potential through administering water with increasing concentrations of PEG-8000 as described by Penfield and co-workers (2001). However, no difference in germination rate was observed when compared to the wild type (85±10 % at 10 % PEG or 70±7 % at 15% PEG at a germination capability of maximally 95% for both wild-type and *rhm2* seeds).

3.2.4 Downregulation of more than one member of the RHM gene family in Arabidopsis

Downregulation of single genes of gene families often pose the problem of genetic redundancy. This can especially be the case if the various gene products are expressed in the same tissue or at the same time in development or if the gene products are very similar in their specificity. All prerequisites for redundancy were met by the RHM proteins due to their broad expression and their high similarity on the protein level suggesting similar substrate specificity. As a result it was not that surprising that *rhm2* plants did not show a difference in their cell wall composition in leaf tissue when compared to wild-type plants.

The redundancy issue can be met by obtaining mutants for every single family member of the gene family and subsequent crossings to create double or triple mutants. Unfortunately knock-out mutants are currently not available for neither *RHM1* nor *RHM3* making this approach not feasible.

Alternatively, an antisense or RNAi approach (Waterhouse *et al*. 2001, Wesely *et al*. 2001, Chuang and Meyerowitz 2000, Fagard *et al*. 2000) can be chosen to downregulate gene families with mutiple members (George Helliwell, CSIRO, Canberra, Australia personal communication). To downregulate the whole *RHM* gene family initially a conserved sequence stretch of approximately 700 bp of *RHM2* was chosen starting from the start codon of *RHM2*. Efficient silencing by double stranded RNA requires stretches of at least 21 nucleotides of total identity with the targeted gene(s). Thus an identity analysis of *RHM2* regions with the corresponding regions of *RHM1* and *RHM3* was conducted. Accordingly a region was chosen which fulfilled this criteria and did not show any significant similarity to any other genes. As shown in figure 17 the chosen region contained a general high conservation on the nucleotide level and totally conserved stretches of at least 21 basepairs in *RHM1* and *RHM3* in at least two instances. The *RHM2* based gene region was subcloned in a head to head fashion into the pHANNIBAL a vector used for RNAi induced gene silencing (Waterhouse *et al*. 2001). The resulting silencing construct was then transferred into the plant transformation vector pART27 to generate the plasmid pRRHM.

```
RHM2        ATGGATGATACTACGTATAAGCCAAAGAACATTCTCATTACTGGAGCTGCTGGATTTATTGCTTCTCATGTTGCCAACAGATTAATCCGTAACTATCCTG 100
RHM1            +++   ++ ++++ +  ++ ++++++++++++++ ++ +++++++++++ ++ +++++ ++++++++ +++++++++ + ++ ++ + ++++++++
RHM3            +++   ++++ ++++++++ ++++++++ +++++ +++++ +++++++++++ ++ ++ +++++++++++ ++++++ ++ + ++ + +++ ++++

RHM2        ATTACAAGATCGTTGTTCTTGACAAGCTTGATTACTGTTCAGATCTGAAGAATCTTGATCCTTCTTTTTCTTCACCAAATTTCAAGTTTGTCAAAGGAGA 200
RHM1        +++++++ ++++++++ RRRRRRRRRRRRRRRRRRRRRRRR ++++ ++++++++  ++++++++      ++ ++ ++ +++++++++++++++++ ++
RHM3        + +++++ ++ +++++ RRRRRRRRRRRRRRRRRRRRRRR  ++++++++++ +++ ++++++++   ++ ++ ++ ++ +++++++++++ ++++++++

RHM2        TATCGCGAGTGATGATCTCGTTAACTACCTTCTCATCACTGAAAACATTGATACGATAATGCATTTTGCTGCTCAAACTCATGTTGATAACTCTTTTGGT 300
RHM1        ++++++ ++++ +++  + ++ ++  + +++++++++++++++   +++++ ++ ++ ++++++++ ++++++++ +++++ ++ ++ ++ ++ ++ +++
RHM3        ++++++ ++++ +++++++++ RRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRR + ++ ++ ++ ++ +++++ ++++++++++++++ +

RHM2        AATAGCTTTGAGTTTACCAAGAACAATATTTATGGTACTCATGTTCTTTTGGAAGCCTGTAAAGTTACAGGACAGATCAGGAGGTTTATCCATGTGAGTA 400
RHM1        ++ ++ ++ ++++++++ +++++ +++++ +++++ ++++++++ +++ + ++ ++ +++++++++++ ++ +++++ +++++++++++ +++++ ++++
RHM3        +++++++ ++ +++++ RRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRR +++++ +++++++++++ ++++++++ ++ ++ ++++++++

RHM2        CCGATGAAGTCTATGGAGAAACCGATGAGGATGCTGCTGTAGGAAACCATGAAGCTTCTCAGCTGTTACCGACGAATCCTTACTCTGCAACTAAGGCTGG 500
RHM1        + ++++++++ +++++ +++++ ++++++++++++  +++ ++ ++++++++ ++++++++++++ + ++++++++++++++++++++ ++ ++ +++++
RHM3        ++++++ ++++++++++++++ +++++ +++++++++++ ++ ++++++++++++ + ++ ++ ++ ++ ++ +++++++++ ++ + ++ ++ +++++

RHM2        TGCTGAGATGCTTGTGATGGCTTATGGTAGATCATATGGATTGCCTGTTATTACGACTCGCGGGAACAATGTTTATGGGCCTAACCAGTTTCCTGAAAAA 600
RHM1        +++++++++++++++ +++++++++++++++++ +++++ ++++++++++++++ +++++ +++++ ++ ++ +++++ ++ ++ +++++++++++ ++
RHM3        +++++ ++ +++++ +++++ ++++++++++++++ +++++ +++++++++++++++++ +++++ +++++ ++ RRRRRRRRRRRRRRRRRRRRRRR +

RHM2        ATGATTCCTAAGTTCATCTTGTTGGCTATGAGTGGGAAGCCGCTTCCCATCCATGGAGATGGATCTAATGTCCGGAGTTACTTGTACTGCGAAGACGTTG 700
RHM1         ++++++++++++++++ +++ ++++ +++++ +++ ++   ++++++++ ++++++++++++++ ++++++ ++++ +++ + +++++ RRRRRRRRRR
RHM3        +++++++++++++++++++  +++++++++++++++++ + +++++ ++++ +++++++++ ++ ++ +++++ +++++++++++++++++  + +++++

RHM2        CTGAGGCTTTTGAGGTTGTTCTTCACAA
RHM1        RRRRRRRRRRRRR ++++++++++++++
RHM3          + +++++++++++ +++++++++++
```

Figure 17 Analysis of totally conserved stretches of nucleotides in the RHM family.

For the analysis the RHM2 nucleotide sequence was used and compared to RHM1 and RHM3 using RNAIfinder, a tool developed during the course of the PhD. Conserved residues are marked with a plus and conserved stretches of 21 or more nucleotides are highlighted by an R.

The obtained construct was used to transform *Arabidopsis* plants by the dipping method (Clough

and Bent 1998). In a first selection round 15 kanamycin resistant T1 plants were obtained. Ten plants out of those were viable on soil and were termed rRHM. Of these ten plants, three plants (55, 69, 71) exhibited a reduced content of seed mucilage as shown by staining with ruthenium red similar to *rhm2-1* and *rhm2-2*. Moreover two of these plants (69 and 71) showed a dwarfed appearance. Since the primary effect of a down-regulation of *RHM2* transcript is a reduction of mucilage (see chapter 3.2.3.1) these three plants were considered potentially effected in *RHM* transcript and therefore further analyzed.

Downregulating *RHM* transcript leads to a reduction of cell wall bound rhamnose

Progeny of these T1 plants were further analyzed. Monosaccharide composition of leaf derived cell wall material from mature plants indicated a reduction in rhamnose of up to 60%. These plants were chosen and further propagated to obtain plants homozygous for the transgene. Three different independent T3 plant lines were obtained showing a rather stable effect in cell wall derived rhamnose reduction in leaf tissues. When hypocotyls of progeny of those plants (T4) were grown on MS medium containing 1% sucrose a reduction of up to 35 % in cell wall derived rhamnose levels was observed whereas the other cell wall sugars remained largely unaltered. (table 7)

Table 7 Monosaccharide composition of cell wall material from 4-day old hypocotyls of three independent rRHM lines

All values are given as weight percent; * denotes a significant difference at the 5% level ** denotes a significant difference at the 1% level by Man-Whitney U test

	WT	*rRHM51*	*rRHM69*	*rRHM77*
Fucose	2.6±0.2	2.7±0.2	3.0±0.4	2.6±0.2
Arabinose	17.8±0.4	19.2±1.2	18.1±0.9	17.3±1.0
Rhamnose	11.6±0.8	10.5±0.6*	10.0±0.3**	9.2±1.5*
Galactose	27.0±2.4	25.1±1.1	27.8±1.1	26.1±3.7
Xylose	17.1±0.9	17.7±0.6	18.7±0.5	17.6±1.4
Mannose	5.3±0.3	5.6±0.2	5.6±0.2	5.5±0.5

When T4 plants germinated on MS medium containing sucrose were later transfered to soil some of them exhibited a dwarfed phenotype (figure 18). Line 55 plants showing the least reduction in cell wall derived rhamnose did not show any visible phenotype .

Figure 18 Six week old rRHM and wild-type plants

T4 Plants were germinated on MS plates supplemented with 1% sucrose from individual seeds, grown for two weeks then transferred to soil and grown for another four weeks in the greenhouse.

In contrast, T4 plants germinated and grown only on soil did show a drastic reduction in viability and growth (figure 19). Moreover, a strong production of anthocyan was observed as indicated by the reddish color of the leaves .

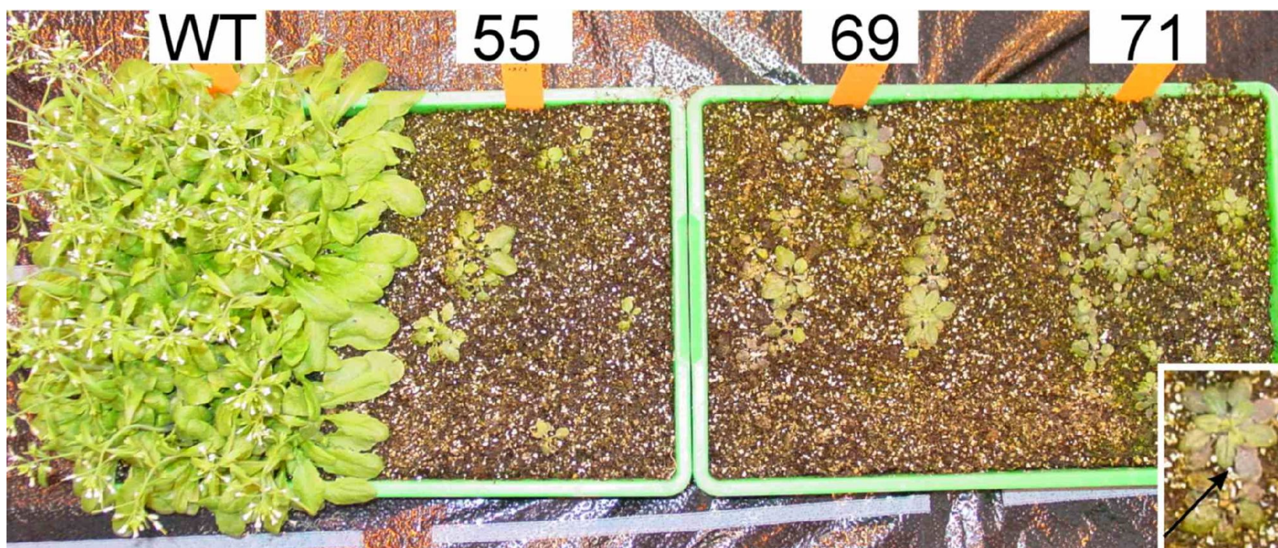


Figure 19 rRHM and wild-type plants germinated and grown only on soil.

T4 Plants were germinated on soil from individual seeds and then transferred to the greenhouse. WT denotes wild-type plants and 55, 69 and 71denotes three independent rRHM lines.

Inset: A blow-up of plants from line 69. The arrow indicates leaves appearing in violet-red, possibly due to anthocyan accumulation.

The effect of the transgene on the transcription level of the *RHM* gene family members had to be elucidated. For this purpose specific primers for each *RHM* transcript were designed. Furthermore *UER1* primers were also designed to check expression level of this protein closely related to the RHMs, which should not be targeted by the RNAi construct. The expression-level of the genes was monitored with real-time RT-PCR. A reduction of transcript levels for the whole family could be monitored in nearly all plants analyzed. Interestingly real-time RT-PCR indicated a small reduction of the *UER1* gene (table 8).

Table 8 Expression level of the different *RHM* and *UER1* in hypocotyls grown on MS medium with 1% sucrose.

Expression level of the different *RHMs* and *UER1* was quantified by real time RT-PCR and levels are indicated as relative to levels in WT plants.

	rRHM55	*rRHM69*	*rRHM71*
RHM1	0.83	0.28	0.25
RHM2	0.44	0.31	0.15
RHM3	1.1	0.19	0.28
UER1	1.3	0.55	0.57

A clear correlation was observed between the rhamnose content of hypocotyls derived cell wall material and the sum of the expression of all *RHM* transcripts without *UER1* (R^2=0.87) in T4 plants from the three independent lines.

The structure of RGI was further explored by extracting the polysaccharide from wild-type and rRHM plants (line #77) through enzymatic degradation. The solubilized products were then separated according to size by gel permeation chromatography. A similar elution profile of the pectic polysaccharides was obtained from rRHM leaf tissue and WT tissue in terms of molecular weight distribution and relative quantity of the fractions. Also, the monosaccharide composition was determined from individual fractions. The rhamnose content fractions was reduced (table 9) in numerous fractions representing RGI..

Table 9 Neutral monosaccharide composition of solubilized fractions

All values are denoted as weight percent ± standard deviations of two independent experiments. * denotes significance at the 5% level and ** denotes significance at the 1% level. The neutral sugars fucose, mannose were measured but they were not detectable.

	#10	*#11*	*#12*	*#16*	*#20*
Galactose (WT)	31.8±0.2	32.9±1.6	34.5±2.0	31.5±2.4	29.4±4.3
(line 77)	35.1±1.7	37.0±1.7	38.2±1.5	38.0±3.1	32.1±0.4
Xylose (WT)	7.1±0.1	5.8±0.5	4.9±0.3	19.4±0.4	41.9±11.0
(line 77)	7.8±0.6	6.9±0.8	6.1±1.0	20.5±0.4	38.0±13.0
Arabinose (WT)	43.4±1.6	45.9±0.4	45.0±2.0	27.1±1.7	28.7±6.6
(line 77)	43.8±1.1	46.2±1.1	46.0±0.8	24.2±1.0	29.3±13.3
Rhamnose (WT)	17.7±1.8	15.5±0.6	14.4±0.3	22.0±0.1	0±0
(line 77)	13.3±0.1	9.9±0.1*	9.6±0.4**	17.3±1.1	0±0

3.2.5 RHM proteins are common and highly conserved in plants

Not only *Arabidopsis thaliana* contains rhamnose in its cell wall, but also other plant species. Therefore the presence of RHM paralogs present in other plant species was investigated. For this purpose the expressed sequence tag clustering databases SPUTNIK and the TIGR plant gene index (Rudd *et al*. 2003, Quackenbush *et al*. 2001) were queried with the RHM2 sequence. The protein sequences encoded for by best scoring EST clusters were aligned with either the N-terminal dehydratase domain or the C-terminal epimerase/reductase domain of RHM2. For both domains very similar sequences were identified in legumes, solanaceous plants, and members of the graminae (table 10). For the crop plants soybean, potato, wheat and barely, high scoring EST clusters encoding for proteins spanning both domains of RHM2 could be identified, indicative of a conservation of the two domain protein architecture in plants in contrast to bacteria.

Table 10 RHM proteins in various plant species

The EST Clustering databases SPUTNIK and TIGR Plant Gene Index were queried with the dehydratase (N-terminal) and epimerase (C-terminal) protein domain sequence of RHM2. The resulting EST cluster hits for various plant species are shown with their corresponding accession numbers. Only hits with an e-value better than $10^{-100}$are reported.

Plant Species	*N-terminal*	*C-terminal*
Prunus persicus	S_BU043981	C_BU043194
Glycine max	C_BI787599	C_BM085701
Zea mays	C_BM660359	C_BE329779
Solanum tuberosum	C_BG888611	C_AW018105
Triticum aestivum	C_BJ268533	C_BF053046
Pinus taeda		C_BG275831
Hordeum vulgare	S_BI779431	C_AV916302
Beta vulgaris	C_BQ585441	

A high sequence similarity of putative full length RHM proteins was observed in a multiple sequence alignment with ClustalW (Thompson *et al*. 1994)(see figure 20). In the alignment the two domain structure of RHM became visible again, since the N-and C-terminal domains of the alignment are generally highly conserved whereas the middle region representing a possible linker is less conserved. In the alignment the very high conservation of the aminoacid sequence of the RHM proteins is striking. Therefore no family tree of the RHM proteins was built as a family tree

could be guided by sequencing errors in the underlying EST projects rather than on real divergence of the family.

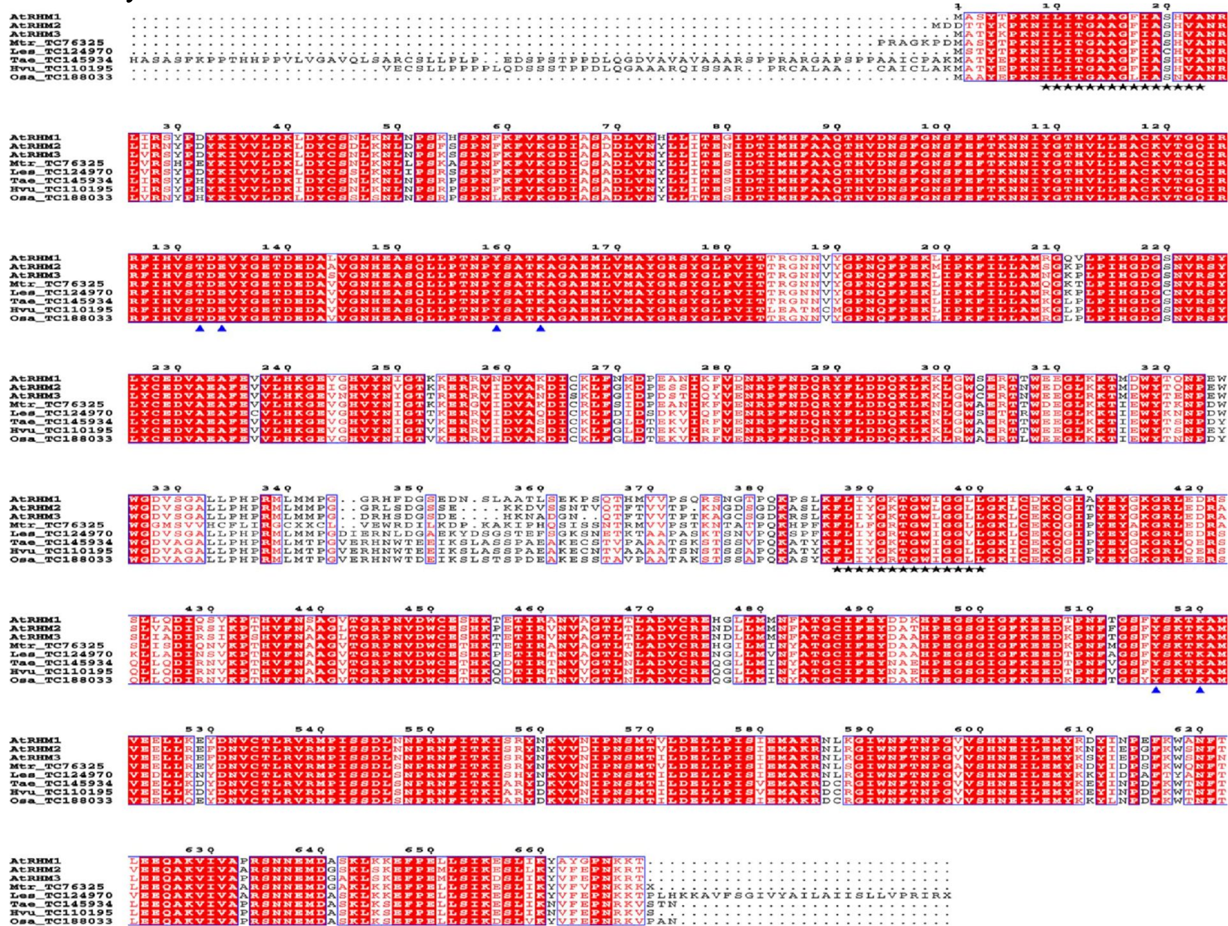


Figure 20 Alignment of the amino acid sequence of the *Arabidopsis* RHM proteins with putative paralogs from other plants species.

The putative Wierenga motives are marked by stars and the putative conserved catalytic signatures are marked by blue triangles. Totally conserved residues are given with a re background and highly conserved residues are marked in red. The alignment was produced by ClustalW (Thompson *et al.*1994) and rendered by ESPript (Gouet *et al.* 1999).

3.2.5.1 RHM proteins in *Lycopersicon esculentum*

Nowadays tomato (*Lycopersicon esculentum*) is gaining more and more attention from cell wall researchers, partly because of ripening issues of this crop plant and particularly fruit texture, which is largely determined by pectins and hence their direct commercial applications. Tomato fruit is also a good model system for pectin structure and synthesis, since it contains up to 30 % pectins. One way to alter fruit wall rhamnose content might thus be by downregulating the transcript of putative *RHM* genes.

The comprehensive analysis of the clustered EST data from SPUTNIK (Rudd *et al*. 2003), the TIGR Plant gene index and the Solanaceae Genomics network database (http://www.sgn.cornell.edu/index.html) retrieved in chapter 3.2.5 pointed out that there is at least one full length *RHM* gene in *Lycopersicon esculentum*. This unigene (SGN-U145639, TC124970) encodes a protein similar in length to the *Arabidopsis* RHM protein and was therefore termed *Le*RHM1. Striking similarity of this protein to the Arabidopsis RHM protein was observed (table 11, figure 20). Moreover, two additional unigenes (SGN-U154673, SGN-U145506) were found, which encode for proteins similar to the C-terminal domain of the RHM family. Another unigene (SGN-U171062) was found covering the N-terminal domain of the RHM proteins only. Based on the data available it remains unclear if SGN-U171062 is part of a larger gene or represents a separate protein.

Table 11 Protein sequence similarity between *At*RHM1-*At*RHM3 and *Le*RHM1.

The protein sequences were aligned pair wise applying the Smith and Waterman algorithm (1981). Similarity was calculated using the BLOSSUM51 matrix.

	***Le*RHM1**	***At*RHM1**	***At*RHM2**	***At*RHM3**
***Le*RHM1**	x	89.8	88.1	88.8
***At*RHM1**		x	91.5	92.5
***At*RHM2**			x	95.1

In silico expression evaluation points to a preferential expression of *Le*RHM1 in the fruit

The expression of the identified unigenes was assessed by counting normalized EST abundance in different tissues. *Le*RHM1 is preferentially expressed in the reproductive organs and the developing tomato fruit, whereas SGN-U145506 seemed to be more confined to the vegetative tissues of the plant (table 12). For both SGN-U154673 and SGN-U171062 only very few ESTs (<4) were available and a meaningful EST based expression analysis could hence not be performed. However, the presence of relatively few ESTs compared to the other unigenes indicates lower expression of these two unigenes.

Table 12 In silico expression analysis of LeRHM1 and SGN-U-145506

Expression of LeRHM1 and SGN-U-145506 was assessed by counting normalized EST transcript abundance in various tissues. - denotes that no EST was found, if ESTs were found they were grouped into three classes of abundance from + low abundance to +++ high relative abundance.

	callus	***fruit***	***flower***	***shoot***	***root***	***seedling***
LeRHM1	+	+++	+++	-	-	-
SGN-U-145506	+	+	+++	+	++	++

When the tomato expression database (http://ted.bti.cornell.edu) was queried with the identified SGN-unigenes for expression during fruit development, *LeRHM1* was stable in expression during the whole process of fruit development. Conversely, both SGN-U154673 and SGN-U145506 showed a drop in expression during fruit development at day 47 and 17 after pollination, respectively.

3.3 Functional characterization of GAE a UDP-D-glucuronic acid epimerase family

3.3.1 Bioinformatic characterization of the GAE family

The GAE gene family consists of six members (figure 21). A first characterization of the GAE protein family was performed using a bioinformatics approach. None of the six *GAE* genes contain any intron in their coding region. A search in the Pfam database (Bateman *et al*. 2002) showed that all six GAE protein family members exhibit highest similarity with the Pfam 1370 domain in their C-terminal part (figure 21). However, their N-terminal part did not show any significant significant similarity to any Pfam domain.

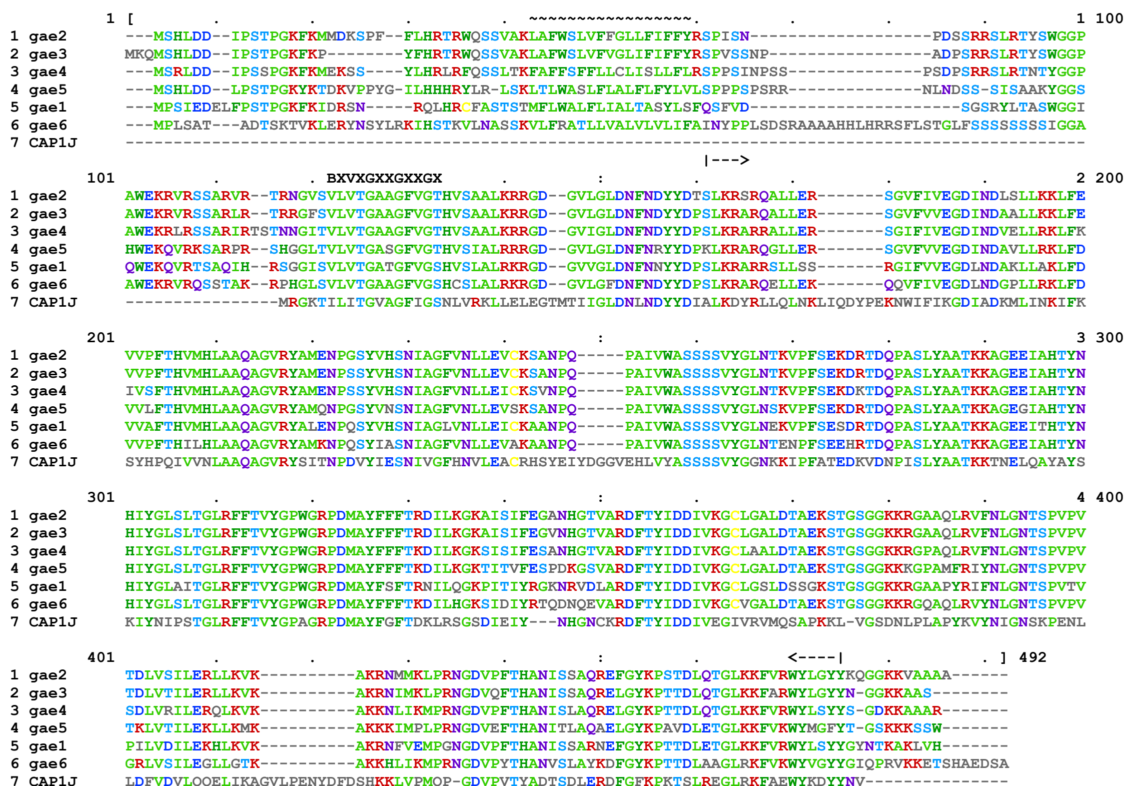


Figure 21 Amino acid sequence alignment between the six GAE proteins and CAP1J.

Amino acids are colored according to their physiochemical group using MVIEW as detailed in Brown *et al.* (1998) if the similarity between the sequences is above 70%. The putative transmembrane region is indicated by a tilde and the start and the end of the PFAM domain PF01370 is indicated by|---> and <---| A putative Wierenga sequence is indicated above the sequence in black.

The occurrence of possible transmembrane segments was investigated by TMHMM (Krogh *et al.* 2001). At least one transmembrane segment was predicted in every family member in the N-

terminal part (figure 21, tilde) whereas the C-terminal part of the GAE proteins was always predicted to be topologically non-cytoplasmatic.

SignalP analysis predicted a signal anchor for every family member. Due to the presence of a signal anchor and a transmembrane domain a localization of all GAE proteins to the endomembrane system such as the golgi-apparatus seems likely. To investigate the relationship of the GAE proteins to each other a global alignment (Needleman and Wunsch 1970) of the protein sequences was performed. It demonstrated that GAE2 and GAE3 were the most similar proteins (93.4% similarity) whereas GAE1 and GAE6 where the most divergent (72.3% similarity) (table 13).

Table 13 Protein sequence similarity in the GAE family.

Pairwise sequence similarity was determined by a global Needleman and Wunsch alignment. Similar residues are given in %.

	GAE1	GAE2	GAE3	GAE4	GAE5	GAE6
GAE1	x	80.4	78.5	79.2	77.4	72.3
GAE2		x	93.4	89.2	83.3	76.6
GAE3			x	89.3	82.5	75
GAE4				x	82.2	75.4
GAE5					x	74.9

3.3.2 Heterologous expression of GAE6 in Spodoptera frugiperda cells

The GAE gene family is a novel gene family showing significant similarity to known bacterial UDP-D-glucuronic acid epimerases such as Cap1J (Muños *et al*. 1999) (figure 22). Therefore, the enzymatic activity of GAE6 as a highly expressed representative of the GAE family was assessed by heterologous expression.

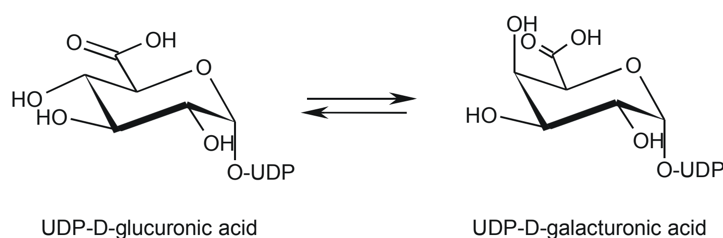


Figure 22 UDP-D-galacturonic acid synthesis

Since heterologous expression of eukaryotic proteins in bacterial systems has often been reported to fail, especially when putative transmembrane segments are present, an eukaryotic expression system was chosen for use.

The entire coding region of *GAE6* from *Arabidopsis* was subcloned into pFastbacHTa thus generating the vector pICGAE6. Subsequently facilitating the Bac-To-Bac system this vector was used to infect Sf21 insect cells to generate recombinant GAE6. Crude lysate of insect cells was then incubated with UDP-D-glucuronic acid and the products of the reaction were analyzed by HPAEC with PAD detection. A conversion of UDP-D-glucuronic acid to a product, which has the same retention time as authentic UDP-D-galacturonic acid was observed (figure 23). Assuming a similar response factor for both UDP-D-glucuronic acid and UDP-D-galacturonic acid a conversion of approximately 10 % was observed after an overnight incubation.

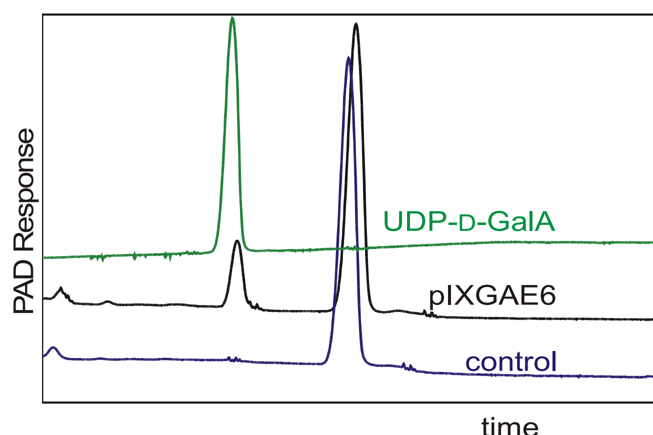


Figure 23 Conversion of UDP-D-glucuronic acid to UDP-D-galacturonic acid

Nucleotide sugars and nucleotide sugar conversion reactions were analyzed by HPAEC with PAD detection. Green trace: authentic UDP-D-galacturonic acid; Black trace: insect cell lysate expressing GAE6 incubated with UDP-D-glucuronic acid; blue trace: vector control insect cell lysate incubated with UDP-D-glucuronic acid.

3.3.3 Heterologous expression of GAE6 in Pichia pastoris

Another system that has been used quite successfully to express plant nucleotide sugar converting enzymes is the *Pichia pastoris* yeast expression system (Burget *et al*. 2003). Due to the rather slow conversion of UDP-D-glucuronic acid to UDP-D-galacturonic acid by the recombinantly expressed GAE6 in insect cells its catalytic properties were established using GAE6 expressed in *Pichia pastoris*.

Crude lysate was prepared from *Pichia pastoris* clones expressing GAE6 (kind gift of Michael Mølhøj). After incubation of crude lysate with UDP-D-glucuronic acid, the reaction products were

separated by HPAEC and monitored by UV detection. As PAD detection was disadvantageous due to interfering substances in *Pichia* extracts. Two peaks with a similar retention time as UDP-D-glucuronic acid and UDP-D-galacturonic acid were observed (figure 24). Identity of their content was further confirmed by mass spectrometric analysis of collected fractions. For both compounds the obtained masses ($(M+Na)^-$ 579.4 m/z) were in good agreement with a UDP-hexuronic acid indicating that the product peak indeed contains UDP- D-galacturonic acid.

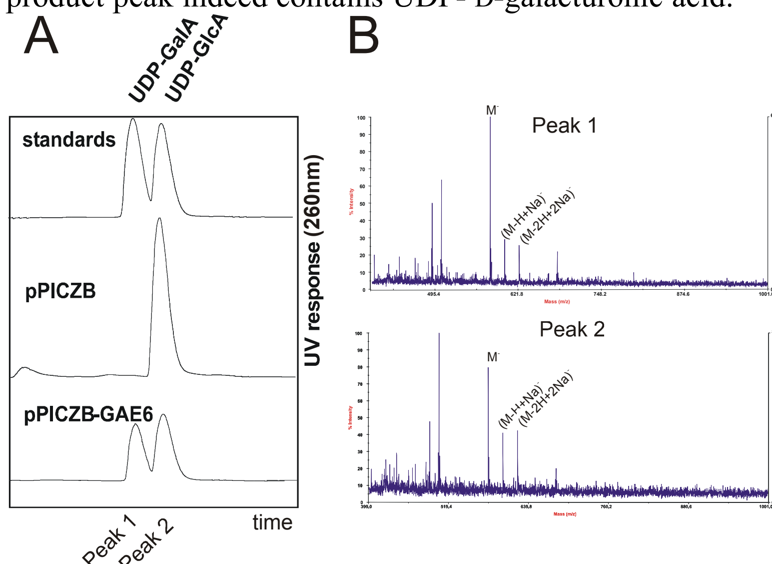


Figure 24 Conversion of UDP-glucuronic acid to UDP-galacturonic acid

Nucleotide sugars and nucleotide sugar conversion reactions were analyzed by HPAEC with UV detection. A) Green trace authentic UDP-D-galacturonic and UDP-D-glucuronic acid. Black trace lysate of *Pichia* expressing GAE6 incubated with UDP-D-glucuronic acid and blue trace control *Pichia* lysate incubated with UDP-D-glucuronic acid. B) The peaks one and two of the pPICZB-GAE6 reaction were collected and analyzed by MALDI-TOF. The resulting spectrum is shown. The labels represent the expected masses of a singly charged UDP-hexuronic acid (M+) and the corresponding sodium adducts.

In this assays a conversion of approximately 50 % of UDP-D-glucuronic acid to UDP-D-galacturonic acid was observed after overnight incubation, assuming similar extinction coefficients for both substances. An incubation for 72 h did not yield significantly more product (data not shown). The reaction equilibrium seems to be a 1:1 ratio.

3.3.4 Transcript levels of the GAE gene family in Arabidopsis plants

The expression of the six *GAE* family members was investigated by quantitative real-time RT-PCR (see 2.3.13) and by querying the MPSS database (http://mpss.udel.edu). A comparison of transcript

levels between the family members by RT-PCR indicated that both *GAE1* and *GAE6* are expressed to the highest level in *Arabidopsis thaliana* (table 14). This is in accordance to earlier observations made by Reiter and Vanzin when assessing expression by EST counting (2001). This is in good agreement with data obtained from the MPSS database, where the sum of all high quality tags in the classical dataset, indicated an order of expression of *GAE1~GAE6>>GAE2>GAE3>GAE5* (for *GAE4* no high-quality tag was obtained). Querying AtGenexpress also showed highest expression of both *GAE1* and *GAE6* (see Appendix).

However, on the individual organ level no more than a five fold difference between two organs could be found (table 14) for any *GAE* family member with RT-PCR. These results suggest a rather ubiquitous expression of each *GAE* under normal conditions or missing spatial resolution of real time RT-PCR.

Table 14 Expression level of the GAEs in different *Arabidopsis* organs as determined by RT-PCR

Upper part: C_t values for different constitutively expressed transcripts and the *GAE* transcripts. Lower part normalized ratios expressed relative to *GAE5* transcript in flowers facilitating the $\Delta\Delta C_t$ method.

Transcript	*stem*	*root*	*flower*	*silique*	*leaf*	*cauline leaf*
actin2	20.69±0.23	17.04±0.03	17.78±0.01	18.65±0.04	19.14±0.09	17.62±0.21
Ubiquitin10	20.40±0.6	16.82±0.14	17.00±0.09	17.37±0.06	19.08±0.05	16.24±0.06
Aprt	23.60±0.56	20.34±0.18	20.82±0.09	19.20±0.16	20.36±0.01	22.30±0.35
β-*6-Tubulin*	23.79±0.47	21.52±0.44	22.91±0.42	20.37±0.14	20.54±0.06	25.59±0.08
GAE1	22.47±0.53	19.42±0.14	20.76±0.69	19.36±0.41	21.25±0.32	20.14±1.17
GAE2	24.06±0.37	21.95±0.19	20.41±0.38	21.81±0.24	23.37±0.01	21.96±0.21
GAE3	26.16±0.042	23.44±0.03	22.53±0.11	23.10±0.22	24.87±0.44	23.39±0.12
GAE4	26.27±0.23	23.40±0.20	22.85±0.01	24.28±0.30	25.34±0.60	24.51±0.31
GAE5	27.89±1.00	25.63±0.20	25.68±0.40	25.63±0.30	27.38±0.73	26.49±0.37
GAE6	22.29±0.22	20.90±0.14	19.50±0.10	18.94±0.10	21.97±0.37	19.87±0.23
GAE1 ratio	106.9	97.5	30.2	125	165	81.9
GAE2 ratio	35.5	16.9	38.7	22.9	38.1	23.2
GAE3 ratio	8.3	6	8.9	9.3	13.5	8.6
GAE4 ratio	7.7	6.2	7.2	4.1	9.7	3.9
GAE5 ratio	2.5	1.3	1.0	1.6	2.4	1
GAE6 ratio	121.5	34.8	72.7	166.7	100.5	98.7

Interestingly, AtGenexpress data pointed out some regulation of *GAE1* and *GAE6* expression in the leaves. Here both *GAE1* and *GAE6* expression peak in leaf number four and then decline and *GAE6* raw expression values remain lower than *GAE1*. However, the situation is reversed in 1 cm long leaves 4 where *GAE6* raw expression is higher (figure 25).

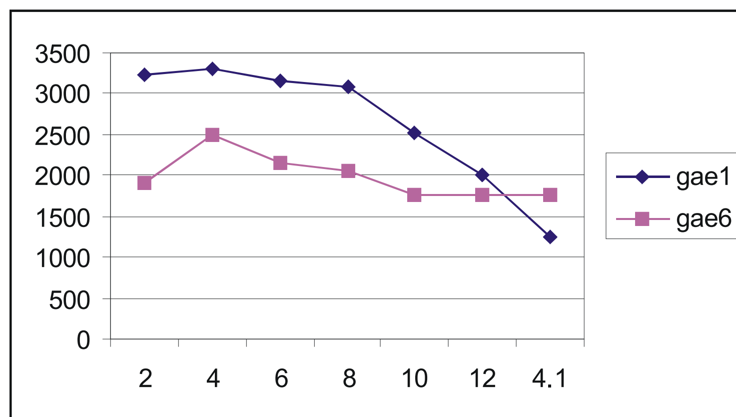


Figure 25 Expression of *GAE1* and *GAE6* through leaf development

Shown are the raw expression values for *GAE1* and *GAE6* from ATGENEXPRESS. The abscissa indicates the number of the leaf where 4.1 indicates leaf number 4 one centimeter long only. The ordinate reflects the raw expression values.

Principal Component Analysis indicates co-expression of *GAE2*, *GAE3*, *GAE4* and *GAE5*

For better comparison of the *GAE* expression, expression of all GAE transcripts was normalized using the ΔΔG method and *GAE5* expression in the flower was set to 1.0 as described by Wandrey and colleagues (2004). The resulting dataset was then used for a principal component analysis (PCA) (figure 26). Principal component analysis is a dimension reduction technique for complex data and thus allows the display of multi-dimensional data in few dimensions. Here PCA results (figure 26) indicated that *GAE3*, *GAE4*, *GAE5* and to a smaller degree *GAE2* had a very similar expression pattern, whereas *GAE1* and *GAE6* had a very dissimilar expression pattern.

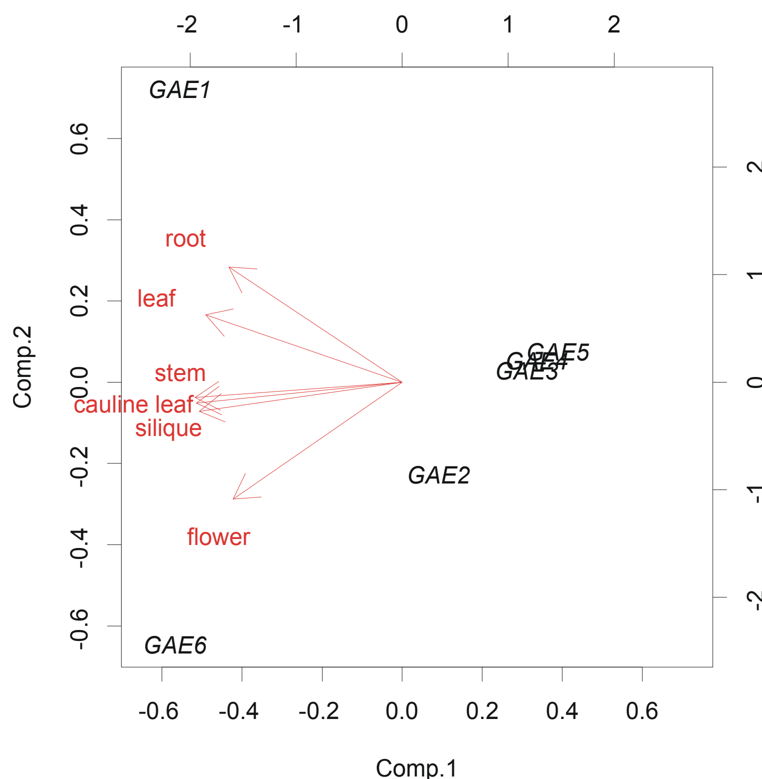


Figure 26 Expression Analysis of *GAE1* through *GAE6*.

Normalized expression values from table 14 were subjected to PCA analysis using the R statistical programming language. The projection of the data points in the first two principal components is shown.

3.3.5 Overexpression of GAE6 in Arabidopsis

Since UDP-D-glucuronic acid epimerase activity of GAE6 was demonstrated in two heterologous expression system, it was of interest, if an overexpression of the endogenous *GAE6* gene in *Arabidopsis thaliana* would lead to an alteration in wall polysaccharide structure, in particular a larger abundance of pectic polysaccharides. A change in cell wall composition could be expected, if UDP-D-galacturonic acid became limiting at some stage, or if this sugar precursor is a major metabolic control point. The coding region of *GAE6* was cloned into the binary vector pBinAR (Höfgen and Willmitzer 1990) behind the strong constitutive CAMV 35S promoter (Guilley *et al*. 1982, Zijlstra *et al*. 1992) thus obtaining the plasmid pAXGAE6. This construct was then used to transform plants by *Agrobacterium tumefaciens* facilitated gene transfer using the dipping method (Clough and Bent 1998). After transformation of *Arabidopsis,* seeds were plated on kanamycin containing MS agar plates. Fourteen kanamycin resistant transformants were screened by Northern-Blotting of leaf RNA for an increase in *GAE6* transcript level. Indeed three lines showed high

upregulation of the *GAE6* transcript (lines 3,4,13) and three lines showed medium upregulation (lines 1, 7, 9) of the transcript as shown by Northern Blot analysis (figure 27) .

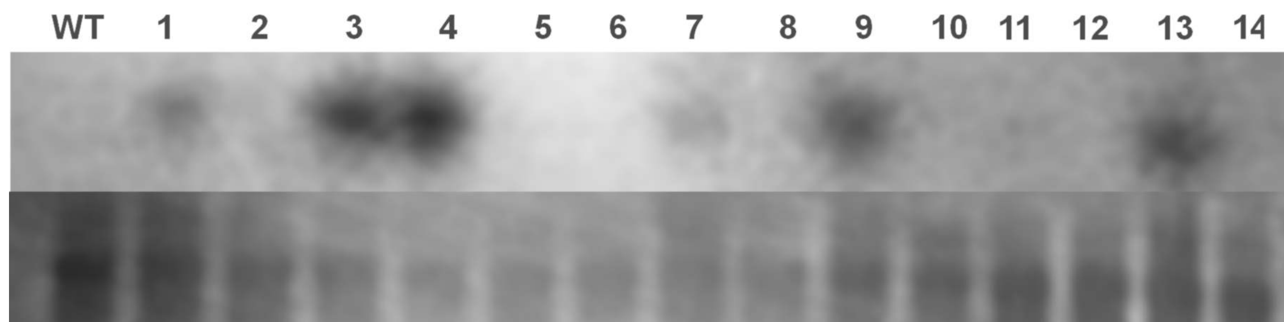


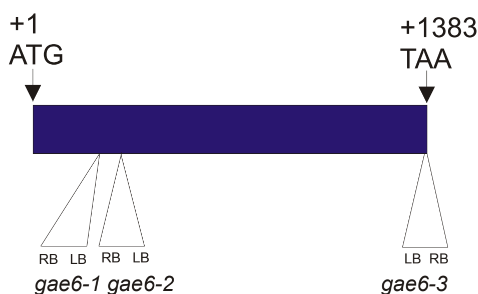
Figure 27 Expression level of *GAE6* transcript in *GAE6* overexpressing lines

RNA was extracted from leaves of mature plants and expression level of GAE6 was assessed by Northern Blotting. Upper panel autoradiography of the blot incbuated with a ^{32}P labeled *GAE6* probe. lower panel methylene blue stained blot. WT represents RNA isolated from wild-type plants and 1 to 14 represent T1 plants carrying the construct for overexpression of GAE6. The approximate size of the signals obtained in the blot was 1.5 kb.

All six overexpression lines were further propagated and progeny (T2) of these lines was reevaluated for overexpression of *GAE6*. In 15 T2 plants (2-3 per line), where an overexpression was still visible by Northern Blotting (data not shown), the cell wall composition of five leaves from five week old plants was determined and compared to WT plants. However, no significant changes in total sugar composition of the cell walls was detectable suggesting that *GAE* is not a major limiting factor or regulating point in pectin synthesis (data not shown).

3.3.6 Phenotypical analysis of gae6 plant lines

As previously described, *GAE1* and *GAE6* are highly expressed in *Arabidopsis*. Therefore a reduction of transcript level for one of these genes might affect cell-wall composition due to limitation of UDP-D-galacturonic acid. Since no insertion line for *GAE1* was available in the common tagged collections (Syngenta, GABI, Genoplante, Szeged, Salk, Stanford, Poetter) *GAE6* insertion lines were obtained from the GABI and Salk collections. In total three different lines were obtained and screened for homozygous insertions of the T-DNA using a PCR based approach. One insertion line was not further analyzed due to an insertion near the stop codon of *GAE6* (*gae6-3*), which is likely to still lead to a catalytically active protein.



TDNA insertions

Figure 28 Structure of gae6 T-DNA insertion lines.

The insertion of the T-DNAs into the *GAE6* gene is shown. The border sequences of the T-DNA are indicated by RB and LB. The single exon of *GAE6* is represented by the blue box.

Of the remaining two insertion lines (*gae6-1* and *gae6-2*) *gae6-1* showed a single insertion as judged by southern-blotting (appendix). The other line, *gae6-2,* carried at least three insertions as judged by southern blotting (figure 29) and therefore a southern blot was performed on all available sub-lines and a line showing only a single band corresponding to the right size (plant number 7) was chosen for further analysis.

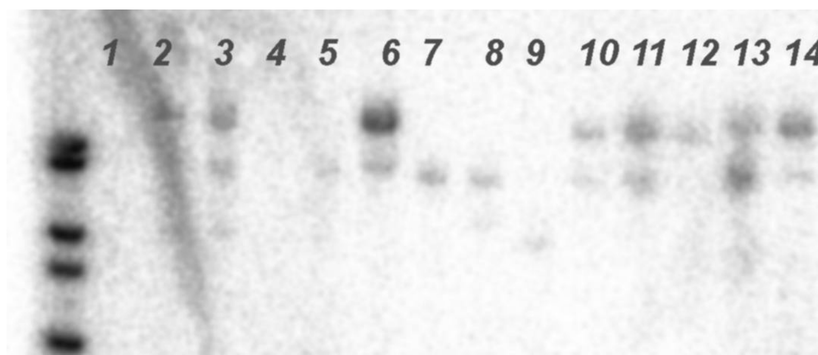


Figure 29 Southern Screen of *gae6-2*

Plants possibly carrying an insertion in GAE6 were screened by Southern blotting using a T-DNA encoded kanamycin as probe which should give rise to a single band only. Here an autoradiography of the blot is shown.

For both lines RT-PCR experiments showed that only an altered or no transcript was assembled *in planta* (figure 30).

Figure 30 RT-PCR analysis of *gae6-1* and *gae6-2*

A-D) -RT control reactions

E-H) RT reactions with primers spanning the putative T-DNA insertion site

A,B,E,F) RNA of wild type plants

C,G) *gae6-1* RNA and D,H) *gae6-2* RNA

Gae6-1 and *gae6-2* plants show no change in cell wall composition

A preliminary analysis of four week old green-house grown *gae6-1* plants showed a reduction of cell wall bound uronic acids of up to 40 % and an En-1 insertion in this gene has been reported to show a reduction of 10 % in leaf derived uronic acids (Gipmans 2001). However, the reduction could not be validated in later generations. In addition, no change in seed mucilage content was observed when imbibed seeds of these plants were stained with ruthenium red (data not shown).

Gae6 plants react like wild-type plants to external stresses

It is possible that structural alterations in the cell wall of *gae6* occurred but were not detected with the methods used here. Therefore, the plants were subjected to external stresses, which might hint subtle structural changes. Plant cell walls pose a primary barrier for pathogens and wall fragments in particular galacturonic acid rich pectic oligogalacturonides released during pathogen attack can act as elicitors and trigger plant defense responses. For example it has been reported that mutations in cell-wall synthetic genes can infer increased pathogen resistance to the plant (Nishimura *et al*. 2003). Therefore, *gae6-2* plants were challenged with pathogens, but no significant difference in fungal growth was observed compared to WT when the plant was challenged with a non-host (*Blumeria graminis f.sp. hordei*) or compatible pathogen (*Erysiphe cichoracearum*) (Dr. Volker Lipka, MPI of plant breeding research, Cologne, Germany).

Figure 31 *Gae6* and WT plants challenged with pathogens.

For the same reasons as above the porosity of the cell wall, which seems to be determined at least in part by the pectin fraction of the cell wall (Baron-Epel *et al*. 1988), was investigated. Ten day old *gae6-1* and *gae6-2* hypocotyls were vertically sliced and freeze-dried material was then subjected to porosity determination. However, no discernible difference was observed in porosity of *gae6* hypocotyls when compared to wild type hypocotyls.

It has been long discussed that changes in the abundance of cellulose can be counterbalanced by change in pectins and vice versa (Shedletzky *et al*. 1992, Wells *et al*. 1994). Therefore challenging plantlets with cellulose inhibitors might increase the abundance of pectic polysaccharides and thus increasing the demand for UDP-D-galacturonic acid. Therefore wild-type and *gae6* plants were grown on plates containing dichlorobenonitrile (DCB) a potent inhibitor of cellulose synthesis (Vaughn *et al*. 1996) (see appendix). No significant difference was observed in plant morphology, development leaf cell wall composition compared to WT plants grown under the influence of DCB (data not shown). In addition, plants were also challenged with different concentration of isoxaben, another inhibitor of cellulose synthesis (Heim *et al*. 1990), since the mechanism of action of DCB is unclear. Again, no difference between the mutant and the wild type was observed when hypocotyl growth was investigated (figure 32).

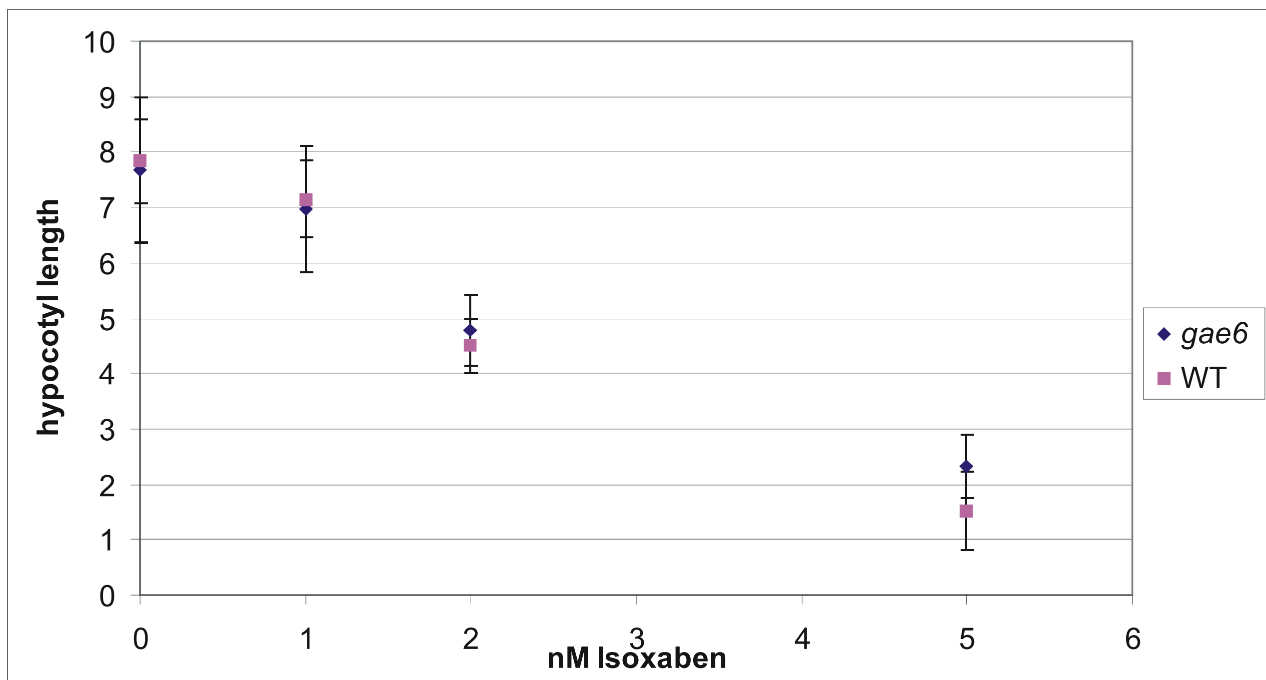


Figure 32 Hypocotyl growth on Isoxaben

WT and *gae6-2* seeds were germinated and grown for 4 days in darkness on MS medium with 1% sucrose containing various concentrations of the cellulose inhibitor isoxaben.

3.3.7 Downregulation of more than one member of the GAE family

A downregulation of expression of one member of the *GAE* gene family did not result in an effect on the cell wall composition. Therefore, a downregulation of the whole *GAE* family was attempted. A segment of the coding region of *GAE6* was chosen, which showed highest similarity to the other family members. This gene segment of approximately 700 bp (representing ca. 50% of the ORF) was cloned into the pHANNIBAL vector in a head to head fashion as in the case of the *RHM* genes (see 3.2.4). After mobilization into the plant competent pART27 vector thus obtaining pRGAE and plant transformation by the the dipping method (Clough and Bent 1998), transformed seeds were plated on kanamycin containing plates. After obtaining kanamycin resistant plants, these were assessed for a reduction in uronic acid content in total cell walls, as screening by Northern-Blotting was not feasible due to problems detecting the endogenous transcript already in WT plants (compare figure 27). No significant reduction of uronic acids in approximately 70 lines compared to the WT was observed, when leaf material of four week old plants was analyzed.

3.3.8 GAE proteins are common in plants

Searches for proteins similar to the *Arabidopsis* GAEs using a tBLASTn based approach in the TIGR plant gene index (TIGR, Rockville, MD) (Quackenbush *et al*. 2001) returned protein sequences that had similar length as the Arabidopsis GAEs. They were aligned with ClustalW (Thompson *et al*. 1994), and the resulting output was used to build a maximum likelihood family tree of the GAEs using the PHYLIP software package (Felsenstein 2004). Interestingly, the family tree indicates three major subgroups. Two branches of the tree are reliably occupied by GAE1 and GAE6, respectively, whereas GAE2 to GAE4 and possibly GAE5 are localized in another branch of the tree. This suggests an ancient role of GAE1 and GAE6 and rather young evolution such as endoduplication events leading to the other GAEs. However, an analysis of segmental duplications in *Arabidopsis* (http://www.tigr.org/tdb/e2k1/ath1/Arabidopsis_genome_duplication.shtml) demonstrated that none of the GAEs fell into the major segmental duplication regions of the *Arabidopsis* genome.

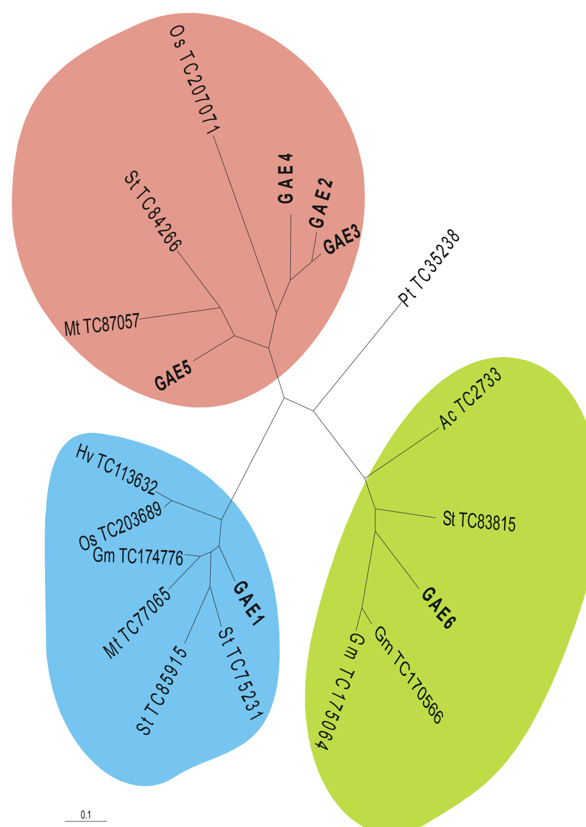


Figure 33 Phylogenetic tree of GAE proteins. Possible groups of GAE proteins are indicated by colored shading.

3.4 MIOX a myo-inositol oxygenase family

3.4.1 Bioinformatic characterization of the MIOX family

A first characterization of the Arabidopsis MIOX proteins was performed using bioinformatics tools. The four predicted MIOX proteins were not predicted to have any transmembrane domains by TMHMM (Krogh *et al*. 2001). They were all predicted to be targeted either to the cytoplasm or the peroxisomes as determined with a PsortI analysis (Nakai and Kanehisa 1992). In accordance, a TargetP (Emanuelsson 2002) analysis did not find any mitochondrial or chloroplastic targeting signals.

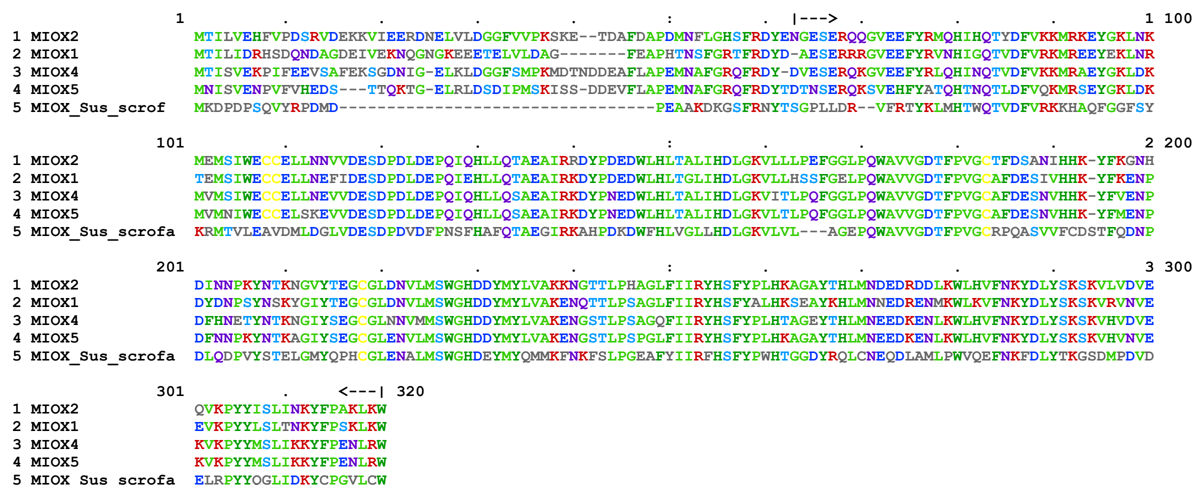


Figure 34 Amino acid sequence alignment between the four Arabidopsis MIOX and the MIOX protein from hog.

Amino acids are colored according to their physiochemical group using MVIEW as detailed in (Brown *et al.* 1998) if the similarity between the sequences is above 70%. The similarity of PFAM domain DUF706 is indicated by |---> and <---| .

An RPS-BLAST (Marchler-Bauer *et al*. 2003) search for conserved domains only returned the domains DUF706 and KOG157, which were both not yet comprehensively annotated. Both domains of both *Arabidopsis* MIOX proteins and proteins encoding for *myo*-inositol oxygenase as shown by biochemical evidence were present. For DUF706 the founding members were annotated as inositol oxygenase, aldehyde reductase-like, renal-specific oxidoreductase and as kidney-specific proteins.

For the MIOX proteins the similarity was between 75.7 % (MIOX1, MIOX5) and 88.8 % (MIOX4, MIOX5) in a global alignment facilitating the Needleman Wunsch algorithm (1970). Moreover in contrast to the *RHM* and *GAE* genes for the *MIOX* genes some different intron-exon structures within the family members was observed.

MIOX proteins are widespread in the plant kingdom

Searches for proteins similar to the MIOXes from Arabidopsis using a tBLASTn based approach in the TIGR plant gene index (TIGR, Rockville, MD) (Quackenbush *et al*. 2001) returned open reading frames that had similar length to the *Arabidopsis* MIOXes. Among them were open reading frames from the crop plants barley, tomato, wheat, soybean and vine indicating that MIOX proteins might play a role in crop plants as well.

The best scoring putative full length open reading frames were aligned with ClustalW (Thompson *et al*. 1994) and the resulting output was used to build a maximum likelihood family tree of MIOX using the PHYLIP software package (Felsenstein 2004) (figure 35).

An analysis of the resulting tree indicates that MIOX1 seems to be more similar to the MIOXes present in monocotyledons whereas MIOX2, 4 and 5 seem to be closely related only to putative proteins from *Lycopersicon esculentum*. *S*ince for the majority of the considered monocots comprehensive genome information is missing the data might be biased.

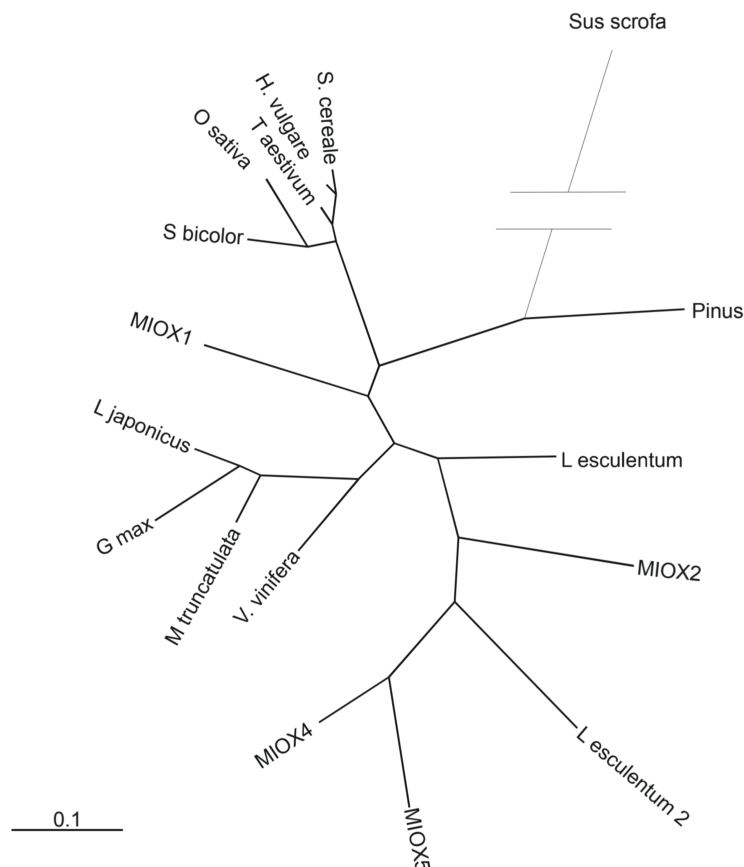


Figure 35 MIOX protein family tree

Putative MIOX proteins were identified in the TIGR Plant gene register and aligned with the *Arabidopsis* MIOX proteins and the MIOX protein from hogs (S. *scrofa*) facilitating ClustalW. The resulting alignment was used to build a family tree with the PHYLIP software package.

The relationship of the *Arabidopsis MIOX* genes was further analyzed by comparing the gene loci with the segmental duplication events. The segmental duplication database (http://www.tigr.org/tdb/e2k1/ath1/Arabidopsis_genome_duplication.shtml) showed that *MIOX4* and *MIOX5* lie in a region of a segmental duplication between chromosomes IV and V. For *MIOX1* and *MIOX2* both genes were in the immediate neighborhood of a segmental duplication between the chromosomes I and II, however, they were not annotated as members of this duplication.

3.4.2 Expression of the MIOX family

The expression of MIOX proteins family was further analyzed by querying the MPSS database (http://mpss.udel.edu) (table 15).

Table 15 Expression of *Arabidopsis MIOX*es

	root	*leaves*	*flower*	*silique*	*callus*
MIOX1	0	2	0	233	1
MIOX2	60	96	90	23	80
MIOX5	8	0	0	0	2

The expression of the *Arabidopsis MIOX* transcripts was investigated by establishing tag abundance of each member in the MPSS database. The normalized occurrence for each tag is shown. For *MIOX4* no high quality tags were obtained.

The results indicate that *MIOX2* seems to be expressed ubiquitously, whereas *MIOX5* does not seem to play a major role. Conversely, *MIOX1* seems to be mainly expressed in the siliques. When the AtGENEXPRESS data (ftp.arabidopsis.org) was queried for expression of the *MIOX* transcripts the data indicated that *MIOX1* was barely expressed at all. In contrast, *MIOX2*, *MIOX4* and *MIOX5* were highly expressed in the pollen. Moreover intermediate expression of *MIOX2* was found in the seedling (see Appendix).

3.4.3 Arabidopsis thaliana plants carrying T-DNA insertions in MIOX

The function of the MIOX proteins was explored by seeking T-DNA insertion lines in the loci. For both *MIOX1* and *MIOX4* insertion lines were identified in the GABI-KAT collection. For *MIOX2*, two insertion lines could be identified in the SALK collection. Two homozygous insertion lines for *miox1* and *miox2* were isolated (see chapter 2.3.14). However, in four to six weeks old plants grown on soil no difference in the cell wall composition was observed in leaves. Additional homozygous insertion lines were obtained for *MIOX4* and *MIOX5* from U. Kanter (University of Frankfurt, Germany) but these did not show a difference in cell wall composition either (U.Kanter, pers. communication).

Due to the prevalent expression of *MIOX1* in the silique, the role for the MIOXes for mucilage development was studied. Seeds from T-DNA insertion lines for each MIOX member were extracted with ammonium oxalate (Goto 1985) to investigate the mucilage composition of these seeds. But no significant difference in uronic acid or total sugar content of extracted seeds was observed.

3.5 Development of new bioinformatic tools

Cell wall research has made considerable progress over the past years in terms of methods (Choo *et al*. 2002, Goubet *et al*. 2003, Lu *et al*. 2003). Thus analyzing many samples and obtaining massive data sets became possible. However, evaluating these data sets can pose serious problems for the researcher. Even though tools for statistical analysis exist (R, S-plus etc.), these are either not user-friendly in terms of data formats accepted, cost extensive, or are not able to perform the desired tests.

To close this gap in available software in the framework of this project new data-analysis software was developed taking into account the specific needs of the user in terms of processable data format and applied analysis.

3.5.1 OLIMP

A software OLIMP (oligosaccharide mass profiler) was developed to work on MALDI TOF generated data, since a method developed in our lab (Choo *et al*. 2002) allowed the quick and easy evaluation of a multitude of samples. However, a quick method for statistical evaluation was lacking.

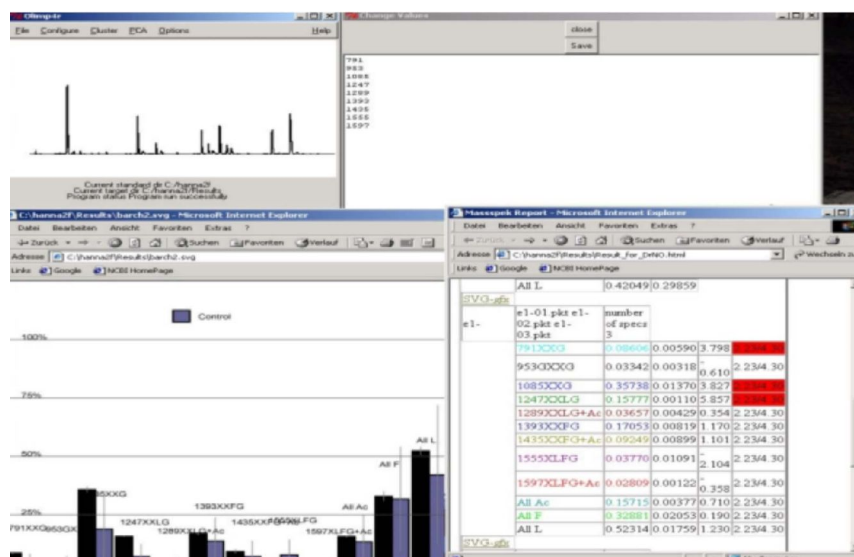


Figure 36 A screenshot of an OLIMP working session.

Upper left OLIMP menu Upper right: peak assignment window;

Lower panel: Results displayed as HTMLs in Internet explorer. Left: automatically produced bar chart and Right: tabulated display of the result set. Significant values are highlighted in red.

The data is evaluated by pooling similar files according to names and comparing these to one or two standard set(s) which can be set by the user during one session. For example MALDI data from xyloglucan derived oligosaccharides (Choo *et al.* 2002) can be directly imported into OLIMP. These are then compared to a WT sample facilitating student's t-test. If a significant difference is detected the user is flagged by highlighting the respective column in red (figure 36 lower right corner). In addition, each individual comparison can be displayed as a bar chart (figure 36 lower left corner). However, OLIMP allows for some flexibility since the user can individually assign, which peaks the software should evaluate.

3.5.2 CSBDB a systems biology database

As already visible from the current explosion in knowledge in cell wall biosynthesis (Seifert 2004, Scheible and Pauly 2004) it seems necessary to develop new easily accessible tools to investigate the pathway connection between novel genes possibly involved in cell wall synthesis. In addition, extending the gained knowledge about nucleotide-sugars networks should give new insights on the action of other cell wall genes. Since substrate-channeling and thus co-operation of some cell wall genes has been shown (Nakayama *et al*. 2003) or is discussed in the case of the UGE proteins (Seifert *et al*. 2002) it was clear that simple sequence similarity based tools would not suffice. Since it has been shown that genes partaking in a common pathway or sharing a function can be detected using clustering technologies based on transcript co-response analysis (Steinhauser *et al*. 2004) a database was developed in co-operation with Dirk Steinhauser (MPI of molecular plant physiology, Golm) which harbors co-response values of expression abundance of three different organisms amongst them *Arabidopsis thaliana* (http://csbdb.mpimp-golm.mpg.de). The infrastructure of the database allows the user to query for transcripts co-regulated with his candidate gene(s) over 51 different micro-array experiments, thus allowing the identification of novel genes potentially involved in similar pathways as the candidate genes.

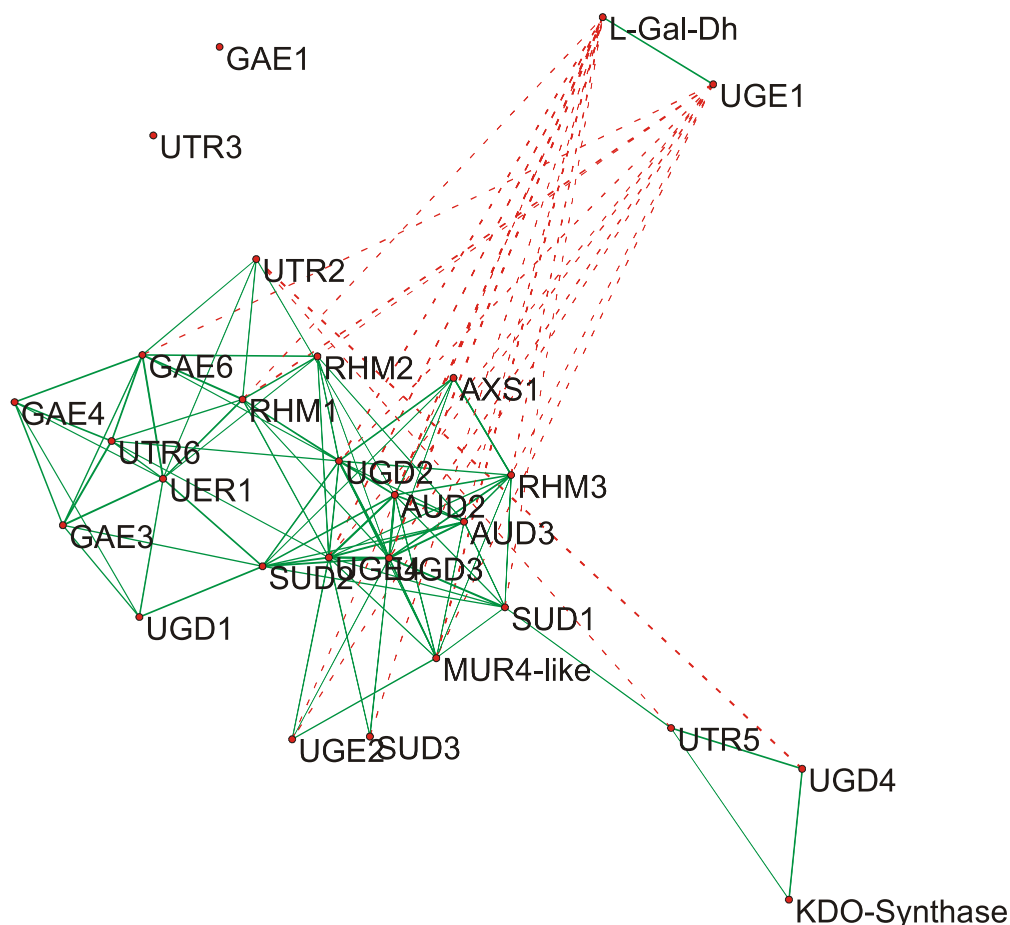


Figure 37 Co-response analysis of most identified nucleotide sugar genes

Nucleotide sugar converting enzymes identified in this thesis (table 1 and table 2) and some other proteins potentially involved in nucleotide sugar synthesis were subjected to a pairwise co-response analysis in the CSB database. The resulting set was then displayed using the pajek layout program. Green lines represent co-regulated genes. Red dotted lines represent negatively co-regulated genes.

As a first application the database was queried with all available predicted nucleotide sugar converting enzymes and related proteins (see table 1, page 39 and Seifert 2004) in a single query to build an interrelationship network. A network analysis could then identify major hubs which might play a central role in nucleotide sugar analysis. Moreover, a network might indicate cooperativity between transcripts being part of a single pathway. The resulting network was then displayed facilitating the pajek display program (figure 37).

Most nucleotide sugar converting enzymes found in the matrix where interconnected to each other. One exception was the separation of *UGE1* from the rest of the nucleotide sugar interconversion enzymes. Interestingly, it showed negatively correlated behavior to most of the other nucleotide sugar converting enzymes, whereas the other detected *UGEs* did display a major role based on their

connectivity. This might indicate that UGE1 has a different role than the other UGEs and might not be involved in nucleotide sugar interconversion at all.

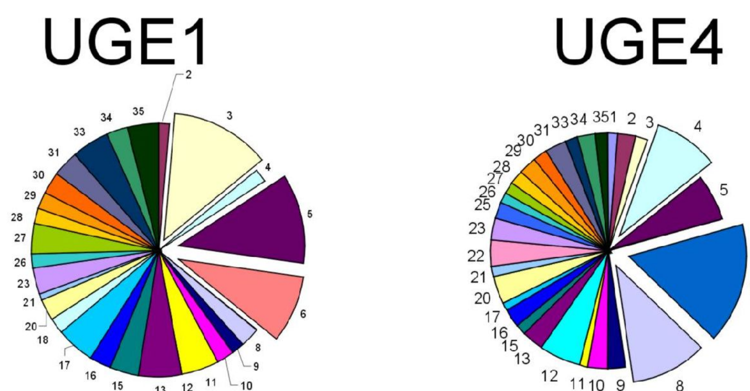


Figure 38 Categories

For both UGE1 and UGE4 a “single gene query” was conducted in the CSB database. Co-regulated genes were classed into bins which were derived from the MapMan program (Thimm *et al*. 2003). After normalization of these bins to their occurrence in the experiments the resulting sets were displayed as pie-charts. The represented bins are 1 photosystem, 2 major CHO metabolism, 3 minor CHO metabolism, 4 glycolysis, 5 fermentation, 6 gluconeogenese/ glyoxylate cycle, 7 oxydative pentose phosphate pathway, 8 tri carboxylic acid cycle, 10 cell wall, 11 lipid metabolism, 12 N-metabolism, 13 amino acid metabolism, 14 S-assimilation, 15 metal handling,16 secondary metabolism, 17 hormone metabolism, 20 stress, 21 redox, 22 polyamine metabolism, 23 nucleotide metabolism, 25 C1-metabolism, 26 misc, 27 RNA, 28 DNA, 29 protein, 30 signaling, 31 cell, 33 development, 34 transporter, 35 not assigned

To further explore the difference between *UGE1* and *UGE4* genes co-regulated with either of them were placed into 35 categories which have been previously defined (Thimm *et al*. 2004). These were divided by their level of occurrence in the matrix. For both genes three categories of over-represented classed could be identified. For *UGE1* these were minor CHO metabolism, fermentation and gluconeogeneses/glyoxylate cycle for *UGE4* the classes were glycolysis, fermentation and TCA cycle. This might give a further indication of the different roles of the two UGE isoforms in *Arabidopsis*.

To further explore the capabilities of the CSB database it was queried with *RHM2*, *GAE6* and *UGD2* which show high interconnection in figure 37 at the same time. Only genes being co-regulated to all three genes were considered (CSB.DB intersection gene query). The resulting dataset of co-regulated genes (following the expression of the above mentioned genes) contains only 26 genes (table 16), however, many of them are related to cell wall synthesis or modification.

Table 16 Genes identified by a CSBDB search

The AGI Codes At1g53500 (*RHM2*), At3g23820 (*GAE6*) and At5g39320 (*UGD2*) were used to query the CSB database with an intersection gene query. The resulting output is reproduced here. The genes are ranked according to their probability of the co-response (cumulative probability).

rank	ID	gene description	Cumultative probability
1	At5g03760	glycosyltransferase family 2; similar to beta-(1-3)-glucosyl transferase GB:AAC62210 GI:3687658 from (Bradyrhizobium japonicum), cellulose synthase from Agrobacterium tumeficiens (gi:710492) and Agrobacterium radiobacter (gi:710493); contains Pfam glycosyl transferase, group 2 family protein domain PF00535	1.0521e-13
2	At2g35860	expressed protein	7.5465e-13
3	At3g20570	plastocyanin-like domain containing protein	2.7372e-10
4	At3g03780	methionine synthase -related; similar to cobalamin-independent methionine synthase GB:AAC50037 (Arabidopsis thaliana)	5.2737e-10
5	At4g02500	transferase - related; low similarity to alpha-1,2-galactosyltransferase, Schizosaccharomyces pombe (SP|Q09174)	1.3376e-09
6	At1g16860	expressed protein; contains similarity to merozoite surface protein 2 (MSP-2) GI:1657451 from (Plasmodium falciparum)	1.3191e-08
7	At1g64390	glycosyl hydrolase family 9 (endo-1,4-beta-glucanase); similar to endo-beta-1,4-glucanase GI:4972236 from (Fragaria x ananassa) (Plant Mol. Biol. 40, 323-332 (1999))	1.2752e-07
8	At1g63000	expressed protein	1.0231e-06
9	At4g31590	glycosyltransferase family 2; similar to cellulose synthase from Agrobacterium tumeficiens (gi:710492) and Agrobacterium radiobacter (gi:710493); contains Pfam glycosyl transferase, group 2 family protein domain PF00535	1.4407e-06
10	At2g34560	katanin -related	1.7488e-06
11	At5g45280	pectinacetylesterase, putative; similar to pectinacetylesterase precursor GI:1431629 from (Vigna radiata)	3.7442e-06
12	At1g50010	tubulin alpha-2/alpha-4 chain (TUA2); identical to tubulin alpha-2/alpha-4 chain SP|P29510 GB:P29510 from (Arabidopsis thaliana)	4.6131e-06
13	At4g12880	plastocyanin-like domain containing protein	1.5184e-05
14	At4g13890		5.4338e-05
15	At5g22740	glycosyltransferase family 2; similar to beta-(1-3)-glucosyl transferase GB:AAC62210 GI:3687658 from (Bradyrhizobium japonicum), cellulose synthase from Agrobacterium tumeficiens (gi:710492) and Agrobacterium radiobacter (gi:710493); contains Pfam glycosyl transferase, group 2 family protein domain PF00535	6.5535e-05
16	At4g13210	polysaccharide lyase family 1 (pectate lyase); similar to pectate lyase GP:14531296 from (Fragaria x ananassa)	6.3146e-05
17	At3g09820	adenosine kinase 1 (ADK1)/adenosine 5'-phosphotransferase 1; identical to adenosine kinase 1 /adenosine 5'-phosphotransferase 1 SP:Q9SF85 from (Arabidopsis thaliana)	0.0002049
18	At1g04680	polysaccharide lyase family 1 (pectate lyase); similar to pectate lyase GP:14531296 from (Fragaria x ananassa)	0.0004851
19	At1g13280	allene oxide cyclase family; similar to ERD12 (GI:15320414), allene oxide cyclase GI:8977961 from (Lycopersicon esculentum)	0.00032547
20	At1g74690	expressed protein	0.0004021
21	At1g30690	expressed protein; Contains the PF|00650 CRAL/TRIO phosphatidyl-inositol-transfer protein domain. ESTs gb|T76582, gb|N06574 and gb|Z25700 come from this gene	0.00058425
22	At5g14790	expressed protein; predicted protein, Arabidopsis thaliana	0.0010045
23	At1g18840	expressed protein; similar to unknown protein GI:6539269 from (Arabidopsis thaliana)	0.0012426
24	At1g34020	transporter-related; low similarity to UDP-sugar transporter (Drosophila melanogaster) GI:14971008, UDP-glucuronic acid transporter (Homo sapiens) GI:11463949	0.0011204
25	At1g10200	transcription factor -related; similar to transcription factor SF3 (pir|IS37656); similar to ESTs gb|T42207, gb|N37716, and emb|Z17491	0.0009319
26	At2g25520	phosphate translocator-related; low similarity to SP|P52178 Triose phosphate/phosphate translocator, non-green plastid, chloroplast precursor (CTPT) {Brassica oleracea}, phosphoenolpyruvate/phosphate translocator precursor (Mesembryanthemum crystallinum) GI:9295275	0.0014853

Interestingly the resulting data set contains some sugar transporter related proteins. These might be good candidate genes for a further elucidation. Moreover the two non-annotated genes At2g35860 and At1g74690 had the highest match to the MIPS functional categories “metabolism of primary metabolic sugars derivatives” and “nucleotide binding”, respectively.

Moreover for the genes with the accession numbers At2g35860, At4g02500, At1g64390, At5g45280, At4g13890, At5g22740, At1g04680 a more than two fold up or downregulation was measured in a microarray experiment (B. Willats, www.arabidopsis.info) where cell cultures were treated with isoxaben a cellulose inhibitor which might also affect pectin synthesis. However, apparently no enrichment of up-or down regulated genes was achieved (approximately 1/3 of each set contains these genes). Nevertheless of the eight genes from the above list containing the keyword lyase or transferase five showed a more than two fold up-or downregulation under isoxaben treatment and one (At5g03760) was not measured.

4 Discussion

Although substantial knowledge about the structure of the plant cell wall has been gathered during the past decades (Cosgrove 1997) little is known about its synthesis and development. A major effort to understand cell wall synthesis and regulation has been a forward genetics approach. This approach yielded various cell wall mutants (Reiter *et al*. 1997) based on their wall derived neutral monosaccharide composition. However, most of the genes responsible for the wall alteration were not mapped at the beginning of this work and still many await positional cloning.

Here a reverse genetics approach was chosen to identify proteins putatively involved in cell wall synthesis with a focus on nucleotide sugar metabolism. This approach was based upon studies of proteins well known to be involved in nucleotide sugar metabolism from bacteria and mammals (Tonetti *et al*. 1998).

4.1 A reverse genetics approach to identify nucleotide sugar converting enzymes

Cell wall polysaccharides are synthesized by sugar transferases (Scheible and Pauly 2004) which might act on presynthesized primers (Peng *et al*. 2002). These transferases facilitate nucleotide sugars as activated, preenergized precursors. Since 14 different nucleotide sugars are necessary for the synthesis of the wall (see Introduction) the plant has assembled a complex nucleotide sugar conversion pathway. Numerous activities of enzymes in this pathway have been shown in plant extracts, but most of the corresponding genes are not known. In order to identify genes for nucleotide sugar precursor modifying enzymes the knowledge about similar bacterial (reviewed by Samuel and Reeves 2003) or mammal enzymes (King *et al*. 2001, Tonetti *et al*. 1998) was facilitated.

Candidate gene pools for this class of enzymes seems to be small and high inter-species conservation of individual genes seems to be applicable. However, up to now only relatively crude methods such as standard BLAST have been used to identify new candidate genes (Vanzin and Reiter 2001). Some candidate genes (namely the genes coding for UDP-D-glucose epimerase and UDP-D-glucose dehydrogenase) in the nucleotide conversion pathway were identified via sequence similarity to their mammalian or bacterial counterparts (Dörmann and Benning 1996, Tenhaken

and Thulke 1996, Kaplan *et al*. 1997, Bonin and Reiter 2000). It is well known that BLAST searches have some limitations (Altschul *et al*. 1990). Therefore even more powerful techniques have been developed such as profile hidden markov models (Eddy 1998) which take conservation of residues between species into account to reveal new genes with high specificity and comparatively little effort. Thus profile hidden markov models were facilitated to uncover genes possibly involved in nucleotide sugar interconversion.

A nucleotide sugar conversion candidate list was obtained (see table 1 and 2, results 3.1). Its content was verified during the course of this study by examinations carried out by Reiter and Vanzin (2001), who also concluded that many genes from the list could potentially encode for nucleotide sugar converting enzymes. However, since their approach was also based on reverse genetics the overlap of candidates cannot be seen as an independent proof. Moreover some gene families such as the *MUR4* gene family, the *AXS* family, one gene annotated as being involved in nucleotide sugar metabolism (At4g00560) and several genes not annotated as acting on nucleotide sugars were not mentioned in their list. The approach used here gained substantial validity by identifying the proteins already known to play a role in nucleotide sugar biosynthesis at the time of its conduction (Dörman and Benning 1996, Tenhaken and Thulke 1996, Bonin *et al*. 1997, Bonin and Reiter 2000). Further candidate genes identified here (Table 1 and 2) were confirmed during the course of this study such as *MUR4*, a UDP-D-xylose epimerase, and its homologs (Burget *et al*. 2003). Interestingly, the rough tree of all nucleotide sugar converting enzymes found here presented in figure 7 indicated that the MUR4 proteins should be similar to both the UGE and GAE protein families and indeed publications of both UGE (Seifert *et al*. 2002) and MUR4 (Burget *et al*. 2003) both mention the other two families as sharing common sequence features. The gene family identified here (table 3) through a similarity to known *myo*-inositol oxygenase from pigs was later confirmed to indeed encode *Arabidopsis myo*-inositol oxygenase (Lorence *et al*. 2004).

Since by now many candidate proteins identified in the original reverse genetics approach have been characterized some other less likely candidates should also be considered such as proteins carrying a non-descriptive annotation like “putative protein” or an annotation unrelated to nucleotide sugar, eg. At1g09340 which is annotated as an RNA binding protein. However, it can be expected that for these proteins functional prediction is more difficult as it becomes increasingly more tedious to infer their function due to their higher dissimilarity to known proteins. Here it could be difficult to assess a possible function especially since loss of function mutants in nucleotide sugar converting enzymes do not necessarily show a revealing phenotype as has been

shown in this thesis. However, due to the identification of many of the nucleotide sugar converting enzymes also conducted as part of this thesis, a characterization of the remaining candidates might become feasible by a combinatorial analysis. This can be done by building on the foundation of the extensive studies conducted about possible nucleotide sugar conversion pathways *in planta* (extensively reviewed by Feingold and Avigad 1980). Since the major pathways have been elucidated (or are in the process of being published) the remaining candidates might code for other minor enzyme functions such as NDP-D-rhamnose synthesis whose activities have been shown *in planta* (Feingold and Avigad 1980) but where no gene has been cloned yet. Thus the work presented here lays the foundations for a thorough analysis of the remaining nucleotide sugar converting enzymes.

4.2 RHM2 encodes an enzyme necessary for mucilage pectin synthesis

One important goal of the thesis was the identification of plants with an altered cell wall structure. Rhamnose is found in the pectic components rhamnogalacturonan I (RGI) and rhamnogalacturonan II (RGII). Therefore, a rhamnose precursor might play a major role in pectin synthesis, especially since all RGI side chains are attached to the rhamnosyl substituents (figure 2, introduction).

One of the candidate gene families, *RHM*, identified by the reverse genetics approach was studied in further detail by T-DNA insertion lines for one of the genes, *RHM2*. No difference in leaf cell wall composition and hence RGI structure nor in the size of the pectic fraction was obtained in mutants for this allele. However, a strong reduction in seed mucilage, which is nearly entirely composed of pectins (Goto 1985), was observed in seeds of the mutant plants. Moreover, a lesion in the *RHM2* locus led to severe developmental effects in the developing seed coat such as a flattened columella (figure 16, results).

Therefore, *rhm2* was the first known synthesis mutant involved in seed development (Usadel *et al*. 2004), highlighting the importance of primary metabolism for proper development. It was later shown that *rhm2* is allelic to *mum4,* a previously identified mucilage mutant (Western *et al*. 2004). However, even though RHM2 probably is involved in NDP-L-rhamnose synthesis (Watt *et al*. 2004) conclusive biochemical evidence is still lacking. Several points indicate a role as an UDP-L-rhamnose synthase. First there is strong similarity of the N-terminal domain with bacterial enzymes involved in NDP-L-rhamnose synthesis. Secondly a heterologously expressed C-terminal domain of

one of the RHM members and UER1 do convert an intermediate of NDP-L-rhamnose synthesis into NDP-L-rhamnose (Watt *et al*. 2004). Moreover, *rhm2* seeds show a defect in mucilage RGI, specifically a downregulation of rhamnose and uronic acids. Here, the downregulation of uronic acids can be explained by the backbone structure of RGI, which consists of an alternating rhamnosyl-galacturonic acid disaccharide unit. Therefore a reduction in the availability of rhamnose substrate will concomitantly limit GalA content. Last but not least RHM2's homolog RHM1 does convert UDP-D-glucose into a product that upon hydrolysis co-migrates with rhamnose upon thin-layer chromatography (Mølhøj and Reiter, University of Connecticut, CT, USA, personal communication). Thus it seems very likely that RHM2 indeed catalyzes the conversion of NDP-D-glucose into NDP-L-rhamnose, which is then incorporated into mucilage RGI. Based on data available from plant extracts, where most NDP-L-rhamnose is present as UDP-L-rhamnose (Feingold and Avigad 1980) it seems likely that *RHM2* encodes a UDP-L-rhamnose synthase.

***Rhm2* provides a new genetic tool to dissect mucilage biosynthesis**

The deposition of mucilage is a highly complex process and only recently some understanding of mucilage deposition in *Arabidopsis* began (Windsor *et al*. 2000, Western *et al*. 2000). *Rhm2* as an enzymatic mutant provides a new genetic tool to dissect the process of mucilage deposition in *Arabidopsis*. Other mapped mucilage mutants usually exhibited pleiotropic effects due to molecular lesion in transcription factors (Western *et al*. 2002, Penfield *et al*. 2001). However, it cannot be excluded that the observed developmental effect seen in the *rhm2* seed coat is not primarily due to the lack of RGI in the seed mucilage. The observed effect could also be the direct or indirect effect of a lack of rhamnose or rhamnose containing structures. For example, some evidence has been published showing that at least rhamnose containing oligosaccharides have some effect on hypocotyl development in *Arabidopsis* (Yokotani-Tomita *et al*. 1998, Hirose *et al*. 2004). In any case, *rhm2* should prove a useful genetic tool to start a dissection of different rhamnose requiring pathways in the developing seed. This is especially true since under laboratory conditions the mucilage is dispensable for the seed (Western *et al*. 2000) and seems to consist of rather pure RGI (Penfield *et al*. 2001). Seeds from *myb61* plants lacking a functional MYB61 transcription factor and showing a strong reduction in mucilage displayed a reduction of germination rate under water-limiting conditions (Penfield *et al*. 2001). Thus Penfield and coworkers speculated that a disruption of mucilage would lead to decreased germination under water stress conditions due to the hydrophilic nature of the pectic RGI of the mucilage (2001). If the observed effect in *myb61*

was due to the stronger reduction of mucilage or due to pleiotropic effects associated with the loss of the transcription factor has still to be determined. However, *rhm2* seeds did not show any reduction in germination rate when compared to wild type plants under normal or water limiting conditions (chapter 3.2.3.2). Western and colleagues showed by an independent approach that a decrease of MYB61 does not seem to affect *RHM2* transcript levels (2004). Therefore they speculated that RHM2 would rather be regulated by other transcription factors such as AP2, TTG1 and GL2 which partly also show mucilage phenotypes in a loss of function situation (Western *et al*. 2002). They showed evidence for such a behavior by demonstrating that reduced *RHM2* transcript was observable in mutants for those transcription factors

Another interesting example highlighting the importance of pectic structures for the mucilage is the impact that the discovery of *rhm2* as a mucilage mutant (*mum4*) had for the current models of mucilage and seed coat formation (compare Western *et al*. 2002 to Western *et al*. 2004). The discovery of pectin modifying enzymes in mucilage mutants was rather unexpected (G. Haughn, University of British Columbia, pers. communication) but at least an additional pectin mutant, a putative pectin methylesterase, seems to be involved in mucilage development (Michelle Facette, Carnegie Institute, CA, USA, personal communication). Therefore, the identification of *rhm2* as *mum4* might start a new era of mucilage research. Interestingly, the defect in *rhm2* lead not only to the reduction of mucilage RGI but also to a 40 % reduction in the molecular weight of the isolated RGI (chapter 3.2.3.1) This suggests that limiting substrate availability has an influence on the processivity of putatively involved rhamnosyltransferases. So far a similar effect had only been shown for starch synthesis (Van den Koornhuyse *et al*. 1996), but could also be more generally applicable for the synthesis of other wall polysaccharides. Thus it can be speculated that a complete understanding of the nucleotide interconversion pathway and flux might eventually lead to altered polysaccharides changed just on the basis of substrate availability and flux.

4.3 The expression of at least one RHM protein seems to be necessary for proper plant development in Arabidopsis

The downregulation of the whole *RHM* gene family expression in *Arabidopsis* (3.2.4) resulted in a strong reduction of growth and an accumulation of antocyanins. This was so far not observed in other pectin cell wall mutants so far, where a similar biochemical phenotype such as a reduction of rhamnose (*mur8*, *mur11,* Reiter *et al*. 1997) but no visible phenotype was observed. This indicates

that the growth reduction in the rRHM plants is not due to the same molecular mechanism as the one causing the observed effect in the *mur8* and *mur11* mutants. Since only a composition of the cell walls of the two *mur* mutants has been published so far (Reiter *et al*. 1997), the growth reduction can be explained either by differences in the cell wall alone or by effects on other rhamnose containing components in *Arabidopsis*. Since there are several structures in the cell wall containing rhamnose even in minor amounts such as RGI, RGII (Visser and Voragen, 1996) and AGPs (Fincher *et al*. 1983), the *mur* mutants might be affected in a transferase specifically affecting only one of these components, whereas a broad limitation of UDP-L-rhamnose might lead to a reduction in all of these three compounds. Both RGII (O'Neill *et al*. 1990) and AGP (Fincher *et al*. 1983) contain only a small percentage of total rhamnose of the cell wall, but both polymers are known to be involved in cell to cell adhesion and communication (Iwai *et al*. 2002, Showalter 2001). Thus even without a drastic reduction of wall rhamnose content a minor reduction/structural defect in either of them could lead to profound effects on plant growth and development. It is therefore necessary to isolate these cell wall components and monitor rhamnose levels of the rRHM plants and the published *mur* mutants. An initial analysis presented here already showed that RGI did show a 40% reduction in rhamnose in one of the rRHM lines. However, as of now it is not known if also other wall polysaccharides are structurally altered.

Interestingly the RNAi construct seems to have lead to the reduction of transcript not only of *RHM1-3* but also *UER1* which encodes a proteins similar to the C-terminal region of the RHM proteins. However, this data could be due to inaccuracy of real-time RT-PCR measurements. Alternatively it could be speculated that UER1 which would need other enzyme activities to synthesize NDP-L-rhamnose from NDP-D-glucose probably found in the N-terminal domain of RHM (Watt *et al*. 2004) might be co-regulated on a transcriptional level with the *RHM* genes which is also detected in the network analysis performed within the CSB.DB framework, where *UER1* transcript was correlated to that of *RHM1* and *RHM2* expression. However, current knowledge about multi domain enzymes such as RHM implicates that the substrates would be channeled from one domain to the other (see e.g. Voet and Voet 1995). This would leave the role of UER1 undefined, because no good candidates exist, which is similar to the N-terminal domains of the RHM proteins. UER1 might act as a safeguard for intermediates released from the RHM enzyme complex or might form interactions with certain RHMs which could be uncovered in a yeast two hybrid screen.

The putative lack of UDP-L-rhamnose supply in the rRHM plant might also influence the level of other rhamnose containing oligosaccharides and/or flavonoids which also use UDP-L-rhamnose as a rhamnose donor (Jones *et al*. 2003). This in turn might influence proper plant growth, development or might lead to triggering plant defense responses such as in the case of oligogalacturonides (Pilling and Höfte 2003). Interestingly, the observed phenotype of increased anthocyan production with concomitant mucilage reduction has also been observed in the pleiotropic mutants in the *TRANSPARENT TESTA GLABRA1* locus (Walker *et al*. 1999). Following the current model of mucilage production proposed by Western and colleagues (2004), TTG1 should act upstream of GL2, which in turn signals to RHM2. The observed effects in the rRHM plants having strong pleiotropic effects might eventually also point to a feed-back inhibition mechanism via TTG1. However, a good epigenetic study for the proposed model is still lacking.

Here it was shown that altering RHM expression levels lead to pectins with a reduced rhamnose content. It will be interesting to apply this knowledge to crop plants such as the tomato (*Lycopersicon esculentum*). Here, a change in pectin structure could have significant impact on the commercial value of fruits (Camara *et al*. 2002). A good candidate for a RHM protein could alreready be identified in *Lycopersicon esculentum*. Based on the extremely high sequence similarity between *Le*RHM1 and the *Arabidopsis* RHM family on amino acid level it can be concluded with some confidence that the identified protein is indeed the best candidate. Due to the preferential expression of *Le*RHM1 in fruits the gene poses an immediate target to modify fruit structure without changing the general appearance of the plant and hence fruit yield.

One possibility is a downregulation of *LeRHM1* using an RNAi construct and subsequently analyzing fruit pectins of the transgenic plants.

4.4 GAE6 encodes a functional UDP-D-glucuronic acid epimerase

Galacturonic acid is the major component of pectins and present in all pectin polysaccharide backbones (Mohnen 1999). The proposed precursor of pectic galacturonic acid is UDP-D-galacturonic acid (reviewed by Feingold and Avigad 1980). However, up to now UDP-D-galacturonic acid had to be generated from UDP-D-glucuronic acid by means of a crude radish root extracts with poor yield (Liljebjelke *et al*. 1995). Here the identification, cloning and functional expression of a putative UDP-D-galacturonic acid epimerase gene (3.3.2 and 3.2.3) was performed and the activity of the enzyme allowed the production of milligrams of UDP-D-galacturonic acid. It

has been shown that using *Pichia pastoris* extracts UDP-D-galacturonic acid can also be produced in the mg scale in overnight reactions. Even though a bacterial *GAE* had also been cloned and functionally expressed in *E. coli* (Muñoz *et al* .1999) it has not been used for generation of UDP-D-galacturonic acid, possibly due to low reaction yields or byproducts.

Despite the recent advance in knowledge about nucleotide sugar metabolism in plants (Seifert 2004) no functionally cloned enzyme has been used to generate new NDP-sugars from commercially available substrates in the mg scale. However, the generation of smaller amounts is possible (e.g. the CCRC substrate service). Moreover for most studied enzymes the precise identity of the products was not unambiguously shown (Mølhøj *et al*. 2003) as usually only hydrolysis products were analyzed. A notable exception is the work of Watt and colleagues who generated dTDP-L-rhamnose and extensively characterized it by nuclear magnetic resonance (2004). However, they did not start from any commercially available product like UDP-D-glucose but they had to first synthesize a dTDP-D-4keto-6deoxy-D-glucose intermediate with the help of a bacterial enzymes from commercially available dTDP-D-glucose. It remains questionable whether the generated substance (dTDP-L-rhamnose) would be useful for studies in plant cell wall synthesis since a compound was generated which so far has not been detected in plants (Feingold and Avigad 1980).

The possibility that radish extracts and insect cell extracts lose their epimerase activity or that both harbor other enzymes further converting UDP-D-galacturonic acid cannot be excluded. However, in the case of the insect cells no other nucleotide sugar peak was detected in an HPAEC trace indicative of no conversion of UDP-D-galacturonic acid into another major nucleotide sugar which would be detectable by HPAEC (Usadel & Pauly unpublished results).

GAE6 when expressed in *Pichia* does convert approximately half of the UDP-D-glucuronic acid to UDP-D-galacturonic acid. Interestingly, a similar case was demonstrated for UDP-D-xylose and UDP-L-arabinose whereas the apparent equilibrium from plant extracts was 4:1 (Pauly *et al*. 1999) and when a gene encoding for this epimerase expressed in *Pichia* was used for *in vitro* conversion a 1:1 ratio was observed (Burget *et al.* 2003). Based on the observations made it could be speculated that some kinetic control mechanism would be exerted in both insect cells and plant extracts thus capping synthesis of the products. However, more experiments are necessary to prove such a control mechanism and elucidate its mechanism.

4.5 A loss of function in GAE6 does not result in any phenotypes

Initial results from the analysis of *gae6* cell walls where a reduction of 40% in leaf cell wall bound uronic acids was found, a persisting difference of *gae6* plants in cell wall composition was not obtained. It is unlikely that the analyzed T-DNA insertion lines still encode a functional UDP-D-galacturonic acid epimerase, since the *GAE6* transcript of *gae6* plants was altered in such a way that either the translation product would be modified by a block of T-DNA inserted in the predicted catalytically active domain of GAE6 or that the transcript was degraded. One possible explanation for the non-reproducable results might be the growth of *gae6* lines in the greenhouse as slight differences in the plant growth environment such as light and humidity conditions, soil composition and/or plant pathogen interaction existed. One of these conditions might be a stress condition under which a structural phenotype becomes visible. Moreover the regulation of *GAE1* and *GAE6* during leaf development where both are expressed to a high level but seem to be differentially regulated in young leaves (see figure 25) might make it difficult to pinpoint cell wall differences. However, the higher raw expression for *GAE6* in small leaves might point to a specific tissue in the *gae6* mutant.

It can be argued that genetic redundancy is responsible for sufficient synthesis of UDP-D-galacturonic acid. To prove this an examination of remaining UDP-D-GlcA epimerase activity *in planta* would be necessary.

Constitutive overexpression of *GAE6* did also not result in an overabundance of pectins. Although it cannot be excluded that the overexpressed protein is rapidly degraded, the synthesis of UDP-D-GalA does not seem to be the limiting factor or a regulation point for pectin synthesis under the growth conditions used here. In contrast, it has been shown for at least one nucleotide sugar converting enzyme, GDP-mannose-epimerase, that tight regulation of the enzyme partly through feed-back inhibition is exerted (Wolucka and Montagu 2003).

Since other members of the GAE family might influence the abundance of GalA present in the wall, the knockout mutants *gae2* and *gae5* were investigated in our laboratory (Beyer 2004). However, in both cases no visible phenotype was observed and in the case of *gae2* plants only a minor reduction in the arabinose level was observed which could be rather due to environmental, random effects, since uronic acids level were unaffected. Further experiments including the creation of double knockout mutants might be necessary to gain further insights into genetic redundancy.

4.6 Quantitative analysis of GAE transcripts reveals three groups of co-expressed genes

Clustering of the different *GAE* transcripts based on their expression level in the different organs yields three different groups. On one hand there are the highly expressed genes *GAE1* and *GAE6* being individually separated from all the other genes and on the other hand *GAE2* to *GAE5* cluster together (see chapter 3.3.4). Even though GUS staining revealed subtle differences in the expression of the latter group, still a co-expression of *GAE2* to *GAE5* might indicate that these are closer related through evolution. This is also the result of a phylogenetic analysis of the GAE proteins sequences by means of tree-building. Taken together these results suggest that phylogenetic groups could be uncovered by means of quantitative RT-PCR analysis. It will be interesting to see if this is generally applicable and if this is due to conserved promoter sequences. From an evolutionary point of view it will be interesting to see what differentiates the expression of such groups and whether GAE2 to GAE5 would have different catalytic properties.

As another example for possible evolutionary relation of expression, Burton and co-workers were able to differentiate two groups of co-expressed putative cellulose synthase genes in barley by means of organ specific real time RT-PCR (2004).

A co-response analysis of *GAE* transcripts using publicly available microarray experiments showed that *GAE6* is co-regulated with *GAE3* and *GAE4*. In contrast, *GAE1* was not co-regulated with any other *GAE* transcript. It could also be speculated that GAE3 and GAE4 whose transcript is co-regulated with GAE6 might take over the role of GAE6 under certain stress conditions which underly the co-response analysis but not the real-time RT-PCR data. It remains to be seen whether this is due to the higher sampling error of microarray based transcript measurements or due to the different environmental circumstances. With the advent of microarray expression profiles for many different plant organs (ftp.arabidopsis.org) produced in Prof Weigel's laboratory (MPI for Development, Tübingen, Germany), it has now become feasible to compare the tissue-specific real-time RT-PCR data with corresponding microarray data and new expression analysis tools as well as new co-response matrices that are currently being developed.

4.7 A loss of MIOX function does not affect leaf cell wall composition

Arabidopsis expresses proteins responsible for the synthesis of D-glucuronic acid from *myo*-inositol (Lorence *et al*. 2004) thus confirming the predicted function of the identified candidate genes (see 3.1). However, a loss of function in any individual member of this family does not result in a change in cell wall composition of 4-8 week old green house grown plants (this thesis and U. Kanter, personal communication). This lack of a visible phenotype could be due to genetic redundancy or pathway redundancy, i.e. the conversion of UDP-D-glucose to UDP-D-GlcA through the UDP-D-glucose dehydrogenase (UGD) pathway. However, there is some evidence that at least under normal conditions the UGD and MIOX pathway seem to occur in different plant organs (Seitz *et al*. 2000). Therefore a comprehensive study of the synthesis of UDP-D-glucuronic acid would involve the generation of many permutations of double and triple mutants to elucidate functional relationships of these genes on a genetic basis. Moreover, studies of the expression levels of all genes in both pathways in the single insertional mutants might be revealing.

The MIOX pathway, even though it has been described as having some importance during plant development (Dalessandro and Northcote 1977, Tenhaken and Thulke 1996, Seitz *et al*. 2000) could only play a minor role in UDP-D-glucuronic acid synthesis as in contrast to UGDs, MIOX genes are often not measured on Affymetrix chips (CSB.database and underlying data, appendix).

D-glucuronic acid generated by the MIOXes still has to be converted into UDP-D-glucuronic acid by the subsequent activity of a C1-kinase and a pyrophosphorylase (Feingold and Avigad 1980) which could also be upregulated to compensate the loss of some MIOX function. In this case the remaining MIOXEes could eventually provide sufficient intermediate which could then be converted faster by upregulation of downstream enzymes yielding the same amount of product. However, a kinetic study of rate constants both in the wild-type and mutant plants and/or epigenetic studies would be necessary to establish such a mechanism.

4.8 Systems biology might reveal new insights into cell wall metabolism

Another tool to gain more insight into nucleotide sugar conversion pathways might be the analysis of co-response networks as already shown above. More specifically, *UGE1* and *UGE4* transcripts seem to be differentially connected to other transcripts in *Arabidopsis* possibly indicating a

different function. Since a functional not further specified difference of both proteins is also assumed by Seifert (2004) this might hint that systems biology might help exploring gene function in the nucleotide sugar field. This gains further strength by the fact that plants having a lesion in *UGE3* which also found to be highly connected also shows a root bulging phenotype as do *uge4* plants (A. Kuschinsky, MPI of molecular plant physiology, Golm, Germany, personal communication). UGE1 on the other hand does not display this observed phenotype (Dörmann and Benning 1998). To gain final insight into the propensities of the network it would be necessary to obtain both a full network consisting of all identified nucleotide sugar converting enzymes and biochemical and genetic evidence for every member of a gene family.

Even though not directly related to the nucleotide sugar conversion pathway positional cloning was performed based on “visual” correlation just recently. Here two cloned proteins encoding for two subunits of a complex were shown to show a correlated expression and to cause the same phenotype. A third mutant also having this phenotype could be positionally cloned based on correlated expression behavior to these two after having narrowed down the candidate gene pool to some tens of candidates by means of mapping (K. Roberts, John Innes Centre, Great Britain, personal communication).

In a different approach the CSB database was used to identify new potential candidate genes possibly involved in cell wall synthesis. The combination of genes from one sub-network of nucleotide sugar modifying enzymes consisting of RHM2, GAE6 and UGD2 was used to obtain a candidate gene list of co-regulated genes. Since many of the obtained genes were indeed cell wall related other fuzzy annotated genes (such as “expressed protein”) might also be interesting to consider for functional analysis in the future. When the data was compared to a cell wall stress experiment where cell cultures were grown in isoxaben, and only the cell wall related genes were considered most of them showed a difference in expression. Hence, the combination with specific related individual microarray data might lead to further specificity of the candidate lists. However, further experiments such as studying knockout plants or expressing the enzymes *in vitro* would be necessary to prove that indeed a refinement of the data set was reached.

CSB.DB can be used as a tool for various other questions such as how correlation networks will behave under different stress situations, and might thus allow the identification of possible stresses under which the mutants such as *gae6* exhibit a developmental or morphological phenotype. The inclusion of the expression data of ATGENEXPRESS enables rapid localization of possible hotspots for gene expression.

In light of the microarray and metabolic profiling data generated by researchers it is even more important to create resources that visualize highly abstracted data about multi-parallel data sets.
Extension of the datasets will allow to consider the nucleotide sugar networks when only cell wall related experiments would be used for matrix building. Even though that was not achieved during the time frame of the thesis due to the lack of sufficient appropriate microarray datasets and the impossibility to generate them in one group, exactly these high amounts of experiments are generated and put into the public domain right now.
In general it can be expected that the multi-parallel datasets which are currently generated will spur new developments and will considerably increase the speed with which progress in plant science is made.

4.9 Concluding remarks

Within this thesis genes were identified and characterized that are involved in the synthesis of cell wall sugar precursors. It was feasible to study these genes in detail using a reverse genetics approach and this resulted in the cloning and functional expression of the genes responsible for the generation of the substrates necessary for the most import sugar in pectin biosynthesis GalA. The role of those genes in cell wall biosynthesis has been thoroughly investigated by modulating the expression level through knockout, RNAi and overexpression techniques as well as investigating into their expression level *in planta*. Facilitating these genetic approaches it was possible to generate plants that exhibit an altered pectin structure confirming the importance of those genes in the nucleotide sugar conversion pathway. More importantly, some of these roles not only of those genes or proteins but also the altered pectin structure in plant growth and development could be elucidated.. Currently experiments are on the way to characterize these genes in crop plants. Moreover, a resource was built which allows the identification of novel candidate genes that might play a role in the nucleotide sugar conversion pathway and/or cell wall biosynthesis.

5 Summary

Even though the structure of the plant cell wall is by and large quite well characterized, its synthesis and regulation remains largely obscure. However, it is accepted that the building blocks of the polysaccharidic part of the plant cell wall are nucleotide sugars. Thus to gain more insight into the cell wall biosynthesis, in the first part of this thesis, plant genes possibly involved in the nucleotide sugar interconversion pathway were identified using a bioinformatics approach and characterized in plants, mainly in *Arabidopsis*. For the computational identification profile hidden markov models were extracted from the Pfam and TIGR databases. Mainly with these, plant genes were identified facilitating the “hmmer” program. Several gene families were identified and three were further characterized, the UDP-rhamnose synthase (*RHM)*, UDP-glucuronic acid epimerase (*GAE)* and the *myo*-inositol oxygenase (*MIOX)* families.

For the three-membered *RHM* family relative ubiquitous expression was shown using variuos methods. For one of these genes, *RHM2*, T-DNA lines could be obtained. Moreover, the transcription of the whole family was downregulated facilitating an RNAi approach. In both cases a alteration of cell wall typic polysaccharides and developmental changes could be shown. In the case of the *rhm2* mutant these were restricted to the seed or the seed mucilage, whereas the RNAi plants showed profound changes in the whole plant.

In the case of the six-membered *GAE* family, the gene expressed to the highest level (*GAE6*) was cloned, expressed heterologously and its function was characterized. Thus, it could be shown that *GAE6* encodes for an enzyme responsible for the conversion of UDP-glucuronic acid to UDP-galacturonic acid. However, a change in transcript level of variuos *GAE* family members achieved by T-DNA insertions (*gae2*, *gae5*, *gae6*), overexpression (*GAE6*) or an RNAi approach, targeting the whole family, did not reveal any robust changes in the cell wall.

Contrary to the other two families the *MIOX* gene family had to be identified using a BLAST based approach due to the lack of enough suitable candidate genes for building a hidden markov model. An initial bioinformatic characterization was performed which will lead to further insights into this pathway.

In total it was possible to identify the two gene families which are involved in the synthesis of the two pectin backbone sugars galacturonic acid and rhamnose. Moreover with the identification of

the *MIOX* genes a genefamily, important for the supply of nucleotide sugar precursors was identified.
In a second part of this thesis publicly available microarray datasets were analyzed with respect to co-responsive behavior of transcripts on a global basis using nearly 10,000 genes. The data has been made available to the community in form of a database providing additional statistical and visualization tools (http://csbdb.mpimp-golm.mpg.de). Using the framework of the database to identify nucleotide sugar converting genes indicated that co-response might be used for identification of novel genes involved in cell wall synthesis based on already known genes.

6 Zusammenfassung

Obwohl der Aufbau der pflanzlichen Zellwand im Großen und Ganzen relativ gut charakterisiert ist, ist relativ wenig über ihre Synthese bekannt. Allgemein akzeptiert ist jedoch, dass die Nukleotidzucker die Vorstufe für den polysaccharidären Teil der Zellwand stellen. Im Rahmen der vorliegenden Arbeit wurden neue Kandidatengene für die Zellwandbiosynthese mittels bioinformatorischer Analysen ermittelt und deren Rolle in Pflanzen, hauptsächlich *Arabidopsis thaliana* untersucht. Zur Identifizierung von *Arabidopsis thaliana* Kandidatengenen des Nukleotidzucker-Stoffwechselweges wurden „hidden Markov Modelle" für Gene desselben aus den Datenbanken Pfam und TIGR extrahiert. Unter anderem wurden diese dann unter Zuhilfenahme des Programms hmmer zur Identifikation von pflanzlichen Genen benutzt. Es wurden einige Genfamilien identifiziert und drei von diesen wurden weiter charakterisiert. Hierbei handelte sich um eine UDP-Rhamnose Synthase Familie (*RHM*), eine UDP-Glucuronsäurepimerase Familie (*GAE*) und eine myo-Inositol Oxygenase Familie (*MIOX*).

Für die *RHM* Kandidatengenfamilie, mit drei Mitgliedern, wurde die relativ ubiquitäre Expression aller Gene mittels verschiedener Methoden gezeigt und für eines der Gene, *RHM2,* konnten T-DNA Linien bezogen werden. Außerdem wurde die Transkription der gesamten Familie mittels eines RNAi Konstruktes herunter geregelt. In beiden Fällen konnte eine Veränderung von zellwandtypischen Polysacchariden sowie schwere Entwicklungsstörungen gezeigt werden. Diese waren bei der *rhm2* Funktionsverlustpflanze auf den Samenschleim bzw. den Samen reduziert, bei den RNAi Pflanzen hingegen war die gesamte Pflanze betroffen.

Im Falle der zweiten Kandidatengenfamilie, *GAE*, wurde das höchst-exprimierte Gen (*GAE6*) kloniert, heterolog exprimiert und die Funktion charakterisiert. So konnte gezeigt werden, dass *GAE6* für ein Enzym kodiert, welches UDP-Glukuronsäure in UDP-Galakturonsäure wandelt. Allerdings zeigten Pflanzen mit veränderter Transkriptmenge, erreicht durch T-DNA Insertionen (*gae2*, *gae5*, *gae6*), Überexpression (*GAE6*) oder RNAi , keine robuste Veränderung der Zellwand.

Die letzte betrachtete Kandiatengenfamilie myo-Inositol Oxygenase wurde im Gegensatz zu den beiden anderen Familien, durch eine BLAST Suche gefunden, da zur Zeit der Durchführung noch zu wenig myo-inositol Oxygenasen bekannt waren, um daraus „hidden Markov Modelle" abzuleiten. Dennoch konnten erste bioinformatorische Analysen zu dieser Genfamilie gemacht werden.

Insgesamt gesehen wurden in diesem Teil der Arbeit die beiden Genfamilien identifiziert und charkterisiert, die bei der Synthese der beiden Pektinrückgradzucker Rhamnose und Galakturonsäure die tragende Rolle spielen. Weiterhin wurde mit der Identifizierung der *MIOX* Genfamilie, eine Genfamilie identifiziert, die wichtige Vorstufen in der Synthese der Nukleotidzucker liefert.

In einem zweiten Teil der Arbeit wurden öffentlich zugängliche Mikroarray-Daten durch ihr Gleich -oder Ungleichverhalten charakterisiert. Dieses erfolgte auf globaler Ebene für zunächst fast 10.000 Gene. Die Daten wurden in Form einer allgemein zugänglichen Datenbank der Allgemeinheit zur Verfügung gestellt (http://csbdb.mpimp-golm.mpg.de). Eine Anwendung der Methode auf Gene des Nukleotidzuckerstoffwechsels, deutet darauf hin, dass so neue Kandiatengene, die bei der Zellwandsynthese eine Rolle spielen, von bereits bekannten Genen abgeleitet werden können.

7 References

Agius F, Gonzalez-Lamothe R, Caballero JL, Munoz-Blanco J, Botella MA, Valpuesta V (2003) Engineering increased vitamin C levels in plants by overexpression of a D-galacturonic acid reductase. Nat Biotechnol **21**: 177-181

Albersheim P, Nevins DJ, English PD, Karr A (1967) A method for the analysis of sugars in plant cell wall polysaccharides by gas-liquid chromatography. Carbohydr Res **5**: 340-345

Allard ST, Beis K, Giraud MF, Hegeman AD, Gross JW, Wilmouth RC, Whitfield C, Graninger M, Messner P, Allen AG, Maskell DJ, Naismith JH (2002) Toward a structural understanding of the dehydratase mechanism. Structure **10**: 81-92

Allard ST, Giraud MF, Whitfield C, Graninger M, Messner P, Naismith JH (2001) The crystal structure of dTDP-D-Glucose 4,6-dehydratase (RmlB) from *Salmonella enterica* serovar Typhimurium, the second enzyme in the dTDP- l-rhamnose pathway. J Mol Biol **307**: 283-295

Allard ST, Giraud MF, Whitfield C, Messner P, Naismith JH (2000) The purification, crystallization and structural elucidation of dTDP-D- glucose 4,6-dehydratase (RmlB), the second enzyme of the dTDP-L-rhamnose synthesis pathway from *Salmonella enterica* serovar typhimurium. Acta Crystallogr D Biol Crystallogr **56 (Pt 2)**: 222-225

Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen H, Shinn P, Stevenson DK, Zimmerman J, Barajas P, Cheuk R, Gadrinab C, Heller C, Jeske A, Koesema E, Meyers CC, Parker H, Prednis L, Ansari Y, Choy N, Deen H, Geralt M, Hazari N, Hom E, Karnes M, Mulholland C, Ndubaku R, Schmidt I, Guzman P, Aguilar-Henonin L, Schmid M, Weigel D, Carter DE, Marchand T, Risseeuw E, Brogden D, Zeko A, Crosby WL, Berry CC, Ecker JR (2003) Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. Science **301**: 653-657

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol **215**: 403-410

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res **25**: 3389-3402

Amor Y, Haigler CH, Johnson S, Wainscott M, Delmer DP (1995) A membrane associated with sucrose synthase and its potential role in synthesis of cellulose and callose in plants. Proc Natl Acad Sci USA **92**: 9353-9357

Andeme-Onzighi C, Sivaguru M, Judy-March J, Baskin TI, Driouich A (2002) The reb1-1 mutation of *Arabidopsis* alters the morphology of trichoblasts, the expression of arabinogalactan-proteins and the organization of cortical microtubules. Planta **215**: 949-58

Arner RJ, Prabhu KS, Thompson JT, Hildenbrandt GR, Liken AD, Reddy CC (2001) myo-Inositol oxygenase: molecular cloning and expression of a unique enzyme that oxidizes myo-inositol and D-chiro-inositol. Biochem J **360 (Pt 2)**: 313-320

Aspinall GO, Krishnamurthy TN, Rosell KG (1977). A fucogalactoxyloglucan from rapeseed hulls. Carbohydr Res **55**: 11-19

Atkinson RG, Schroder R, Hallett IC, Cohen D, MacRae EA (2002) Overexpression of polygalacturonase in transgenic apple trees leads to a range of novel phenotypes involving changes in cell adhesion. Plant Physiol **129**: 122-33

Bacic A, Harris PJ, Stone BA (1988) Structure and function of plant cell walls. In: The biochemistry of plants (ed. Priess, J.), Academic Press, London, Vol. 14, pp. 297-371

Baldwin TC, Hanford MG, Yussef MI, Orellana A, Dupree P (2001) Identification and characterization of GONST1, a golgi localized GDP-mannose transporter in *Arabidopsis*. Plant Cell **13**: 2283-2295

Bar-Peled M, Lewinsohn E, Fluhr R, Gressel J (1991) UDP-rhamnose:flavanone-7-O-glucoside-2''-O-rhamnosyltransferase. Purification and characterization of an enzyme catalyzing the production of bitter compounds in citrus. J Biol Chem **266**: 20953-20959

Baron-Epel O, Gharyal PK, Schindler M (1988) Pectins as mediators of wall porosity in soybean cells. Planta **175**: 389-395

Bateman A, Birney E, Cerruti L, Durbin R, Etwiller L, Eddy SR, Griffiths-Jones S, Howe KL, Marshall M, Sonnhammer EL (2002) The Pfam protein families database. Nucleic Acids Res **30**: 276-280

Baucher M, Chabbert B, Pilate G, Van Doorsselaere J, Tollier MT, Petit-Conil M, Cornu D, Monties B, Van Montagu M, Inze D, Jouanin L, Boerjan W (1996) Red Xylem and Higher Lignin Extractability by Down-Regulating a Cinnamyl Alcohol Dehydrogenase in Poplar. Plant Physiol **112**: 1479-1490

Bedinger P (1992) The remarkable biology of pollen. Plant Cell **4**: 879-887

Bedouet L, Courtois B, Courtois J (2003) Rapid quantification of O-acetyl and O-methyl residues in pectin extracts. Carbohydr Res **338**: 379-383

Bergmeyer HU, Möllering H (1974) In Methoden der enzymatischen Analyse (Bergmeyer HU, ed) 3rd edition, VCH, Weinheim, Germany

Bellincampi D, Dipierro N, Salvi G, Cervone F, De Lorenzo G (2000) Extracellular H_2O_2 induced by oligogalacturonides is not involved in the inhibition of the auxin-regulated rolB gene expression in tobacco leaf explants. Plant Physiol **122**: 1379-1385

Beyer C (2004) Charakterisierung von *Arabidopsis thaliana* T-DNA-Insertionsmutanten für die putativen Nukleotidzucker-Konvertierungsgene *GAE5*, *SUD3* und *UER1* Diploma Thesis, Potsdam University

Blankenfeldt W, Kerr ID, Giraud MF, McMiken HJ, Leonard G, Whitfield C, Messner P, Graninger M, Naismith JH (2002) Variation on a theme of SDR. dTDP-6-deoxy-L- lyxo-4-hexulose reductase (RmlD) shows a new Mg^{2+}-dependent dimerization mode. Structure **10**: 773-786

Blumenkrantz N, Asboe-Hansen G (1973) New method for quantitative determination of uronic acids. Anal Biochem **54**: 484-489

Boerjan W, Ralph J, Baucher M (2003) Lignin biosynthesis. Annu Rev Plant Biol **54**: 519–546

Bonetta DT, Facette M, Raab TK, Somerville CR (2002) Genetic dissection of plant cell-wall biosynthesis. Biochem. Soc Trans **30**: 298-301

Bonin CP, Freshour G, Hahn MG, Vanzin GF, Reiter WD (2003) The GMD1 and GMD2 Genes of *Arabidopsis* Encode Isoforms of GDP-D-Mannose 4,6-Dehydratase with Cell Type-Specific Expression Patterns. Plant Physiol **132**: 883-892

Bonin CP, Potter I, Vanzin GF, Reiter W-D (1997) The MUR1 gene of *Arabidopsis thaliana* encodes an isoform of GDP-D-mannose-4,6-dehydratase, catalyzing the first step in the de novo synthesis of GDP-L-fucose. Proc Natl Acad Sci USA **94**: 2085-2090

Bonin CP, Reiter W-D (2000) A bifunctional epimerase-reductase acts downstream of the MUR1 gene product and completes the *de novo* synthesis of GDP-L-fucose in *Arabidopsis*. Plant J **21**: 445-454

Bonnin E, Dolo E, Le Goff A, Thibault JF (2002) Characterisation of pectin subunits released by an optimised combination of enzymes. Carbohydr Res **337**: 1687-1696

Bouton S, Leboeuf E, Mouille G, Leydecker MT, Talbotec J, Granier F, Lahaye M, Hofte H, Truong HN (2002) QUASIMODO1 encodes a putative membrane-bound glycosyltransferase required for normal pectin synthesis and cell adhesion in *Arabidopsis*. Plant Cell **14**: 2577-2590

Brett C, Waldron K (1996) Physiology and biochemistry of plant cell walls. Chapman and Hall, London

Brown RM (1996) Cellulose biosynthesis in higher plants. Trends Plant Sci **1**: 149-155

Brown NP, Leroy C, Sander C (1998). MView: A Web compatible database search or multiple alignment viewer. Bioinformatics **14**: 380-381.

Brugliera F, Holton TA, Stevenson TW, Farcy E, Lu CY, Cornish EC (1994) Isolation and characterization of a cDNA clone corresponding to the Rt locus of Petunia hybrida. Plant J **5**: 81-92

Burget EG, Reiter WD (1999) The mur4 mutant of *Arabidopsis* is partially defective in the *de novo* synthesis of uridine diphospho L-arabinose. Plant Physiol **121**: 383-389

Burget EG, Verma R, Mølhøj M, Reiter W-D (2003) The Biosynthesis of L-Arabinose in Plants: Molecular Cloning and Characterization of a Golgi-Localized UDP-D-Xylose 4-Epimerase encoded by the *MUR4* Gene of *Arabidopsis*. Plant Cell **15**: 523-531

Burton RA, Gibeaut DM, Bacic A, Findlay K, Roberts K, Hamilton A, Baulcombe DC, Fincher GB (2000) Virus-induced silencing of a plant cellulose synthase gene. Plant Cell **12**: 691–705

Burton RA, Shirley NJ, King BJ, Harvey AJ, Fincher GB (2004) The CesA gene family of barley. Quantitative analysis of transcripts reveals two groups of co-expressed genes. Plant Physiol **134**: 224-236

Brummel DA, Harpster MH (2001) Cell wall metabolism in fruit softening and quality and its manipulation in transgenic plants. Plant Mol Biol **47**: 311-340

Camara Hurtado M, Greve LC, Labavitch JM (2002) Changes in cell wall pectins accompanying tomato (*Lycopersicon esculentum* Mill.) paste manufacture. J Agric Food Chem **50**: 273-8.

Cano-Delgado A, Penfield S, Smith C, Catley M, Bevan M (2003) Reduced cellulose synthesis invokes lignification and defense responses in *Arabidopsis thaliana*. Plant J **34**: 351-362

Carpita NC, Gibeaut DM (1993) Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. Plant J **3**: 1-30

Chang S, Puryear J, Cairney J (1993) A Simple and Efficient Method for Isolating RNA from Pine Trees. Plant Mol Biol Rep **11**: 113-116

Chanliaud E, Burrows KM, Jeronimidis G, Gidley MJ (2002) Mechanical properties of primary plant cell wall analogues. Planta **215**: 989-996

Charalampous FC (1959) Biochemical studies on inositol. V. Purification and properties of the enzyme that cleaves

inositol to D-glucuronic acid. J Biol Chem **234**: 220–227
Chen L, Carpita NC, Reiter WD, Wilson RH, Jeffries C, McCann MC (1998) A rapid method to screen for cell-wall mutants using discriminant analysis of Fourier transform infrared spectra. Plant J **16**: 385-392
Chia DW, Yoder TJ, Reiter WD, Gibson SI (2000) Fumaric acid: an overlooked form of fixed carbon in *Arabidopsis* and other plant species. Planta **211**: 743-751
Chuang CF, Meyerowitz EM (2000) Specific and heritable genetic interference by double-stranded RNA in *Arabidopsis thaliana*. Proc Natl Acad Sci USA **97**: 4985-4990
Choo TS, Lerouxel O, Seveno M, Usadel B, Faye L, Lerouge P, Pauly M (2002) Rapid structural phenotyping of plant cell wall mutants by enzymatic oligosaccharide fingerprinting. Plant Physiol **130**: 1754-1763
Claros MG, von Heijne G (1994) TopPred II: an improved software for membrane protein structure predictions. Comput Appl Biosci **10**: 685-686
Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. Plant J **16**: 735-743
Cole CV, Ross C (1966) Extraction, separation, and quantitative estimation of soluble nucleotides and sugar phosphates in plant tissues. Anal Biochem **17**: 526-539
Conklin PL, Pallanca JE, Last RL, Smirnoff N (1997) L-ascorbic acid metabolism in the ascorbate-deficient *Arabidopsis* mutant vtc1. Plant Physiol **115**: 1277-85.
Cosgrove, DJ (1997) Assembly and enlargement of the primary cell wall in plants. Annu Rev Cell Dev Biol **13**: 171-201
Cosgrove DJ (2000) Expansive growth of plant cell walls. Plant Physiol Biochem **38** :109-124
Czechowski T, Bari RP, Stitt M, Scheible WR, Udvardi MK (2004) Real-time RT-PCR profiling of over 1400 *Arabidopsis* transcription factors: unprecedented sensitivity reveals novel root- and shoot-specific genes. Plant J **38**: 366-379
Darvill AG, McNeill M, Albersheim P, Delmer D (1980) The primary cell wall of flowering plants. In Tolbert, N. (ed.), The biochemistry of plants. Academic Press, London, Vol. 1, pp. 91-162
Dawe RK (2003) RNA interference, transposons, and the centromere. Plant Cell **15**: 297-301
Desprez T, Vernhettes S, Fagard M, Refregier G, Desnos T, Aletti E, Py N, Pelletier S, Hofte H (2002) Resistance against herbicide isoxaben and cellulose deficiency caused by distinct mutations in same cellulose synthase isoform CESA6. Plant Physiol **128**: 482-490
Dhugga KS, Bareirro R, Whitten B, Stecca K, Hazebroek J, Randhawa GS, Dolan M, Kinney AJ, Tomes D, Nichols S, Anderson P (2004) Guar seed β-mannan synthase is a member of the cellulose synthase super gene family Science **303**: 363-366
Dickinson H (2003) Plant cell cycle: cellularisation of the endoderm needs spätzle. Curr Biol **13**: R146-R148
Doblin MS, Vergara CE, Read S, Newbigin E, Bacic A (2003) Plant cell wall synthesis: making the bricks. In Annual Plant Reviews, vol 8. The Plant Cell Wall. Edited by Rose JCK. Sheffield: Blackwell Publishing – CRC Press
Dörmann P, Benning C (1996) Functional expression of uridine 5'-diphospho-glucose 4-epimerase (EC 5.1.3.2) from *Arabidopsis thaliana* in *Saccharomyces cerevisiae* and *Escherichia coli*. Arch Biochem Biophys **327**: 27-34.
Dörmann P, Benning C (1998) The role of UDP-glucose epimerase in carbohydrate metabolism of *Arabidopsis*. Plant J **13**: 641-652
D'Ovidio R, Mattei B, Roberti S, Bellincampi D (2004) Polygalacturonases, polygalacturonase-inhibiting proteins and pectic oligomers in plant-pathogen interactions. Biochim Biophys Acta **1696**: 237-44
Driouich A, Faye L, Staehelin A (1993) The plant Golgi apparatus: a factory for complex polysaccharides and glycoproteins. Trends in Biochemical Science **18**: 210-214
Dumville JC, Fry SC (2000) Uronic acid-containing oligosaccharins: their biosynthesis, degradation and signalling roles in non-diseased plan tissues. Plant Physiol Biochem **38**: 125-140
Eddy SR (1998) Profile hidden Markov models. Bioinformatics **14**: 755-763
Edwards ME, Dickson CA, Chengappa S, Sidebottom C, Gidley MJ, Reid JSG (1999). Molecular characterization of a membrane-bound galactosyltransferase of plant cell wall matrix polysaccharide biosynthesis. Plant J **19**: 691-697
Ellis C, Karafyllidis I, Wasternack C, Turner JG (2002) The *Arabidopsis* mutant cev1 links cell wall signaling to jasmonate and ethylene responses. Plant Cell **14**: 1557-1566
Emanuelsson O (2002) Predicting protein subcellular localisation from amino acid sequence information. Brief. Bioinform **3**: 361-376
Emanuelsson O, Nielsen H, Brunak S, von Heijne G (2000) Predicting subcellular localization of proteins based on their N- terminal amino acid sequence. J Mol Biol **300**: 1005-1016
Emanuelsson O, Nielsen H, von Heijne G (1999) ChloroP, a neural network-based method for predicting chloroplast transit peptides and their cleavage sites. Protein Sci **8**: 978-984

Fagard M, Boutet S, Morel JB, Bellini C, Vaucheret H (2000) AGO1, QDE-2, and RDE-1 are related proteins required for post- transcriptional gene silencing in plants, quelling in fungi, and RNA interference in animals. Proc Natl Acad Sci USA **97**: 11650-11654

Fagard M, Höfte H, Vernhettes S (2000) Cell wall mutants. Plant Physiol Biochem **38**: 15–25

Faik A, Price NJ, Raikhel NV, Keegstra K (2002) An *Arabidopsis* gene encoding an alpha-xylosyltransferase involved in xyloglucan biosynthesis. Proc Natl Acad Sci USA **99**:7797-7802

Feingold DS, Avigad G (1980) Sugar nucleotide transformations in plants. In PK Stumpf, EE Conn, eds Carbohydrates-structure and function. Academic Press, New York, pp 101-170

Felsenstein J (2004) PHYLIP (Phylogeny Inference Package). [3.6b] Distributed by the author.

Ferrandiz C, Pelaz S, Yanofsky MF (1999) Control of carpel and fruit development in *Arabidopsis*. Annu Rev Biochem **68**: 321-354

Fincher GB, Stone BA, Clarke AE (1983) Arabinogalactan proteins: structure, biosynthesis and function. Annu Rev Plant Physiol **34**: 47-70

Franco TT, Rodrigues NR, Serra GE, Panegassi VR, Buckeridge MS (1996) Characterization of storage cell wall polysaccharides from Brazilian legume seeds and the formation of aqueous two-phase systems. J Chromatogr B Biomed Appl **680**:255-261.

Frey-Wyssling A (1976) The Plant Cell Wall. Gebrüder Borntraeger, Berlin

Fry SC (1988) The Growing Plant Cell Wall: Chemical and Metabolic Analysis. Wiley, New York

Galway ME, Masucci JD, Lloyd AM, Walbot V, Davis RW, Schiefelbein JW (1994) The *TTG* gene is required to specify epidermal cell fate and cell patterning in the *Arabidopsis* root. Dev Biol **166**: 740-754

Gao M, Showalter AM (1999) Yariv reagent treatment induces programmed cell death in *Arabidopsis* cell cultures and implicates arabinogalactan protein involvement. Plant J **19**: 321-31

Gillmor CS, Poindexter P, Lorieau J, Palcic MM, Somerville C (2002) Alpha-glucosidase I is required for cellulose biosynthesis and morphogenesis in *Arabidopsis*. J Cell Biol **156**: 1003-1013

Gipmans M (2001) Molecular genetic approaches to study cell wall biosynthesis in *Arabidopsis thaliana*. PhD thesis, University of Potsdam

Giraud MF, Gordon FM, Whitfield C, Messner P, McMahon SA, Naismith JH (1999) Purification, crystallization and preliminary structural studies of dTDP-6-deoxy-D-xylo-4-hexulose 3,5-epimerase (RmlC), the third enzyme of the dTDP-L-rhamnose synthesis pathway, from *Salmonella enterica* serovar typhimurium. Acta Crystallogr D Biol Crystallogr **55 (Pt 3)**: 706-708

Giraud MF, Leonard GA, Field RA, Berlind C, Naismith JH (2000) RmlC, the third enzyme of dTDP-L-rhamnose pathway, is a new class of epimerase. Nat Struct Biol **7**: 398-402

Giraud MF, Naismith JH (2000) The rhamnose pathway. Curr Opin Struct Biol **10**: 687-696

Golovkin M, Reddy AS (2003) A calmodulin-binding protein from *Arabidopsis* has an essential role in pollen germination. Proc Natl Acad Sci USA **100**: 10558-10563

Goto N (1985) A mucilage polysaccharide secreted from *testa* of *Arabidopsis thaliana*. Arabidopsis Inf Serv **22**: 1-4

Goubet F, Morriswood B, Dupree P (2003) Analysis of methylated and unmethylated polygalacturonic acid structure by polysaccharide analysis using carbohydrate gel electrophoresis. Anal Biochem **321**: 174-182

Gouet P, Courcelle E, Stuart DI, Metoz F (1999) ESPript: analysis of multiple sequence alignments in PostScript. Bioinformatics **15**: 305-308

Goujon T, Minic Z, El Amrani A, Lerouxel O, Aletti E, Lapierre C, Joseleau JP, Jouanin L (2003) AtBXL1, a novel higher plant (*Arabidopsis thaliana*) putative β- xylosidase gene, is involved in secondary cell wall metabolism and plant development. Plant J **33**: 677-690

Graninger M, Kneidinger B, Bruno K, Scheberl A, Messner P (2002) Homologs of the Rml enzymes from *Salmonella enterica* are responsible for dTDP-β-L-rhamnose biosynthesis in the gram-positive thermophile *Aneurinibacillus thermoaerophilus* DSM 10155. Appl Environ Microbiol **68**: 3708-3715

Graninger M, Nidetzky B, Heinrichs DE, Whitfield C, Messner P (1999) Characterization of dTDP-4-dehydrorhamnose 3,5-epimerase and dTDP-4- dehydrorhamnose reductase, required for dTDP-L-rhamnose biosynthesis in *Salmonella enterica* serovar Typhimurium LT2. J Biol Chem **274** : 25069-25077

Grignon C, Sentenac H (1991) pH and ionic conditions in the apoplast. Annual Review of Plant Physiology and Plant Molecular Biology **42**: 102-125

Guex N, Peitsch MC (1996) Swiss-PdbViewer: A Fast and Easy-to-use PDB Viewer for Macintosh and PC. Protein Data Bank Quaterly Newsletter 77, pp. 7.

Guex N (1996) Swiss-PdbViewer: A new fast and easy to use PDB viewer for the Macintosh. Experientia **52**: A26

Guex N, Peitsch MC (1997) SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. Electrophoresis **18**: 2714-2723

Guillen R, York WS, Pauly M, An J, Impallomeni G, Albersheim P, Darvill AG (1995) Metabolism of xyloglucan generates xylose-deficient oligosaccharide subunits of this polysaccharide in etiolated peas. Carbohydr Res **277**: 291-311

Guilley H, Dudley RK, Jonard G, Balazs E, Richards KB (1982) Transcription of cauliflower mosaic virus DNA; Detection of promoter sequences and characterization of transcripts. Cell **30**: 763-773.

Hadi MZK, Wendeler E, Reiss B (2002) Simple and versatile selection of *Arabidopsis* transformants. Plant Cell Rep **21**: 130-135

Han Y, Griffiths A, Li H, Grierson D (2004) The effect of endogenous mRNA levels on co-suppression in tomato. FEBS Lett **563**: 123-128

Hanahan, D (1983) Studies on transformation of bacteria with plasmids. J Mol Biol **166** :557-580

Handford MG, Baldwin TC, Goubet F, Prime TA, Miles J, Yu X, Dupree P (2003) Localisation and characterisation of cell wall mannan polysaccharides in *Arabidopsis thaliana*. Planta **218**: 27-36

Hanke DE, Northcote DH (1975) Molecular visualization of pectin and DNA by ruthenium red. Biopolymers **14**: 1-17

Harper AD, Bar-Peled M (2002) Biosynthesis of UDP-Xylose. Cloning and Characterization of a Novel *Arabidopsis* Gene Family, UXS, Encoding Soluble and Putative Membrane- Bound UDP-Glucuronic Acid Decarboxylase Isoforms. Plant Physiol **130**: 2188-2198

Hayashi T, Baba K, Ogawa K (1994) Macromolecular complexes of xyloglucan and cellulose obtained by annealing. Plant Cell Physiol **35**: 219-223

He L, Bickerstaff GF, Paterson A, Buswell JA (1994) Evaluation of catalytic activity and synergism between two xylanase isoenzymes in enzymic hydrolysis of two separate xylans in different states of solubility. Enzyme Microb Technol **16**:696-702

Heim DR, Skomp JR, Tschabold EE, Larrinua IM (1990). Isoxaben inhibits the synthesis of acid insoluble cell wall materials in *Arabidopsis thaliana*. Plant Physiol **93**: 695-700

Henrissat B, Coutinho PM, Davies GJ (2001) A census of carbohydrate-active enzymes in the genome of *Arabidopsis thaliana*. Plant Mol Biol **47**: 55-72

Heyer GH, Lloyd JR, Kossmann J (1999). Production of modified polymeric carbohydrates. Curr Opin Biotechnol **10**: 169-174

Hinterberg B, Klos C, Tenhaken R (2002) Recombinant UDP-glucose dehydrogenase from soybean. Plant Physiol Biochem **40**: 1011-1017

Hirose K, Endo K, Hasegawa K (2004) A convenient synthesis of lepidimoide from okra mucilage and its growth-promoting activity in hypocotyls. Carbohydr Res **339**: 9-19

His I, Driouich A, Nicol F, Jauneau A, Hofte H (2001) Altered pectin composition in primary cell walls of korrigan, a dwarf mutant of *Arabidopsis* deficient in a membrane-bound endo-1,4-β-glucanase. Planta **212**: 348-358

Hoth S, Morgante M, Sanchez JP, Hanafey MK, Tingey SV, Chua NH (2002) Genome-wide gene expression profiling in *Arabidopsis thaliana* reveals new targets of abscisic acid and largely impaired gene regulation in the abi1-1 mutant. J Cell Sci **115**: 4891-4900

Höfgen R, Willmitzer L (1990) Biochemical and genetic analysis of different patatin isoforms expressed in various organs of potato *Solanum tuberosum*. Plant Sci **66**, 221-230

Hughes J, McCully ME (1975) The use of an optical brightener in the study of plant structure. Stain Technol **50** : 319-329

Hulskamp M, Kopczak SD, Horejsi TF, Kihl BK, Pruitt RE (1995) Identification of genes required for pollen-stigma recognition in *Arabidopsis thaliana*. Plant J **8**: 703-714

Huttermann A, Mai C, Kharazipour A (2001) Modification of lignin for the production of new compounded materials. Appl Microbiol Biotechnol **55**: 387-94.

Ishii T (1995) Pectic polysaccharides from bamboo shoot cell walls. Mokuzai Gakkaishi 41: 669–676.

Ishii T (1997) *O*-acetylated oligosaccharides from pectins of potato tuber cell walls. Plant Physiol **113**: 1265–1272.

Ishii T, Matsunga T (2001) Pectic polysaccharides rhamnogalacturonan II is covalently linked to homogalacturonan. Phytochemistry **57**:969-974

Iwai H, Masaoka N, Ishii T, Satoh S (2002) A pectin glucuronyltransferase gene is essential for intercellular attachment in the plant meristem. Proc Natl Acad Sci USA **99**: 16319-16324

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J **6**: 3901-3907

Jencks WP (1976) In Handbook of Biochemistry and Molecular Biology G. Fasman Ed, 3rd ed., Vol I, pp. 302-303. Chem Rubber Publ Co, Cleveland, OH

Jenkins J, Mayans O, Smith D, Worboys K, Pickersgill RW (2001) Three-dimensional structure of *Erwinia chrysanthemi* pectin methylesterase reveals a novel esterase active site. J Mol Biol **305**: 951-960

Johanson H, Sterky F, Amini B, Lundeberg J, Kleczkowski LA (2002) Molecular cloning and characterization of a cDNA encoding poplar UDP-glucose dehydrogenase, a key gene of hemicellulose/pectin formation. Biochem Biophys Acta **1576**: 53-58
Johnson KL, Jones BJ, Bacic A, Schultz CJ (2003) The fasciclin-like arabinogalactan proteins of *Arabidopsis*. A multigene family of putative cell adhesion molecules. Plant Physiol **133**: 1911-1925
Johnson CS, Kolevski B, Smyth DR (2002) *TRANSPARENT TESTA GLABRA2*, a trichome and seed coat development gene of *Arabidopsis*, encodes a WRKY transcription factor. Plant Cell **14**: 1359-1375
Jones L, McQueen-Mason S (2004) A role for expansins in dehydration and rehydration of the resurrection plant *Craterostigma plantagineum*. FEBS Lett **559**: 61-65
Jones L, Milne JL, Ashford D, McQueen-Mason SJ (2003) Cell wall arabinan is essential for guard cell function. Proc Natl Acad Sci USA **100**: 11783-11788
Jones P, Messner B, Nakajima J, Schaffner AR, Saito K (2003) UGT73C6 and UGT78D1, glycosyltransferases involved in flavonol glycoside biosynthesis in *Arabidopsis thaliana*. J Biol Chem **278**: 43910-43918
Kalamaki MS, Powell AL, Struijs K, Labavitch JM, Reid DS, Bennett AB (2003) Transgenic overexpression of expansin influences particle size distribution and improves viscosity of tomato juice and paste. J Agric Food Chem **51**: 7465-71
Kamsteeg J, van Brederode J, Kuipers-Tierie E, van Nigtevecht G (1979) A modified procedure for the preparation of UDP-β-L-(U-14C)rhamnose. Prep Biochem **9**: 457-464
Kamsteeg J, van Brederode J, van Nigtevecht G (1978) The formation of UDP-L-rhamnose from UDP-D-glucose by an enzyme preparation of red campion (*Silene dioica* (L) Clairv) leaves. FEBS Lett **91**: 281-284
Kanter U, Becker M, Friauf E, Tenhaken R (2003) Purification, characterization and functional cloning of inositol oxygenase from *Cryptococcus* Yeast **20**: 1317-1329
Kartel MT, Kupchik LA, Veisov BK (1999) Evaluation of pectin binding of heavy metal ions in aqueous solutions. Chemosphere **38**: 2591-2596
Kawabata T, Nishikawa K (2000) Protein structure comparison using the markov transition model of evolution. Proteins **41**: 108-122
Keegstra K, Raikhel N (2001) Plant glycosyltransferases. Curr Opin Plant Biol **4**: 219-224
Keller R, Brearley CA, Trethewey RN, Müller-Röber B (1998) Reduced inositol content and altered morphology in transgenic potato plants inhibited for 1D-myo-inositol 3-phosphate synthase. Plant J **16**: 403-410
King CD, Rios GR, Green MD, Tephly TR (2000) UDP-glucuronosyltransferases. Curr Drug Metab **1**:143-61
Kleiger G, Eisenberg D (2002) GXXXG and GXXXA motifs stabilize FAD and NAD(P)-binding Rossmann folds through C(alpha)-H... O hydrogen bonds and van der waals interactions. J Mol Biol **323**: 69-76
Komalavilas P, Mort AJ (1989) The acetylation at O-3 of galacturonic acid in the rhamnose-rich region pectins. Carbohydr Res **189**: 261-272
Koncz C, Schell J (1986) The promoter of TL-DNA gene 5 controls the tissue-specific expression of chimaeric genes carried by a novel type of *Agrobacterium* binary vector. Mol Gen Genet **204**: 383-396
Kreuger M, van Holst GJ (1993) Arabinogalactan proteins are essential in somatic embryogenesis in *Daucus carota* L. Planta **189**: 243-248
Krogh A, Larsson B, von Heijne G, Sonnhammer EL (2001) Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. J Mol Biol **305**: 567-580
Kuschinsky AM (2002) Functional analysis of *RHM2,* a gene possibly involved in the synthesis of UDP-L-rhamnose in *Arabidopsis thaliana.* Diploma Thesis, Technical University Berlin
Labavitch JM, Ray PM (1978) Relationship between promotion of xyloglucan metabolism and induction by IAA Plant Physiol **54**: 499-502
Labesse G, Vidal-Cros A, Chomilier J, Gaudry M, Mornon JP (1994) Structural comparisons lead to the definition of a new superfamily of NAD(P)(H)-accepting oxidoreductases: the single-domain reductases/ epimerases/ dehydrogenases (the 'RED' family). Biochem J **304**: 95-99
Lahaye M, Rondeau-Mouro C, Deniaud E, Buleon A (2003)Solid-state 13C NMR spectroscopy studies of xylans in the cell wall of *Palmaria palmata* (L. Kuntze, Rhodophyta) Carbohydr Res **338**:1559-69.
Lai-Kee-Him J, Chanzy H, Muller M, Putaux JL, Imai T, Bulone V (2002) In vitro versus in vivo cellulose microfibrils from plant primary wall synthases: structural differences. J Biol Chem **277**: 36931-36939
Langley KR, Martin A, Stenning R, Murray AJ, Hobson GE, Schuch WW, Bird CR (1994) Mechanical and optical assessment of the ripening of tomato fruit with reduced polygalacturonase activity. J Sci Food and Agriculture **66**: 547-554
Lapasin R, Pricl S (1995) The Rheology of Industrial Polysaccharides. Theory and Applications. London: Blackie
Larkin JC, Walker JD, Bolognesi-Winfield AC, Gray JC, Walker AR (1999) Allele-specific interactions between

ttg and gl1 during trichome development in *Arabidopsis thaliana*. Genetics **151**: 1591-1604

Lau JM, McNeill M, Darvill AG, Albersheim P (1985) Structure of the backbone of rhamnogalacturonan I. A pectic polysaccharide in the primary cell wall of plants. Carbohydr Res **137**: 111-125

Lazar K, Walker S (2002) Substrate analogues to study cell-wall biosynthesis and its inhibition. Curr Opin Chem Biol **6**: 786-793

Lehrach H, Diamond D, Wozney JM, Boedtker H (1977) RNA molecular weight determinations by gel electrophoresis under denaturing conditions, a critical re-examination. Biochemistry **16**: 4743-4751

Lenartowska M, Rodriguez-Garcia MI, Bednarska E (2001) Immunocytochemical localization of esterified and unesterified pectins in unpollinated and pollinated styles of *Petunia hybrida* Hort. Planta **213**: 182-191

Letunic I, Copley RR, Schmidt S, Ciccarelli FD, Doerks T, Schultz J, Ponting CP, Bork P (2004) SMART 4.0: towards genomic data integration. Nucleic Acids Res **32**: D142-144

Levy S, Maclachlan G, Staehlin LA (1997) Xyloglucan sidechains modulate binding to cellulose during in vitro binding assays as predicted by conformational dynamics simulations. Plant J **11**: 373-386

Li Y, Jones L, McQueen-Mason S (2003) Expansins and cell growth. Curr Opin Plant Biol **6**: 603-610

Li X, Cordero I, Caplan J, Molhoj M, Reiter WD (2004) Molecular analysis of 10 coding regions from *Arabidopsis* that are homologous to the MUR3 xyloglucan galactosyltransferase. Plant Physiol **134**: 940-50.

Lide DR (1999) Handbook of Chemisty and Physics 79th edition CRC Press, Boca Raton, FL

Liljebjelke K, Adolphson R, Baker K, Doong RL, Mohnen D (1995) Enzymatic synthesis and purification of uridine diphosphate [14C]galacturonic acid: a substrate for pectin biosynthesis. Anal Biochem **225**: 296-304

Liu H, Sachidanandam R, Stein L (2001) Comparative genomics between rice and *Arabidopsis* shows scant collinearity in gene order. Genome Res **11**: 2020-2026

Loewus F, Dickinson DB (1982) Cyclitols. In: *Plant Carbohydrates* (eds. Loewus, F. and Tanner, W.). Springer-Verlag, Berlin, pp. 193-216

Lolle SJ, Berlyn GP, Engstrom EM, Krolikowski KA, Reiter WD, Pruitt RE (1997) Developmental regulation of cell interactions in the *Arabidopsis fiddlehead-1* mutant: a role for the epidermal cell wall and cuticle. Dev Biol **189**: 311-321

Lord E (2000) Adhesion and cell movement during pollination: Cherchez la femme. Trends Plant Sci **5**: 368-373

Lord EM (2003) Adhesion and guidance in compatible pollination. J Exp Bot **54**: 47-54

Lorence A, Chevone BI, Mendes P, Nessler CL (2004) myo-inositol oxygenase offers a possible entry point into plant ascorbate biosynthesis. Plant Physiol **134**: 1200-1205

Lu F, Ralph J (2003) Non-degradative dissolution and acetylation of ball-milled plant cell walls: high-resolution solution-state NMR. Plant J **35**: 535-544

Lukowitz W, Nickle TC, Meinke DW, Last RL, Conklin PL, Somerville CR. (2001) Arabidopsis *cyt1* mutants are deficient in a mannose-1-phosphate guanylyltransferase and point to a requirement of N-linked glycosylation for cellulose biosynthesis. Proc Natl Acad Sci U S A. **98**:2262-2267

Madduri K, Waldron C, Merlo DJ (2001) Rhamnose biosynthesis pathway supplies precursors for primary and secondary metabolism in Saccharopolyspora spinosa. J Bacteriol **183**: 5632-5638

Madson M, Dunand C, Li X, Verma R, Vanzin GF, Caplan J, Shoue DA, Carpita NC, Reiter WD (2003) The *MUR3* gene of *Arabidopsis* encodes a xyloglucan glycosyltransferase that is evolutoniary related to animal exostosins. Plant Cell **15**:1662-670

Malinowski R, Filipecki M (2002) The Role of Cell Wall in Plant Embryogenesis. Cellular & Molecular Biology Letters **7**: 1137-1151

Mankarios AT, Jones CFG, Jarvis MC, Threlfall DR, Friend J (1979) Hydrolysis of plant polysaccharides and glc analysis of their constituent neutral sugars. Phytochemistry **18**:419-422

Marchler-Bauer A, Anderson JB, DeWeese-Scott C, Fedorova ND, Geer LY, He S, Hurwitz DI, Jackson JD, Jacobs AR, Lanczycki CJ, Liebert CA, Liu C, Madej T, Marchler GH, Mazumder R, Nikolskaya AN, Panchenko AR, Rao BS, Shoemaker BA, Simonyan V, Song JS, Thiessen PA, Vasudevan S, Wang Y, Yamashita RA, Yin JJ, Bryant SH (2003) CDD: a curated Entrez database of conserved domain alignments. Nucleic Acids Res **31**: 383-387

Matar D, Catesson AM (1988). Cell plate development and delayed formation of the pectic middle lamella in root meristems. Protoplasma **146**: 10-17

Matern U, Griesebach H (1977) UDP-apiose/UDP-xylose synthase. Eur J Biochem **74**: 303-312

Mato M, Ozeki Y, Itoh Y, Higeta D, Yoshitama K, Teramoto S, Aida R, Ishikura N, Shibata M (1998) Isolation and characterization of a cDNA clone of UDP-galactose: flavonoid 3-O-galactosyltransferase (UF3GaT) expressed in *Vigna* mungo seedlings. Plant Cell Physiol **39**: 1145-1155

Mauro ML, De Lorenzo G, Costantino P, Bellincampi D (2002a) Oligogalacturonides inhibit the induction of late

but not of early auxin-responsive genes in tobacco. Planta **215**: 494-501
Meier H(1985) Localization of polysaccharides in wood cell walls. In: T. Higuchi, Editor, Biosynthesis and Biodegradation of Wood Components, Academic Press, New York, pp. 43–50.
McCann MC, Bush M, Milioni D, Sado P, Stacey NJ, Catchpole G, Defernez M, Carpita NC, Höfte H, Ulvskov P, Wilson RH, Roberts K (2001) Approaches to understanding the functional architecture of the plant cell wall. Phytochemistry **57**: 811-821
McCartney L, Knox JP (2002) Regulation of pectic polysaccharide domains in relation to cell development and cell properties in the pea testa. J Exp Bot **53**: 707-713
McCartney L, Steele-King CG, Jordan E, Knox JP (2003) Cell wall pectic (1-->4)-β-D-galactan marks the acceleration of cell elongation in the *Arabidopsis* seedling root meristem. Plant J **33** : 447-454
McNeil M, Darvill AG, Albersheim P (1980) Plant Physiol **66**: 1128–1134
Mette MF, Aufsatz W, van der WJ, Matzke MA, Matzke AJ (2000) Transcriptional silencing and promoter methylation triggered by double- stranded RNA. EMBO J **19**: 5194-5201
Moffatt BA, McWhinnie EA, Agarwal SK, Schaff DA (1994) The adenine phosphoribosyltransferase-encoding gene of *Arabidopsis thaliana*. Gene **143**: 211-216
Mohnen D (1999) Biosynthesis of pectins and galactomannans. In BM Pinto, ed Carbohydrates and Their Derivatives Including Tannins, Cellulose, and Related Lignins Vol 3. Elsevier, Oxford, pp 497-527
Mølhøj M, Johansen B, Ulvskov P, Borkhardt B (2001a) Expression of a membrane-anchored endo-1,4-β-glucanase from Brassica napus, orthologous to KOR from Arabidopsis thaliana, is inversely correlated to elongation in light-grown plants. Plant Mol Biol **45**: 93-105
Mølhøj M, Ulvskov P, Dal Degan F (2001b) Characterization of a functional soluble form of a Brassica napus membrane-anchored endo-1,4-β-glucanase heterologously expressed in Pichia pastoris. Plant Physiol **127**: 674-684
Mølhøj M, Verma R, Reiter WD (2003) The biosynthesis of the branched-chain sugar d-apiose in plants: functional cloning and characterization of a UDP-d-apiose/UDP-d-xylose synthase from *Arabidopsis*. Plant J **35**: 693-703
Mollet JC, Park SY, Nothnagel EA, Lord EM (2000) A lily stylar pectin is necessary for pollen tube adhesion to an in vitro stylar matrix. Plant Cell **12**: 1737-1750
Morel JB, Mourrain P, Beclin C, Vaucheret H (2000) DNA methylation and chromatin structure affect transcriptional and post- transcriptional transgene silencing in Arabidopsis. Curr Biol **10**: 1591-1594
Mulder NJ, Apweiler R, Attwood TK, Bairoch A, Barrell D, Bateman A, Binns D, Biswas M, Bradley P, Bork P, Bucher P, Copley RR, Courcelle E, Das U, Durbin R, Falquet L, Fleischmann W, Griffiths-Jones S, Haft D, Harte N, Hulo N, Kahn D, Kanapin A, Krestyaninova M, Lopez R, Letunic I, Lonsdale D, Silventoinen V, Orchard SE, Pagni M, Peyruc D, Ponting CP, Selengut JD, Servant F, Sigrist CJA, Vaughan R, Zdobnov EM. (2003) The InterPro Database, 2003 brings increased coverage and new features. Nucleic Acids Res **31**:315-318.
Mulichak AM, Bonin CP, Reiter WD, Garavito RM (2002) Structure of the MUR1 GDP-Mannose 4,6-Dehydratase from Arabidopsis thaliana: Implications for Ligand Binding and Specificity. Biochemistry **41**: 15578-15589
Muños R, Lopez R, de Frutos M, Garcia E (1999) First molecular characterization of a uridine diphosphate galacturonate 4-epimerase: an enzyme required for capsular biosynthesis in Streptococcus pneumoniae type 1. Mol Microbiol **31**: 703-713
Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologica Plantarum **15**: 473-497
Murray MG, Thompson WF (1980) Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res **8**: 4321-4325
Nakai K, Kanehisa M (1992) A knowledge base for predicting protein localization sites in eukaryotic cells, Genomics **14**: 897-911.
Nakayama KI, Maeda Y, Jigami Y (2003) Interaction of GDP-4-keto-6-deoxymannose-3,5-epimerase-4-reductase with GDP-mannose-4,6-dehydratase stabilizes the enzyme activity for formation of GDP-fucose from GDP-mannose. Glycobiology **13**: 673R–680R
Needleman SB, Wunsch CD (1970) A general method applicable to the search for similarities in the amino acid sequence of two proteins. J Mol Biol **48**: 443-453
Neish AC (1958) The biosynthesis of cell wall carbohydrates. IV. Further studies on cellulose and xylan in wheat. Can J Biochem Physiol **36**:187-193
Nesi N, Debeaujon I, Jond C, Stewart AJ, Jenkins GI, Caboche M, Lepiniec L (2002) The TRANSPARENT TESTA16 locus encodes the ARABIDOPSIS BSISTER MADS domain protein and is required for proper development and pigmentation of the seed coat. Plant Cell **14**: 2463-2479
Nickle TC, Meinke DW (1998) A cytokinesis-defective mutant of *Arabidopsis* (*cyt1*) characterized by embryonic lethality, incomplete cell walls, and excessive callose accumulation. Plant J **15**:321-32.

Nicol F, His I, Jauneau A, Vernhettes S, Canut H, Höfte H (1998) A plasma membrane-bound putative endo-1,4-β-D-glucanase is required for normal wall assembly and cell elongation in *Arabidopsis*. EMBO J **17**: 5563-5576
Nielsen H, Engelbrecht J, Brunak S, von Heijne G (1997) A neural network method for identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Int J Neural Syst **8**: 581-599
Nishimura MT, Stein M, Hou BH, Vogel JP, Edwards H, Somerville SC. (2003) Loss of a callose synthase results in salicylic acid-dependent disease resistance. Science **301**:969-72.
Norambuena L, Marchant L, Berninsone P, Hirschberg CB, Silva H, Orellana A (2002) Transport of UDP-galactose in plants. Identificationand functional characterization of AtUTR1, an *Arabidopsis thaliana* UDP-galactose/UDP-glucose transporter. J Biol Chem **277**:32923-32929
O'Brien TP, Feder N, McCully ME (1964) Polychromatic Staining of Plant Cell Walls by Toluidine Blue O. Protoplasma **59**: 367-373
O'Connell A, Holt K, Piquemal J, Grima-Pettenati J, Boudet A, Pollet B, Lapierre C, Petit-Conil M, Schuch W, Halpin C (2002) Improved paper pulp from plants with suppressed cinnamoyl-CoA reductase or cinnamyl alcohol dehydrogenase. Transgenic Res **11**: 495-503.
O'Neill MA, Albersheim P, Darvill A (1990) The pectic polysaccharides of primary cell walls. In: Methods in Plant Biochemistry, Vol. 2 (D.M. Dey, ed.), pp. 415-441. Academic Press, London.
O'Neill MA, Eberhard S, Albersheim P, Darvill AG (2001) Requirement of borate cross-linking of cell wall rhamnogalacturonan II for *Arabidopsis* growth. Science **294**: 846-849
O'Neill MA, Ishii T, Albersheim P, Darvill AG (2004) RHAMNOGALACTURONAN II: Structure and Function of a Borate Cross-Linked Cell Wall Pectic Polysaccharide. Annu Rev Plant Physiol Plant Mol Biol **55**: 109-139
Oomen RJ, Doeswijk-Voragen CH, Bush MS, Vincken JP, Borkhardt B, van den Broek LA, Corsar J, Ulvskov P, Voragen AG, McCann MC, Visser RG (2002) In muro fragmentation of the rhamnogalacturonan I backbone in potato (*Solanum tuberosum* L.) results in a reduction and altered location of the galactan and arabinan side-chains and abnormal periderm development. Plant J **30**: 403-413
Orellana A, Mohnen D (1999) Enzymatic synthesis and purification of [(3)H]uridine diphosphate galacturonic acid for use in studying Golgi-localized transporters. Anal Biochem **272**: 224-231
Orsel M, Krapp A, Daniel-Vedele F (2002) Analysis of the NRT2 nitrate transporter family in *Arabidopsis*. Structure and gene expression. Plant Physiol **129**: 886-896
Papp I, Mette MF, Aufsatz W, Daxinger L, Schauer SE, Ray A, van der WJ, Matzke M, Matzke AJ (2003) Evidence for nuclear processing of plant micro RNA and short interfering RNA precursors. Plant Physiol **132**: 1382-1390
Park YW, Tominaga R, Sugiyama J, Furuta Y, Tanimoto E, Samejima M, Sakai F, Hayashi T (2003) Enhancement of growth by expression of poplar cellulase in *Arabidopsis thaliana*. Plant J **33**: 1099-1106
Patra HK, Sen NK, Sarangi C, Mishra D (1985) Acid phosphatase and pyrophosphatases in leaves during development and senescence. Beitr Biol-Pflanz. Berlin **60**: 1-13
Paton AW, Paton JC (1999) Molecular characterization of the locus encoding biosynthesis of the lipopolysaccharide O antigen of *Escherichia coli* serotype O113. Infect Immun **67**: 5930-5937
Pauly M (2003) Im Dschungel der Zucker Genome Express 01/03
Pauly M, Albersheim P, Darvill A, York WS (1999) Molecular domains of the cellulose/xyloglucan network in the cell walls of higher plants. Plant J **20**: 629-639
Pauly M, Eberhard S, Albersheim P, Darvill A, York WS (2001a) Effects of the *mur1* mutation on xyloglucans produced by suspension- cultured *Arabidopsis thaliana* cells. Planta **214**: 67-74
Pauly M, Qin Q, Greene H, Albersheim P, Darvill A, York WS (2001b) Changes in the structure of xyloglucan during cell elongation. Planta **212**: 842-850
Pauly M, Scheller HV (2000) O-Acetylation of plant cell wall polysaccharides: identification and partial characterization of a rhamnogalacturonan O-acetyl-transferase from potato suspension-cultured cells. Planta **210**: 659-667
Pedersen JS, Hein J (2003) Gene finding with a hidden Markov model of genome structure and evolution. Bioinformatics **19**: 219
Peña MJ, Ryden P, Madson M, Smith AC, Carpita NC (2004) The Galactose Residues of Xyloglucan Are Essential to Maintain Mechanical Strength of the Primary Cell Walls in *Arabidopsis* during Growth. Plant Physiol **134**: 443-451
Peng L, Kawagoe Y, Hogan P, Delmer D (2002) Sitosterol-beta-glucoside as primer for cellulose synthesis in plants Science **295**: 147-150
Penfield S, Meissner RC, Shoue DA, Carpita NC, Bevan MW (2001) *MYB61* is required for mucilage deposition and extrusion in the *Arabidopsis* seed coat. Plant Cell **13**: 2777-2791
Pennell RI, Roberts K (1990) Sexual development in pea is presaged by altered expression of arabingalactan protein.

Nature **344**: 547-549
Perrin RM, DeRocher AE, Bar-Peled M, Zeng W, Norambuena L, Orellana A, Raikhel NV, Keegstra K (1999) Xyloglucan fucosyltransferase, an enzyme involved in plant cell wall biosynthesis. Science **284**: 1976-1979.
Perrin R, Wilkerson C, Keegstra K (2001) Golgi enzymes that synthesize plant cell wall polysaccharides: finding and evaluating candidates in the genomic era. Plant Mol Biol **47**: 115-130
Pilling E, Höfte H (2003) Feedback from the wall Curr Opin Plant Biol **6**: 611-616
Powell AL, Kalamaki MS, Kurien PA, Gurrieri S, Bennett AB (2003) Simultaneous transgenic suppression of *LePG* and *LeExp1* influences fruit texture and juice viscosity in a fresh market tomato variety. J Agric Food Chem **51**: 7450-7455
Prade RA, Zhan D, Ayoubi P, Mort AJ (1999) Pectins, pectinases and plant-microbe interactions. Biotechnol Genet Eng Rev **16**: 361–391
Quackenbush J, Cho J, Lee D, Liang F, Holt I, Karamycheva S, Parivizi B, Pertea G, Sultana R, White J (2001) The TIGR Gene Indices: analysis of gene transcript sequences in highly sampled eukaryotic species. Nucleic Acids Res **29**: 159-164
Quirino BF, Reiter W-D, Amasino RD (2001) One of two tandem *Arabidopsis* genes homologous to monosaccharide transporters is senescence-associated. Plant Mol Biol **46**: 447-457
Rabina J, Maki M, Savilahti EM, Jarvinen N, Penttila L, Renkonen R (2001) Analysis of nucleotide sugars from cell lysates by ion-pair solid-phase extraction and reversed-phase high-performance liquid chromatography. Glycoconj J **18**: 799-805
Radzio JA, Lorence A, Chevone BI, Nessler CL (2003) L-Gulono-1,4-lactone oxidase expression rescues vitamin C-deficient *Arabidopsis* (*vtc*) mutants. Plant Mol Biol **53**: 837-844
Raiola A, Camardella L, Giovane A, Mattei B, De Lorenzo G, Cervone F, Bellincampi D (2004) Two *Arabidopsis thaliana* genes encode functional pectin methylesterase inhibitors. FEBS Lett **557**: 199-203
Ramachandran GN, Ramakrishnan C, Sasisekharan V (1963) Stereochemistry of polypeptide chain configurations. J Mol Biol **7**: 95-99
Ray A, Langer M (2002) Homologous recombination: ends as the means. Trends Plant Sci **7**: 435-440
Rayon C, Cabanes-Macheteau M, Loutelier-Bourhis C, Salliot-Maire I, Lemoine J, Reiter W-D, Lerouge P, Faye L (1999) Characterization of N-glycans from *Arabidopsis*. Application to a fucose-deficient mutant. Plant Physiol **119**: 725-734
Reddy CC, Swan JS, Hamilton GA (1981) Myo-Inositol oxygenase from hog kidney. I. Purification and characterization of the oxygenase and of an enzyme complex containing the oxygenase and D-glucuronate reductase. J Biol Chem **256**: 8510–8518
Reich R (1993) Thermodynamik, VCH, 2. Auflage, Weinheim Germany
Reid JS, Edwards ME, Dickson CA, Scott C, Gidley MJ (2003) Tobacco transgenic lines that express fenugreek galactomannan galactosyltransferase constitutively have structurally altered galactomannans in their seed endosperm cell walls. Plant Physiol **131**: 1487-1495
Reiter WD (2002) Biosynthesis and properties of the plant cell wall. Curr Opin Plant Biol **5**: 536
Reiter WD, Chapple C, Somerville CR (1997) Mutants of *Arabidopsis thaliana* with altered cell wall polysaccharide composition. Plant J **12**: 335-345
Reiter WD, Vanzin GF (2001) Molecular genetics of nucleotide sugar interconversion pathways in plants. Plant Mol Biol **47**: 95-113
Rhee SY, Osborne E, Poindexter PD, Somerville CR (2003) Microspore separation in the *quartet* 3 mutants of *Arabidopsis* is impaired by a defect in a developmentally regulated polygalacturonase required for pollen mother cell wall degradation. Plant Physiol **133**: 1170-1180
Rhee SY, Somerville CR (1998) Tetrad pollen formation in quartet mutants of *Arabidopsis thaliana* is associated with persistence of pectic polysaccharides of the pollen mother cell wall. Plant J **15**: 79-88
Ridley BL, O'Neill MA, Mohnen DA (2001) Pectins: Structure, Biosynthesis, and Oligogalacturonide-Related Signaling. Phytochemistry **57**: 929-967.
Rihouey C, Morvan C, Borissova I, Jauneau A, Demarty M, Jarvis M (1995) Structural features of CDTA-soluble pectins from flax hypocoyls. Carbohydr Polym **28**: 159-166
Ringli C, Keller B, Ryser U (2001) Glycine-rich proteins as structural components of plant cell walls. Cell Mol Life Sci **58**: 1430-41
Rizzi M, Tonetti M, Vigevani P, Sturla L, Bisso A, Flora AD, Bordo D, Bolognesi M (1998) GDP-4-keto-6-deoxy-D-mannose epimerase/reductase from *Escherichia coli*, a key enzyme in the biosynthesis of GDP-L-fucose, displays the structural characteristics of the RED protein homology superfamily. Structure **6**: 1453-1465
Roberts RM (1967) The incorporations of 14C labelled D-glucuronate and D-galactose into segments of the root-tip

of corn. Phytochemistry **6**:525-533
Roberts RM (1968) The metabolism of L-fucose by sycamore (*Acer pseudoplatanus* L.) cell cultures. Arch Biochem Biophys **128**: 818-820
Roos C, Kolmer M, Mattila P, Renkonen R (2002) Composition of *Drosophila melanogaster* proteome involved in fucosylated glycan metabolism. J Biol Chem **277**: 3168-3175
Rose JKC, Braam J, Fry SC, Nishitani K (2002) The XTH family of enzymes involved in xyloglucan endotransglycosylation and endohydrolysis: current perspectives and a new unifying nomenclature. Plant Cell Physiol **43**: 1421-1435
Rosso MG, Li Y, Strizhov N, Reiss B, Dekker K, Weisshaar B (2003) An *Arabidopsis* thaliana T-DNA mutagenized population (GABI-Kat) for flanking sequence tag-based reverse genetics. Plant Mol Biol **53**: 247-59.
Rudd S, Mewes HW, Mayer KF (2003) Sputnik: a database platform for comparative plant genomics. Nucleic Acids Res **31**: 128-132
Ryden P, Sugimoto-Shirasu K, Smith AC, Findlay K, Reiter W-D, McCann MC (2003) Tensile properties of *Arabidopsis* cell walls depend on both a xyloglucan cross-linked microfibrillar network and rhamnogalacturonan II-borate complexes. Plant Physiol **132**: 1033-1040
Salnikov VV, Grimson MJ, Delmer DP, Haigler CH (2001) Sucrose synthase localizes to cellulose synthesis sites in tracheary elements. Phytochemistry **57**: 823-33.
Sambrok J, Fritsch EF, Maniatis T (1989) Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
Samuel G, Reeves P (2003) Biosynthesis of O-antigens: genes and pathways involved in nucleotide sugar precursor synthesis and O-antigen assembly. Carbohydr Res **338**: 2503-19
Santos HP, Purgatto E, Mercier H, Buckeridge M (2004) The Control of Storage Xyloglucan Mobilization in Cotyledons of *Hymenaea courbaril*. Plant Physiol 2004 **135**: 287-99.
Schweikert C, Liszkay A, Schopfer P (2000) Scission of polysaccharides by peroxidase-generated hydroxyl radicals. Phytochemistry **53**: 565-570
Schopfer P, Liszkay A, Bechthold M, Frahry G, Wagner A (2002) Evidence that hydroxyl radicals mediate auxin-induced extension growth. Planta **214**: 821-82
Schroeder JI, Kwak JM, Allen GJ (2001) Guard cell abscisic acid signalling and engineering drought hardiness in plants. Nature **410**: 327-330
Sedgley M (1979) Structural changes in the pollinated and unpollinated avocado stigma and style. J Cell Sci **38**: 49-60
Seifert GJ (2004) Nucleotide sugar interconversions and cell wall biosynthesis: How to bring the inside to the outside. Curr Opin Plant Biol **7**: 277-284
Seifert GJ, Barber C, Wells B, Dolan L, Roberts K (2002) Galactose biosynthesis in *Arabidopsis*: genetic evidence for substrate channeling from UDP-D-galactose into cell wall polymers. Curr Biol **12**: 1840-1845
Seifert GJ, Barber C, Wells B, Roberts K (2004) Growth regulators and the control of nucleotide sugar flux. Plant Cell **16**: 723-30
Seitz B, Klos C, Wurm M, Tenhaken R (2000) Matrix polysaccharide precursors in *Arabidopsis* cell walls are synthesized by alternate pathways with organ-specific expression patterns. Plant J **21**: 537-46.
Selvendran RR, March JF, Ring SG (1979) Determination of aldoses and uronic acid content of vegetable fiber. Anal Biochem **96**: 282-292
Séné CFB, McCann MC, Wilson RH, Grinter H (1994) Fourier-transform Raman and Fourier-transform infrared spectroscopy. An investigation of five higher plant cell walls and their components. Plant Physiol **106**: 1623-1631
Serpe MD, Nothnagel EA (1994) Effects of Yariv phenylglycosides on Rosa cell suspensions: evidence for the involvement of arabinogalactan-proteins in cell proliferation. Planta **193**: 542-550
Shani Z, Dekel M, Tsabary G, Shoseyov O (1997) Cloning and characterization of elongation specific endo-1,4-β-glucanase (cel1) from *Arabidopsis thaliana*. Plant Mol Biol **34**: 837-842
Shedletzky E, Shmuel M, Delmer DP, Lamport DTA (1990) Adaptation and growth of tomato cells on the herbicide 2,6-dichlorobenzonitrile (DCB) leads to the production of unique cell walls virtually lacking a cellulose-xyloglucan network. Plant Physiol **100**: 980-987
Sherson SM, Alford HL, Forbes SM, Wallace G, Smith SM (2003) Roles of cell-wall invertases and monosaccharide transporters in the growth and development of *Arabidopsis*. J Exp Bot **54**: 525-531
Shinohara N, Fukuda H (2002) Isolation of monoclonal antibodies recognizing rare and dominant epitopes in plant vascular cell walls by phage display subtraction. J Immunol Methods **264**: 187-194
Showalter AM (1993) Structure and function of plant cell wall proteins. Plant Cell **5**: 9-23
Showalter AM (2001) Arabinogalactan-proteins: structure, expression and function. Cell Mol Life Sci **58**:1399-1417
Skjøt M, Pauly M, Bush MS, Borkhardt B, McCann MC, Ulvskov P (2002) Direct interference with

rhamnogalacturonan I biosynthesis in Golgi vesicles. Plant Physiol **129**: 95-102
Smith TF, Waterman MS (1981) Identification of common molecular subsequences. J Mol Biol **147**: 195-197
Somerville CR, Bonetta D (2001) Plants as factories for technical materials. Plant Physiol **125**: 168-171
Sorensen MB, Mayer U, Lukowitz W, Robert H, Chambrier P, Jurgens G, Somerville C, Lepiniec L, Berger F (2002) Cellularisation in the endosperm of *Arabidopsis thaliana* is coupled to mitosis and shares multiple components with cytokinesis. Development **129**: 5567-5576
Sørensen SO, Pauly M, Bush M, Skjot M, McCann MC, Borkhardt B, Ulvskov P (2000) Pectin engineering: modification of potato pectin by *in vivo* expression of an endo-1,4-β-D-galactanase. Proc Natl Acad Sci USA **97**: 7639-7644
Stajich JE, Block D, Boulez K, Brenner SE, Chervitz SA, Dagdigian C, Fuellen G, Gilbert JG, Korf I, Lapp H, Lehvaslaiho H, Matsalla C, Mungall CJ, Osborne BI, Pocock MR, Schattner P, Senger M, Stein LD, Stupka E, Wilkinson MD, Birney E (2002) The bioperl toolkit: perl modules for the life sciences. Genome Res **12**: 1611-1618
Steffan W, Kovac P, Albersheim P, Darvill AG, Hahn MG (1995) Characterization of a monoclonal antibody that recognizes an arabinosylated (1-->6)-beta-D-galactan epitope in plant complex carbohydrates. Carbohydr Res **275**: 295-307
Stein A, Kula MR, Elling L (1998) Combined preparative enzymatic synthesis of dTDP-6-deoxy-4-keto-D- glucose from dTDP and sucrose. Glycoconj J **15**: 139-145
Stephenson MB, Hawes MC (1994) Correlation of Pectin Methylesterase Activity in Root Caps of Pea with Root Border Cell Separation. Plant Physiol **106**: 739-745
Stolle-Smits T, Beekhuizen JG, Kok MTC, Pijnenburg M, Recourt K, Derksen J, Voragen AJG (1999) Changes in Cell Wall Polysaccharides of Green Bean Pods during Development. Plant Physiol **121**: 363–372
Stolz J, Munro S (2002) The Components of the *Saccharomyces cerevisiae* Mannosyltransferase Complex M-Pol I Have Distinct Functions in Mannan Synthesis. J Biol Chem **277**: 44801-44808
Stuart JM, Segal E, Koller D, Kim SK (2003) A gene-coexpression network for global discovery of conserved genetic modules. Science **302**: 249-255
Szittya G, Silhavy D, Molnar A, Havelda Z, Lovas A, Lakatos L, Banfalvi Z, Burgyan J (2003) Low temperature inhibits RNA silencing-mediated defence by the control of siRNA generation. EMBO J **22**: 633-640
Szyjanowicz PM, McKinnon I, Taylor NG, Gardiner J, Jarvis MC, Turner SR (2004) The *irregular xylem 2* mutant is an allele of *korrigan* that affects the secondary cell wall of *Arabidopsis thaliana*. Plant J **37**: 730-740
Taiz L, Zeiger L (2002) Plant Physiology 3rd edition, Sinauer Associates Inc, Sunderland, MA
Tajner-Czopek A (2003) Changes of pectic substances concentration in potatoes and French fries and the effect of these substances on the texture of the final product. Nahrung **47**: 228-31
Talbott LD, Ray PM (1992) Molecular size and separability features of pea cell wall polysaccharides. Implications for models of primary wall structure. Plant Physiol **92**: 357–368
Tenhaken R, Thulke O (1996) Cloning of an enzyme that synthesizes a key nucleotide sugar precursor of hemicellulose biosynthesis from soybean: UDP-glucose dehydrogenase. Plant Physiol **112**: 1127-1134
Terashima H, Fukuchi S, Nakai K, Arisawa M, Hamada K, Yabuki N, Kitada K (2002) Sequence-based approach for identification of cell wall proteins in *Saccharomyces cerevisiae*. Curr Genet **40**: 311-316
Thakur BR, Singh RK, Handa AK (1997) Chemistry and uses of pectin--a review. Crit Rev Food Sci Nutr **37**: 47-73
The Arabidopsis Genome Initiative (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature **408**: 796-815
Thibault J-F, Renard CMGC, Axelos MAV, Roger P, Creépeau M-J (1993) Studies of the length of the homogalacturonan regions in pectins by acid hydrolysis. Carbohydr Res **238**: 271-286
Thimm O, Blasing O, Gibon Y, Nagel A, Meyer S, Kruger P, Selbig J, Muller LA, Rhee SY, Stitt M (2004) MAPMAN: a user-driven tool to display genomics data sets onto diagrams of metabolic pathways and other biological processes. Plant J **37**:914-39
Thomas G, Zoran M, Abdelhak EA, Olivier L, Estelle A, Catherine L, Jean-Paul J, Lise J (2003) *AtBXL1*, a novel higher plant (*Arabidopsis thaliana*) putative β-xylosidase gene, is involved in secondary cell wall metabolism and plant development. Plant J **33**: 677-690
Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res **22**: 4673-4680
To JP, Reiter W-D, Gibson SI (2002) Mobilization of seed storage lipid by *Arabidopsis* seedlings is retarded in the presence of exogenous sugars. BMC Plant Biol **2**: 4
Tomiya N, Ailor E, Lawrence SM, Betenbaugh MJ, Lee YC (2001) Determination of nucleotides and sugar nucleotides involved in protein glycosylation by high-performance anion-exchange chromatography: sugar nucleotide

contents in cultured insect cells and mammalian cells. Anal Biochem **293**: 129-137

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

Tonetti M, Sturla L, Bisso A, Zanardi D, Benatti U, De Flora A. (1998) The metabolism of 6-deoxyhexoses in bacterial and animal cells. Biochimie **80**: 923-31

Turner W, Botha FC (2002) Purification and kinetic properties of UDP-glucose dehydrogenase from sugarcane. Arch Biochem Biophys **407**: 209-216

Updegraff DM (1969) Semi-micro determination of cellulose in biological material. Anal Biochem **32**: 420-424

Usadel B, Kuschinsky AM, Rosso MG, Eckermann N, Pauly M (2004) *RHM2* Is Involved in Mucilage Pectin Synthesis and Is Required for the Development of the Seed Coat in *Arabidopsis*. Plant Physiol **134**: 286-295

Van den Koornhuyse N, Libessart N, Delrue B, Zabawinski C, Decq A, Iglesias A, Carton A, Preiss J, Ball S (1996) Control of starch composition and structure through substrate supply in the monocellular alga *Chlamydomonas reinhardtii*. J Biol Chem. **271**: 16281-16287

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol **3**: 1-11

Vanzin GF, Madson M, Carpita NC, Raikhel NV, Keegstra K, Reiter W-D (2002) The *mur2* mutant of *Arabidopsis thaliana* lacks fucosylated xyloglucan because of a lesion in fucosyltransferase *AtFUT1*. Proc Natl Acad Sci USA **99**: 3340-3345

Vaughn KC, Hoffman JC, Hahn MG, Staehelin LA (1996). The herbicide dichlobenil disrupts cell plate formation: immunogold characterization. Protoplasma **194**: 117-32

Vara JA, Hutchinson CR (1988) Purification of thymidine-diphospho-D-glucose 4,6-dehydratase from an erythromycin-producing strain of *Saccharopolyspora erythraea* by high resolution liquid chromatography. J Biol Chem **263**: 14992-14995

Vaucheret H, Beclin C, Fagard M (2001) Post-transcriptional gene silencing in plants. J Cell Sci **114**: 3083-3091

Verma DP, Hong Z (2001) Plant callose synthase complexes. Plant Mol Biol 2001 **47**: 693-701

Vidal S, Doco T, Williams P, Pellerin P, York WS, O'Neill MA,Glushka J, Darvill AG, Albersheim P (2000) Structural Characterization of the Pectic Polysaccharide Rhamnogalacturonan II: Evidence for the Backbone Location of the Aceric Acid-Containing Oligoglycosyl Side Chain. Carbohydr Res **326**: 277-294.

Vincken JP, Schols HA, Oomen RJ, McCann MC, Ulvskov P, Voragen AG, Visser RG (2003) If homogalacturonan were a side chain of rhamnogalacturonan I. Implications for cell wall architecture. Plant Physiol **132**: 1781-1789

Vincken JP Wijsman AJM, Beldman G Niessen WMA, Voragen AGJ (1996) Potato xyloglucan is built from XXGG-type subunits Carbohydr Res **288**: 219-232

Visser J, Voragen AGJ (1996) Progress in Biotechnology 14: Pectins and Pectinases. Elsevier, Amsterdam

Vogan EM, Bellamacina CR, He X, Foxman BM, Ringe D, Liu HW, Petsko GA (2002) Purification, crystallization and molecular symmetry of CDP-D-glucose 4,6-dehydratase from Yersinia pseudotuberculosis. Acta Crystallogr D Biol Crystallogr **58**: 370-373

Voet D, Voet JG (1995) Biochemistry 2nd edition Wiley- Text Books

Voragen AG, Le Goff A, Bonnin E, Thibault JF, Knox JP (2004) A xylogalacturonan epitope is specifically associated with plant cell detachment. Planta **218**: 673-681

Voragen AGJ, Schols HA, Pilnik W (1986). Determination of the degree of methylation and acetylation of pectins by h.p.l.c. Food Hydrocolloids **1**: 6570

von Heijne G (1992) Membrane protein structure prediction. Hydrophobicity analysis and the positive-inside rule. J Mol Biol **225**: 487-494

Wainwright SA, Biggs WD, Curry JD, Gosline JM (1976) Mechanical Design in Organisms. London: Arnold pp 423 ff

Walker AR, Davison PA, Bolognesi-Winfield AC, James CM, Srinivasan N, Blundell TL, Esch JJ, Marks MD, Gray JC (1999) The *TRANSPARENT TESTA GLABRA1* locus, which regulates trichome differentiation and anthocyanin biosynthesis in *Arabidopsis*, encodes a WD40 repeat protein. Plant Cell **11**: 1337-1350

Wandrey M, Trevaskis B, Brewin N, Udvardi MK (2004) Molecular and Cell Biology of a Family of Voltage-Dependent Anion Channel Porins in *Lotus japonicus*. Plant Physiol **134**: 182-193

Waterhouse PM, Wang MB, Lough T (2001) Gene silencing as an adaptive defence against viruses. Nature **411**: 834-842

Watt G, Leoff C, Harper AD, Bar-Peled M (2004) A Bifunctional 3,5-Epimerase/4-Keto Reductase for Nucleotide-Rhamnose Synthesis in *Arabidopsis*. Plant Physiol **134**: 1337-1346

Wesley SV, Helliwell CA, Smith NA, Wang MB, Rouse DT, Liu Q, Gooding PS, Singh SP, Abbott D, Stoutjesdijk PA, Robinson SP, Gleave AP, Green AG, Waterhouse PM (2001) Construct design for efficient, effective and high-throughput gene silencing in plants. Plant J **27**: 581-590
Western TL, Burn J, Tan WL, Skinner DJ, Martin-McCaffrey L, Moffatt BA, Haughn GW (2001) Isolation and characterization of mutants defective in seed coat mucilage secretory cell development in *Arabidopsis*. Plant Physiol **127**: 998-1011
Western TL, Skinner DJ, Haughn GW (2000) Differentiation of mucilage secretory cells of the *Arabidopsis* seed coat. Plant Physiol **122**: 345-356
Western TL, Young DS, Dean GH, Tan WL, Samuels AL, Haughn GW (2004) *MUCILAGE MODIFIED4* encodes a putative pectin biosynthetic enzyme developmentally regulated by *APTELA2, TRANSPARENT TESTA GLABRA1*, and *GLABRA2* in the *Arabidopsis* Seed Coat Plant Physiol **134**: 296-306
Wierenga RK, de Mayer MC, Hol WG (1985) Interaction of Pyrophosphate Moieties with alpha-Helixes in Dinucleotide Binding Proteins. Biochemistry **24**: 1346-1357
Willats WG, Marcus SE, Knox JP (1998) Generation of monoclonal antibody specific to (1-->5)-alpha-L-arabinan. Carbohydr Res **308**: 149-52
Willats WG, Knox JP (1999) Immunoprofiling of pectic polysaccharides Anal Biochem. **268**: 143-146
Willats WG, McCartney L, Knox JP (2001a) In-situ analysis of pectic polysaccharides in seed mucilage and at the root surface of *Arabidopsis thaliana*. Planta **213**: 37-44
Willats WG, Orfila C, Limberg G, Buchholt HC, van Alebeek GJ, Voragen AG, Marcus SE, Christensen TM, Mikkelsen JD, Murray BS, Knox JP (2001b) Modulation of the degree and pattern of methyl-esterification of pectic homogalacturonan in plant cell walls. Implications for pectin methyl esterase action, matrix properties, and cell adhesion. J Biol Chem **276**: 19404-19413
Willats WG, Steele-King CG, Marcus SE, Knox JP (1999) Side chains of pectic polysaccharides are regulated in relation to cell proliferation and cell differentiation. Plant J **20**: 619-628
Wellmann E, Griesebach H (1971) Purification and properties of an enzyme preparation from Lemna minor L. catalyzing the synthesis of UDP-apiose and UDP-D-xylose from UDP-D-glucuronic acid. Biochem Biophys Acta **235**:389-397
Williams MN, Freshour G, Darvill AG, Albersheim P, Hahn MG (1996) An antibody Fab selected from a recombinant phage display library detects deesterified pectic polysaccharide rhamnogalacturonan II in plant cells. Plant Cell **8**: 673-685
Williamson RE, Burn JE, Hocart CH (2001) Cellulose synthesis: mutational analysis and genomic perspectives using *Arabidopsis thaliana*. Cell Mol Life Sci **58**: 1475-1490
Williamson RE, Burn JE, Hocart CH (2002) Towards the mechanism of cellulose synthesis. Trends Plant Sci **7**: 461-467
Windsor JB, Symonds VV, Mendenhall J, Lloyd AM (2000) *Arabidopsis* seed coat development: morphological differentiation of the outer integument. Plant J **22**: 483-493
Wobus U, Baumlein H, Conrad U, Muntz K, Weber H (2000) Seeds of change. Trends Plant Sci **5**: 512-513
Wolucka BA, van Montagu M (2003) GDP-mannose 3,5-epimerase forms GDP-L-gulose, a putative intermediate for the *de novo* biosynthesis of vitamin C in plants J Biol Chem **278**: 47483-47490
Wolucka BA, Persiau G, van Doorsselaere J, Davey MW, Demol H, Vandekeckhove J, van Montagu M, Zabeau M, Boerjan W (2001) Partial purification and identification of GDP-D-mannose 3,5-epimerase of *Arabidopsis thaliana*, a key enzyme of the plant vitamin C pathway Proc Natl Acad Sci USA **98**: 14843-14848
Wulff, C, Norambuena, L, Orellana, A (2000) GDP-fucose uptake into the Golgi apparatus during xyloglucan biosynthesis requires the activity of a transporter-like protein other than the UDP-glucose transporter. Plant Physiol **122**: 867-877
Wyatt PJ (1993) Light scattering and the absolute characterization of macromolecules. Analytica Chimica Acta **272**: 1-40
Ye ZH, Varner JE (1991) Tissue-Specific Expression of Cell Wall Proteins in Developing Soybean Tissues. Plant Cell **3**: 23-37
Yokotani-Tomita K, Goto N, Kosemura S, Yamamura S, Hasegawa K (1998) Growth-promoting allelopathic substance exuded from germinating *Arabidopsis thaliana* seeds. Phytochemistry **47**: 1-2
York WS, Darvill AG, McNeill M, Stevenson TT, Albersheim P (1985) Isolation and characterization of cell walls and cell wall components. Methods in Enzymology **118**: 3-40
Zablackis E, York WS, Pauly M, Hantus S, Reiter W-D, Chapple CCS, Albersheim P, Darvill A (1996) Substitution of L-Fucose by L-Galactose in Cell Walls of *Arabidopsis* mur1. Science **272**: 1804-1808
Zijlstra C, Hohn T (1992) Cauliflower mosaic virus gene VI controls translation from dicistronic expression units in

transgenic Arabidopsis plants. Plant Cell **4**: 1471-1484

8 Appendix

8.1 Additional RHM data

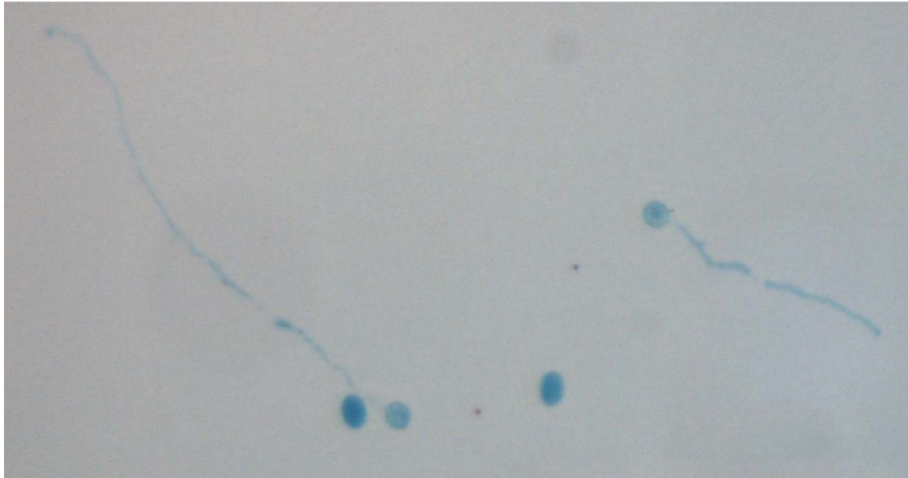


Figure I GUS staining of RHM2 promoter::GUS plant pollens germinated on basic medium.

Pollen from six to eight week old plants carrying the RHM2 promoter::GUS were harvested and put on basic medium. They were incubated for up to 24h and then for 1h for stained for GUS activity as described by Jefferson *et al.*(1987).

8.2 Additional GAE data

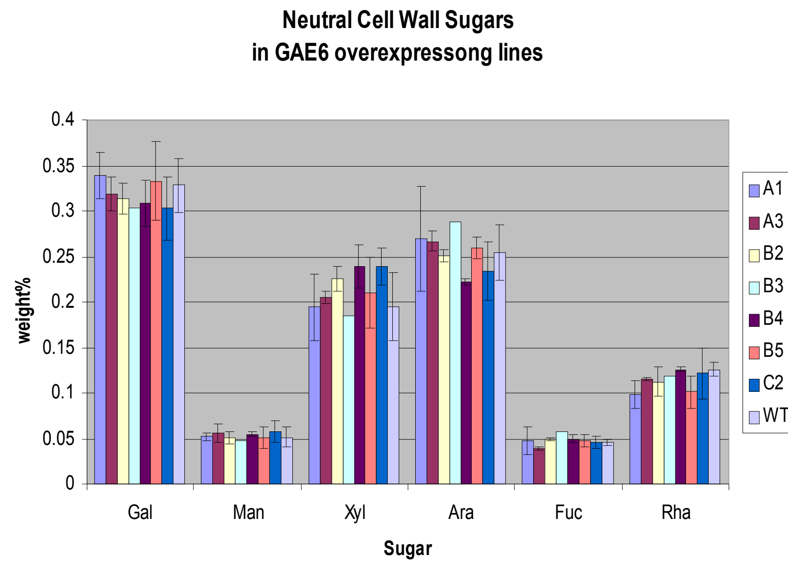


Figure II Cell wall sugars

Cell wall material from leaves of 6-8 week old plants was extracted and its sugar composition analyzed by GC-MS. Shown are the weight percentages for the different overexpression plant lines. WT denotes wild-type plants whereas A1, A3, B2, B3, B4, B5, C2 are the different overexpression plant

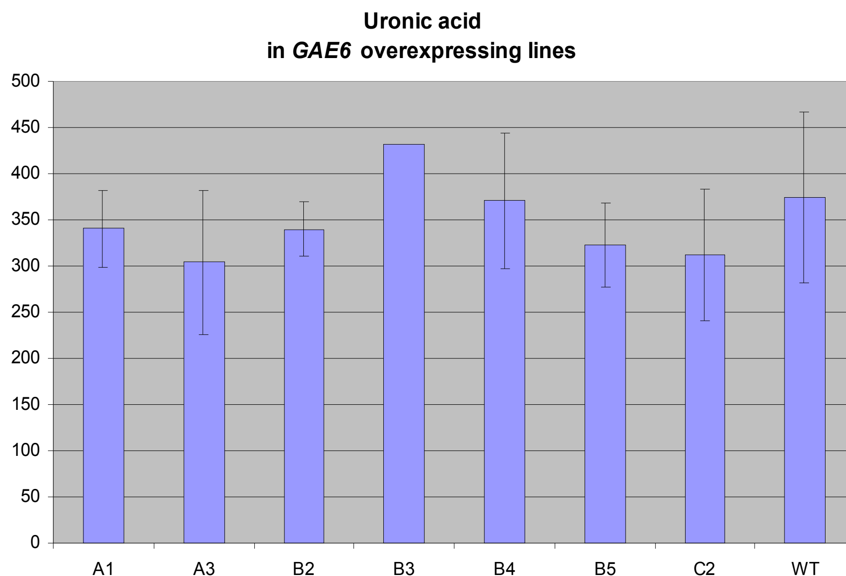


Figure III Uronic acid content

Cell wall material from leaves of 6-8 week old plants was extracted and its sugar composition analyzed by GC-MS. Shown are the weight percentages for the different overexpression plant lines. WT denotes wild-type plants whereas A1, A3, B2, B3, B4, B5, C2 are the different overexpression plant

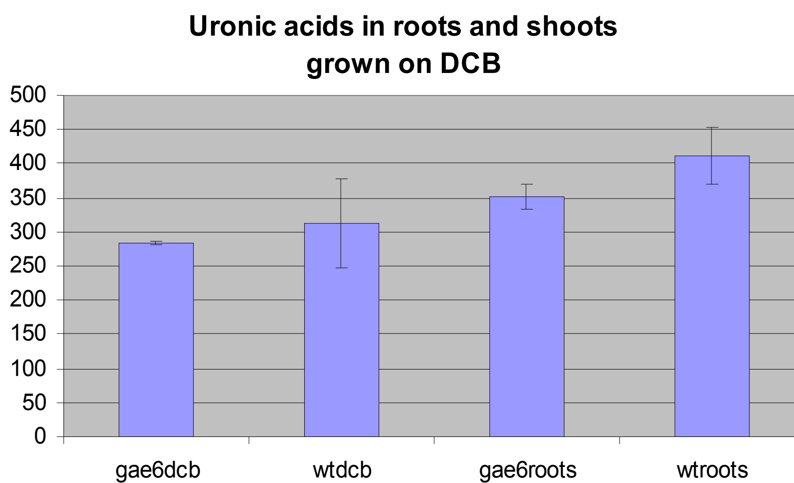


Figure IV Uronic acid content in GAE6 and WT plants.

Plants were grown on 10-6M DCB on MS medium or on Hoagland medium without DCB.

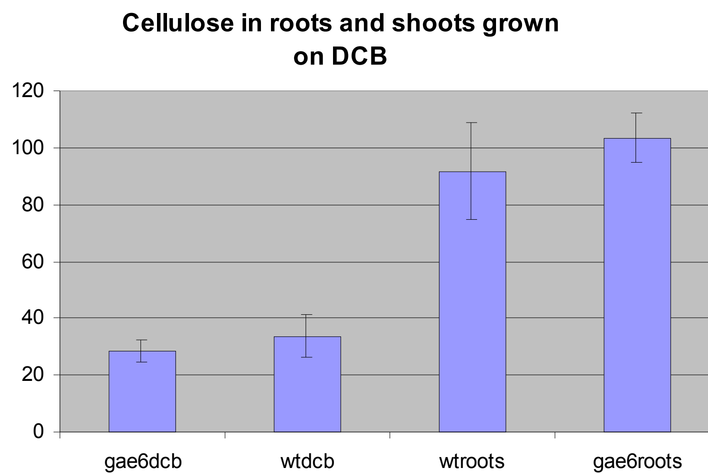


Figure V Cellulose content in *gae6* and WT plants

WT and *gae6* plants were grown on MS plates containing 1% sucrose. The cellulose content was estimated using the Updegraff method (1969) and is given in arbitrary units. Shoots were harvested from plates containing 10^{-6} M DCB (gae6dcb and WTdcb).

8.3 Additional MIOX data

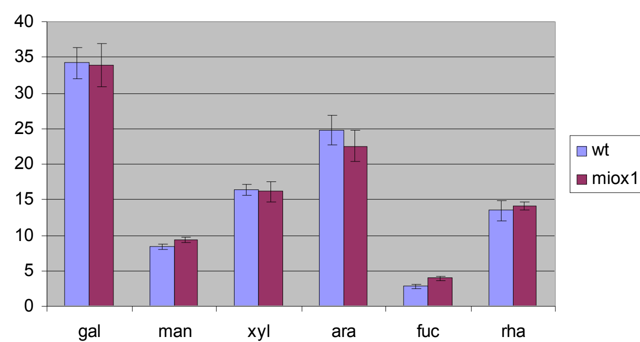


Figure VI Relative cell wall composition of *miox1* and WT leaves

Cell wall material from leaves of 6-8 week old plants was extracted and its sugar composition analyzed by GC-MS. Shown are the weight percentages for both the WT and *miox1* plants.

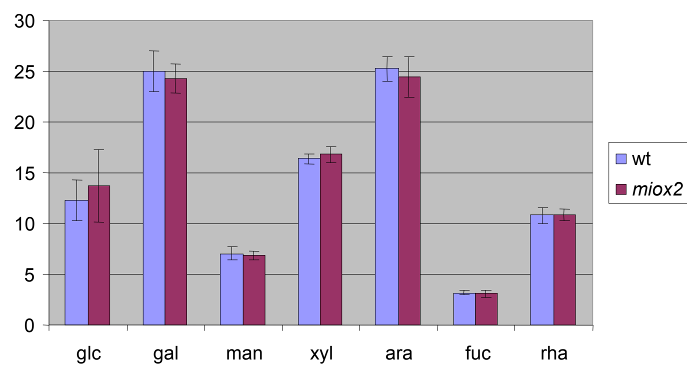


Figure VII Relative cell composition of *miox2* and WT hypocotyls

Cell wall material from hypocotyls of 10-14 day old plants was extracted and its sugar composition analyzed by GC-MS. Shown are the weight percentages for both the WT and *miox2* plants.

8.4 *Expression Data for genes involved in cell wall synthesis*

Table 17 Microarray ATGENEXPRESS Expression Data for identified nucleotide sugar genes

Given are the mean values of three different experiments. The values are color coded according to their expression from green (low expression) to red (high expression). Exceptionally high expression values are marked in blue. The values were obtained from the non-public alpha version of the expression mapping module in CSBDB.

Experiment	100	101	10	11	12	13	14	15	16	17	18	19	1	20	21	22	23	24	25	26	27	28	29	2	31
AT1G79500	125	129	203	231	100	123	177	180	224	240	233	256	190	171	130	195	218	170	45	97	123	236	242	197	222
AT1G16340	0	89	162	150	97	130	109	151	185	176	182	181	129	143	107	132	144	124	0	82	92	132	198	170	156
AT5G39320	111	182	233	169	47	103	143	231	272	318	284	463	83	218	132	250	258	209	0	25	707	788	402	364	412
AT3G29360	840	886	749	856	860	1035	1110	1215	1210	968	893	1506	914	1288	1307	1052	925	914	400	550	2714	2882	732	1261	724
AT5G15490	242	316	339	301	185	250	281	385	418	477	444	686	124	330	219	374	440	378	250	221	1893	1772	246	840	364
AT1G26570	153	131	222	199	126	137	192	261	296	325	188	322	167	237	217	185	177	163	0	70	415	502	252	269	277
AT1G12780	1457	1349	249	338	998	689	518	582	443	434	229	900	903	563	605	493	383	339	642	885	4195	2944	257	537	287
AT4G23920	77	85	108	76	80	58	55	64	84	97	82	60	61	67	54	64	61	69	232	109	46	27	78	42	117
AT1G63180	219	163	69	56	52	52	50	59	70	75	51	110	54	62	57	54	51	48	61	44	80	114	45	52	105
AT1G64440	95	106	155	107	99	104	102	105	96	116	179	110	115	104	100	115	122	123	117	131	127	151	142	143	191
AT4G10960	62	99	0	33	159	69	50	41	0	0	48	0	80	46	68	50	56	78	922	392	53	0	0	37	61
AT4G20460	37	42	33	33	23	26	35	23	28	43	25	0	24	20	25	22	23	30	39	33	25	25	24	58	135
AT2G34850	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	47	17	0
AT1G30620	148	143	79	96	134	153	137	142	134	103	113	95	84	115	168	145	97	120	157	116	105	142	184	143	225
AT5G44480	50	57	0	0	46	40	0	36	53	0	0	0	0	0	0	0	45	52	141	44	65	45	33	72	37
AT4G30440	2529	2302	1238	1926	3220	3298	3156	3096	2510	2010	1257	2276	2326	3059	3218	2204	1185	1131	684	1445	2050	2199	406	1475	440
AT1G02000	89	90	49	80	99	77	88	74	70	66	60	71	99	80	86	79	70	68	69	82	95	101	50	62	56
AT4G00110	150	162	160	153	162	165	179	146	143	146	145	152	171	150	154	154	160	157	121	142	212	182	190	167	235
AT2G45310	40	54	100	82	42	46	55	54	64	71	90	59	56	58	52	60	71	63	26	53	42	55	107	83	110
AT4G12250	108	98	0	0	85	90	85	75	64	44	76	84	91	79	89	85	78	93	135	111	170	169	103	135	131
AT3G23820	1175	1203	1760	1518	1907	2494	2142	2055	1753	1773	1757	2318	2528	2558	2754	1837	1730	2014	425	1739	1500	4109	874	2435	964
AT1G53500	171	225	386	326	180	261	278	354	323	334	375	420	225	316	284	307	414	446	173	318	518	616	370	434	381
AT3G14790	186	214	411	353	201	228	263	311	347	321	328	369	295	358	330	310	390	350	154	228	383	420	450	315	431
AT1G63000	342	446	724	654	495	669	755	808	762	752	724	1058	601	768	537	708	873	775	442	512	923	1221	584	1065	582
AT2G47650	342	380	241	297	348	338	312	356	301	322	347	375	357	337	342	372	356	329	441	291	663	653	435	730	531
AT3G53520	215	236	276	253	259	223	235	259	254	299	302	333	218	262	253	271	313	309	331	284	574	495	327	342	455
AT3G46440	161	212	175	197	243	231	201	212	190	253	252	278	198	196	192	279	271	275	489	257	1091	986	280	362	412
AT5G59290	343	475	299	252	146	220	223	307	313	324	287	449	229	296	258	293	313	295	165	157	2951	2276	199	589	371
AT2G28760	117	142	161	159	72	115	153	193	246	208	170	323	87	180	121	136	166	142	49	122	1617	1467	53	557	81
AT5G46340	183	239	111	115	158	122	125	119	117	104	125	104	147	108	115	136	145	156	202	196	1483	1103	125	222	105
AT2G34410	111	115	141	132	95	87	123	109	125	139	139	132	119	93	85	120	138	134	138	141	1135	736	141	161	144
AT2G02810	70	86	134	158	112	189	173	156	192	163	199	121	118	127	118	158	155	176	230	148	63	88	156	188	293
AT4G23010	1093	716	262	416	1159	907	802	686	537	380	337	443	825	709	802	570	445	589	1880	844	299	264	83	324	604
AT1G14360	193	216	260	321	244	338	332	319	297	288	420	236	266	252	218	289	325	323	422	256	167	285	323	388	521
AT1G12600	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	111
AT3G46180	42	45	58	48	44	43	41	41	45	50	55	39	46	46	49	41	56	62	60	65	45	53	66	45	91
AT3G59360	146	127	186	179	200	183	198	188	173	172	176	167	205	169	169	190	210	216	240	211	339	287	129	193	130
AT1G14520	23	23	29	29	26	24	27	23	22	31	16	22	23	30	0	20	25	26	26	22	28	14	28	24	19
AT2G19800	1103	331	45	51	90	80	88	131	121	77	0	290	37	86	112	97	39	40	49	39	315	102	467	239	282
AT5G56640	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
AT4G26260	0	46	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	29	0	73	30	740	34	323
AT2G27860	2061	1876	1800	1839	1662	1918	1866	1975	1934	1897	2023	2367	1682	2108	2081	1991	1933	1908	824	1238	2258	2275	1368	1761	1539

	32	33	34	35	36	37	39	3	40	41	43	45	46	47	48	49	4	50	51	52	53	54	55	56	57
AT1G79500	202	199	106	336	186	273	119	221	277	36	56	191	243	249	249	280	281	288	229	270	182	257	226	238	248
AT1G16340	155	151	65	203	96	179	96	187	167	0	0	201	198	209	170	233	177	230	216	219	158	171	166	184	178
AT5G39320	316	398	101	706	829	481	178	948	489	21	112	421	449	321	331	323	402	288	412	451	466	312	287	349	345
AT3G29360	677	945	638	1208	1734	997	610	1129	1189	383	597	934	696	761	625	746	968	888	949	734	779	734	515	675	798
AT5G15490	333	739	359	922	2397	495	359	753	729	170	270	493	272	247	233	316	555	300	263	238	758	305	318	490	423
AT1G26570	301	458	108	303	728	514	156	76	332	61	84	405	273	345	250	279	402	310	383	250	372	290	276	401	386
AT1G12780	446	1095	1292	941	3387	226	1448	316	2096	1142	3279	405	245	136	220	190	253	154	129	192	1172	170	557	524	299
AT4G23920	149	109	76	26	198	61	70	54	57	105	126	72	79	86	78	86	101	82	82	85	123	93	179	133	66
AT1G63180	117	246	84	73	997	46	237	95	106	138	449	63	39	19	21	24	53	16	33	22	284	33	126	112	33
AT1G64440	159	206	158	310	347	262	184	243	169	149	223	215	184	153	134	166	143	142	152	127	184	152	176	163	172
AT4G10960	65	153	263	54	932	0	272	66	0	943	389	55	0	0	0	0	0	0	0	0	154	39	83	76	44
AT4G20460	280	308	63	69	1457	49	79	225	32	78	133	85	19	20	25	28	42	20	22	22	345	27	243	147	34
AT2G34850	30	0	0	0	0	0	0	51	0	0	0	0	51	45	35	43	0	51	46	37	0	45	29	0	29
AT1G30620	235	241	110	169	385	297	214	176	109	312	211	232	201	244	218	251	166	267	248	206	226	229	290	270	338
AT5G44480	47	72	0	34	87	40	135	483	0	103	72	138	36	0	0	0	81	0	0	32	57	0	44	47	28
AT4G30440	841	875	2241	2032	1465	1583	1085	1433	1235	922	1859	1399	471	533	486	567	1672	409	459	515	830	628	670	678	672
AT1G02000	68	121	108	74	762	53	337	74	92	155	1538	114	60	41	72	60	39	50	57	55	170	57	74	84	74
AT4G00110	185	218	115	110	729	181	228	220	185	140	618	208	200	180	204	174	149	132	181	183	220	167	167	211	177
AT2G45310	82	90	51	81	369	76	149	108	51	48	722	73	93	94	86	105	87	116	112	88	95	101	95	110	98
AT4G12250	143	131	87	93	121	150	122	106	82	106	96	157	105	106	115	127	58	102	110	92	136	122	115	140	141
AT3G23820	1390	1235	2752	2421	538	492	1200	1548	1400	1089	1988	412	1111	810	742	617	1383	920	897	886	1480	974	976	598	1105
AT1G53500	405	591	297	1020	1615	495	423	343	528	205	796	458	363	398	373	396	283	443	388	389	595	428	443	451	545
AT3G14790	341	527	235	606	1351	489	284	421	417	206	414	461	447	486	472	474	412	506	525	470	479	420	277	447	432
AT1G63000	508	707	512	812	1834	600	641	1940	952	355	1368	648	527	597	532	607	737	646	627	546	602	542	353	597	600
AT2G47650	438	572	294	522	1233	573	561	987	409	335	239	671	470	421	429	496	438	424	395	466	684	437	475	526	566
AT3G53520	459	523	298	459	932	451	363	420	435	324	446	406	334	347	346	395	295	403	365	361	514	342	438	473	479
AT3G46440	467	584	331	404	1906	337	350	603	229	479	338	342	342	258	282	336	260	335	235	257	704	263	440	500	369
AT5G59290	323	454	312	596	607	320	232	598	511	141	371	347	198	215	178	187	274	204	238	200	487	254	287	314	273
AT2G28760	148	308	225	260	509	67	90	371	231	111	77	100	42	48	42	47	134	61	69	43	272	85	183	125	74
AT5G46340	134	213	164	120	288	152	190	270	125	171	191	167	155	122	150	162	98	137	122	120	261	112	184	190	171
AT2G34410	131	134	129	171	156	130	160	123	173	86	94	162	124	119	173	150	149	128	145	111	131	121	125	177	130
AT2G02810	247	270	185	230	612	300	219	160	170	327	264	195	173	166	132	155	158	172	184	151	271	211	272	242	242
AT4G23010	271	499	630	119	170	1149	563	523	107	1255	366	526	88	69	96	130	323	66	55	58	502	177	395	358	797
AT1G14360	414	452	344	488	732	539	326	406	261	453	108	381	322	327	263	290	297	274	355	321	517	407	438	349	415
AT1G12600	36	115	0	56	503	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	120	0	69	53	0
AT3G46180	64	77	49	66	161	65	58	72	63	50	56	73	85	52	67	64	42	67	71	71	94	63	75	76	60
AT3G59360	160	183	179	220	218	158	192	243	250	176	282	182	134	142	167	154	159	167	150	149	178	154	156	175	194
AT1g14520	19	28	21	20	19	27	21	23	25	21	24	22	17	18	31	21	22	20	28	27	23	21	25	26	16
AT2G19800	78	122	66	82	651	393	504	423	418	55	493	749	484	290	347	245	311	267	295	551	147	159	102	166	48
AT5G56640	0	118	0	51	979	0	153	0	0	41	922	60	0	0	0	0	0	0	0	0	86	0	0	0	0
AT4G26260	141	280	97	147	2779	89	1054	43	60	195	4913	300	462	139	166	181	89	443	236	841	389	75	125	139	56
AT2G27860	1513	1549	1666	2374	2049	1560	1310	1919	1954	1076	1410	1595	1420	1323	1298	1272	1825	1299	1520	1307	1684	1419	1297	1352	1494

	58	59	5	6	73	75	76	77	78	79	7	81	82	83	84	87	89	8	90	91	92	93	94	95	96
AT1G79500	232	209	229	270	90	318	259	154	133	187	171	148	46	46	39	204	243	257	243	237	262	129	110	106	95
AT1G16340	191	168	151	178	0	185	197	121	102	113	137	0	0	0	0	136	130	244	156	150	185	112	104	0	0
AT5G39320	335	316	192	374	477	365	447	347	243	124	274	81	42	0	0	203	255	385	305	195	362	1257	986	1057	123
AT3G29360	823	600	832	1047	1327	525	785	823	520	421	724	462	388	392	406	609	502	988	633	499	740	1179	813	1051	717
AT5G15490	386	283	262	669	1341	270	591	1415	1331	1210	394	1095	551	420	380	277	346	313	457	205	489	965	749	1051	203
AT1G26570	258	284	244	341	301	304	392	304	309	405	202	701	258	234	188	203	244	321	260	137	285	53	52	73	150
AT1G12780	321	544	661	165	816	1381	576	1182	1562	1821	200	1934	1694	1540	1018	283	284	163	256	615	435	221	737	267	1186
AT4G23920	66	83	94	102	0	73	58	64	56	54	91	138	83	116	103	123	123	80	110	91	184	88	45	65	75
AT1G63180	25	88	96	43	717	248	92	46	30	37	58	65	616	703	769	45	88	27	83	77	170	27	227	31	189
AT1G64440	157	165	139	154	123	70	127	107	67	59	190	61	90	74	84	110	143	164	138	89	156	310	253	222	72
AT4G10960	59	49	0	0	177	60	46	149	150	41	36	83	386	559	639	0	0	0	0	57	82	333	165	197	64
AT4G20460	32	84	25	71	1235	37	48	63	129	254	30	325	173	177	184	25	26	36	22	24	222	421	312	427	61
AT2G34850	0	35	0	0	0	0	0	0	36	68	0	141	92	90	0	0	0	0	32	0	0	0	0	0	0
AT1G30620	251	259	66	165	559	119	105	156	137	173	105	257	190	209	201	76	102	238	121	72	250	164	118	148	140
AT5G44480	37	33	0	139	67	49	43	402	245	191	63	84	0	0	0	49	52	59	48	0	44	782	616	732	45
AT4G30440	811	753	2224	1275	7915	751	1138	2590	2786	1524	818	1262	1739	1521	1543	905	748	632	763	1414	632	683	647	779	1429
AT1G02000	72	56	75	58	14134	229	224	106	102	62	60	84	118	116	145	70	49	56	49	78	72	100	90	92	75
AT4G00110	159	170	141	173	5746	173	194	168	167	151	150	119	113	70	110	146	129	184	143	165	161	232	269	247	147
AT2G45310	113	84	69	106	6349	134	156	40	36	27	114	23	17	0	0	82	88	86	85	65	94	74	51	57	48
AT4G12250	122	123	0	58	195	119	131	265	377	423	48	475	430	417	367	0	69	101	65	0	134	151	109	114	108
AT3G23820	1275	1011	1914	1168	1082	656	1091	2914	2933	922	2392	837	108	122	111	2957	1937	919	1883	1824	1005	1640	1569	1672	1585
AT1G53500	607	439	330	310	2784	191	384	2194	2150	821	506	652	130	133	162	381	356	331	358	251	407	308	331	407	221
AT3G14790	422	308	349	428	2023	199	354	440	336	235	437	267	170	166	173	303	359	413	375	312	418	454	426	354	266
AT1G63000	650	416	583	808	4592	594	755	2107	1770	924	730	666	386	318	347	694	652	625	742	578	614	2301	1595	1984	373
AT2G47650	548	430	274	667	629	309	404	994	967	632	292	492	324	343	356	244	273	537	290	242	463	1344	1013	1145	347
AT3G53520	409	416	239	300	268	342	442	548	498	515	278	534	396	454	417	239	256	317	323	233	448	415	371	359	234
AT3G46440	326	359	144	265	1490	300	242	625	616	445	214	419	256	230	212	151	199	333	192	187	428	796	705	735	123
AT5G59290	285	240	252	238	375	267	387	754	346	162	298	150	65	63	97	231	249	211	259	307	369	693	619	617	244
AT2G28760	101	71	164	131	0	154	201	517	117	62	146	70	0	0	101	107	108	44	110	116	160	248	247	320	86
AT5G46340	133	132	109	124	85	147	179	419	230	171	113	177	140	156	168	116	94	135	110	155	201	261	279	254	130
AT2G34410	118	122	119	145	112	55	78	109	124	105	156	109	98	89	124	137	120	123	139	123	139	213	201	174	92
AT2G02810	204	261	95	180	1929	133	220	124	114	187	101	194	135	154	117	153	192	193	186	83	234	139	146	144	122
AT4G23010	249	600	428	339	125	380	429	1084	1496	1857	262	2147	877	697	459	244	278	126	252	356	431	1268	1024	1213	1320
AT1G14360	464	433	226	341	444	282	454	270	286	587	257	648	423	460	293	304	308	315	293	231	398	390	397	424	233
AT1G12600	0	0	0	0	327	52	0	115	192	276	0	343	172	266	136	0	0	0	0	0	88	0	0	0	0
AT3G46180	57	69	42	63	184	42	59	42	40	57	60	71	84	85	85	64	61	59	56	62	63	67	53	57	50
AT3G59360	171	159	167	159	113	129	164	201	168	178	208	228	181	208	186	154	125	157	130	165	128	225	231	199	151
AT1G14520	19	18	30	24	0	34	28	29	18	34	13	35	43	58	64	30	20	20	20	31	22	21	12	21	18
AT2G19800	69	97	185	127	4501	1519	235	60	67	77	32	84	404	497	503	89	96	99	125	83	153	371	1671	735	2581
AT5G56640	0	0	0	0	6514	176	146	93	101	129	0	121	0	0	62	0	0	0	0	0	0	0	0	0	0
AT4G26260	0	103	0	56	26602	1021	691	85	79	0	0	0	0	0	63	0	0	50	0	0	148	0	47	0	78
AT2G27860	1649	1277	1871	1647	2146	1229	1582	1287	1163	895	1651	790	485	471	427	2019	1845	1470	1867	1886	1391	1742	1653	1643	2135

	97	98	99	9
AT1G79500	132	88	117	196
AT1G16340	112	92	113	185
AT5G39320	176	519	758	1157
AT3G29360	704	685	818	1473
AT5G15490	268	577	715	884
AT1G26570	180	49	67	107
AT1G12780	579	881	531	542
AT4G23920	89	70	125	66
AT1G63180	83	161	40	105
AT1G64440	90	151	188	316
AT4G10960	69	208	337	80
AT4G20460	48	194	245	170
AT2G34850	0	0	52	0
AT1G30620	96	142	165	189
AT5G44480	81	482	523	440
AT4G30440	1346	724	845	2076
AT1G02000	70	86	102	96
AT4G00110	140	216	238	203
AT2G45310	49	53	57	120
AT4G12250	101	144	177	99
AT3G23820	1473	1354	1381	1578
AT1G53500	276	218	244	302
AT3G14790	250	303	297	470
AT1G63000	483	1252	1448	1960
AT2G47650	363	845	1046	920
AT3G53520	249	374	406	389
AT3G46440	164	542	678	556
AT5G59290	252	556	546	638
AT2G28760	119	240	278	330
AT5G46340	158	284	311	261
AT2G34410	98	160	152	159
AT2G02810	191	88	116	125
AT4G23010	932	891	844	507
AT1G14360	404	252	338	344
AT1G12600	0	0	0	0
AT3G46180	33	56	74	63
AT3G59360	141	174	198	224
AT1G14520	28	0	35	23
AT2G19800	592	1301	521	332
AT5G56640	0	0	0	0
AT4G26260	0	37	0	28
AT2G27860	1730	1512	1544	2016

Experiment Legend:

1 cotyledons; 2 hypocotyl; 3 roots; 4 shoot apex, vegetative + young leaves; 5 leaves 1 + 2; 6 shoot apex, vegetative; 7 seedling, green parts; 8 shoot apex, transition (before bolting); 9 roots; 10 rosette leaf #4, 1cm long; 11 rosette leaf #4, 1cm long; 12 rosette leaf # 2; 13 rosette leaf # 4; 14 rosette leaf # 6; 15 rosette leaf # 8; 16 rosette leaf # 10; 17 rosette leaf # 12; 18 rosette leaf # 12; 19 leaf 7, petiol; 20 leaf 7, proximal half; 21 leaf 7, distal half; 22 developmental drift; whole plant after transition, but before bolting; 23 developmental drift; whole plant after transition, but before bolting; 24 developmental drift; whole plant after transition, but before bolting; 25 senescing leaves; 26 cauline leaves; 27 stem, 2nd internode; 28 1st node; 29 shoot apex, inflorescence (after bolting); 31 flowers stage 9; 32 flowers stage 10/11; 33 flowers stage 12; 34 flowers stage 12, sepals; 35 flowers stage 12, petals; 36 flowers stage 12, stamens; 37 flowers stage 12, carpels; 39 flowers stage 15; 40 flowers stage 15, pedicels; 41 flowers stage 15, sepals; 42 flowers stage 15, petals; 43 flowers stage 15, stamen; 45 flowers stage 15, carpels; 46 shoot apex, inflorescence (after bolting); 47 shoot apex, inflorescence (after bolting); 48 shoot apex, inflorescence (after bolting); 49 shoot apex, inflorescence (after bolting); 50 shoot apex, inflorescence (after bolting); 51 shoot apex, inflorescence (after bolting); 52 shoot apex, inflorescence (after bolting); 53 flower stage 12; multi-carpel gyneoceum; enlarged meristem; increased organ number; 54 flowers converted to leaf-like structures; some shoot characteristics; 55 flowers without sepals, petals replaced by sec. flowers; 56 flowers without sepals, petals; carpeloid structures on sepals; 57 flowers without petals, stamens; 58 flowers without stamens, carpels; replaced by sepals and petals; indeterminate; 59 filamentous organs in whorls two and three; 73 mature pollen; 76 siliques, w/ seeds stage 3; 77 siliques, w/ seeds stage 4; 78 siliques, w/ seeds stage 5; 79 seeds, stage 6, w/o siliques; 81 seeds, stage 7, w/o siliques; 82 seeds, stage 8, w/o siliques; 83 seeds, stage 9, w/o siliques; 84 seeds, stage 10, w/o siliques; 87 veg rosette (for phase change); 89 veg rosette (for phase change); 90 veg rosette (for phase change); 91 leaf; 92 flower; 93 root; 94 root; 95 root; 96 seedling, green parts; 97 seedling, green parts; 98 root; 99 root; 100 seedling, green parts; 101 seedling, green parts

Curriculum Vitae

Björn Usadel

Research Experience

Jul. 2001- July 2004	PhD Student of the University Potsdam at the Max-Planck Institute of molecular Plant Physiology Project: Analysis of nucleotide sugars in the plant cell wall
Feb. 2001-Apr. 2001	Foreign Researcher at the Rockefeller University, NY Project: Role of the feng protein in the development of the adult visual system of Drosophila melanogaster
(Jul. 2000-Feb. 2001)	Foreign Research Intern at the Rockefeller University, NY
Jul. 2000-Feb. 2001	Master-student of Free University Berlin, Berlin, Germany Project: Role and expression of the plexin/semaphorin and roundabout/slit receptor-ligand pairs in the development of the adult visual system of Drosophila melanogaster
Sep.1999- Oct 1999	Internship at the University of California at Berkeley, CA Project: Influence of estrogens and related compounds on astrocyte and glial cell viability
Jun.1998- Jul 1998	Internship at the Max Delbrück Center for molecular medicine, Berlin, Germany Project: Characterization of AN-2 positive neuronal cells
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Education

1994-1996	High School “städtisches Ruhrgymnasium Witten”
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Publications

Usadel B, Steinhauser D, Luedemann A, Thimm O, Kopka J (2004) CSB.DB: A comprehensive systems-biology database. Bioinformatics accepted

Usadel B, Schlüter U, Molhoj M, Gipmans M, Verma R, Kossmann J, Reiter W-D, Pauly M (2004) Identification and characterization of a UDP-D-glucuronate 4-epimerase in Arabidopsis. FEBS Lett. 569:327-31.

Usadel B, Kuschinsky AM, Rosso MG, Eckermann N, Pauly M (2004) RHM2 is involved in mucilage pectin synthesis and is required for the development of the seed coat in Arabidopsis. Plant Physiol 134:286-95

Lerouxel O, Choo TS, Seveno M, Usadel B, Faye L, Lerouge P, Pauly M (2004) Rapid structural phenotyping of plant cell wall mutants by enzymatic oligosaccharide fingerprinting. Plant Physiol 130:1754-63.

Invited Talks

Novel pectin mutants through genetic engineering of pectin synthesis, X Cell Wall Meeting, 29.8-3.9, Sorrento, Italy

Poster Presentations at Scientific Meetings

Usadel, B., Guerineau F., Pauly M. Functional analysis of the nucleotide sugar conversion pathway in Arabidopsis 15th International Conference on Arabidopsis Research, July 11-14, 2004 Berlin, Germany

Steinhauser, D., Usadel, B., Luedemann, A., Thimm, O., Kopka, J. CSB.DB-A comprehensive Systems-Biology Database 15th International Conference on Arabidopsis Research, July 11-14, 2004 Berlin, Germany

Usadel, B., Steinhauser, D., Nagel A., Svenja M., Schmid M., Lohmann J., Weigel D., Thimm O., Stitt M. A MapMan-based web-application that extracts gene expression level of your favourite gene from databases like AtGenExpress and NASC, and visualises it in false-colour code on simple diagrams, 15th International Conference on Arabidopsis Research, July 11-14, 2004 Berlin, Germany

Usadel, B., Kuschinsky, A.M., Beyer, C., Pauly, M. Biological Functions of Plant Cell Wall Polysaccharides: Systematic analysis of the nucleotide sugar conversion pathways, Siebzehnte Tagung Molekularbiologie der Pflanze, Dabringhausen, March, 9-12, 2004

Usadel, B., Kuschinsky, A.M., Obel, N., Pauly, M., Changing the nucleotide interconversion pathway, Gordon Research Conference: Plant Cell Walls, Kimball Union Aademy, Meriden, New Hampshire, USA, August 10-15, 2003

Kuschinsky, A.M., Usadel, B., Scheible, W.R., Pauly, M., Changing the structure of plant cell walls through genetic interference in the nucleotide sugar conversion pathway, 7th International Congress of Plant Molecular Biology, Barcelona, Spain, June 23-28, 2003

Witucka-Wall, H., Usadel, B., Erben, V., Obel, N., Pauly, M., Oligosaccharide profiling of plant cell wall polysaccharides via MALDI-TOF MS as a tool for chromosomal mapping of cell wall related genes, 5th Carbohydrate Bioengineering Meeting, University of Groningen, Netherlands, April 6-9, 2003-10-22

Obel, N., Usadel, B., Gibon, Y., Pauly, M., Identification and characterization of novel cell wall mutants, 3rd GABI status seminar, Bonn, Germany, February 11-12, 2003

Usadel, B., Kuschinsky, A.M., Pauly, M., Changing the structure of plant cell walls through modulation of the nucleotide conversion pathway, 22nd Symposium: Frontiers of Plant Cell Biology: Signals and Pathways, System-Based Approaches, UCLA Riverside, Riverside, California, USA, January 15-18, 2003

Choo, T.S., Usadel, B., Pauly, M., Plant cell wall oligosaccharide profiling using MALDI-TOF mass spectrometry, XXI International Carbohydrate Symposium, Cairns, Queensland, Australia, July 7-12, 2002

Choo, T.S., Lerouxel, O., Usadel, B., Lerouge, P., Pauly, M., Profiling of plant cell wall oligosaccharides by enzymatic fingerprinting, Plant Polysaccharide Workshop, Palm Cove, Queensland, Australia, July 4-6, 2002

Usadel, B., Kuschinsky, A.M., Pauly, M., Plant polysaccharide biosynthesis: Further insights into the pectic component rhamnogalacturonan, XIII International conference on Arabidopsis research, Seville, Spain, June 28- July 2, 2002

Pauly, M., Choo, T.S., Usadel, B., Phenotyping wall structure by oligosaccharide profiling using MALDI-TOF MS. Plant Cell Wall Biosynthesis Meeting, UCLA Lake Arrowhead, California, USA, May 12-15, 2002

Pauly, M., Choo, T.S., Usadel, B., The identification of genes involved in cell wall biosynthesis using oligosaccharide profiling by MALDI-TOF MS. 2nd GABI status seminar, Bonn, Germany, February 19-20, 2002

Eidesstattliche Erklärung

Ich versichere, die vorliegende Arbeit selbstständig angefertigt und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt zu haben. Ich versichere ebenfalls, dass die Arbeit an keiner anderen Hochschule als der Universität Potsdam eingereicht wurde.

Potsdam, den 1.10.2004

Björn Usadel

Danksagung

Ich danke Dr. Markus Pauly sowohl für die Bereitstellung und die Betreuung meines Projektes, als auch für die Freiheit eigene Ideen zu entwickeln und weiter voranzutreiben.
Ich danke Prof. Dr. Lothar Willmitzer für die Betreuung und die Begutachtung meines Projektes, sowie für weitere Anregungen und seine ständige Diskussionsbereitschaft. Ebenso danke ich Dr. Wolf-Rüdiger Scheible und Prof. Dr. Mark Stitt für Ihre Diskussionsbereitschaft und weitergehende Ideen.
Weiterhin danke ich Prof. Dr. Thomas Altmann und PD Dr. Raimund Tenhaken für Ihre Bereitschaft, meine Arbeit zu begutachten.
At this point I also have to say thank you to Prof. Dr. Tobias Baskin who also kindly agreed to referee my thesis and who always had a joke to cheer up the lab while he was here in Golm.
Je voudrais également remercier François Guinerau pour son aide et le bon temps passé en sa compagnie. (And Stéphanie for correcting my bad french).
Mein Dank gilt außerdem unseren zahlreichen Kooperateuren, ohne deren Hilfe und Input wir dies alles nicht geschafft hätten.

Ebenso danke ich allen Mitarbeitern (und hier besonsers Julia) der AG Pauly, für Ihre ständige Hilfsbereitschaft und die gute Arbeitsatmosphäre. Weiterhin gilt mein besonderer Dank in der Arbeitsgruppe sowohl Anja, Claudia als auch Salma, für Ihre Mitarbeit. Außerdem danke ich den Gärtnern des Max-Planck Institutes und hier besonders Torsten für die Sorge um meine Pflanzen. Weiterhin geht mein Dank am Institut sowohl an das IT Team, und hier besonders an Eckhard, für den Support, den Technikern, hier besonders Toralf, für Ihre Unterstützung als auch an Josef für gute Bilder und manch nette Unterhaltung.
Weiterer Dank für Unterstützung geht an Raju, Stefan, Dirk S. und nicht zuletzt an Mahmoud.

Desweiteren möchte ich mich bedanken bei Dr. Markus Pauly, Stefan, Stéphanie, Dirk B. und Andy für die ermüdende Korrektur meiner Arbeit.
Dirk, Junker und Stefan S. möchte ich für die Zusammenarbeit und so manches Bier zusammen danken. Erst so können neue Ideen geboren werden.

Außerdem danke ich Herrn Prof. emeritus Dr. Schweiger, ohne ihn stände ich heute nicht da wo ich stehe.

Ich möchte hier auch Felix und Dr. Kortakis danken, die mich während meines Studiums immer weiter vorantrieben.

Ein ganz besonderer Dank gilt meinen Eltern für Ihre nicht-enden wollende Unterstzützung. Außerdem danke ich Borjana, für Ihre Liebe und Zuwendung und dass sie mich in der Zeit des Zusammenschreibens ertragen hat.