Recherche et caractérisation de glycosyltransférases impliquées dans la biosynthèse des polysaccharides de la paroi chez Arabidopsis thaliana

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UNIVERSITÉ DE GRENOBLE

THÈSE

Pour obtenir le grade de

DOCTEUR DE L'UNIVERSITÉ DE GRENOBLE

Spécialité : **BIOLOGIE VEGETALE**

Arrêté ministériel : 7 août 2006

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préparée au sein du **Laboratoire Centre de Recherches sur les Macromolécules Végétales** dans **l'École Doctorale Chimie Science du Vivant**

Recherche et Caractérisation de glycosyltransférases impliquées dans la biosynthèse des polysaccharides de la paroi chez *Arabidopsis thaliana*

Thèse soutenue publiquement le **« 4 Novembre 2011 »**,
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ACKNOWLEDGEMENTS

I carried out my thesis under the auspices of CERMAV, University of Grenoble France. I can say without exaggeration that the three years spent in my PhD make the most memorable time of my life. During this span of time, I have observed tremendous positive changes in my personal and professional development. I am of the opinion that everyone that we come across in life influences us one way or the other. The bunch of people that I came to know during my PhD was simply wonderful. While I remain the sole responsible for imprecisions and omissions, there are a lot of people to whom I am indebted.

My utmost gratitude goes to my supervisor Dr. Christelle Breton. Her cool and calm personality had been a source of inspiration for me. During this period, Dr. Christelle not only provided all the lab material necessary for carrying out day to day research but also spent a lot of time for the analysis of the results. I have greatly benefitted from her scientific competence and had a chance of fruitful scientific discussions with her.

I am highly indebted to Dr. Olivier Lerouxel. He has always been cool, encouraging, kind and patient. He will always remain special for me for the special reason that he showed confidence in me when I needed it the most. I take great pride in having worked with him who is the specialists of his field. I have learnt a lot from him. I am grateful to him for having always kept his office door open for answering to all my questions: sometime stupid. Without doubt, he showed great patience to have scientific, religious and social discussions with me. He has always been a source of hope, courage and confidence for me during the ups and downs of my professional and personal life. He invested a lot of time for the explanation of the theoretical aspects of experiments. Above all it was a pure pleasure to work and discuss with him. I am extremely short of words to pay gratitude to you Olivier for all you have done for me. I wish you a life of unlimited pleasures and success.

I am highly thankful to Azeddine Durioch and Marie-Laure Follet-Gueye for allowing and helping me to carry out microscopy experiments under their kind supervision at Glycomev, University of Rouen.

I am extremely grateful to the members of my jury who gave their consent to analyze and consequently validate my work. I feel honored that my work got approved by such well renowned specialists.

I feel greatly indebted to all other researchers Anne Imberty, Annabelle varrot and Aline Thomas at CERMAV for nice working environment and company. I would like to thanks to all my colleagues Geraldine, Gaelle, Joanna and Aymeric who made my life colorful. I feel lucky to have known such gentle and nice people. I am also grateful to Valerie Chazalet for technical assistance during the thesis. I am thankful to Vincent Grassot M2R student who helped me a lot during his internship and he taught me French language too. I also say thanks to Anita Sarker for her time to time help to resolve the computer problems.

I am also thankful to Higher Education Commission Pakistan for providing me the scholarship of 4 years for getting higher education in France.

Along with the wonderful professional life, I have experienced four best years of my personal life during my stay in France. I dedicate all this to my friends especially „Grenobloise" (I would like to write the individual names but the list is very long) whose company or contact had been a source of enjoyment, pleasure and peace of mind.

I am thankful to Mr Nawazish Hameed, my beloved husband for his patience and support all along the period of my thesis. I am grateful to my parents, brothers and sisters and friends in Pakistan for their best wishes, prayers and love.

Above all I am thankful to Almighty Allah for providing me the wit and health without which nothing would have been possible.

ABSTRACT

The plant cell wall not only defines the unique biology of the plants but also have practical applications as feedstock for biomaterials and for the production of biofuels. Plant primary cell wall is mainly composed of cellulose, hemicelluloses and pectins. Significant progress has been made recently in identifying the enzymes involved in plant cell wall biosynthesis, but only a handful of those have been involved in pectin biosynthesis. With the aim of identifying new putative glycosyltransferases (GTs), in lab Hansen et al 2009 designed a bioinformatic strategy and identified a new group of 24 genes called "NGT" for (Novel Glycosyltransferase) which were considered "strong" candidates for putative glycosyltransferase activities. In order to determine the putative role of these NGT genes in plant cell wall biosynthesis, we designed a functional genomics strategy, analysing in parallel Arabidopsis T-DNA mutant lines and performing heterologous expression of candidate genes.

I have characterized 15 homozygous mutant lines among the group of 24 putative NGT genes through PCR. We analysed the homozygous mutants for phenotypic alteration such as dwarfing or organ malformation and found that some of mutant lines have narrow leaves as compared to Wild type plants. In parallel I have carried out the cell wall chemical analysis of 12 homozygous mutant lines and did not get any strong difference in neutral monosaccharide composition. The detailed and complete analysis (chemical, expression and microscopic analysis) of all the above mentioned genes could have been time consuming and an overwhelming work, so I focused on At5g28910 (named NGT1) which harbours a fucosyltransferase peptide signature and on At5g14550 (named P), a gene belonging to the DUF266 gene family.

Homozygous T-DNA mutant lines *ngt1-1* and *ngt1-2* lines were analyzed and showed a reduced growth phenotype (leaf area). Leaf area was quantified at various development stages using ImageJ, and showed a 38% reduction in mutants. Additionally, biochemical characterization of the cell wall was performed showing a reduction in neutral monosaccharide contents, like arabinose, rhamnose and galactose in mutant cell wall. Furthermore glycosyl linkage analysis of mutant lines *ngt1-1* and *ngt1-2* has shown that 5-Arabinofuranose (5-Araf) and 3,5-Arabinofuranose (3,5-Araf) contents were decreased as compared to Wild type Col0 cell wall. These results were also confirmed by immunolabeling of stem cross section of mutant and wild type plants. The complementation of the mutant plants through Agrobacterium transformation resulted in the complete restoration of plant phenotype. Taken together, these data suggest that NGT1 could be an arabinosyltransferase. In order to characterize its biochemical activity, the NGT1 protein was heterologously expressed in *Pichia pastoris.* The recombinant protein was used to perform in vitro activity tests, but we were unable to demonstrate any neither fucosyltransferase (on the basis of peptide signature) nor arabinosyltransferase activity.

In parallel to this study, I contributed to the heterologous expression and characterization of two biochemically characterized Arabidopsis GTs involved in xyloglucan synthesis: the fucosyltransferase (AtFUT1) and xylosyltransferase (AtXT1). I have successfully expressed a truncated and active form of AtFUT1, which represents an essential step for further structural studies that will be undertaken in the lab.

Résumé

La paroi végétale assure des fonctions biologiques majeures définissant la singularité des plantes ; elle est également à l'origine de multiples applications en tant que ressource agro-alimentaire, source de biomatériaux ou encore pour la production de biocarburants. Malgré cette importance fondamentale et pratique de la paroi végétale, la connaissance de sa biosynthèse apparaît à ce jour toujours très limitée. En effet, la faible abondance des glycosyltransférases (GTs) responsables de sa biosynthèse, l'absence de substrat spécifique et les difficultés à obtenir certains nucléotides-sucres nécessaires aux tests enzymatiques, a souvent rendu difficile les approches de biochimie classiques. Cependant, le séquençage de génomes (*Arabidopsis thaliana*, *Oryza sativa*, *Poplar populus*), la création de banques de mutants d'insertion et la classification des activités glycosyltransférases dans la base de données CAZy (www.cazy.org) sont autant d'outils récents ayant permis des avancées significatives vers la compréhension de la biosynthèse de la paroi des végétaux.

Le CERMAV a participé à ce type d'avancée en 2009, en publiant une liste de 24 gènes candidats, nommés « NGT » pour « Nouvelles GlycosylTransférases », présentant des signatures caractéristiques des glycosyltransférases. Afin de démontrer l'implication des gènes NGT dans les processus d'édification de la paroi végétale, nous avons développé une approche de génomique fonctionnelle, analysant en parallèle des lignées mutantes d'Arabidopsis altérées pour les gènes NGT et testant l'activité GT de ces protéines exprimées en systèmes hétérologues. Durant mes travaux de thèse j'ai pu caractériser 15 lignées mutantes à l'état homozygote pour 7 des 24 gènes NGT. Ces lignées homozygotes ont été criblées afin de rechercher un phénotype d'altération du développement ou de la composition en sucres de leur paroi qui soit corrélé à l'altération des gènes NGT. Ce travail de criblage a conduit à s'intéresser plus particulièrement aux mutants *ngt1-1* et *ngt1-2* altérés pour le gène NGT1 (At5g28910).

La caractérisation des lignées mutantes *ngt1-1* et *ngt1-2* a permis de quantifier un phénotype de croissance foliaire réduit de 38%, par comparaison au développement des feuilles de la plante sauvage. Par ailleurs, la caractérisation biochimique de la paroi des mutants a révélé des réductions significatives et quantitatives de l'arabinose, du galactose et du rhamnose dans la paroi des mutants, ainsi que des modifications qualitatives marquées principalement des arabinanes. L'altération des arabinanes a d'ailleurs pu être confirmée par microscopie après immuno-marquage de sections d'hypocotyle de mutants à l'aide des anticorps monoclonaux LM6 et LM13 dirigés contre des épitopes α-1,5-arabinanes. Il a pu être montré également que la complémentation des mutants par une construction 35S::NGT1 permet de restaurer un phénotype sauvage à ces mutants. Par ailleurs, de façon à tester l'activité glycosyltransférase de la protéine NGT1, nous avons réalisé son expression en système hétérologue. A ce jour, malgré des résultats préliminaires encourageants, il n'a pas été possible de déterminer des conditions de tests permettant d'observer une activité glycosyltransférase suffisante et reproductible pour la protéine NGT1, que ce soit une activité fucosyltransférase (correspondant à la signature de la séquence du gène) ou bien une activité arabinosyltransférase (correspondant au phénotype biochimique des mutants *ngt1*).

Préface

Mes travaux de thèse ont été financés principalement par la “ Higher education commission of Pakistan » et supportés par le CNRS. Cette bourse de thèse m’a été allouée afin de poursuivre un master2, puis une thèse dans l’université de mon choix. J’ai choisi de rejoindre l’Université de Grenoble qui offrait l’assurance d’une excellente formation scientifique, dans le cadre d’une université habituée à recevoir un nombre important d’étudiants étrangers, ceci afin de faciliter mon intégration. La mission de la « Higher Education Commission” (HEC) est dédiée à faciliter la constitution au Pakistan d’une base de personnels très qualifiés, qui serviront le développement socio-économique national. Afin de poursuivre les objectifs de HEC, j’ai décidé de conduire des travaux de biologie végétale au sein de l’équipe Glycobiologie Moléculaire du CERMAV-CNRS (UPR 5301).

L’équipe Glycobiologie Moléculaire s’intéresse à deux groupes de protéines particulièrement importantes en glycobiologie, les glycosyltransférases qui synthétisent les structures glucidiques complexes et les lectines qui reconnaissent ces structures glucidiques. Mes travaux de recherche, présentés dans ce manuscrit de thèse s’inscrivent dans la thématique de l’identification et la caractérisation de nouvelles glycosyltransférases qui seraient impliquées dans la biogénèse de la paroi végétale. La paroi végétale est une structure complexe, tant du point de vue de sa composition biochimique, que de la variété de fonctions physiologiques essentielles qui sont assurées par cette matrice extracellulaire, telle que la régulation de l’élongation cellulaire ou bien la participation aux mécanismes de défenses contre les phytopathogènes. La composition biochimique de la paroi végétale est maintenant bien documentée pour quelques plantes modèles (*Arabidopsis thaliana, Physcomitrella patens, Poplar, etc.*…) ainsi que de plusieurs espèces d’intérêt agronomique (*Oryza sativa, Zea mays,* …) ; et si de nombreuses variations existent entre-espèces et même entre différents organes d’une espèce, il se dégage de l’ensemble de ces études un modèle de la paroi composé de deux réseaux interdépendant de polysaccharides. Le premier réseau serait composé de microfibrilles de cellulose associées les unes aux autres par l’intermédiaire de molécules d’hémicelluloses adsorbées à leur surface. Ce premier réseau confèrerait la majeure partie des propriétés mécaniques de la paroi, et serait interpénétré par un second réseau composé des polymères pectiques. Ce second réseauserait lui plutôt impliqué dans la cohésion intercellulaire. En effet, il est généralement admis que parmi les pectines, les homogalacturonanes participent à la réticulation des pectines, *via* la formation de ponts calciques tout comme les molécules de rhamnogalacturonanes II qui forment des ponts inter-moléculaires via des atomes de bore.

Ainsi, la paroi végétale assure des fonctions biologiques majeures définissant la singularité des plantes ; elle est également à l'origine de multiples applications en tant que ressource agro-alimentaire, source de biomatériaux ou encore pour la production de biocarburants. Malheureusement, malgré cette importance fondamentale et pratique de la paroi végétale, la méconnaissance de sa biosynthèse limite le développement de la valorisation de la biomasse notamment en tant que ressource énergétique. En effet, on estime qu'environ 10% du génome d'*Arabidopsis thaliana* (plus de 2000 gènes) serait impliqué dans l'édification, l'assemblage et le maintien de la cette paroi végétale ; mais à ce jour seule une poignée a pu être caractérisée.

A titre d'exemple, à la vue de la diversité des liaisons entre les unités monosaccharidiques qui constituent les polysaccharides de la paroi, on estime que plus d'une centaine de glycosyltransférases seraient impliquées ; cependant moins d'une dizaine a pu être caractérisée de façon biochimique à ce jour. Mes travaux de thèse s'inscrivent dans ce contexte difficile de caractérisation d'activités glycosyltransférases de la paroi végétale : la faible abondance de glycosyltransférases dans les cellules, l'absence de molécule acceptrice spécifique de chaque GTs, la variété de monosaccharides qui composent la paroi et les difficultés d'obtention de certains nucléotides-sucres donneurs ont rendu extrêmement difficile les approches « classiques » de caractérisation biochimique de ces enzymes. Cependant, le séquençage de génomes, la création de banques de mutants d'insertion et la classification des activités glycosyltransférases dans la base de données CAZy (www.cazy.org) sont autant d'outils récents ayant permis des avancées significatives vers la compréhension de la biosynthèse de la paroi des végétaux en suivant une approche de génétique inverse. C'est cette stratégie de génomique fonctionnelle qui constitue la clef de voute de mes travaux de thèse.

Mes travaux se basent donc sur la caractérisation de gènes codant de nouvelles glycosyltransférases végétales potentielles chez Arabidopsis que l'on a nommés gènes NGT pour « New GlycosylTransferase ». Ces 24 gènes NGT ont été préalablement identifiés au cours de la thèse de doctorat de Sara Fasmer Hansen dans l'équipe Glycobiologie Moléculaire du CERMAV, à l'aide d'une approche bio-informatique originale (Ph.D. Université Grenoble, 2009). J'ai donc entrepris une étude de génomique fonctionnelle axée d'une part sur la caractérisation de mutants d'Arabidopsis pour ces gènes et d'autre part sur l'expression de ces gènes en systèmes hétérologues afin de caractériser l'activité potentielle de ces glycosyltransférases.

Le manuscrit de thèse est divisé en 4 chapitres.

Le **chapitre I** est une introduction générale sur l'état de l'art de mon sujet de recherche. Ce chapitre commence par une description de la paroi végétale et de son importance chez la plante

modèle en physiologie végétale, *Arabidopsis thaliana*. Ce chapitre décrit ensuite les caractéristiques des enzymes qui synthétisent les polysaccharides dans le vivant, nommées glycosyltransférases, en s'attachant à leurs mécanismes d'action. Enfin, un dernier paragraphe recense les acteurs moléculaires actuellement identifiés comme étant impliqués dans la biosynthèse des différents polysaccharides de la paroi végétale, pour finalement conclure sur l'objectif de mes travaux de thèse.

Le **chapitre II** décrit principalement la première année de mes travaux de thèse, durant laquelle je me suis attachée à caractériser un maximum de lignées mutantes d'Arabidopsis concernant les gènes NGT. Ce travail a été entrepris afin de rechercher à l'aide d'études de phénotypes, qu'ils soient développementaux ou bien biochimiques, les mutants et donc les gènes qui sembleraient impliqués dans la mise en place de la paroi végétale. Cette partie des travaux m'a conduit à identifier par PCR 35 lignées T-DNA affectant 16 gènes NGT différents. Ce travail préalable, relativement fastidieux, m'a permis par la suite d'étudier le développement en serre de ces 35 mutants, ainsi que d'analyser leur composition en sucres, afin de choisir sur la base de ce criblage de caractériser deux lignées mutantes affectant le gène At5g28910 (renommé NGT1). Le chapitre II se poursuit et termine finalement sur les clonages que j'ai effectués de 6 gènes de la famille NGT, dont le gène At5g28910, afin de démontrer une activité glycosyltransférase *in-vitro* pour la protéine NGT1 exprimée en système hétérologue.

Le **chapitre III** est l'utilisation de la génomique fonctionnelle pour démontrer l'implication du gène NGT1 dans les processus de biosynthèses de la paroi végétale. Ce chapitre débute par la caractérisation de deux lignées mutantes nommées *ngt1-1* et *ngt1-2* qui sont respectivement altérés dans le premier exon et dans la région 5'- non traduite du gène NGT1. Ces deux lignées mutantes présentent un phénotype qui se traduit par un développement ralenti des feuilles. Ce phénotype peut être restauré par la transformation de nos lignées mutantes par le gène NGT1 sous contrôle d'un promoteur fort, ce qui permet de corréler de façon certaine ce phénotype à l'altération du gène NGT1 chez nos mutants. Par ailleurs, la caractérisation de la paroi des mutants *ngt1-1* et *ngt1-2* a révélé des diminutions significatives de certains sucres présents notamment au niveau des pectines, ce qui nous a conduit à étudier plus finement la composition de la paroi des mutants et à démontrer l'implication du gène NGT1 dans l'élaboration de la paroi. Le chapitre III se termine sur de nombreux tests de caractérisation *in-vitro* de l'activité glycosyltransférase de la protéine NGT1, qui malgré des résultats parfois encourageants n'ont pas permis à ce jour de définir de façon indiscutable le type d'activité qui serait catalysée par le gène NGT1.

Le **chapitre IV** décrit une partie annexe de mes travaux de thèse, durant laquelle je me suis intéressée à exprimer de façon hétérologue deux glycosyltransférases impliquées dans la biosynthèse du xyloglucane (une xylosyltransférase et une fucosyltransférase), afin de permettre une étude structurale d'une première glycosyltransférase végétale par diffraction aux rayons-X. Des versions tronquées (délétées de leur partie N-terminale) de la xylosyltransférase (AtXT1) ainsi que de la fucosyltransférase (AtFUT1) ont pu être exprimées en système hétérologue mais seule AtFUT1 a été démontrée active et caractérisée. Ces travaux ouvrent la perspective d'une purification prochaine de cette glycosyltransférase afin de cribler des conditions permettant sa cristallisation et son étude structurale.

Le **chapitre V** est une conclusion générale des résultats marquants de mes travaux qui ouvre sur une discussion des perspectives à court terme, notamment du point de vue expérimental, concernant l'étude des gènes NGT.

Finalement, les trois derniers chapitres du manuscrit sont respectivement la partie « matériels et méthodes », la partie « bibliographie » et une partie « annexes » de mes travaux de thèses. Il est à noter que cette partie « annexes » présente entre-autres documents un projet de revue intitulé « Golgi-mediated synthesis and secretion of matrix polysaccharides of the primary cell wall of higher plants » qui sera publiée prochainement.

List of Abbreviations

2-AB	2-Aminobenzamide
3D	Three-dimensional
aa	Amino acid
ABRC	*Arabidopsis* Biological Resource Center
AGA	ApioGAlacturonan
AGP	Arabinogalactan protein
ANTS	8-Aminonaphthalene-1,3,6-TriSulfonate
Api	Apiose
APS	Ammonium Per Sulphate
Ara	Arabinose
AtFUT1	*A. thaliana* Fucosyltransferase1
AtpFut	*A. thaliana* putative Fucosyltransferase
AtXT1	*A. thaliana* Xylosyltransferase1
BAR	Bio-Array Resource
BSA	Bovine serum albumin
BMMY	Buffered Methanol-complex Medium
BMGY	Buffered Glycerol-complex Medium
CalS	Callose synthase
CATMA	The Complete Arabidopsis Transcriptome MicroArray
CAZy	Carbohydrate Active EnZyme
CesA	Cellulose-synthase
CMP	Cytidine monophosphate
Col0	Columbia ecotype
CSB.DB	Comprehensive Systems-Biology Database
CSC	Cellulose synthase complex
Csl	Cellulose-synthase-like
CTAB	Hexa-decyl-trimethyl ammonium bromide
Dha	3-deoxy-D-lyxo-2-heptulosaric acid
DTT	Di-thio-threitol
EDTA	Ethylene Diamine Tetra-Acetic acid
ER	Endoplasmic reticulum
EST	Expressed sequence tag
EXTs	Extensins
FTIR	Fourier-transformed infrared spectroscopy
Fuc	Fucose
FACE	Fluorophore-Assisted Carbohydrate Electrophoresis
Gal	Galactose
GalNAc	N-Acetylgalactosamine
GATL	GAlacturonic acid Transferase-Like
GAUT	GAlactUronic acid Transferase
GAX	Glucurono ArabinoXylan
GDP	Guanidine diphosphate
GH	Glycosylhydrolase
Glc	Glucose

GlcA	Glucuronic acid
GlcNAc	N-Acetylglucosamine
GPI	glycosylphosphatidylinositol
GSL	Glucan synthase-like
GT	Glycosyltransferase
GUS	β-glucuronidase
HCA	Hydrophobic Cluster Analysis
HG	Homogalacturonan
HPLC	High-Performance Liquid Chromatography
HRGP	Hydroxyproline-rich glycoproteins
HRP	Horseradish peroxidase
HyP	Hydroxyproline
irx	Irregular xylem
IHF	Integration Host Factor
Int	Integrase
Kdo	2-keto-3-deoxy-D-manno-octulosonic acid
Man	Mannose
ManS	Mannan synthase
MALDI-TOF	Matrix Assisted Laser Desorption Ionisation-Time Of Flight
MLG	Mixed Linked Glucans
MS	Mass Spectrometer
NASC	Nottingham Arabidopsis Stock Center
NDP	Nucleotide diphosphate
NeuAC	*N*-acetyl neuraminic acid
NGT	Novel GlycosylTransferase
NMR	Nuclear Magnetic Resonance
OGA	OligoGalacturonides
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCW	Plant cell wall
PME	Pectine methylesterase
PRPs	Proline-rich proteins
QUA	Quasimodo
RG I	Rhamnogalacturonan I
RG II	Rhamnogalacturonan II
Rha	Rhamnose
rsw	Radial swelling mutant
SDS	Sodium dodecyl sulfate
TAIR	The Arabidopsis Information Resource
TBE	Tris-borate-EDTA buffer
TBS	Tris buffered saline
TFA	Tri-Fluoroacetic Acid
TMS	Tri-Methyl Silylation

TMD	Transmembrane domaine
Tris	Tris (hydroxymethyl)aminomethane
UDP	Uridine diphosphate
UGTs	UDP-glycosyltransferases
WT	Wild-type
XET/XTH	Xyloglucan Endotransglycosylases
XGA	Xylogalacturonan
Xis	Excisionase
XyG	Xyloglucan
Xyl	Xylose
YPD	Yeast Peptone Dextrose
YPDS	Yeast Peptone Dextrose Sorbitol

Chapter 1

Introduction

1 Introduction

1.1 Importance of the plant cell wall

Plant cells are enclosed by a dynamic multilayered structure, which is a unique and characteristic feature of plants, called cell wall that differentiates them from animals. Plant cell wall receives a lot of attention as it serves multiple purposes for the plant physiology and development, but also because of the many applications it has for human uses. For the plant, cell wall plays a central role in determining plant shape, growth, development, provides tensile strength and mechanical support. In addition, it has a significant role in plant defense against pathogens and responses to environmental stresses. Cell wall is also involved in other processes like cell adhesion, cell signaling and cell-cell interaction (Carpita & Gibeaut 1993, Gibeaut & Carpita 1994, Vorwerk *et al.*, 2004). The plant cell wall has many commercial uses, it serves for example as a raw material in wood, paper, textile and food industries (Farrokhi *et al.*, 2006) but it is also envisioned as a major source of renewable biomass for sustainable biofuel production .

The structural and functional properties of cell wall depend on polysaccharides, proteins, lignin and some other compounds like suberin and cutin that make up the plant cell wall (Figure 1.1). Owing to the diversity of cell shapes and functions, the molecular composition and arrangement of cell wall exhibits a great diversity. Based on ultrastructural observation and biochemical composition the plant cell wall consists of three types of layers in higher plants. The first layer, called middle lamella, is the outermost layer to the cell and is mostly made up of pectic polysaccharides. The middle lamella is found at the interface of two adjacent cells (which develop from the cell plate present at division) and hold them together thanks to divalent cations bridging anionic pectic polysaccharides from each cell. Primary walls (the 2nd layer) are formed in developing, growing and enlarging cells. They are composed of 90% of polysaccharides and 10% of proteins (McNeil *et al.*, 1984, Showalter 1993; 2001). Primary cell wall mainly provides mechanical support and dynamic strength to allow cell expansion. Depending on the composition, two different types of primary cell walls (type I and type II) are found in angiosperm (or flowering plants) (Carpita & Gibeaut 1993). Dicots and non-commelinoids have type I primary cell walls that consists of cellulose microfibrills interconnected by xyloglucan (XyG) polysaccharides in a network (Carpita & Gibeaut 1993, Yokoyama & Nishitani 2004). Then, this cellulose-XyG network is embedded in a pectic network consisting of

homogalacturonan (HGA), rhamnogalacturonan I (RG-I) and rhamnogalacturonan II (RG-II) (Carpita & Gibeaut 1993, Carpita & McCann 2000). The percentage composition of different components of type I primary cell walls (on a dry weight basis) is typically cellulose-XyG ~50%, Pectin ~30% and structural proteins are ~20%.

Type II walls are found in commelinoid monocotyledons, i.e. in cereals such as rice, wheat, oat and barley (Carpita & Gibeaut 1993). They are organized like type I walls except that they have lower amount of XyG and pectin (Carpita & Gibeaut 1993). The major hemicellulose is glucuronoarabinoxylans (GAX) and mixed linked glucans (MLG). The percentage composition of different components of type II primary cell walls (on a dry weight basis) is cellulose ~30%, GAX ~30%, MLG ~30%, Pectin ~5%, XyG ~4% and structural proteins are almost 0.5% (Fry & Stephen 1988) .

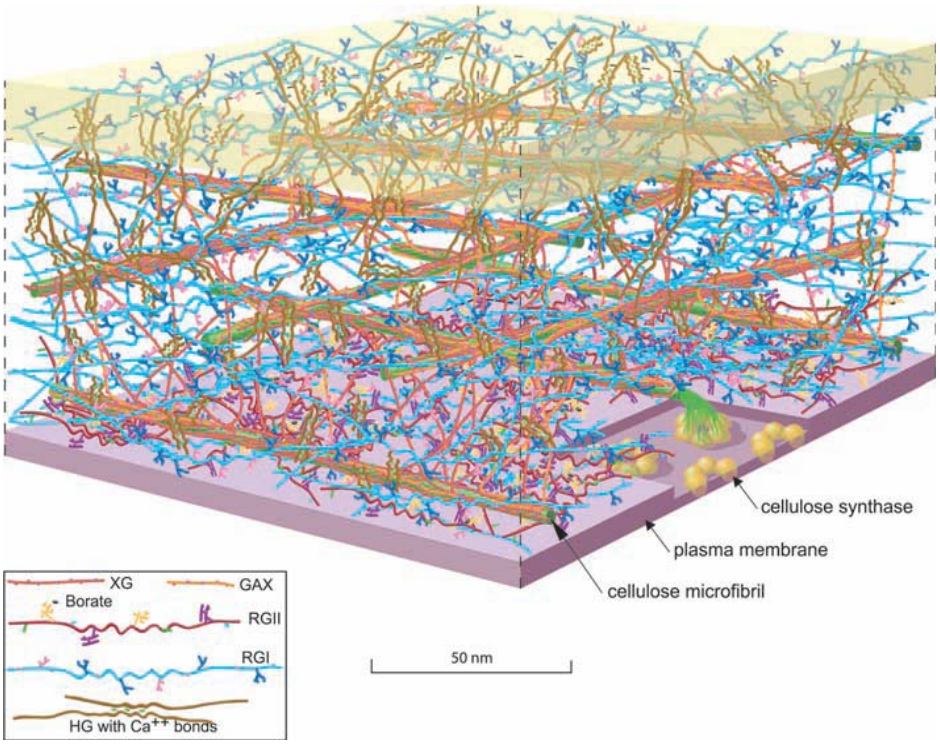


Figure 1.1: Scale model of the polysaccharides organisation in an Arabidopsis leaf cell wall. The amount of the various polymers is shown based approximately on their ratio to the amount of cellulose. The amount of cellulose shown was reduced, for clarity. Because of the exaggerated distance between microfibrils, the hemicellulose cross-links [shown in dark orange (xyloglucan, XyG) or light orange (glucoronoarabinoxylan, GAX)] are abnormally extended (Somerville *et al.*, 2004).

Secondary cell wall is formed when the cells have ceased enlarging and fully expanded and is laid down between primary cell wall and plasma membrane. Secondary cell walls provide

strength and contribute to specialized functions related to specific cell types such as xylem fibers, tracheids and sclerides. It is mainly composed of cellulose but also have some other polysaccharides like hemicelluloses. In addition, it has lignin and glycoproteins which are responsible for mechanical strength (Carpita & McCann 2000). Pectins and structural proteins or enzymes may be absent in secondary cell walls.

1.2 *Arabidopsis thaliana* as a model plant to study cell wall biosynthesis

Arabidopsis is a small flowering plant that completes its life cycle in six weeks. In addition, this plant is of very small size which makes it easy to cultivate in a small space in labs. Individual plant can produce several thousand seeds. It has one of the smallest plant genome, estimated at 26735 genes spreads over five chromosomes and encoding approximately 31392 proteins (http://genome.jgi.doe.gov/), and was the first plant genome to be completely sequenced by the Arabidopsis genome Initiative in 2000. All these features lead to *Arabidopsis thaliana* as a unique model in plant biology, in order to unravel genetics mechanisms underlying many plant traits. Although Arabidopsis was one of the first lands plant species whose genome sequencing project was completed it was followed by the sequencing of the genome of many other plant species like *Medicago truncatula*, *Oryza sativa*, *Zea mays*, *Citrus sinensis* etc. Genome sequencing of the plants is an important genetic tool which facilitates the scientific community to a greater extent for the following reasons:

- Genome sequencing not only provides sequence information of all the genes but also provides sequence information of the regulatory regions outside the genes.
- This sequence information can be very useful to predict the function of these genes by homology with already characterized genes of the related species.
- Last but not least, genome sequencing has helped to reduce the time needed for the molecular/genetic characterization of the plant species and to identify genes for crop improvement.

Data regarding the sequenced plant genomes are freely available to the scientific community all over the world on the genome data base phytozome (http://www.phytozome.net/). This database is powered by the joint project of the Department of Energy's Joint Genome Institute and the Center for Integrative Genomics

(http://www.jgi.doe.gov/). It facilitates comparative genomic studies amongst green plants. Till date 25 nuclear genomes (*Arabidopsis thaliana, Medicago truncatula, Oryza sativa, zea mays, citrus sinensis, chlaymydomonas reinhardtii* etc) have been completely sequenced and annotated. Some of the plant species whose genome have been sequenced serve as model plants for other genus of the same species i.e. their genome is representative of the genomes of other genera.

There are many databases which provide the molecular, genetic and physiological information about the genes of different species. For example, Bio-Array Resource" (BAR) which can be used to explore large scale data sets available from microarrays of Arabidopsis and other species is currently serving a scientist community. It comprises of various tools that facilitates the community of researchers by providing information about the expression of a gene, co-expression of genes, interaction of other proteins with your gene of interest, localization of the gene, and much other useful information (http://bar.utoronto.ca/affydb/BAR_instructions.html). Another database, publicly available, is "Genevestigator" (https://www.genevestigator.ethz.ch/). It contains gene expression data available from many transcriptome experiments and gives information about the regulation of gene expression i.e. spatial and temporal localization, response to stimuli, drug treatment, disease or genetic modification. An Arabidopsis specific database for gene sequence tags (GSTs) is CATMA. CATMA stands for (The Complete Arabidopsis Transcriptome MicroArray) (http:// www.catma.org/). It contains gene model sequences for over 70% of the predicted genes in the *Arabidopsis thaliana* genome as well as primer sequences for GSTs amplification and a wide range of supplementary information. The Comprehensive Systems-Biology Database (CSB.DB) is hosted at the Max Planck Institute of Molecular Plant Physiology, Golm, Germany. It presents the biostatistical analyses on numeric gene expression data which is associated with current biological knowledge. It also provides Co-Response Databases of various model organisms, like *Escherichia coli*, *Saccharomyces cerevisiae* and *Arabidopsis thaliana*.

After the completion of genome sequencing for many plant species, the important work was to assign function to identified genes. Then the scientists have focused attention to the functional genomics. An important tool while doing functional genomics is insertional mutagenesis which is used to disrupt the gene function to obtain knock out mutants. It provides direct route to determine gene function. T-DNA of *Agrobacterium tumefaciens* is commonly

used as mutagen to create knock-out mutants. Hundreds of thousands T-DNA insertion mutants are available at ABRC (*Arabidopsis* Biological Resource Center) and NASC (Nottingham Arabidopsis Stock Center) that are helpful to link DNA sequence to its phenotype. The main knock-out mutant resources are SALK (http://signal.salk.edu/cgi-bin/tdnaexpress), SAIL (Syngenta Arabidopsis Insertion Line, available on SALK website), GABI-KAT (http://www.mpiz-koeln.mpg.de/GABI-Kat/) and FLAG (http://flagdb-genoplante-info.infobiogen.fr/).

It has been estimated that in Arabidopsis almost 10% of the genes are involved in different aspects of plant cell wall metabolism like polysaccharides biosynthesis, their transport and deposition and remodeling and regulation of these processes (McCann & Carpita 2008). Arabidopsis is one good model plant for cell wall studies because its cell wall is similar to many other crop plants and trees (Liepman *et al.*, 2010). In order to determine the putative function of candidate genes involved in cell wall biosynthesis, a functional genomics strategy is then commonly used. This strategy consists of two approaches (Figure 1.2).

1- First approach is the characterization and identification of the T-DNA mutant and then determination of the alterations in phenotype and chemotype of the mutants to find out the putative role of the gene.
2- Second is the cloning of gene of interest for heterologous expression of protein and then to perform activity test *in vitro* to find out its function.

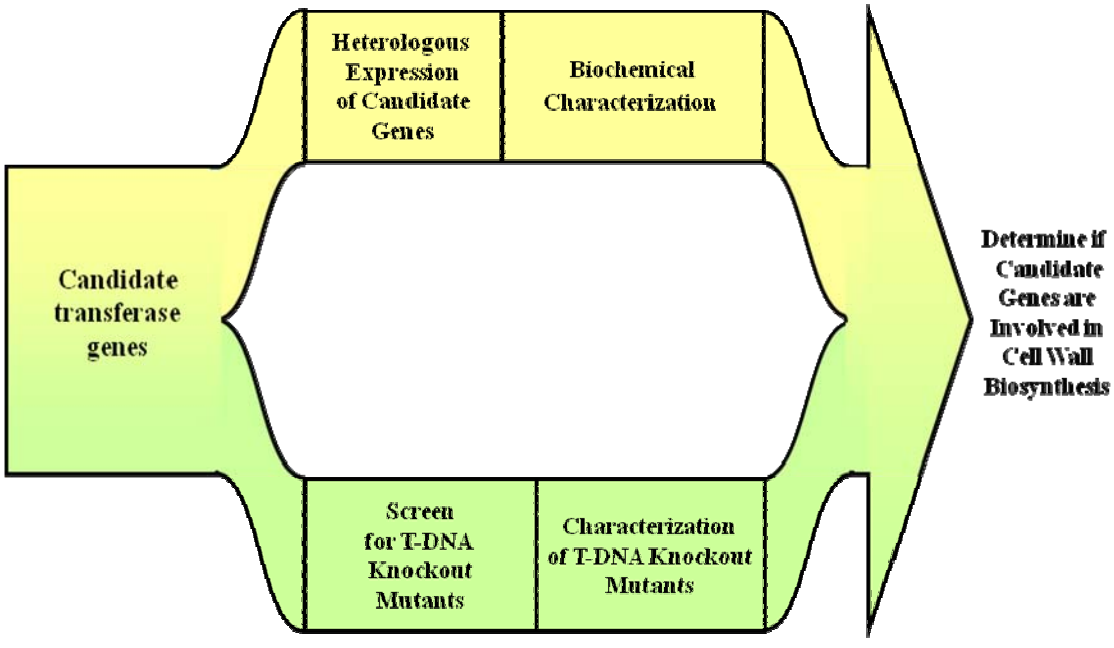


Figure 1.2: A functional genomic strategy for the characterization of putative genes involved in plant cell wall biosynthesis. This approach consists of parallel identification of the protein activity using hetereologous expression and activity tests and characterization of cell wall features from altered mutants.

1.3 Plant cell wall components

1.3.1 Cellulose

Cellulose is mostly synthesized by vascular plants, but many species from algae to bacteria, including the animal tunicate are naturally able to produce cellulose. As cellulose is one of the major components of plant cell wall, it is also the world's most abundant macromolecule found in nature (Somerville 2006). Up to one third of the total dry mass of many plants is often contributed by cellulose alone. It is the major load bearing component of plant cell wall. It not only provides the strength to resist the turgor pressure in plant cell walls but also has a very important role in maintaining the size, shape and division/differentiation potential of most plant cells and ultimately determines the direction of plant growth.

Chemically, cellulose is a simple linear polymer of β-(1→4) linked glucose residues. The repetitive building block in cellulose is cellobiose which consists of a pair of glucose linked in β-(1→4), where successive glucosyl units are rotated of 180° with respect to the other (Figure 1.3). The flat conformation of the glucopyranose ring and the linkage pattern provide a ribbon shape and semi-rigid properties to the cellulose, finally permitting the molecules to crystallize into rods named microfibrils.

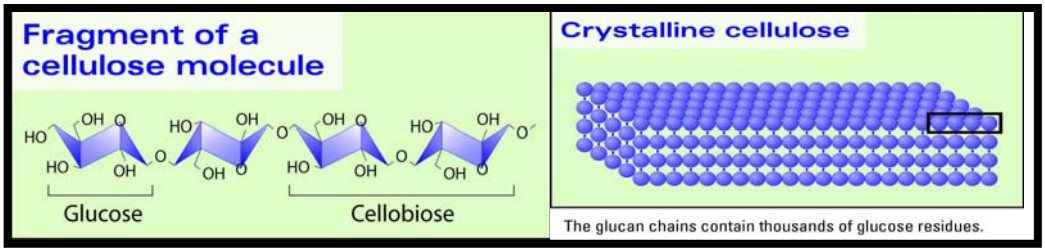


Figure 1.3: Schematic representation of cellulose composition and organization to form a microfibril. (Modified from http://genomics.energy.gov)

- **Different cellulose substructures**

Cellulose appears a simple structure but its physical properties vary remarkably in term of degree of crystallinity and molecular weight, because of the diversity of the living source from which it could be obtained. Cellulose can be found in nature as noncrystalline, crystalline form I (cellulose I) and crystalline form II (cellulose II). Cellulose I is found in higher plants and characterized with glucan chains parallel to each other and packed to form the microfibril. NMR and X-ray studies showed that cellulose I exists in two different forms named allomorph Iα and allomorph Iβ (Brown *et al.*, 1976, Brown 1996). These two allomorphs of cellulose differ in their physical properties because of different molecular conformation, their crystal packing and hydrogen bonding but can still co-exist together within a microfibril (Nishiyama *et al.*, 2003). Cellulose from plant (cotton fiber) was shown to be enriched in cellulose Iβ whereas bacteria and algae were rich in cellulose Iα. Cellulose II, the most thermodynamically stable form of cellulose, is rarely found in nature and was studied using *Acetobacter Xylinum* mutants.

- **Biogenesis of cellulose I**

The substrate for cellulose synthesis is UDP-Glucose which is channeled through a plasma-membrane localized enzymatic complex named cellulose synthase complex (CSC). Although the detailed mechanism of the polymerization of glucose units into a linear cellulose chain have not been established (regarding how successive glucosyl units will be flipped by 180° from its neighbor unit), it remains that cellulose I is synthesized processively with the non-reducing end (growing end) of the glucan chains attached with the catalytic enzyme of the CSC (Koyama *et al.*, 1997). Parallel chains are then synthesized and held together by hydrogen bonding to form crystalline microfibrils. Cellulose microfibrils vary in width from 25-30 nm in Valonia and other green algae to approximately 5-10 nm in most of the plants (Herth 1983, Ha *et al.*, 1998). The secondary cell wall has higher molecular weight cellulose with the degree of polymerization of 14000-15000 units (Brett 2000) whereas low molecular weight cellulose is present in primary cell wall with a degree of polymerization of 8000 units (Brown 2004).

Freeze fracture electron microscopy showed that CSC harbors a hexagonal structure with a six-fold symmetry, also named rosette or terminal complex, which is present at the plasma membrane surface in algae and vascular plants (Mueller *et al.*, 1976, Giddings *et al.*, 1980). This hexagonal structure is believed to contain 6 rosette subunits, each subunit being formed of the assembly of 6 cellulose synthase (CESA) catalytic polypeptide chains (the products of three

different CesA genes). This hypothetical organization is deduced from immunogold labelling assays using an antibody raised against cotton CESA (Figure 1.4; (Kimura *et al.*, 1999). This organization suggests that a rosette would be responsible for the simultaneous elongation of 36 β-(1→4)-glucan chains that would co-crystallize to form a microfibril (Delmer 1999).

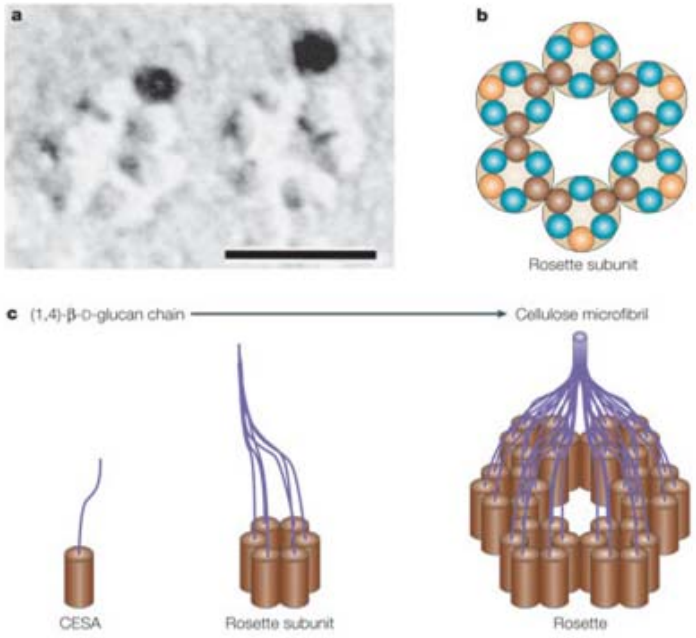


Figure 1.4: The cellulose-synthesizing machinery of the cell wall. A: Immunogold labeling shows that CESA is localized to hexameric 'particle rosettes' in the plasma membrane (Kimura *et al.*, 1999). The black circles represent gold nanoparticles that are attached to antibody against CESA. The smallest subunit in the particle rosette is believed to be made of six CESA proteins. Particle rosettes are sometimes found attached to cellulose microfibrils. Scale bar, 30 nm. B: This model of a hexameric particle rosette shows how three different CESA proteins (shown in three different colours: α, orange; β, brown; γ, green) might be organized into rosette subunits and then into a hexameric synthase complex (Doblin *et al.*, 2002).CESA assembly into rosette subunits C: A model of how CESA complexes synthesize a cellulose microfibril. Each CESA protein can synthesize a single β- (1→4)-linked-D-glucan chain. Cellulose is formed as a crystalline ribbon that is composed of many such glucans. In this model, 36 β-D-glucan chains are formed by a particle rosette, which is composed of a hexamer of CESA hexamers.

This number of 36 chains was actually compatible with the lateral size of the microfibrils isolated from primary cell wall of most of the plants (Delmer 1999). However, others studies propose the presence of 18 glucan chains or even less per microfibril (Chanzy 1978, Chanzy *et al.*, 1979, Ha *et al.*, 1998, Thimm *et al.*, 2002, Kennedy *et al.*, 2007). Actually the determination of the number of glucan chains depend on the number of active catalytic subunits per rosette, but how many active enzyme molecules are present per rosette have not been experimentally demonstrated (Guerriero *et al.*, 2010). Although the precise composition of

the cellulose synthase complex, the way cellulose is synthesized and the number of glucan chains within a microfibril are still under debate, our understanding of cellulose biosynthesis has moved forward thanks to genetics using Arabidopsis mutants impaired for cellulose biosynthesis. This aspect of the "genetic" of cellulose biosynthesis, using Arabidopsis mutants, will be developed in paragraph 1.5.1.

1.3.2 Hemicelluloses

Hemicelluloses are a heterogeneous group of polysaccharides present in various proportions in the cell wall, depending on plants. Their composition is also variable in quantity between primary and secondary walls, between species and even within different plant organs (O'Neill & York 2003). They are grouped into xyloglucan, xylans, mannans and glucomannans, and mixed-linked β-(1→3, 1→4)-glucans (but mixed-linked glucans are present only in cereals and grasses and absent in Arabidopsis cell wall).

- **Xyloglucans**

Xyloglucan (XyG) is one of the principal hemicellulose in the primary cell walls of dicots and present in almost all vascular plant species but has not been found in charophytes (Popper & Fry 2003, Moller *et al.*, 2007, Popper 2008). The XyG interacts with cellulose microfibrills through hydrogen bonding between xyloglucan backbone and the cellulose chain and this network is considered a major load bearing element in plant primary cell wall (Somerville *et al.*, 2004). Xyloglucan is composed of a backbone of β-(1→4)-linked glucose residues most of which are substituted by α-(1→6)-linked xylose side chains. These xylosyl residues can bear β-D-galactosyl (1→2) at O-2 position and some of which are further substituted by α-L-fucosyl (1→2) units (Figure 1.5) (McNeil *et al.*, 1984, Fry 1989a; b). Previous studies showed that XyG is not fucosylated in grasses (Hayashi 1989). But later on fucosylated XyG has been found in *Festuca arundinaceae* (McDougall & Fry 1994) and low xyloglucan amount was also detected in rice (Pena *et al.*, 2008). This indicates that, at least at early stages of XyG synthesis, fucose would be present but may be removed at late stages, for example during deposition into the cell wall. Xyloglucan has mainly two structural arrangements XXGG and XXXG where G represents unsubstituted glucosyl residues and X represents a glucosyl residue substituted with a xylosyl residue (Fry *et al.*, 1993). Most common among plant is the XXXG-type characteristic gymnosperm and found in dicots, like Arabidopsis (Fry *et al.*, 1993, Lerouxel *et al.*, 2002, Cavalier *et al.*, 2008). The XXGG-type is characteristic of some plant species like Poaceae

(monocots) and Solanaceae where xylosyl residues can be also substituted by α-L-Ara*f*. For these species XyG represents only 1-5% of the primary cell wall whereas it represents up to 20% in dicots cell wall (Scheller & Ulvskov 2010; Bonin *et al.*, 1997).

One letter code for xyloglucan substructure											
β-D-Glc*p*	α-L-Fuc*p* 1 ↓ 2 β-D-Gal*p* 1 ↓ 2 α-D-Xyl*p* 1 ↓ 6 β-D-Glc*p*	β-D-Gal*p* 1 ↓ 2 α-D-Xyl*p* 1 ↓ 6 β-D-Glc*p*	α-D-Xyl*p* 1 ↓ 6 β-D-Glc*p*	α-L-Gal*p* 1 ↓ 2 β-D-Gal*p* 1 ↓ 2 α-D-Xyl*p* 1 ↓ 6 β-D-Glc*p*	α-L-Ara*f* 1 ↓ 2 α-D-Xyl*p* 1 ↓ 6 β-D-Glc*p*	β-L-Ara*f* 1 ↓ 3 α-L-Ara*f* 1 ↓ 2 α-D-Xyl*p* 1 ↓ 6 β-D-Glc*p*	α-D-Xyl*p* 1 ↓ 6 β-D-Glc*p* 2 ↑ 1 α-L-Ara*f*	α-D-Xyl*p* 1 ↓ 6 β-D-Glc*p* 2 ↑ 1 β-D-Xyl*p*	α-D-Xyl*p* 1 ↓ 6 β-D-Glc*p* 2 ↑ 1 β-D-Xyl*p* 3 ↑ 1 α-L-Ara*f*	β-D-Xyl*p* 1 ↓ 2 α-D-Xyl*p*	D-Glcol
G	F	L	X	J	S	T	A	B	C	U	Gol

Table 1: Nomenclature for Xyloglucan Oligosaccharides. This table is based on the nomenclature (Fry *et al.*, 1993); modified with (Ray *et al.*, 2004) in which each of the differently substituted β-D-glucosyl residues is indicated by a single letter. For commodity, the pattern of xyloglucan substitution of each glucose residue is represented using a single letter nomenclature corresponding to the outermost substitution. Reducing glucose residues that have been converted to alditol moieties are indicated by the code "Gol". Xyloglucan oligosaccharides are unambiguously named by listing the code letters for each glucosyl residue, starting with the non-reducing end.

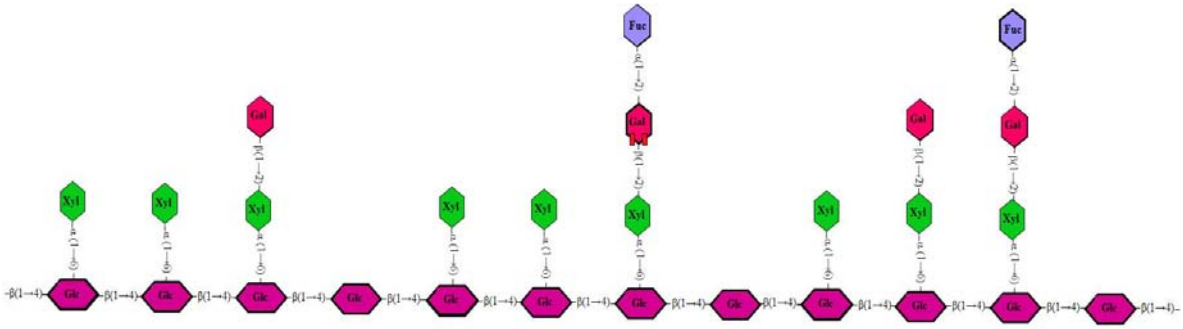


Figure 1.5: Schematic representation of xyloglucan structure. Xyloglucan glucan backbone is branched with xylose, galactose and fucose. (According to previous nomenclature this polysaccharide would be coded as: XXLG-XXFG-XLFG.)

Xyloglucan is currently described as a cross-linking polymer bridging cellulose microfibrils, and thus forming a load-bearing network responsible for most cell wall stiffness. This interaction between xyloglucan and cellulose was firstly based upon the observation that the two molecules remain associated during extraction procedures but could also be modelled at the atomic scale. Molecular dynamics simulations indicated that xyloglucan can interact with cellulose through its side chains as well as through its backbone (Hanus & Mazeau 2006). It has been observed that in case of less substituted XXXG direct interaction of all its residues occur due to flat conformation of xyloglucan and cellulose as it is difficult for XXLG and almost impossible for highly substituted XXFG to adopt a flat conformation that make the interaction difficult for all of the residues with the cellulose surface. These results are in accordance with experimental data as NMR experiments on tamarind xyloglucan and cellulose have shown that for the interaction of XXFG fragment, all backbone and side chain residues are in the close proximity of cellulose (Hanus & Mazeau 2006).

- **Xylans**

Xylans are the main hemicellulosic polysaccharides in the secondary wall of dicots. They consist of β-(1→4)-D-xylosyl residues for the backbone that could be substituted by arabinose, glucuronic acid and 4-O-methyl glucuronic acid residues, depending on plant species. Xylans are involved in the cross-linking of cellulose microfibrils and lignin (Awano *et al.,* 2002). When xylan backbone is substituted with arabinofuranose (Ara*f*) they are called arabinoxylans and glucuronoarabinoxylans (Figure 1.6). Arabinoxylans are more common in primary wall of grasses where they may be acetylated on C-2 and C-3 position of the GlcA residue (McNeil *et al.,* 1984, Ebringerova & Heinze 2000, Teleman *et al.,* 2000).

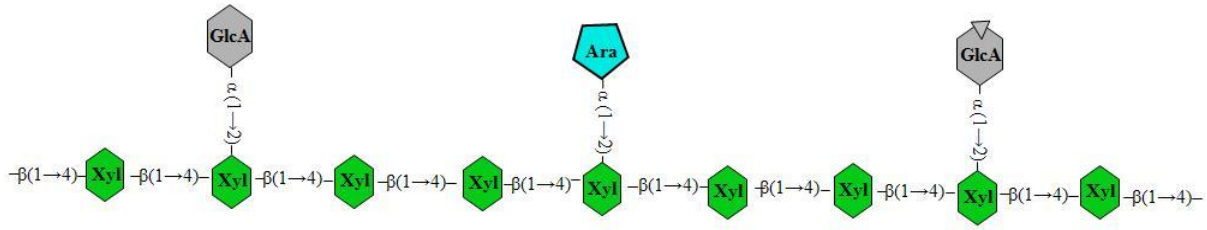


Figure 1.6: Schematic representation of glucuronoarabinoxylan structure. Xylose residues are substituted with Glucuronic acid and Arabinose.

- **Galacto-gluco-mannans**

Galacto-gluco-mannans are hemicellulosic polysaccharides with a backbone of β-(1→4)-linked mannosyl and β-(1→4)-linked glucose residues, which backbone is substituted with α-(1→6)-linked galactosyl residues. They are present in seeds, as a storage carbohydrate, in different plant species like legumes and palms but also exist in cell wall harbouring a structural role, specifically demonstrated in secondary cell wall (Buckeridge *et al.,* 2000; Maeda *et al.,* 2000). Other types of mannans are galactomannans and glucomannans mainly present in secondary cell walls; depending on plant type (Heredia *et al.,* 1995). The absence of the major glucomannan synthase in seeds of Arabidopsis results in a severe embryo lethal phenotype (Goubet *et al.,* 2003) which confirms the importance of mannan for seeds development.

- **Mixed-Linked Glucans**

Mixed linked β-(1→3, 1→4)-glucans (MLG) are found in poaceae (grasses) (Smith & Harris 1999) but not in dicots. MLG is composed of β-D-(1→4) linked glucans with interspersed β-D-(1→3)-linkages. In primary cell walls they are involved in cell expansion but their quantity is variable at different stages of growth (Obel *et al.,* 2002, Gibeaut *et al.,* 2005).

1.3.3 Pectins

Pectin is one of the major components of plant primary cell wall and middle lamella. Like other polysaccharides pectin has many commercial uses and approximately 40,000 tons of pectins are produced every year to be used in food industry mainly as a gelling agent, thickening agent and stabilizer. Some pectic polymers are even studied as pharmaceuticals for prostate cancer treatment (Jackson *et al.,* 2007). The cell wall of Arabidopsis leaves contains approximately 50% of pectin but the content varies according to the environment, tissue and species (Zablackis *et al.,* 1995). Pectin have a very important role for plant growth and development, cell wall strength, defense, morphogenesis, signaling, cell expansion, pollen tube growth, leaf abscission, seed hydration and fruit development (Ridley *et al.,* 2001, Willats *et al.,* 2001, Mohnen 2008). Pectins are also involved in defence mechanisms as they can detect pathogen attack and trigger signaling pathways that induce defence responses in the plants. Plant pathogens cause degradation of cell wall by releasing cell wall degrading enzymes. It has been suggested that degradation of homogalacturonan produce oligogalacturonides (OGA) which act as elicitors to trigger plant defences. Notably, it has been established that plants

treated with OGA produce reactive oxygen species (ROS) and plant defence hormones like ethylene (ET) and jasmonic acids (JA) (Moscatiello *et al.*, 2006). In addition modifications of pectic polymer also affect the plant growth and development. Peaucelle and his colleagues showed that pectin de-methyl-esterification plays an important role in the formation of flower primordia in the Arabidopsis shoot apical meristem (Peaucelle *et al.*, 2008). Pectin is a structurally complex molecule with high heterogeneity that could be subdivided in five (different classes, i.e. homogalacturonan (HG), xylogalacturonan (XGA), apiogalacturonan (AGA), rhamnogalacturonan-I (RG-I) and rhamnogalacturonan-II (RG-II), all having in common the presence of a high content of galacturonic acid.

- **Homogalacturonan**

HG is the most abundant polysaccharide, constituting about 65% of the total pectin (Mohnen 2008), is a linear polymer of α-(1→4)- linked D-galacturonic acid (GalA) residues (Figure 1.7) that are often methyl-esterified at the C-6 carboxyl position and possibly acetylated at the O-2 and O-3 of the GalA residues but degree of acetylation varies a lot among species (Carpita & Gibeaut 1993).

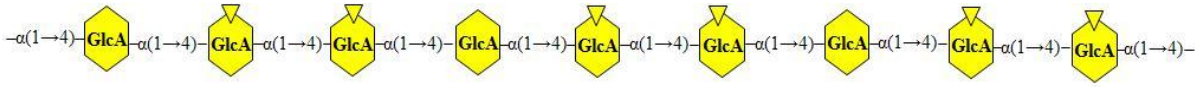


Figure 1.7: Schematic representation of homogalacturonan backbone substituted at the C-6 carboxyl position with methyl ester groups.

- **Xylogalacturonan and Apiogalacturonan**

Xylogalacturonan (XGA) has a backbone of GalA residues like HG but it is substituted with a single D-xylose residues at the C-3 of the GalA backbone residues (Schols *et al.*, 1990, Nakamura *et al.*, 2002, O'Neill & York 2003) but additional Xyl residues can be attached to the first Xyl with β-(1→4) linkage (Figure 1.8(Zandleven *et al.*, 2006). XGA is mostly abundant in reproductive tissues but to some extent present in other tissues, such as Arabidopsis leaves (Zandleven *et al.*, 2007).

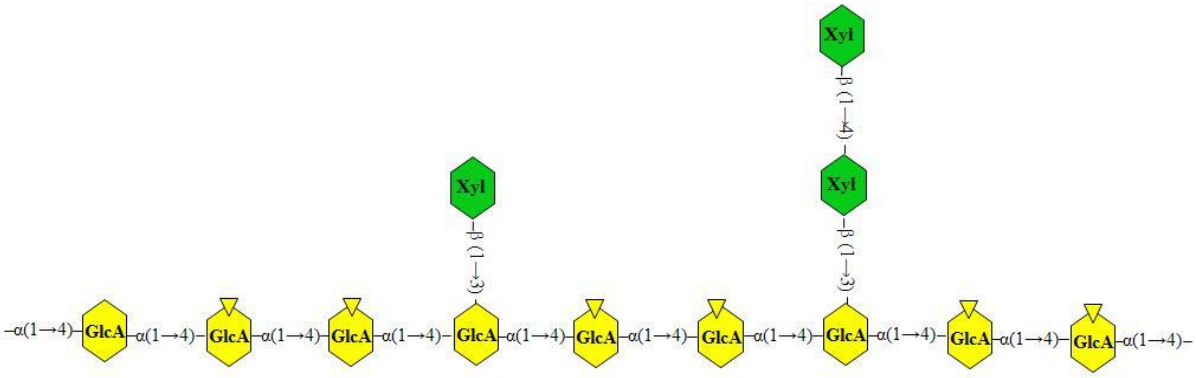


Figure 1.8: Schematic representation of xylogalacturonan (XGA), HG backbone substituted with xylose residues.

Apiogalacturonan is also similar to HG except that it is substituted with D-apiose residues at the C-2 or C-3 of GalA backbone residues. It has been described so far in aquatic plants such as duck weeds and the marine sea grasses (Hart & Kindel 1970, Ovodov *et al.*, 1971). Sometimes substitution can also occur with apiose, with the disaccharide of apiose (Api*f*-(1→3)-Api*f*-(1- found in lemna walls (O'Neill & York,2003)

- **Rhamnogalacturonan-I**

Rhamnogalacturonan-I (RG-I) has a different backbone from other pectic polysaccharides. It is made up of repeating disaccharide units of [α-(1→4)-GalA-α-(1→2)-Rha]. The rhamnose residues are often substituted with galactan, arabinan and type I arabinogalactan (Figure 1.9). Galactans are linear chains of β-(1→4)-linked galactose residues, while arabinans are chains of α-(1→5)-linked arabinofuranose residues that are mostly branched at C-3 and sometimes at C-2. RG-I is often acetylated at O-3 position of galacturonic acid (Ishii 1995; 1997). Type I arabinogalactans (AGs) are associated with RG-I. Type I AG has a β-(1→4)-linked linear chain of D-galactose which is substituted with single arabinose unit or shorter chains of L-arabinose units while type II AGs are highly branched chains with backbones of variously linked α-D-galactose units, which are terminated by L-arabinose residues and they are found in association with arabinogalactan proteins and xylans.

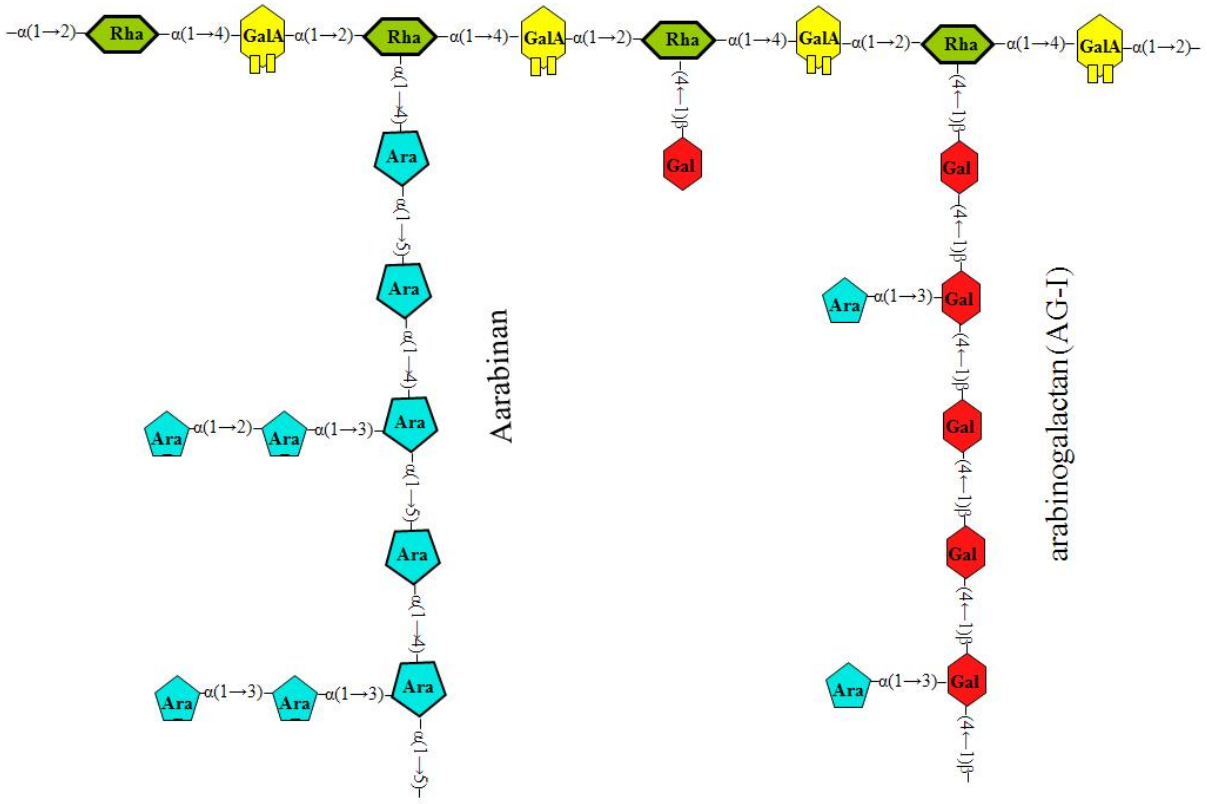


Figure 1.9: Schematic representation of substituted rhamnogalacturonan I (RG-I) with arabinan and arabinogalactan side chains

- **Rhamnogalacturonan-II**

Rhamnogalacturonan-II (RG-II) has a backbone of GalA residues but it is substituted at the C-2 and C-3 with four complex side chains (A to D), composed of 12 different types of glycosyl residues including some unique sugars like 2-O-methyl-xylose, 2-O-methyl-fucose, aceric acid (AceA), 2-keto-3-deoxy-D-lyxo heptulosaric acid (Dha) and 2-keto-3-deoxy-D-manno-octulosonic acid (kdo) (Figure 1.10). These glycosyl residues are linked together at least with 22 different glycosidic linkages, but despite of its complex nature the structure of RG-II is highly conserved among vascular plants (Matsunaga *et al.*, 2004, O'Neill *et al.*, 2004) which suggest that it has an important role in wall integrity and functions.

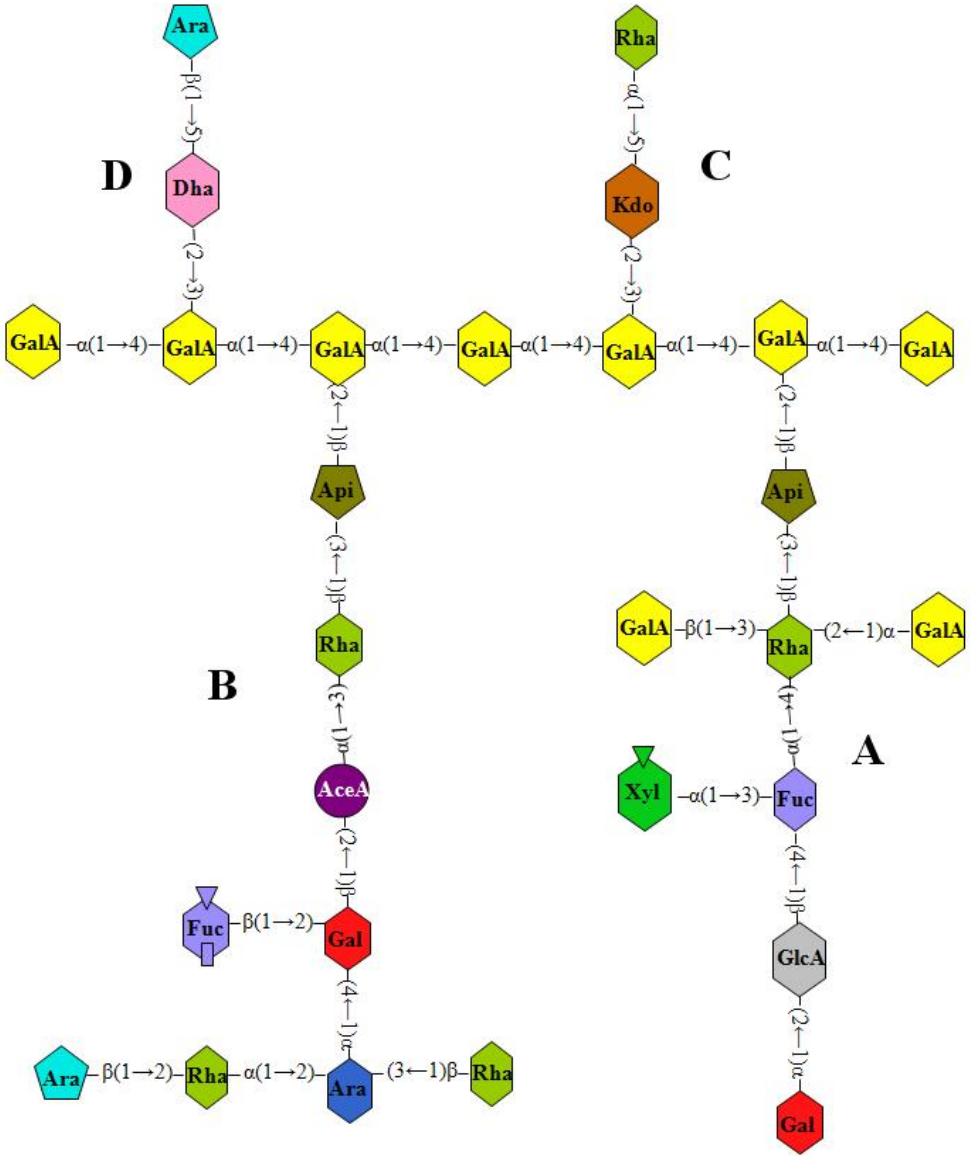


Figure 1.10: Schematic representation of rhamnogalacturonan II (RG-II) backbone which is substituted with four side chains. These side chains harbor rare and specific sugars.

The important characteristic of RG-II is its ability to dimerize with another RG-II molecule. In planta, 95 % of RG-II molecules exist in dimers where two apiosyl residues of side chain A are cross-linked by a borate diester bond between (O'Neill *et al.*, 2001). RG-II dimerization was first demonstrated *in vitro* by NMR spectroscopy in sugar beet cell wall, but its importance for plant development was demonstrated later thanks to Arabidopsis mutant studies (Ishii & Matsunaga 1996; O'Neill *et al.*, 2001). Indeed, characterization of RG-II from *mur1* mutant had shown that the lack of fucose in RG-II side chain B ultimately led to the alteration of RG-II overall structure and ability to dimerize (O'Neill *et al.*, 2001). While boron has been referenced as an essential micronutriment in plant physiology since the fifties, only

recently the analysis of Arabidopsis mutants altered for RG-II has been able to suggest that boron is essential for cell wall integrity and required for cell-cell adhesion and plant development (O'Neill *et al.*, 2001).

1.3.4 Callose

Callose is a plant polysaccharide, present at particular stages of growth and differentiation in cell walls or cell wall-associated structures (Bruce & Clarke 1992). It is composed of linear β-(1→3)-linked glucose residues and sometimes has β-(1→6) branches.

Callose is laid down at plasmodesmata, at cell plates during cytokinesis and is involved in pollen development (Bruce & Clarke 1992). It is also produced in response to wounding, infection by pathogens, aluminium, abscissic acid and other physiological stresses (Bruce & Clarke 1992). Callose is involved in multiple aspects of plant growth and development and response to biotic and abiotic stress (Figure 1.11).

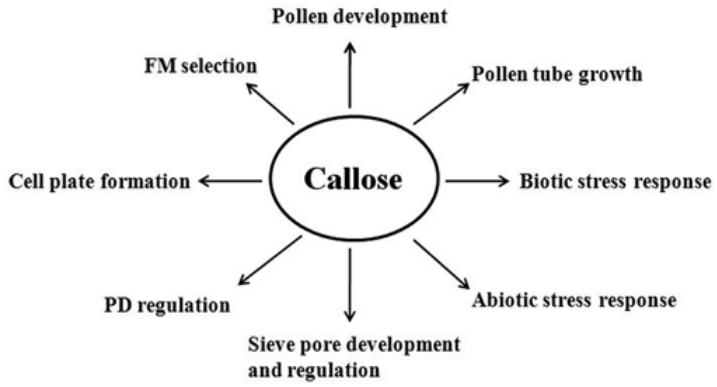


Figure 1.11: Callose is involved in multiple aspects of plant growth and development and response to biotic and abiotic stress (Chen & Kim 2009).

1.3.5 Lignin

Lignin is the second most abundant biopolymer on earth after cellulose. Lignin is found in plant mostly in secondary cell wall. It has a complex chemical structure based on the association of three monolignol monomers, i.e. ρ-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol. It performs many biological functions, e.g. it provides mechanical strength to the cell wall by cross-linking different plant polysaccharides as it is covalently linked to hemicellulose and cellulose and makes a lignin polysaccharide complex (Sarkanen 1998b, Sarkanen 1998c, Whetten *et al.*, 1998, Anterola & Lewis 2002, Boerjan *et al.*, 2003). It fills the

spaces in the cell wall between cellulose, hemicellulose and pectin components especially in tracheids, sclereids and xylem cells. It helps to conduct water in plant stems. It has some economic values as lignified wood serves as raw material for many applications and it can also be used as fuel. It also provides resistance to insects and pathogens.

1.3.6 Cell wall proteins

Proteins account usually for 10% of dry weight of plant cell wall and ubiquitous proteoglycans on the cell surface in plants. These proteins play a structural role, provide strength, control rate of cell growth and prevent or protect the cells from pathogen attack. They are involved in all aspects of plant development such as cell division and differentiation, pollen recognition and fertilization, flower organ formation etc (Wu *et al.,* 2001). Arabidopsis plant cell wall contains a super-family of hydroxyproline-rich glycoproteins which includes hyperglycosylated arabinogalactan proteins (AGPs), moderately glycosylated extensins (EXTs), and lightly glycosylated proline-rich proteins (PRPs).

The main structural protein in the cell wall of higher plants is extensin which is a class of hydroxyproline-rich glycoproteins (HRGP) (Showalter 1993). These proteins provide rigidity and strength to the wall by cross-linking with themselves or to other cell wall components and involved in the process of cell extension (Brady *et al.,* 1996, MacDougall *et al.,* 2001). Interestingly, one gene XEG113 belonging to GT family 77 was identified through forward chemical genetic approach which could putatively encode extensin arabinosyltransferase (Gille *et al.,* 2009). As analysis of T-DNA insertional mutant *xeg113* showed that etiolated hypocotyls are more elongated as compared to WT plants, so it provides the genetic evidence that extensins play an important role in the process of cell elongation and moreover the reduction of arabinose in *xeg113* have shown that extensin arabinosylation is important for normal plant growth and development (Gille *et al.,* 2009).

Arabinogalactan proteins (AGPs) consist of a core protein backbone (10%) that is decorated by arabinogalactan polymer chains and arabinoside oligomers as their carbohydrate components (90%) (Showalter, 2001). Most of the carbohydrate chains contain β-(1→3)-linked galactan and β-(1→6)-linked galactan chains those are connected to each other with (1→3, 1→6)-linked at O-3 and O-6 positions with side chains mainly composed of arabinose residues but sometimes also contain glucuronic acid, rhamnose, xylose, fucose (Figure 1.12) (Gaspar *et al.,* 2001, Seifert & Roberts 2007). Interestingly, many AGPs have been described to have a

glycosylphosphatidyinositol (GPI) lipid anchor, leading to suggest a role in signaling this molecule.

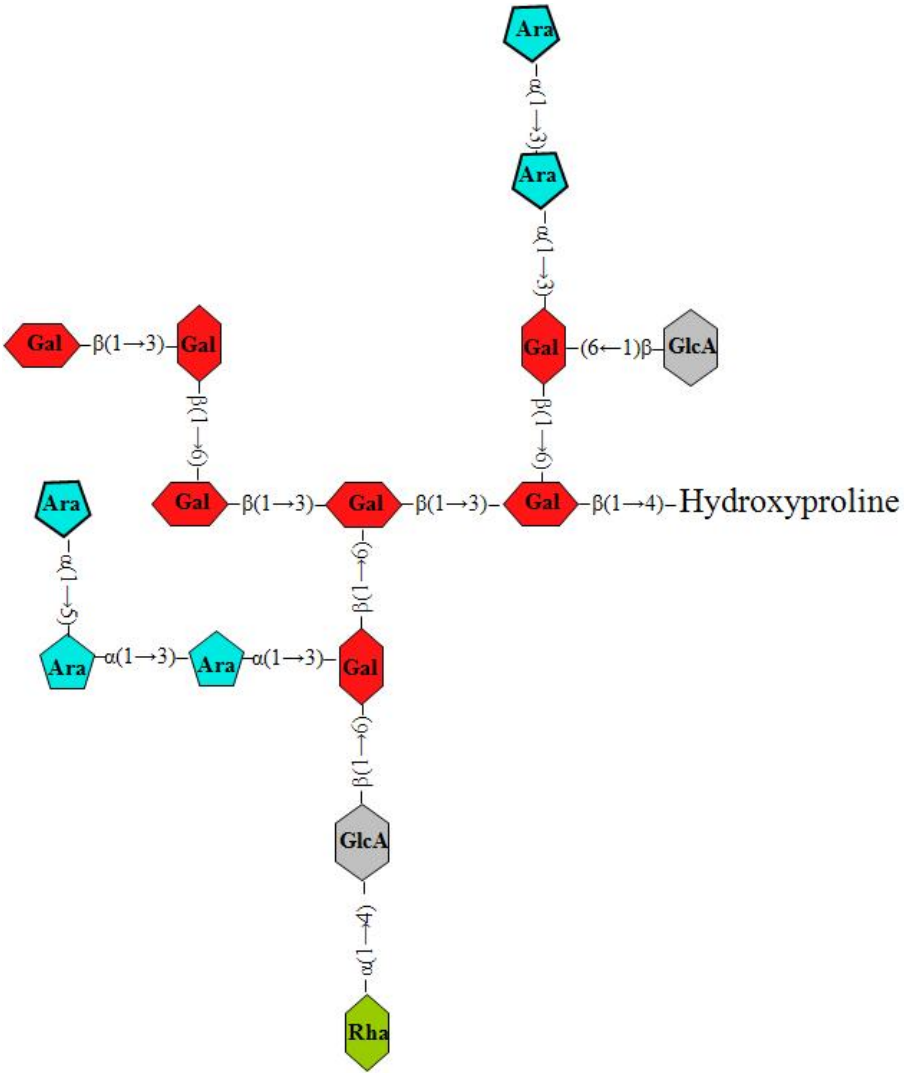


Figure 1.12**:** Schematic representation of arabinogalactan protein (AGP). The glycan is linked to hydroxyprolin of the peptide chain

They are classified into classical and non-classical AGPs. Classical AGPs consist of a central domain rich in Proline, Alanine, Serine, Threonine flanked by an N-terminal signal peptide and C-terminal GPI anchor, whereas non-classical AGPs contains Lysine-rich domains and Fasciclin-like AGPs (Gaspar *et al.*, 2001, Showalter 2001). Arabidopsis genome has 47 genes that encode AGP protein backbones and most of them are predicted to be GPI anchored out of which 13 are classical AGPs, three AGPs containing Lys-rich region, approximately 21 fasciclin-like AGPs and approximately 10 arabinogalactan peptides consisting of only 10-17

amino acid residues (Schultz *et al.*, 2002). They have a role in plant growth and development and recent studies proposed their role in many other biological processes like cell proliferation and survival and in plant pathogen interaction.

Plant cell wall also contains expansins which refer to a family of closely-related non-enzymatic proteins, which play important roles in plant cell growth, fruit softening, abscission, and emergence of root hairs, pollen tube invasion of the stigma and style, and other developmental processes where cell wall loosening occurs (Cosgrove 2000). It has been suggested that it disrupts the hydrogen bonding between xyloglucan and cellulose and believe to regulate cell expansion.

1.4 Glycosyltransferases

The biosynthesis of oligosaccharides, polysaccharides and glycoproteins requires the existence of a specific class of enzymes that catalyse the formation of the glycosidic bond by transferring a monosaccharide unit from a donor to a specific acceptor substrate. These enzymes are named glycosyltransferases (GTs). GTs commonly use activated donor sugar substrates like nucleoside diphosphate sugars that contain a phosphate leaving group (e.g., GDP-Fuc, UDP-Gal) but also some other donor molecules like nucleoside monophosphate sugars (CMP-NeuAc), or dolichol phosphate sugars (Lairson *et al.*, 2008). GTs that use nucleotide sugars as donors are called Leloir enzymes in honor of Luis F. Leloir who discovered the first nucleotide sugar. The acceptor molecules more commonly used by GTs are sugars but can also be some other molecules like lipids, proteins, nucleic acid, antibiotic or any other small molecule (Lairson *et al.*, 2008). These enzymes are present in both eukaryotes and prokaryotes and show exquisite specificity for both the glycosyl donor and the acceptor substrate.

1.4.1 Classification

A classification has been proposed that groups GTs into families based on amino acid sequence similarities. It is kept updated in the Carbohydrate-Active enZyme database (CAZy, available at http://www.cazy.org/). The database currently comprises over 75000 GT sequences that have been divided over 94 GT families. The GT families are created based on an experimentally proven protein function of at least one GT, and then populated by sequences of significant sequence similarity that are extracted from public databases. Within each family, a similar three-dimensional structure should be expected. The vast majority of enzymes in these

families remain biochemically uncharacterized ORFs. Plants tend to have far more GT genes (and other carbohydrate-active enzymes) than any other organism sequenced to date. More than 450 Arabidopsis GT genes have already been listed in CAZy that are spread into 42 families but less than 20% of these genes have been biochemically characterized. By comparison, less than 230 GT genes have been identified in the human genome, of which more than 80% are annotated. Multiplicity of GT genes in plants is mainly attributed to the highly complex network of cell wall polysaccharides that requires the participation of numerous GTs (Lerouxel *et al.*, 2006, Mohnen *et al.*, 2008), and to the large number of glycosylated secondary metabolites (Lim & Bowles 2004).

1.4.2 Mechanism

Glycosylation reactions proceed either through processive transfer reactions with multiple addition of the same monosaccharide (e.g., cellulose synthesis), or through non-processive single transfer reactions, as it is observed in the synthesis of most of the glycoconjugates. The transfer of saccharides by GTs is regiospecific and stereospecific. Glycosyltransferase reactions follow two mechanistically distinct pathways which result in either inversion or retention of the anomeric configuration of the transferred sugar (Figure 1.13). In the case of an inverting enzyme, a monosaccharide α-linked to its donor becomes β-linked in the final product (or vice-versa) whereas there is no change in the anomericity of the transferred sugar with a retaining enzyme.

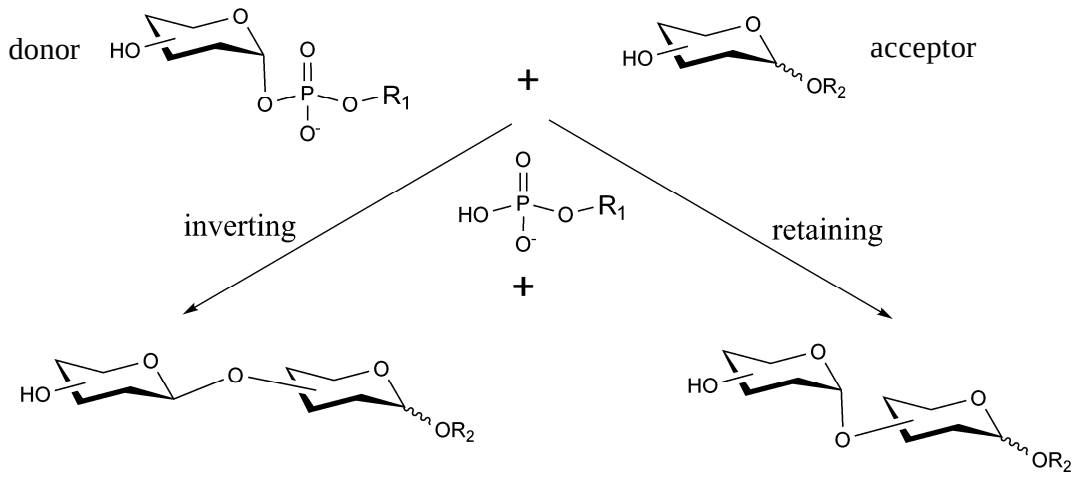


Figure 1.13: GT reactions occurring with either inversion or retention of configuration at the anomeric center of the donor sugar.

Inverting reactions are believed to follow a single displacement mechanism that involves nucleophilic attack of the OH-group of the acceptor on the anomeric center of the donor sugar (Lairson *et al.,* 2008). Structural and enzymatic studies strongly suggest that inverting GTs utilize a single S_N2-like displacement mechanism that involves nucleophilic attack of the OH-group of the acceptor on the anomeric center of the donor sugar (Lairson *et al.,* 2008). Reaction occurs with the formation of an oxocarbenium-ion transition state and is concomitant with departure of the nucleotide leaving group. The mechanism of retaining GTs is less clear. Although some members of this class may utilize a S_N2-like double-displacement mechanism that leads to the formation of a covalent glycosyl-enzyme intermediate (Monegal & Planas 2006, Soya *et al.,* 2011), a S_Ni-like mechanism involving the formation of a short-lived ion pair intermediate has also been proposed that seems likely for the majority of retaining GTs (Lairson *et al.,* 2008, Wilson *et al.,* 2008, Errey *et al.,* 2010).

1.4.3 Structure

In contrast to other classes of enzymes like glyosylhydrolases which have wide variety of folds, nucleotide-sugar-dependent glycosyltransferases solved to date have revealed only two structural folds called GT-A and GT-B (or variants) (Breton *et al.,* 2006).

- **GT-A fold**

The first 3D X-ray crystal for GT-A fold was identified for SpsA, an inverting enzyme from *Bacillus subtilis* (Charnock & Davies 1999). The GT-A fold consists of a α/β/α sandwich (a mixed seven-stranded β-sheet with 3214657 topology where strand 6 is antiparallel to the rest) that resembles the Rossmann fold. The central β-sheet is flanked by a smaller one, and the association of both creates the active site (Figure 1.14). The first region mostly corresponds to the nucleotide binding domain, encompassing the first 100-120 residues, and that is usually terminated by a characteristic Asp-X-Asp (often referred to as DxD motif) (Breton *et al.,* 1998, Breton & Imberty 1999) where X represents any aminoacid. The DxD motif is a degenerated sequence that is shown in all crystal structures to interact primarily with the phosphate groups of the nucleotide donor through the coordination of a divalent cation, typically Mn^{2+}. However one example of GT-A enzyme was shown recently to not possess this characteristic DxD motif (Pak *et al.,* 2006). The C-terminal part of GT-A shows more structural variability than the N-terminal and is responsible for the recognition of acceptor molecules (Breton *et al.,* 2006).

The two sialyltransferases of family GT-42 showed almost the same canonical GT-A topology but with different order of β-strands, hence these structures were considered either as a new fold or as a variant of the GT-A fold (Chiu *et al.,* 2004, Chiu *et al.,* 2007). These enzymes utilize a nucleotide monophosphosugar (CMP-NeuAc) as a donor and do not have DxD motif.

- **GT-B fold**

The first 3-D structure reported was for the GT-B fold enzyme, a DNA modifying β-glucosyltransferase from bacteriophage T4 (Vrielink *et al.,* 1994). It was found to be structurally homologous to glycogen phosphorylase. The GT-B fold is characterized by two separate Rossmann-type domains with a connecting linker region and a catalytic site located between the domains. Both domains show a α/β/α structure formed by a central parallel β-sheet with the topology 321456 (Figure 1.14). Members of GT-B family have a structurally conserved C-terminal domain which is responsible for binding the nucleotide sugar donor substrate whereas the N-terminal domain has more pronounced variations in the loops and α-helices which is responsible for the recognition of the wide range of sugar acceptors. GT-B are metal-ion independent and do not possess a DXD motif.

The 3D structure of a fucosyltransferase from *Helicobacter pylori* revealed a different fold type that can be considered as variant of GT-B fold because it exhibited the same 2-domain architecture as other GT-B members but they have some differences in the connectivity of β-strands (Sun *et al.,* 2007). A second fucosyltransferase that has been crystallized is the human α–(1→6)-fucosyltransferase (Ihara *et al.,* 2007). This enzyme displays an unusual modular architecture, consisting of a coiled *N*-terminal coil region, a catalytic domain, and a SH3 domain at the C-terminal. The catalytic domain is formed by two sub-structures, an open α/β sheet structure and a Rossmann domain.

- **Other GT folds**

Very recently, completely different folds have been observed for glycosyltransferases that utilize lipid-phosphate activated donor substrates. The crystal structures of the peptidoglycan glycosyltransferase domains (GT51) from *Staphylococcus aureus* and *Aquifex aeolicus* display an intriguing structural similarity to the bacteriophage λ-lysozyme (Lovering *et al.,* 2007; Yuan *et al.,* 2007). The STT3 catalytic subunit of oligosaccharyltransferase (GT66) from *Pyrococcus furiosus* shows very different and modular protein architecture (Igura *et al.,* 2008). These enzymes are not constrained by the need to bind a nucleotide, which might explain

the absence of a Rossmann domain. The discovery of novel folds is therefore likely in other lipid-phospho-sugar dependent GT families (Lairson *et al.*, 2008).

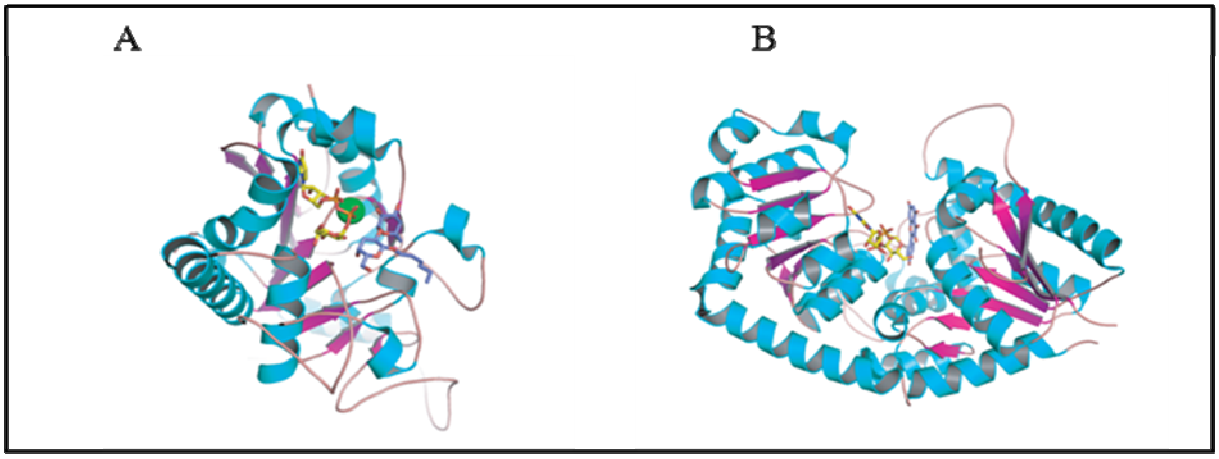


Figure 1.14: Ribbon diagram of GTs representative of the two general GT-A and GT-B folds. : (a) Classical GT-A fold (*PDB code 2RJ7*) (Alfaro *et al.*, 2008) (B) Classical GT-B fold (*PDB code 2CIZ*) (Offen *et al.*, 2006)

1.4.4 Localization of glycosyltransferases

In eukaryotes most of the glycosylation reactions take place in the endoplasmic reticulum (ER) and in the Golgi apparatus. In plants, cellulose and callose are synthesized at plasma membrane and deposited directly into the wall, whereas hemicellulose and pectin are synthesized into the Golgi and then transported to the wall in secretory vesicles. Most of the ER- and Golgi-resident GTs are transmembrane proteins with either type II or type III topology. The type II topology is by far the most common protein architecture among Golgi GTs, consisting of a short N-terminal cytoplasmic tail followed by a transmembrane domain, a stem region and a large C-terminal globular catalytic domain facing the luminal side (Paulson & Colley 1989, Breton *et al.*, 2001). Other metabolites such as substrates and ions like manganese, magnesium and calcium (for the activation of enzyme) are also required for the biosynthesis of non-cellulosic polysaccharides in the Golgi apparatus. Most of the nucleotide-sugars which act as donors for GTs are synthesized in cytoplasm and therefore must be transported to the Golgi by nucleotide sugar transporters (NSTs) (Keegstra & Raikhel 2001). Some polysaccharides are also modified to various degrees by the addition of methyl or acetyl groups to the nascent polysaccharide in the Golgi by methyl and acetyltransferases.

1.5 Biosynthesis of cell wall polysaccharides

The polysaccharide synthesis process is divided mainly into four different stages (1) production of activated nucleotide-sugar donors, (2) initiation of polymer synthesis, (3) polymer elongation, and (4) termination of polymer synthesis (Delmer & Stone 1988). The processes of production of nucleotide-sugar donors and the polymer elongation have been well studied but the mechanism of initiation and termination of polymer synthesis is not known. The biosynthesis of cellulose and callose takes place directly at the plasma membrane through polysaccharide synthases (Kudlicka & Brown 1997, Delmer 1999) whereas other non-cellulosic and pectic polysaccharides are synthesized by using both polysaccharide synthases and glycosyltransferases within the endoplasmic reticulum (ER) and Golgi apparatus (Figure 1.15) (Fincher & Stone 1981, Gibeaut & Carpita 1994). Then these polysaccharides are transported to the cell surface via Golgi-derived secretory vesicles and deposited into the cell wall.

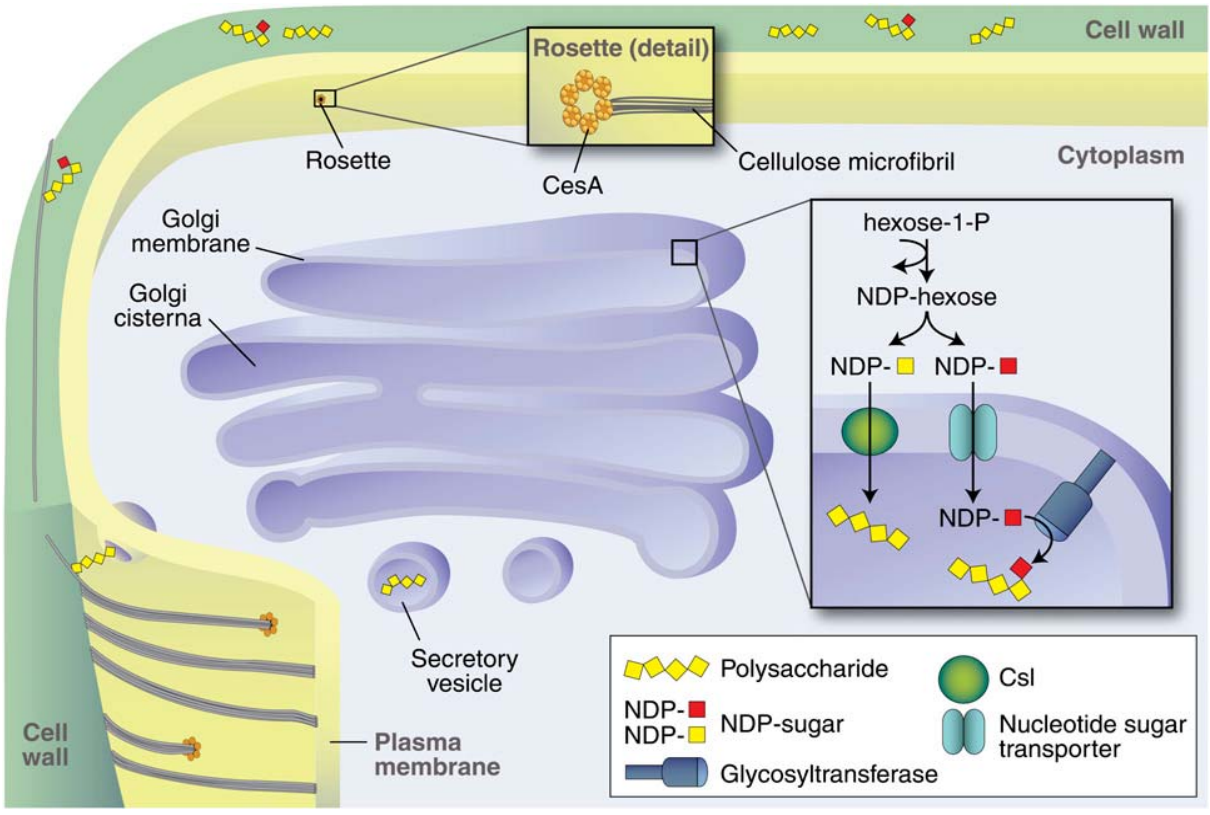


Figure 1.15: Schematic representation of the key events in cell wall biosynthesis. Cellulose biosynthesis occurs at the plasma membrane in large complexes visualized as rosettes. The synthesis of matrix polysaccharides and glycoproteins occurs in the Golgi where the products accumulate in the lumen before transport to the cell wall via vesicles. The regulation of these biosynthetic events is an important issue that needs more study. Abbreviations used in the figure: CesA, cellulose synthase proteins that form the rosette; NDP-sugar, nucleotide sugars that act as

donors for the sugars that go into polysaccharides; Csl, cellulose synthase-like proteins that are known to be involved in hemicellulose biosynthesis (Keegstra 2010).

1.5.1 Cellulose biosynthesis in plants

The catalytic subunit of plant cellulose synthases are encoded by CesA genes which are involved in cellulose biosynthesis. These genes were first identified in a cotton fiber cDNA library by weak homology with bacterial cellulose synthase (Pear *et al.,* 1996) and revealed the presence of three peptides conserved regions with respect to the proteins encoded by bacterial celA genes. Interestingly, the two putative CesA genes identified were expressed at high levels during secondary cell wall cellulose synthesis in cotton fibers. The completion of Arabidopsis genome sequence revealed that Arabidopsis has 10 CesA genes on the basis of sequence similarity (Holland *et al.*, 2000, Richmond 2000). In other plant species, maize has at least 12 (Appenzeller *et al.,* 2004), rice 9, barley has at least 8 (Burton *et al.,* 2004) and poplar has at least 7 (Joshi *et al.,* 2004).

- **Genetic characterization of cellulose biosynthesis in Arabidopsis**

Molecular characterization of mutants with defects in cell wall biogenesis has confirmed the participation of the CesA proteins in cellulose synthesis (Arioli *et al.,* 1998b, Taylor *et al.,* 1999, Fagard *et al.*, 2000). First direct evidence for the involvement of cellulose synthase in cellulose biosynthesis came from the analysis of *rsw1* (radial swelling 1) mutants due to mutation in AtCesA1. Mutation in CesA1 gene caused the disassembly of cellulose synthase and rosettes dissociation into individual lobes so it showed that CESA1 proteins were required for proper cellulose synthesis in Arabidopsis (Arioli *et al.,* 1998b).

Tissue specific expression of various *CesA* genes, combined with knowledge of the mutant phenotypes, has led to the proposal that at least three different CesA proteins are required for the formation of a functional CesA complex (Taylor *et al.,* 2000, Perrin *et al*., 2001, Gardiner *et al.,* 2003). The current view emphasizes two triplexes of CesAs, one consisting of CesA1, 3 and 6 and the other of CesA4, 7 and 8, which are active during primary and secondary cell wall formation, respectively.

In Arabidopsis AtCesA1, AtCesA3 and AtCesA6 would be required for primary cell wall cellulose synthesis as the mutation in these genes affected cellulose production in primary

walls. (Sarkanen 1998, Fagard *et al.,* 2000, Scheible *et al.,* 2001, Burn *et al.,* 2002a). Mutation in CesA1 and CesA3 resulted in severely retarded growth phenotypes which showed that both are non-redundant (Arioli *et al.,* 1998b, Beeckman *et al.,* 2002, Gillmor *et al.,* 2002) whereas mutation in CesA6 exhibited anisotropic cell swelling phenotypes and a growth phenotype for etiolated seedling but not in light-grown condition (Fagard *et al.,* 2000). This observation led to the hypothesis that other CesA subunits may be functionally redundant to CesA6. Later on double mutants of other CesAs like CesA6, 2, 5 and 9 showed redundancy in their functions and suggested that CESAs 2, 5 and also 9 might be able to substitute for the CESA6 sub-unit (Desprez *et al.,* 2007, Persson *et al.,* 2007b). Recently the null mutation in cellulose synthase 9 (CesA9) resulted in 25% reduction in cellulose contents in seeds but unaltered composition of cellulose in leaves and stems (Stork *et al.,* 2010). Scanning electron micrograph studies have shown that in *cesa9* mutant seeds, the epidermal layer is de-shaped and moreover irregular seed coat size, shape and internal angle uniformity was observed. So these results showed that CesA9 plays a non-redundant role for secondary cell wall biosynthesis in radial cell walls of epidermal seed coat and important for seed morphogenesis (Stork *et al.,* 2010).

The occurrence of triplet of CesA proteins involved in cellulose biosynthesis was biochemically demonstrated using co-immunoprecipitation and bimolecular fluorescence confocal microscopy. Because mutation in three genes AtCesA4, AtCesA7 and AtCesA8 resulted in the impairment of cellulose formation in secondary cell wall, it was hypothesized that CesA4, CesA7 and CesA8 work together in a complex and demonstrated later on thanks to co-immuno precipitation experiments (Turner & Somerville 1997, Taylor *et al.,* 1999, Taylor *et al.,* 2000, Taylor *et al.,* 2003). Similar results were obtained for primary cell wall CESAs by the co-immuno precipitation of CesA1, CesA3and CesA6 (Desprez *et al.,* 2007). It was also showed that CesA3 and CesA6 are expressed in the same cell at the same time in dark growing seedlings when fused to GFP and have a similar cellular distribution. Time-lapse spinning disk microscopy showed that both CesA3 and CesA6 proteins migrate at the cell surface with the same velocities along linear trajectories (Desprez *et al.,* 2007). These results provided strong evidence for the presence of three distinct catalytic subunits in plants primary cell wall cellulose synthase complex, with two positions being invariably occupied by CesA1 and CesA3 whereas there is competition between CesA6, CesA2 and CesA5 isoforms for the third position, because of partial functional redundancy among these genes.

Recent advances in visualizing the Cellulose synthase complex (CsC), using a functional YFP (Yellow Fluorescent Protein)-tagged CesA6, have greatly improved our understanding for how primary wall cellulose is synthesized (Paredez *et al.*, 2006). Time average images of the fluorescently labelled primary wall CesA complexes *in vivo* lead to the observation that these complexes move with an average velocity estimated at 300nm/min. This corresponds to the addition of 300 to 1000 glucose molecules/min, assuming that the microfibril is immobilized in the cell wall. In addition, Paredez et al. (2006) also showed that the migrating primary wall CesA complexes are aligned with the microtubules in rapidly expanding cells (Chan *et al.*, 2010).

Very recently, Sullivan and his colleagues have shown that in Arabidopsis CesA5 is involved in the biosynthesis of cellulose in seed adherent mucilage (Sullivan *et al.*, 2011). They observed that birefringent microfibrils are absent from adherent mucilage hence crystalline cellulose is reduced in *cesa5* mutant seeds. Furthermore, labeling experiments of adherent mucilage residues indicated that *cesa5* mutant seeds have less cellulose and less pectin methyl esterification of HG was observed (Sullivan *et al.*, 2011).

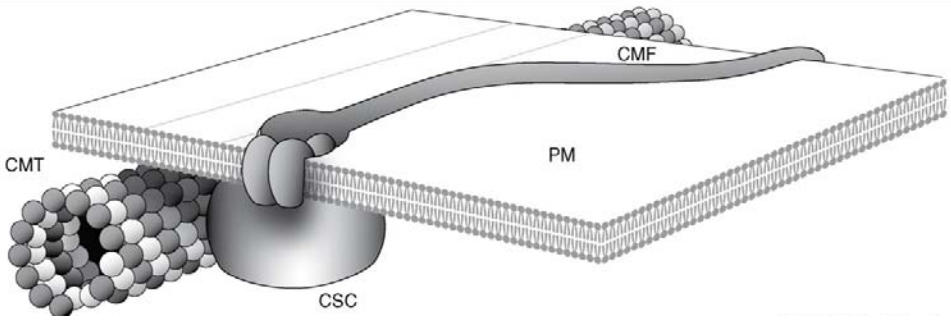


Figure 1.16: Cartoon showing a cellulose synthase complex that is moving inside the plasma membrane leaving a cellulose microfibril in its wake. This particular cellulose synthase became active while unattached to a microtubule. It then bumped into a microtubule and followed it further. Abbreviations: CMF, cellulose microfibril; CMT, cortical microtubule; CSC, cellulose synthase complex; PM, plasma membrane. (Emons *et al.*, 2007)

- **Other genes involved in cellulose biosynthesis in Arabidopsis**

In addition to CesA proteins, some other proteins were found to be involved in cellulose biosynthesis but their specific functions still remain to be identified. Mutations in the KORRIGAN (KOR) gene which encodes an endo-β-(1→4) glucanases, resulted in lateral organ swelling, and reduced cellulose production (Zuo *et al.*, 2000). Another gene, COBRA, was

found out in a screen of mutants that encodes a small protein present on the plasma membrane (Benfey *et al.,* 1993). *Cob* mutants showed defects in cellulose microfibrils orientations but exact role of this protein is still unclear in polymerization or cell wall deposition (Roudier *et al.,* 2002, Roudier *et al.,* 2005). Arabidopsis genome has 11 COBRA-LIKE (COBL) genes. Like other CesA proteins COBL4 is involved in secondary cell wall biosynthesis because the cobl4 mutants showed secondary cell wall defects (Brown *et al.,* 2005). COBL6 has a role in the biosynthesis of cellulose in pollens and anthers like CesA9 (Persson *et al.,* 2007a). Some other cellulose deficient mutants like *kobito, pom1, rsw3, fragile fibre1* (*fra1*) and *fragile fiber2* (*fra2*) were studied but for none of them clear functional mechanism for cellulose biosynthesis was identified (Hauser *et al.,* 1995, Burk *et al.,* 2001, Burn *et al.,* 2002b, Pagant *et al.,* 2002, Zhong *et al.,* 2002, Mouille *et al.,* 2003).

Recently, one non-CesA, cellulose synthase-interactive protein 1(CSI1) was identified by using a two-hybrid screen system. This protein was found to interact with cellulose synthase isoforms that are involved in primary cell wall (Gu *et al.,* 2011). Mutation in CSI1 reduced cellulose contents and caused defects in cell elongation in hypocotyls and roots. Red fluorescent fusion protein showed that CSI1 is associated with CESA complex in the plasma membrane.

1.5.2 Hemicellulose biosynthesis

- **Backbone biosynthesis: The CSL hypothesis**

Plants contain a large number of genes encoding cellulose synthase-like (Csl) proteins that share sequence similarity with CesA proteins. It has been hypothesized that the backbone of non-cellulosic polysaccharides including glucuronoarabinoxylan, xyloglucan, galactoglucomannan, and mixed-linked glucan may be synthesized by Csl proteins that are predicted to be Golgi-resident proteins (Richmond & Somerville 2001). This assumption was proposed based on the observation that cellulose backbone was structurally related to the backbone of hemicellulose β-(1→4)-linked sugar. These Csl genes are members of GT2 family in CAZy database. The Arabidopsis genome contains multiple Csl genes that have been subdivided into six groups (CslA, B, C, D, E, and G) (Richmond & Somerville 2001). But some other groups like CslF and CslH are also present in grasses. Characterization of mutant plants and recombinant enzymatic activities provided valuable information about the functions of these Csl genes. Many groups have shown that members of CslA family are involved in the biosynthesis of either mannan and glucomannan backbone depending on the type of substrate

provided to the heterologously expressed proteins (Dhugga *et al.,* 2004, Liepman *et al.,* 2005, Suzuki *et al.,* 2006, Liepman *et al.,* 2007b, Goubet *et al.,* 2009). Heterologous expression of rice CslF gene in Arabidopsis which lacks mixed linked glucans (MLGs) in its wall, showed detectable MLGs in transformed plants and they confirmed their presence in epidermal cells by using specific MLG monoclonal antibody (Burton *et al.,* 2006). Similarly Doblin and colleagues have shown that in barley MLG polysaccharides are synthesized by another CslH gene subfamily. They cloned CSLH gene from barley and expressed it in Arabidopsis and showed that transgenic plants have detectable MLGs in their walls (Doblin *et al.,* 2009). Moreover their presence was confirmed through immunoelectron microscopy with the use of specific MLG antibody. Later on Bernal and colleagues studied the CslD group of Arabidopsis and demonstrated that CslD2, CslD3 and CslD4 are localized in Golgi when their N-terminus was fused with yellow fluorescent protein (YFP) (Bernal *et al.,* 2008).

- **Xyloglucan biosynthesis**

Many of the biosynthetic enzymes involved in XyG biosynthesis have been determined by using different approaches. Its biosynthesis requires at least four different glycosyltransferase activities like α-(1→2)-fucosyltransferase, β-(1→2)-galactosyltransferase, β-(1→4)-glucan synthase and α-(1→6) xylosyltransferase (Faik *et al.,* 2000). The Arabidopsis CELLULOSE SYNTHASE-LIKE C4 (CslC4) gene may encode β-(1→4)-glucan synthase that is involved in XyG backbone biosynthesis (Cocuron *et al.,* 2007) but characterization of XyG in *cslc4* mutant plants is still lacking.

XyG fucosyltransferase was one of the first XyG biosynthetic enzymes to be identified (Perrin *et al.,* 1999). It was purified from the pea epicotyls using biochemical purification techniques (Perrin *et al.,* 1999). It was named as PsFUT1.Then the amino acid sequence was used to find out and characterize the homologous gene (AtFUT1) in *Arabidopsis thaliana* (Faik *et al.,* 2000) which belongs to GT37 family in CAZy database. In *in vitro* assays AtFUT1 catalyzes the addition of L-fucose at the 2-position of galactose residue into XyG in the presence of non-fucosylated xyloglucan acceptor by using GDP-L-fucose as a donor. Afterwards a genetic screen of Arabidopsis mutants showed that *mur2* was also affected in the same AtFUT1 gene (Vanzin *et al.,* 2002). The *mur2* plants were completely lacking L-fucose in cell wall and showed 99% reduction in xyloglucan fucosylation thus indicating that AtFUT1 is the only fucosyltransferase responsible for XyG fucosylation, at least in Arabidopsis (Reiter *et al.,* 1997, Vanzin *et al.,* 2002, Perrin *et al.,* 2003).

The Arabidopsis genome has nine AtFUT1-like genes (named AtFUT2 to AtFUT10) on the basis of amino acid sequence similarities. Wu and colleagues have shown recently that AtFUT4 and AtFUT6 genes putatively encode α-(1→2)-fucosyltransferases (FUTs) that are responsible for the fucosylation of arabinogalactan proteins (Wu *et al.,* 2010b).

From the same collection of mutants, some other fucose-deficient mutants like *mur1* and *mur3* were identified (Reiter *et al.,* 1997). *mur1* plants were defective in the interconversion of GDP-D-mannose to GDP-L-fucose and were completely deficient in cell wall fucose content (Bonin *et al.,* 1997),whereas *mur2* and *mur3* plants were identified having a 50% reduction in cell wall fucose content (Reiter *et al.,* 1997). MUR3 protein was shown to encode a XyG β-(1→2)-galactosyltransferase (Madson *et al.,* 2003). The mutant plants have altered XyG structure because α-L-fucose (1→2) β-D-galactosyl side chains were completely absent (Lerouxel *et al.,* 2002, Madson *et al.,* 2003). This galactosyltransferase is specific for the addition of the third galactose in the repeating XXXG unit in XyG. This galactose is often fucosylated explaining why the mutants are also fucose-deficient (Scheller & Ulvskov 2010). Ten predicted coding regions in the Arabidopsis genome are closely related to the MUR3 XyG galactosyltransferase and one of them has been proposed to add a galactose onto the second position of the repeating unit, but the final evidence is still lacking (Li *et al.,* 2004).

Seven Arabidopsis genes belonging to family GT34 were previously annotated as putative galactosyltransferases on the basis of sequence similarity to the previously characterized fenugreek galactomannan α-(1→6)-galactosyltransferase (Faik *et al.,* 2002). All of these candidate genes were expressed into *Pichia pastoris* to evaluate this hypothesis. As a result of this heterologous expression one of the candidate genes showed xylosyltransferase activity in the presence of cellopentaose acceptor substrate. This gene was first named as AtXT1 but was recently renamed XXT1. Later on, a second gene displaying 85% similarity to the XXT1 was shown to also encode an α-(1→6)-xylosyltransferase activity when expressed into insect cells and was named XXT2 (Cavalier & Keegstra 2006). Both XXT1 and XXT2 were capable of transferring Xyl from UDP-Xyl onto cellopentaose and cellohexaose acceptor substrates. In fact in the presence of cellohexaose acceptor both enzymes catalyzed the addition of second xylose residues next to the first one forming dixylosylated cellohexaose. Later on by the use of functional genomics it was proved that both XXT1 and XXT2 genes are involved in XyG biosynthesis. Single T-DNA insertion mutant *xxt1* and *xxt2* did not show remarkable

phenotype and almost no reduction in xyloglucan contents, whereas *xxt1 xxt2* double mutant showed a severe root hair phenotype and lacked detectable xyloglucan (Cavalier *et al.,* 2008).

A third GT34 enzyme was found to be involved in XyG biosynthesis named as XXT5 (Zabotina *et al.,* 2008). It is believed to encode α-(1→6) xylosyltransferase and T-DNA insertion showed that mutants have shorter root hairs and less xyloglucan quantity in cell wall. But no *in vitro* xylosyltransferase activity was observed when XXT5 protein was heterologously expressed either in *Pichia pastoris* or Sf21 insect cells.

- **Xylan Biosynthesis**

Xylans are structurally diverse plant polysaccharides with a backbone of β-(1→4)-linked D-Xylosyl residues which are often substituted with glucuronic acid and 4-O-methyl glucronic acid (glucuronoxylan) and with arabinose (arabinoxylans). The backbone substitution is greatly dependent on plant species and tissues of origin. Glucuronoxylans are major hemicelluloses in secondary cell wall of dicotylenous plants whereas arabinoxylans are major components of cell wall of grasses. At least five types of enzymes are required for Xylan backbone and side chain biosynthesis and for their modifications including xylosyltransferase (XylT), glucuronosyltransferase (GlcATs), arabinosyltransferase (AraT), acetyltransferase and methyltransferase.

- **Backbone Biosynthesis**

Despite a lot of work, very little was known till recently about the mechanism underlying xylan biosynthesis. To date, a number of putative glycosyltransferase genes have been identified that are involved in the synthesis of either xylan backbone, side chains or the reducing end sequence which are summarized in figure 1.17. Because of the structural similarity of xylan to the β-(1→4)-linked backbones of other hemicelluloses, it has been assumed that members of the Csl protein group might probably be involved in xylan backbone synthesis. However, heterologous expression of various Csl genes failed to identify any xylan synthase activity (Richmond & Somerville 2001). However some genetic evidences about xylan biosynthesis arose from the characterization of mutants harbouring irregular xylem (*irx*) structure, and altered for genes named *irx*9, *irx*14, I9H (*irx*9-LIKE), I14H (*irx*14-LIKE), *irx*10 and *irx*10-LIKE (which are discussed in detail in the next paragraphs) (Bauer *et al.,* 2006, Brown *et al.,* 2007, Lee *et al.,* 2007a, Brown *et al.,* 2009, Wu *et al.,* 2009). All of these genes

encode putative glycosyltransferases belonging to families GT43 and GT47 (Scheller & Ulvskov 2010). They are expressed in cells undergoing secondary cell wall biosynthesis and their encoded proteins are targeted to the Golgi where xylan is synthesized (Jensen *et al.,* 2011, Zhong *et al.,* 2005). It has been shown experimentally for IRX9 to be localized in Golgi (Pena *et al.,* 2007).

Analysis of mutants in each of these genes indicates that *IRX9, IRX10,* and *IRX14* encode enzymes that function as xylosyltransferases in the synthesis of the *β*-1-4-xylan backbone while *IRX8, FRA8,* and *PARVUS* appear to be involved in synthesis of the reducing end tetrasaccharide structure (for a review see Scheller and Ulvskov 2010). However, till now no xylan synthase activity has been observed for any of these proteins when heterologously expressed, which leads to the suggestion that the enzymes may not work alone and require other proteins in a protein functional complex for xylan synthesis (Brown *et al.,* 2007, Pena *et al.,* 2007). Previous studies have already proposed that glucuronoxylan (GX) biosynthesis requires the cooperative actions of XylT and GlcAT (Baydoun *et al.,* 1989). Interestingly, very recently in wheat, a Glucuronoarabionoxylan (GAX) synthase complex was identified by using proteomic and transcriptomic approches (Zeng *et al.,* 2010). This complex contains three putative glycosyltransferases (from GT43, GT47 and GT75 CAZy families), a xylosyltransferase (XylT), an arabinosyltransferase (AraT), and a glucuronosyltransferase (GlcAT) which would be required for the biosynthesis of GAX.

Mutation in the Arabidopsis *irx9* gene, which belongs to family GT43, resulted in plants with decreased amounts of wall GX contents, suggesting that this gene is required for GX synthesis (Brown *et al.,* 2005, Bauer *et al.,* 2006) and further characterization of *irx9* mutants have shown the decrease in chain length of glucuronoxylan (GX) (Pena *et al.,* 2007). Later on Lee and his colleagues showed that the *irx9* mutant is deficient in xylan xylosyltransferase activity (Lee *et al.,* 2007a). They first measured the XylT and GlcAT activities from the microsomal extraction of the stems of wild-type Arabidopsis rich in xylan in the presence of exogenous acceptor (1→4)-linked β-D-xylooligomers. Then they have shown that XylT activity was substantially reduced in the *irx9* mutant compared with the wild type but GlcAT remained unchanged in the *irx9* mutants (Lee *et al.,* 2007a). These observations confirmed the previous results and showed that IRX9 is a xylosyltransferase that is responsible for the elongation of the xylan backbone. Another mutation in IRX14, homologous of IRX9 and also belonging to family GT43 has shown the reduction in GX contents in mutant cell wall (Brown *et al.,* 2007) and

biochemical assays have shown that *irx14* is defective in the incorporation of radiolabelled UDP- ^{14}C-xylose onto β-(1→4) xylooligosaccharides which proposed that IRX14 would be needed for the elongation of xylan back bone.

Arabidopsis GT43 family has four GT members, two of them IRX9 and IRX14 have been previously characterized but recently two independent groups (Lee *et al.,* 2010, Wu *et al.,* 2010) have studied two other members of this family and have used different nomenclature for the same genes, for example Lee et al called them I9H (homolog of IRX9, also called IRX9-L) and I14H (homolog of IRX14, also called IRX14-L) by Wu *et al*. (2010). Genetic analysis showed that both genes are expressed in cells undergoing secondary wall thickening and predicted to be Golgi-localized. Lee *et al* (2010) have shown that defects in GX synthesis caused by *irx9* mutation can be rescued by the overexpression of I9H but not by either IRX14 or IRX14-L. Similarly, overexpression of I14H complemented the defects caused by *irx14* mutation but not by either IRX9 or IRX9-L. The functional redundancy of this gene was actually confirmed by the analysis of double mutants which showed a severe reduction in GX contents and loss of secondary wall thickening in fibre cells as compared to single mutants. To summarize, IRX9, IRX9-L and IRX14, IRX14-L genes are involved in GX backbone elongation, but complementation studies showed that there exist two functionally non-redundant groups in this GT43 family. Wu and colleagues, confirmed these results through complementary studies and showed that IRX9 are IRX14 are more important and major genes while IRX9-L are IRX14-L are less important and minor genes that are involved in GX synthesis (Wu *et al.,* 2010).

Two other Arabidopsis genes belonging to the family GT47, IRX10 and IRX10-LIKE have been identified that are involved in xylan backbone elongation (Wu *et al.,* 2009). Single mutation did not show any visible phenotype but the double mutants showed a severe phenotype as the mutants have shorter rosettes and infertile inflorescence. NMR (Nuclear Magnetic Resonance) analysis showed that degree of polymerization of the xylan backbone was decreased in mutant plants as compared with the wild type. Very recently, two articles were published studying the DUF579 domain in Arabidopsis and they found that 5 genes out of 10 are co-expressed with genes involved in secondary cell wall biosynthesis (Brown *et al.,* 2011). They showed that two members of the DUF579 gene family named as IRREGULAR XYLEM 15 (IRX15) and IRREGULAR XYLEM 15 LIKE (IRX15L) are essential for normal xylan deposition during secondary cell wall biosynthesis. Sugar composition analysis of double

mutant *irx15/irx15l* exhibited reduction in xylan content as compared to wild type and furthermore the xylem vessels are distorted like other xylan deficient mutants. Localization experiments with the use of fluorescent fusion proteins have shown that both proteins are located into Golgi apparatus (Brown *et al.*, 2011).

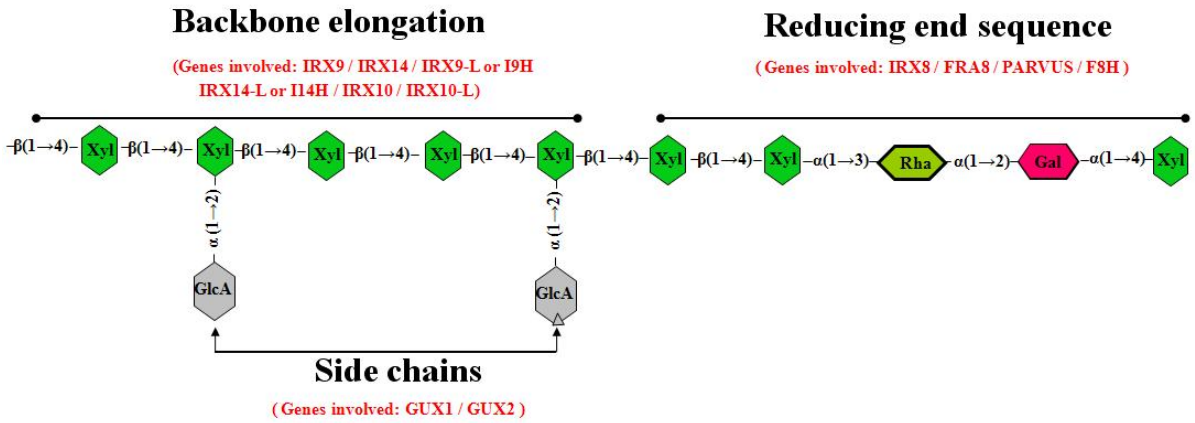


Figure1.17: Basic structure of glucuronoxylan indicating reducing end sequence, backbone elongation and side chains. The genes involved in the biosynthesis of different parts of glucuronoxylan are represented in red colour.

- **Side chains biosynthesis**

The side chains of xylans should require the action of α-glucuronsyltransferases and α-arabinofuranosyltransferases. In dicots xylan is substituted with glucuronic acid (GlcA) and 4-O-methylglucuronic acid (MeGlcA), and recently two putative glycosyltransferases named GUX1and GUX2 (GlucUrono acid substitution of Xylan) were identified to add GlcA and MeGlcA to xylan backbone in Arabidopsis stem cell walls (Mortimer *et al.*, 2010). Double mutants of *gux1 gux2* have shown no change in xylan backbone contents but GlcA and MeGlcA contents were almost completely absent. Moreover, the glucuronosyltransferase activity was also strongly diminished in double mutants because the stem microsomes were unable to transfer GlcA from UDP-GlcA onto xylooligosaccharide acceptors. These results suggest that GUX1 and GUX2 are responsible for the substitution of xylan backbone in Arabidopsis stem glucuronoxylan.

- **Reducing end oligosaccharide synthesis**

Xylans have a reducing end with a unique structure β-D-Xyl*p*-(1→4)-β-D-Xyl*p*-(1→3)-α-L-Rha*p*-(1→2)-α-D-Gal*p*A-(1→4)-D-Xyl*p* (Shimizu *et al.*, 1976, Pena *et al.*, 2007) mostly in dicots. A number of mutations in different genes IRX8, PARVUS, FRA8 (IRX7), and F8H (homolog of FRA8) have been demonstrated to be involved in the biosynthesis of the reducing end of GX (Brown *et al.,* 2007, Lee *et al.,* 2007b, Pena *et al.,* 2007). FRA8, IRX8 and F8H all are Golgi localized proteins except PARVUS that is located in endoplasmic reticulum.

Arabidopsis, IRX8 also known as GAUT12, a close homolog of GAUT1 belongs to family GT8 that encodes an α-(1→4) galacturonosyltransferase activity, possibly involved in the biosynthesis of the backbone of pectic homogalacturonan (Sterling *et al.,* 2006). Mutation in IRX8 resulted in significant reduction in secondary cell wall thickness and moreover the plant height was reduced. Cell wall analysis of mutants showed that level of xylan and homogalacturonan were significantly reduced which was further confirmed by immuno-histochemical analysis. Structural fingerprinting of cell wall polymers revealed the remarked reduction in glucuronoxylan chain length or almost loss of GX reducing end tetrasaccharide sequence but it did not cause a complete disruption in GX biosynthesis. Double mutants of *irx9/irx8* exhibited collapsed xylem vessels and reduction in xylose and cellulose in cell wall as compared to *irx8* and *irx9* single mutants (Persson *et al.,* 2007b). Protein encoded by PARVUS is co-localized with endoplasmic reticulum and belongs to GT8 family. It is thought to be involved in the initiation of biosynthesis of the GX reducing end tetrasaccharide sequence by catalyzing the transfer of the reducing Xyl residues onto an unknown acceptor in the endoplasmic reticulum (Lee *et al.,* 2007a). Mutation of the PARVUS gene resulted in secondary wall thickening and reduced GX contents. NMR analysis showed the absence of tetrasaccharide primer sequence at the reducing end of GX in mutants and loss of glucuronic acid side chain in GX. These data proposed the putative role of PARVUS in the initation of biosynthesis of the GX tetrasaccharide primer sequence.

Arabidopsis thaliana fragile fiber8 (*fra8*) mutant was shown to have reduced levels of xylan and resulted in fiber wall thickness and decreased in stem strength (Zhong *et al.,* 2005). FRA8 was suggested to encode glucuronosyltansferase on the basis of amino acid sequence similarity to tobacco (*Nicotiana plumbaginifolia*) pectin glucuronosyltransferase which belongs to family GT47 (Zhong *et al.,* 2005). Structural analysis of cell wall of *fra8* showed the absence of substituted glucuronic acid but the presence of only 4-O-methylglucuronic acid. These data suggested that FRA8 encodes a glycosyltransferase that is involved in the addition of glucuronic

acid residues onto the backbone of xylan. Recently a close homolog of FRA8, F8H was identified in Arabidopsis and was shown to be involved in GX biosynthesis (Lee *et al.*, 2009). The Gus reporter gene expression indicated that the F8H expression is associated with secondary cell walls in xylem cells which are rich in GX and F8H protein is located in the Golgi where GX is synthesized. To check that either these homologs are functionally redundant, mutants of *fra8* were complemented with F8H construct and its overexpression completely rescued the mutant phenotypes i.e. reduction in secondary cell wall thickness and a deformation of xylem vessels (Zhong *et al.*, 2005). NMR spectrometry also showed the recovery of structural defects in *fra8* mutant that was the loss of GlcA side chains in GX and reduction in the GX reducing end tetrasaccharide structure. Double mutant of *fra8/fh8* has shown more severe defects that include deformation of vessels and retarded growth as compared to single mutant of *fra8*. All these findings proposed that FR8 and F8H are functional paralogs and functionally redundant in GX biosynthesis.

P. Ulvskov has proposed that reduction of α-D-GlcA in *fra8* could be an indirect effect of mutation as all the GTs in family 47 are inverting enzymes whereas a xylan specific α-glucuronosyltransferase would be a retaining enzyme (Ulvskov 2011). So if it is true that FRA8 is involved in xylan biosynthesis, another alternative might be that FRA8 encodes β-1,3-xylosyltransferase involved in synthesis of reducing end oligosaccharide (Scheller & Ulvskov, 2010).

- **Galacto-glucomannan Biosynthesis**

Galacto-gluco-mannans (GGM) are polysaccharides found in many tissues and cell types in Arabidopsis (Handford *et al.*, 2003, Moller *et al.*, 2007, Liepman *et al.*, 2007a). It has been demonstrated by Liepman and coll. (2007) that GGM are synthesized by CslA protein from GDP-mannose and GDP-glucose activated-sugar. A second enzyme at least is required for GGM biosynthesis, an α-(1→6)-galactosyltransferase adding side chains to backbone (Edwards *et al.*, 1999). The study of GGM biosynthesis could be considered as land mark, as the first proof of a CslA gene to be responsible for the biosynthesis of hemicellulose backbone, was the characterization of a guar Csl gene encoding a mannan synthase synthesizing activity (Dhugga *et al.*, 2004). This work actually validates a long standing hypothesis of Cellulose-synthase-like genes family to be involved in hemicelluloses biosynthesis. As all members of Csl genes family, CslA genes involved in GGM biosynthesis are grouped in family GT2. Heterologous expression studies have shown that CslA is responsible for the synthesis of mannan and glucomannan

polysaccharides in Arabidopsis but the type of polysaccharide synthesized depend on the type of substrate used (Dhugga *et al.,* 2004, Liepman *et al.,* 2005, Suzuki *et al.,* 2006, Liepman *et al.,* 2007b). Mutation in CslA7 resulted in embryo lethality and reduced transmission of pollens thus demonstrating that CslA7 is necessary for embryogenesis and pollen tube growth (Goubet *et al.,* 2003). Later on to determine the role of other CslA proteins involved in glucomannan synthesis in Arabidopsis, insertional single, double and triple mutants were characterized. Out of which one single *csla7* and triple *csla2-csla3-csla9* mutants have reduced glucomannan which confirmed the role of CslA in glucomannan synthesis (Goubet *et al.,* 2009).

1.5.3 Pectin biosynthesis

Because of complexity of pectic polysaccharides, it has been proposed that at least 67 different glycosyltransferases, acetyltransferases, and methyltransferases (Mohnen 2008) are required for its biosynthesis. Till now very little is known about its biosynthesis and only a few pectic biosynthetic enzymes have been identified unambiguously. Because of the complex nature of pectic polysaccharides it has also been hypothesized that GTs that are involved in pectin biosynthesis work in supramolecular complexes. However some of the genes have been identified that could be involved in pectin biosynthesis.

- **HG biosynthesis**

Study of HG biosynthesis *in vitro* has been a challenging field for researchers: starting in the sixties, (Villemez *et al.,* 1965) had first shown the synthesis of the D-galacturonic acid chain of pectin with a cell free enzyme preparation from mung beans but it finally took a very long time for the first characterization of a HG galacturonosyltransferase (GAUT1; GT8 family) enzyme from Arabidopsis (Sterling *et al.,* 2006). Before characterization of GAUT1 activity, some evidence came from the screening of Arabidopsis mutants that GT8 gene family could be responsible for homogalacturonan biosynthesis. Indeed, in 2002, Bouton and coll. characterized an Arabidopsis mutant quasimodo1 (*qua1*) with pectin alteration phenotypes including a large decrease in homogalacturonan contents (HGA), a reduction in cell adhesion and stunted plant growth. Interestingly, scanning electron microscopy also confirmed cell adhesion defect, showing the rupture lines and gaps between epidermal cells by comparison to wild type plants. These findings suggested that QUASIMODO1 could encode a glycosyltransferase that is involved in pectin biosynthesis. However the activity of QUA1/GAUT8 has not been determined but the *qua1* mutants showed a 23% decreased in HG galacturonosyltransferase

activity (Orfila *et al.,* 2005), consistent with QUA1 protein being involved in pectin biosynthesis. However, the authors also noticed a 40% decrease in activity for β-(1→4)-D-xylan synthesis in *qua1* mutant stem as compared to WT. So, QUA1 activy remains to be determined unambigously.

Later on, in Arabidopsis, the first pectin biosynthetic enzyme, the HG α-(1→4)-Galacturonosyltransferase was identified using a traditional biochemical approach (Sterling *et al.,* 2006). GalAT activity was partially purified from *Arabidopsis* suspension-cultured cells and bioinformatics together with peptide sequence data were used to identify two putative GalATs that were named GAUT1 and GAUT7. Biochemical characterization of GAUT1 (At3g61130) provides compelling evidence that this protein is a HG:GalAT when expressed in human kidney cells (HEK293 cells). GAUT7 did not show GalAT activity when expressed in HEK293 cells, but recent results suggest that both proteins interact with each other and are present in the same pectic biosynthetic complex *in vivo* (Mohnen, 2008).

GAUT1 and GAUT7 are members of a multigenic family of 25 genes in Arabidopsis that classifies into CAZy family GT8, and which comprises 15 GAUT genes and 10 GAUT-like (GATL) with, respectively, 56-84 and 42-53% amino acid sequence similarity to GAUT1 (Sterling *et al.,* 2006). In order to determine the function of these genes in Arabidopsis, 26 homozygous T-DNA insertion mutants for 13 out of 15 GAUT genes were studied (Caffall & Mohnen 2009). Some *gaut* mutants like *gaut6, 8, 9, 10, 11, 12, 13* and *14* showed a significant reduction in different cell wall glycosyl residues composition like galacturonic acid, xylose, rhamnose, galactose and arabinose. The observed phenotypes for different *gaut* mutants allowed proposing a role in pectin or xylan biosynthesis for GAUT genes. GAUT 11 has been found to be implicated in the synthesis of mucilage polysaccharides because mutants have reduced mucilage expansion and less GalA contents in extracted mucilage and in testa (Caffall & Mohnen 2009).

Another functional homolog of QUA1, in Arabidopsis referred as QUA2 putatively encodes a pectin methyltransferase because it has no sequence similarity to glycosyltransferases but having a known methyltransferase domain (Mouille *et al.,* 2007). *qua2-1* mutant has revealed a 50% reduction in HG contents as compared to wild type but no change was found in other cell wall polysaccharides.

Recently Miao and colleagues have shown that QUA3 is a Golgi localized type II integral membrane protein. It encodes a putative homogalacturonan methyltransferase which plays an important role in controlling the pectin methylation and as well as cell wall biosynthesis in Arabidopsis suspension cultured cells (Miao *et al.,* 2011).

- **RG-I Biosynthesis**

The biosynthesis of RG-I requires multiple glycosyltransferase activities to synthesize a backbone of [→2)-α-L-Rha*p*-(1,4)-α-D-Gal*p*A-(1→] disaccharide repeats that are branched at the C-4 of approximately half of the rhamnose residues by 5-linked and 3,5-linked arabinan, 4-linked and 4,6-linked galactan, as well as Type-I and Type-II arabinogalactans (Caffall & Mohnen 2009). Potentially 34 specific activities may be required to synthesize RG-I. Only two GTs have been identified as involved in RG-I biosynthesis, namely ARAD1 (ARABINAN DEFICIENT 1) and XGD1 (XYLOGALACTURONAN DEFICIENT 1) (Harholt *et al.,* 2006). Both enzymes belong to family GT47 and were shown to be located in the Golgi. ARAD1 is a putative arabinosyltransferase as suggested by the analysis of *arad1* mutant showing a reduced amount of Ara in the cell wall. These assays showed that T-DNA mutants leaves and stems have 75% and 46% less arabinose contents as compared to wild type and showed decrease in arabinan when labeling was done with the LM6 anti-arabinan antibody (Harholt *et al.,* 2006). They further confirmed these results by linkage analysis which also exhibited reduction in arabinan contents in mutants as compared to wild type and this phenotype was restored with the complementation of wild type gene ARAD1 under the 35S promoter. These results suggested that ARAD1 is an α-(1→5)- arabinosyltransferase but all attempts to demonstrate AraT activity by *in vitro* or *in vivo* assays have so far been unsuccessful. The Arabidopsis genome has seven homologs of ARAD1; the closest homolog to ARAD1 is referred as ARAD2. ARAD1 and ARAD2 are not redundant in function because expression of ARAD2 in *arad1* mutants does not complement the arabinan defiency. Immunohistochemical analysis also showed different patterns of labeling in single *arad1*, *arad2* and double *arad1 arad2* mutants when compared to wild type reviewed in (Harholt *et al.,* 2010b).

In pectic arabinan and galactan, arabinose is almost exclusively present in the furanose configuration. Recently it was demonstrated that plants have mutases that convert UDP-arabinopyranose to UDP-arabinofuranose. (Konishi *et al.,* 2007; Konishi *et al.,* 2010).

XGD1 is thought to be involved in pectin biosynthesis since sugar composition and linkage analysis of loss-of-function mutants showed a substantially decreased content of XGA (Jensen *et al.*, 2008). Transient expression of XGD1 in *N. benthamiana* cells and *in vitro* assays showed transfer of xylose from UDP α-D-xylose onto homogalacturonan oligogalacturonides and endogeneous acceptors, thereby confirming the function of XGD1 as a xylogalacturonan β-(1→3)-xylosyltransferase.

- **RG-II biosynthesis**

RG-II is the most structurally complex polysaccharide in the plant cell wall so it has been hypothesized that at least 24 biosynthetic activities are required, but its biosynthesis has been poorly studied. Three genes RGXT1, RGXT2 and RGXT3 belonging to family GT77 were found to be involved in its biosynthesis (Egelund *et al.*, 2006, Egelund *et al.*, 2008). These proteins encode α-(1→3) Xylotransferases and can transfer Xyl from UDP-Xyl onto Fucose. This glycosidic linkage is only present in RG-II so suggesting that these proteins are involved in RG-II biosynthesis. However the mutants of *rgxt1* and *rgxt2* do not exhibit any changes in sugar cell wall composition, so the lack of visible phenotype could be attributed to the functional redundancy among the members of RGXT family (Egelund *et al.*, 2006, Harholt *et al.*, 2010a). The RGXT family in Arabidopsis has 4 members, and the fourth member, designated RGXT4, has been very recently characterized (Fangel *et al.*, 2011, Liu *et al.*, 2011). When expressed in *Pichia pastoris*, RGXT4 exhibited xylosyltransferase activity and transferred xylose onto fucose (Liu *et al.*, 2011).

A cell adhesion mutant in *Nicotiana plumbaginifolia* was a decade ago reported to be deficient in GlcA residues of RG-II, suggesting that the protein (named NpGUT1) was involved in RG-II biosynthesis (Iwai *et al.*, 2002). However recent results tend to demonstrate that the effect on RG-II in the *Nicotiana plumbaginifolia* mutant was a pleiotropic effect and that NpGUT1 is not directly involved in RG-II biosynthesis (Wu *et al.*, 2009). NpGUT1 that is an ortholog of IRX10/IRX10L is expected to be involved in xylan biosynthesis. No gene encoding RG-II acetyltransferase and methyltransferase has been identified to date.

1.6 Callose biosynthesis

Callose synthases are encoded by multigene families, and different isoforms have different locations and functions in the plant. The enzymes which are responsible for callose

biosynthesis are called callose synthase and they use UDP-glucose as a substrate. In Arabidopsis, twelve genes encoding putative callose synthases have been identified as a result of work of two independent groups. They were named as CalS (Callose synthase), AtCalS1-AtCalS12 according to Desh Verma group and named as GSL (Glucan synthase-like), and AtGSL1 to AtGSL12 by Somerville group. These genes encode proteins with a molecular mass of 200 kDa which is larger than most plant genes (Verma & Hong 2001, Enns *et al.,* 2005). These proteins contain multiple transmembrane domains that are grouped into N-terminal and C-terminal regions. Most of the genes were identified through mutational analysis of mutants of callose synthase genes in Arabidopsis.

AtGSL1 and AtGSL5 are found to be involved in pollen development and fertility (Enns *et al.,* 2005). Both of these genes are responsible for the synthesis of callose deposition at the primary wall of meiocytes, tetrads and microspores, and these genes also play a role in pollen grain germination. Mutation in GSL2 (Cals5) resulted in remarked reduction in fertility. However this mutation resulted in male sterility but the female gametes were not affected and were fertile. Callose were almost absent in meiocytes, tetrads, microspores and mature plloen of these mutants (Dong *et al.,* 2005).

Mutants of other two GSL family members like GSL8 and GSL10 resulted in lethal male gametophytic phenotype so no homozygous mutants were obtained for either of these genes (Toller *et al.,* 2008). Mutation leads to failure of microspores division symmetry and as well entry of microspores into mitosis. Further the authors have suggested their indirect functions through interaction with other proteins, rather than through their catalytic activity alone. After that it has been found that amino terminus of AtGSL6 could interact with lectin-containing receptor-like kinase so it can be hypothesized that GSL8 and GSL10 may interact with receptor-like kinase for the regulation of pollen entry during mitosis.

Gene AGI no	Name	Function	Mutant	Mutant phenotypes	References
Cellulose biosynthesis (CESA genes)					
At4g32410	AtCesA1	Primary cell wall cellulose synthase catalytic subunit	*rsw1*	Reduced cellulose in primary cell wall	(Arioli *et al.*, 1998b)
At4g39350	AtCesA2	Cellulose synthase subunit	*cesa2*	Shorter hypocotyl of dark grown seedlings	(Arioli *et al.*, 1998a, Desprez *et al.*, 2007)
At5g05170	AtCesA3	Primary cell wall cellulose synthase catalytic subunit	*ixr1*	Reduced growth and cellulose contents	(Scheible & Pauly 2004)
At5g64740	AtCesA6	Primary cell wall cellulose synthase catalytic subunit	*procuste1/ ixr2*	Growth defects, reduced cellulose contents	(Fagard *et al.*, 2000, Desprez *et al.*, 2002)
At5g44030	AtCesA4	Secondary cell wall cellulose synthase subunit	Irx5	Reduced cellulose and irregular xylem	(Taylor *et al.*, 2003)
At5g17420	AtCesA7	Secondary cell wall cellulose synthase subunit	Irx3	Collapsed xylems	(Taylor *et al.*, 1999)
At4g18780	AtCesA8	Secondary cell wall cellulose synthase subunit	*irx1*	Reduced cellulose and collapsed xylem	(Zhong *et al.*, 2003) (Taylor *et al.*, 2000)
At2g21770	AtCesA9	Secondary cell wall synthesis in seed coat	*cesa9*	25% reduction in cellulose contents in seeds	(Stork *et al.*, 2010)
Cellulose biosyhthesis (Non-CESA genes)					
At5g49720	KORRIGAN	Endo-1,4-β-D-glucanase	*kor1*	Lateral organ swelling and reduction in secondary cell wall cellulose	(Zuo *et al.*, 2000, Lane *et al.*, 2001)
At3g08550	KOBITO 1	Cellulose biosynthesis during cell expansion	*kobito1*	Reduced cellulose content	(Pagant *et al.*, 2002)
At5g49720	COBRA	Cellulose microfibril organization	*Cob*	defects in cellulose microfibrils orientation and reduction in crystalline cellulose in roots	(Schindelman *et al.*, 2001, Roudier *et al.*, 2002, 2005)
At2g22125	CSI1	Primary cell wall cellulose biosynthesis	*csi1*	reduced cellulose content ; defects in cell elongation in hypocotyls and roots	(Gu *et al.*, 2010)

Gene AGI no	Name	Function	Mutant	Mutant phenotypes	References
Pectin biosynthesis genes					
At3g25140	AtQUA1	putative α-1,4-galacturonosyltransferase	*qua1*	Dwarf, reduced cell adhesion, 25% reduction in cell wall GalA content	(Bouton *et al.*, 2002)
At3g61130	AtGAUT1	α -1,4-galacturonosyltransferase	None	None	(Sterling *et al.*, 2006)
At4g01770	AtRGXT1	α -1,3-xylosyltransferase	*rgxt1*	RGII from mutant (but not from WT) is an acceptor for a-1,3-xylosyltransferase acitvity	(Egelund *et al.*, 2006)
At4g01750	AtRGXT2	α -1,3-xylosyltransferase	*rgxt2*	RGII from mutant (but not from WT) is an acceptor for a-1,3-xylosyltransferase acitvity	(Egelund *et al.*, 2006)
At1g56550	AtRGXT3	α -1,3-xylosyltransferase	None	None	(Egelund *et al.*, 2008)
At4g01220	AtRGXT4	α -1,3-xylosyltransferase	*mgp4*	Defect in pollen tube growth	(Fangel *et al.*, 2011, Liu *et al.*, 2011)
At2g35100	AtARAD1	α -1,5-arabinosyltransferase	*arad1*	Cell wall composition altered, decrease in RGI arabinose content	(Harholt *et al.*, 2006)
At3g55830	AtEPC1	Unknown	*epc1*	Reduced growth habit, defects in vascular formation and reduced cell-cell adhesion in hypocotyls	(Singh *et al.*, 2005)
At1g78240	AtQUA2	putative methyltransferase	*qua2*	Dwarf, reduced cell adhesion, 50% reduction in HG content	(Mouille *et al.*, 2007)
At5g33290	AtXGD1	Xylogalacturonan xylosyltransferase	*xgd1*	Reduction in pectic xylogalacturonan	(Jensen *et al.*, 2008)
At1g18580	AtGAUT11	unknown	*gaut11*	reduced mucilage expansion and less GalA contents in extracted mucilage and in testa	(Caffall & Mohnen 2009)

Gene AGI no	Name	Function	Mutant	Mutant phenotypes	Refrences
Hemicelluloses (Xyloglucan syhthesis genes)					
At2g03220	AtFUT1	Xyloglucan α-1,2-fucosylT	*mur2*	99% reduction in fucosylation of xyloglycan	(Faik *et al.*, 2002 ; Vanzin *et al.*, 2002a)

At2g20370	Mur3	Xyloglucan β-1,2 galactosyltransferase	*mur3*	50% reduction in fucose contents and α-L-fucose (1→2) β-D-galactosyl side chains absent	(Lerouxel *et al.*, 2002{Madson *et al.*, 2003)
At3g62720	XXT1	α-1,6- xylosyltransferase	*xxt1*	Decreased Xyloglucan contents	(Cavalier & Keegstra 2006)
At4g02500	XXT2	α-1,6-xylosyltransferase	*xxt2*	Decreased Xyloglucan contents	(Cavalier & Keegstra 2006)
At1g74380	XXT5	Putative α-1,6-xylosyltransferase	*xxt5*	shorter root hairs and less xyloglucan contents	(Zabotina *et al.*, 2008)
Xylan syhthesis genes					
At2g37090	IRX9	Putative β-1,4-xylosyltransferase (elongation of xylan backbone)	*irx9*	Shorter xylan chain length, less activity and irregular xylem	(Lee *et al.*, 2007a, Pena *et al.*, 2007))
At4g36890	IRX14	Putative β-1,4-xylosyltransferase (elongation of xylan backbone)	*irx14*	Defective in the incorporation of radiolabelled UDP- ^{14}C-xylose	(Brown *et al.*, 2007)
At1g27600	IRX9-L/ I9H	Elongation of glucuronoxylan backbone	*Irx9-l*	Reduction in GX contents and loss of secondary wall thickening	(Lee *et al.*, 2010, Wu *et al.*, 2010a)
At5g67230	IRX14-L/ I14H	Elongation of glucuronoxylan backbone	*Irx14-l*	Reduction in GX contents and loss of secondary wall thickening	(Wu *et al.*, 2010a) (Lee *et al.*, 2010)
AT1G27440	IRX10	Elongation of glucuronoxylan backbone	*Irx10 irx10-like*	Double mutants have shorter rosettes and infertile inflorescence	(Wu *et al.*, 2009)
	IRX10-LIKE	Elongation of glucuronoxylan backbone	*Irx10 irx10-like*	Double mutants: shorter rosettes and infertile inflorescence	(Wu *et al.*, 2009)
AT3G50220	IRX15	Xylan synthesis	*irx15 irx15l*	Double mutants: less xylan contents and irregular xylem vessels	(Brown *et al.*, 2011)

AT5G67210	IRX15-LIKE	Xylan synthesis	*irx15 irx15l*	Double mutants: less xylan contents and irregular xylem vessels	(Brown *et al.*, 2011)
AT3G18660	GUX1	α-glucuronsyltransferases (glucuronoxylan side chains synthesis)	*gux1 gux2*	Absent of side chains (GlcA and MeGlcA contents reduced)	(Mortimer *et al.*, 2010)
AT4G33330	GUX2	α-glucuronsyltransferases (glucuronoxylan side chains synthesis)	*gux1 gux2*	Absent of side chains (GlcA and MeGlcA contents reduced)	(Mortimer *et al.*, 2010)
AT5G54690	IRX8/GAUT 12	Putative α-(1→4) galacturonosyltransferase	*irx8*	Shorter plants, loss of GX reducing end tetrasaccharide sequence	(Persson *et al.*, 2007a)
AT2G28110	FRA8	Glucuronosyltransferase	*fra8*	fiber wall thickness , decreased in stem strength and absence of substituted glucuronic acid	(Zhong *et al.*, 2005)
AT5G22940	F8H	Glucuronoxylan biosynthesis	*fra8/fh8*	Deformation of vessels and retarded growth	(Lee *et al.*, 2009)

1.7 The objective of thesis work

Cell walls play many important roles in defining the unique biology of plants; they also have practical applications as a feedstock for biomaterials and for the production of biofuels and are important sources of human and animal nutrition. Despite the fundamental and practical importance of cell walls, little is known about the biosynthesis of the macromolecular components that comprise them. This is difficult because only mutational studies are not sufficient as cell wall related genes expressed at low levels between cells of different tissues (Sarria *et al.,* 2001) and secondly because of gene redundancy and compensatory mechanism to maintain cell wall function in the absence of a particular gene (Somerville *et al.,* 2004). Thus, complexity of cell wall polysaccharides required a combination of various tools in order to fully understand the cell wall structure and its function

Plant primary cell wall is mainly composed of polysaccharides like cellulose, hemicelluloses and pectins. Glycosyltransferases (GTs) are the enzymes which are involved in cell wall polysaccharides biosynthesis. These enzymes are classified into 94 families on the basis of amino acid sequence similarities into CAZy database. With the completion of Arabidopsis genome project, approximately 450 GT Arabidopsis sequences have already been identified. Significant progress has been made recently in identifying the enzymes involved in plant cell wall biosynthesis through bioinformatics and biochemical approaches but only a handful of those are involved in polysaccharide biosynthesis. As the plant cell wall polysaccharide network is very complex, it can be expected that this number of genes remain incomplete.

So in our lab a bioinformatic strategy has been developed to search new genes encoding putative GT sequences in the Arabidopsis genome that could be responsible for polysaccharide synthesis and their modification. As most of the GTs display only two structural folds, named GT-A and GT-B folds, so bioinformatic strategy was designed to search the Arabidopsis translated genome or proteom for new candidates GT genes. This strategy was based on the combination of several bioinformatics tools. First, the profile Hidden Markov Model (HMM) method, which acts at 1D level, was used to find out remote homologues in the Arabidopsis proteome based on amino acid sequences comparison with the known GT families. Secondly the use of PSI-BLAST programme linked to structural overlap

approach (Sov), which acts at 2D level, and thirdly ProFit, a fold recognition program which acts at 3D level, were used. These methods helped to find out more than 150 putative GT candidate genes out of which 24 were considered “strong” candidates after extensive sequence analysis. Among these genes, At5g28910 showed structural similarity to mammalian fucosyltransferase that belongs to family GT23 of CAZy database. In addition, three conserved motifs were identified that are specific for the large fucosyltransferase family (Figure 1.18). The other 23 genes belong to Domain of Unknown Function 266 (DUF266) family (Hansen *et al.*, 2009).

Figure1.18: The three conserved peptide regions of the large fucosyltransferase superfamily (Hansen *et al.*, 2009).

The objective of my thesis work at CERMAV is to assign function to these candidate genes that encode putative glycosyltransferases that could be possibly involved in the biosynthesis of plant cell wall polysaccharides, particularly in pectin biosynthesis which are not reported in CAZy database. One of the selected genes At5g28910 is unique in the Arabidopsis genome and presents the characteristic features of known fucosyltransferases, making this gene a likely candidate to be involved in RGII biosynthesis, named as NGT1 (Novel Glycosyltransferase 1) and 23 other selected genes belong to a new gene family, so far annotated with a “Domain of Unknown Function 266” (DUF266) for which implication in polysaccharides / pectin biosynthesis is hypothesized (Hansen *et al.*, 2009). Pectins have different classes of cell wall polymers, namely homogalacturonan, rhamnogalacturonan I and

rhamnogalacturonan II and xylogalacturonan that are essential for plant development and physiology, but also have economical interest as commonly used in food industry and recently developed as pharmaceuticals (Jackson *et al.*, 2007). Because of the diversity and complexity of pectic polysaccharides it has been determined that at least 67 different glycosyltransferases are expected to be required for its biosynthesis (Caffall & Mohnen 2009).

Thus complexity of cell wall polysaccharides required a combination of various tools in order to fully understand the cell wall structure and its function. So in our lab, functional genomic strategy was developed to unravel the function of putative fucosyltransferase At5g28910. This strategy mainly consists of two tasks to be done in parallel. First is the characterization of T-DNA mutants of gene of interest and then determination of phenotype and chemotype of these mutants. We have used GC-MS linkage analysis of permethylated sugars derived from the cell wall to screen each mutant for pectin and cell wall defects. This technique will provide us a “two-dimensional biochemical characterization” of the cell wall, as it will be informative about the type of sugar and the type of linkage binding units. Second is the cloning of gene of interest for heterologous expression of protein and then to perform activity test with different acceptors and donors available at Cermav Glycolibrary.

During first year of my PhD at CERMAV, I characterized homozygous mutant lines for putative GT identified displaying a fucosyltransferase signature and for some of the genes belonging to DUFF-266 family and cloning of these genes was done into donor and expression vector for expressing them into heterologous expression system.

More specifically, I focused during my thesis on the characterization of putative fucosyltransferase named as NGT1 for “Novel GlycosylTransferase 1” and evaluated for being a putative GT involved in plant cell wall biosynthesis.

In parallel, I also worked on the expression of ATFUT1 (Arabidopsis fucosyltransferase) which is responsible for the fucosylation of xyloglucan in *Arabidopsis thaliana*, for its structural characterization. Truncated version of AtFUT1 (without transmembrane domain) was expressed in insect cells and in *pichia pastoris* to test its activity *in-vitro* and to undergo enzyme purification.

Chapter 2

Developing functional genomics on putative “Novel Glycosyltransferase” genes

2 Developing functional genomics on putative "Novel Glycosyltransferase"genes

2.1 Introduction

Plant cell wall is a complex network of polysaccharides and proteins for which biosynthesis remains poorly understood. One likely hypothesis for the lack of information about the genes involved in polysaccharide biosynthesis inferred that some of these genes have been missed during Arabidopsis genome annotation, and thus not referenced in CAZy database. In our lab, a bioinformatics strategy was developed to search new candidate Glycosyltransferase genes (GTs) from the Arabidopsis proteome, in order to identify novel GTs responsible for polysaccharide synthesis and their modification (Hansen *et al.,* 2009). It is interesting to note that such strategy was undertaken in the past and led to the characterization of several novel non-CAZy glycosyltransferases which are involved in RG II biosynthesis (Egelund *et al.,* 2004, Egelund *et al.,* 2006). Our bioinfomatics strategy to identify novel glycosyltransferases was expected to be complementary as the criteria for the identification of genes of interests were different. Taking advantage of this approach, more than 150 candidate genes have been identified (Hansen *et al.,* 2009). Among them, a new group of genes called "NGT" for (Novel Glycosyltransferase) has been identified. It comprises 24 genes that are considered likely candidates to encode GT activity on the basis of clear GT signatures that could be identified in their sequences with high prediction.

Out of these 24 genes, At5g28910 gene was unique and contained a specific signature, different from the 23 others genes all belonging to one family of gene called DUF266 (Domain of Unknown Function 266). The **At5g28910** gene was named "**NGT1**" (Novel Glycosyltransferase1) during the course of our study. Interestingly, NGT1 gene harbors a fucosyltransferase signature thus providing a testable function to that candidate gene. The presence of the fucosyltransferase signature makes this gene, for example, a possible candidate to be involved in pectin RGII biosynthesis as this complex cell wall polysaccharide contains both fucose and 2-O-Me-fucose. Moreover, the other fucosyltransferase involved in N-glycan, arabinogalactan proteins and xyloglucan biosyntheses are already known and have been characterized (Perrin *et al.,* 1999, Vanzin *et al.,* 2002b, Wu *et al.,* 2010). On the

contrary, the fucosyltransferases which are responsible for the addition of fucose and 2-O-Me-fucose during pectin RG-II biosynthesis remains unknown. This expectation of NGT1 to encode a fucosyltransferase activity involved in RG-II biosynthesis based on the protein sequence signature strengthens our interest in the functional characterization of this gene among others. It is noteworthy that this gene has a close homolog At5g28960 in Arabidopsis genome but the analysis of comprehensive database revealed this gene has no EST (Hansen *et al.*, 2009).

Additionally, a second set of genes was identified, comprising 23 members belonging to the Domain of Unknown Function 266 family (DUF266 family). This DUF266 family contains 23 genes with overall amino acid sequence similarity ranging from 15-80% and can be clustered into 3 clades as represented in phylogenetic tree (Figure 2.1). We used an alphabetical one letter code (A→ V) to reference them in the lab, to facilitate ourselves and data handling. It has been suggested that these 23 selected genes could encode GTs (as NGT1) involved in polysaccharide biosynthesis (Hansen *et al.*, 2009).

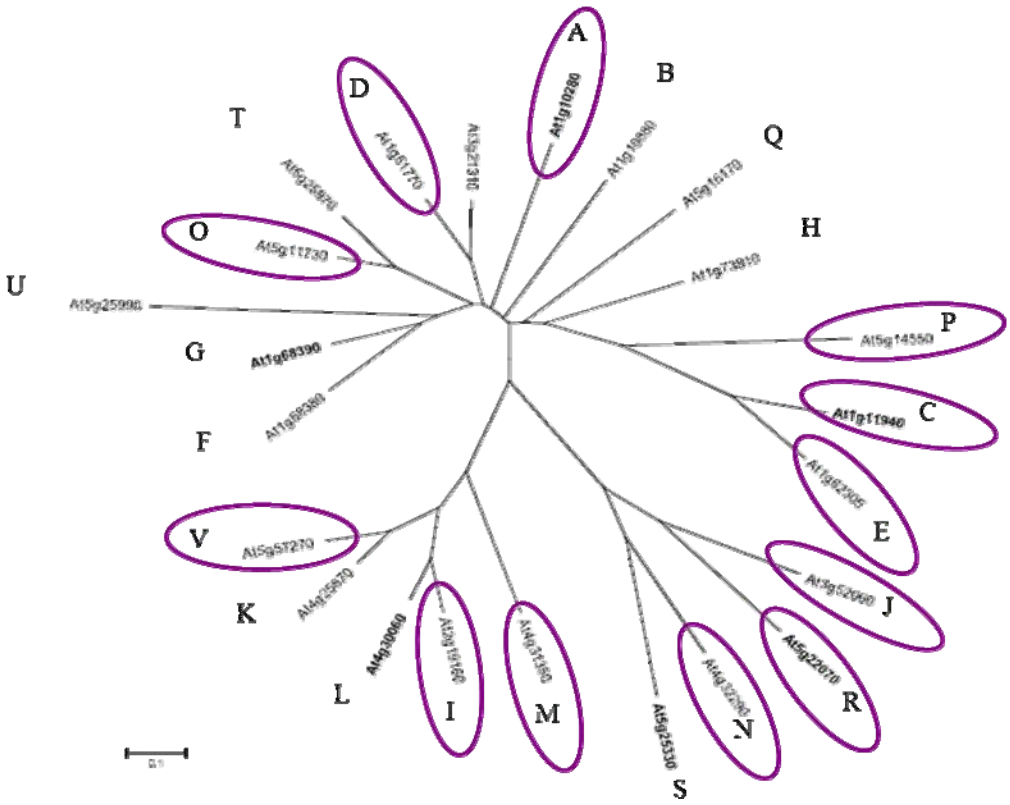


Figure 2.1: Phylogenetic tree of DUF266 genes. 23 genes grouped into 3 different clades. Each gene was annotated with a one letter code with respect to its AGI number. Genes circled in purple were studied for mutant characterization.

A functional genomics approach was then developed in the lab in order to determine if the selected genes could encode GTs. This approach mainly consists of two tasks currently performed in parallel (as shown in figure 2.2).

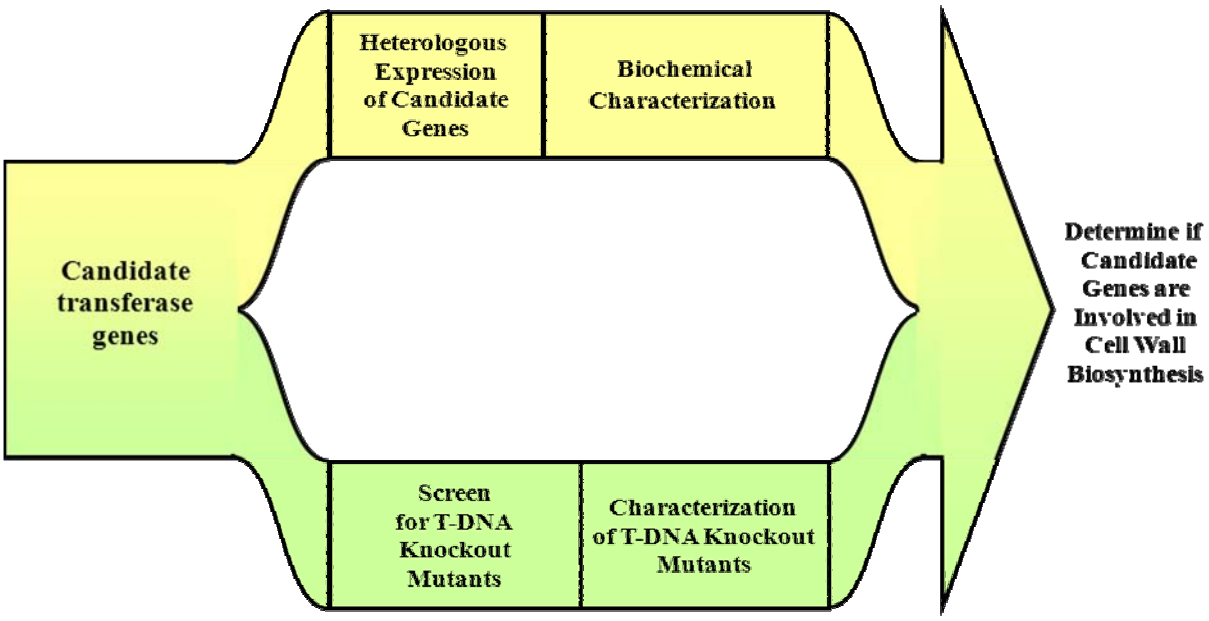


Figure 2.2: Functional genomics strategy for the characterization of putative genes involved in plant cell wall biosynthesis.

- First task is the characterization of homozygous T-DNA mutants of gene of interest. Gene knockout (or null) mutants are very useful in reverse genetics because they provide direct information about the gene function *in vivo*. There are many ways to introduce targeted mutagenesis into specific gene of interest. Out of these, insertional mutagenesis is a very common and valuable tool for studying function of a gene in *Arabidopsis thaliana*. This is mainly based on the insertion of foreign DNA into the gene of interest which disrupts the gene function and then the effect of this deficiency on the plant is investigated. During my thesis, out of these 24 putative glycosyltransferases, I have selected 13 genes of interest (A-At1g10280, C- At1g11940, D- At1g51770, E- At1g62305, I- At2g19180, J- At3g52060 M-At4g31350, N-At4g32290, O-At5g11730, P- At5g14550, R- At5g22070 and V- At5g57270 from the DUF266 gene family and W-At5g28910 for NGT1) on the basis of mutant seed stock availability and ordered Arabidopsis mutant seeds for one or two alleles. Over the 13 genes selected were representatives of the three different clades of DUF266 family shown in figure 18. It was our choice to try to characterize various Arabidopsis mutants over the three

different clades expecting that among each clade these genes may have evolved related but distinct functions.

-The characterization of mutant lines for 13 genes (12 genes from DUF266 family and NGT1) represented a labour intensive work, but it offered the opportunity to compare phenotype and cell wall composition of plants having various but related genotypes in a comprehensive manner, and ultimately to draw hypothesis about their implication in cell wall biogenesis.

- Second task is the cloning of gene of interest into a vector for its heterologous expression in the yeast *Pichia pastoris* or insect cells. For this purpose cDNAs were ordered for the DUF266 family genes (A, C, D, E, I, J, L, M, N, O, P, R and V) and for the putative fucosyltransferase NGT1. These cDNAs were cloned into Gateway cloning system. This versatile cloning system offers the advantage of expressing the proteins into multiple expression systems like *Pichia pastoris* and insect cells. Heterologously expressed proteins could then be used to perform radioactive activity test *in vitro*, with different acceptors and donors currently available at CERMAV glycolibrary (comprising commercial and home-made oligosaccharides prepared at CERMAV). Ultimately, if a radioactive sugar transfer is assessed from the nucleotide-sugar donor onto the acceptor, a product characterization would be performed.

2.2 Identification of homozygous T-DNA lines

The main objective of this work was to address the following questions for the 13 selected genes.

1- Is there any specific developmental phenotype related to impairment of the selected gene?
2- What is the effect of T-DNA knockout mutation of the selected gene on plant cell wall composition?

2.2.1 Selection of homozygous mutant lines

For the evaluation and determination of the putative biological function of candidate genes, 28 T-DNA knockout mutant lines for 13 candidate genes (in order to characterize 2 or

more different alleles of a gene mutation in parallel when possible) were ordered from Nottingham Arabidopsis Stock Centre (NASC).

Table 2.1: List of alleles of each mutant and additional information i.e. gene name, letter code, order, T-DNA category and T-DNA line status. W is the one letter code used for NGT1.

Gene name	**Letter code**	**Order**	**TDNA category**	**PCR screening**
At1g10280	A1	N613287	SALK_113287	Yes
	A2	N566646	SALK_066646	Yes
	A3	N859542	SALK_066024	Yes
At1g10880	B1	N25026	SALK_014515	No
	B2	N517312	SALK_017312	No
At1g11940	C1	N801531	SAIL_31_G08	Yes
At1g51770	D1	N511445	SALK_011445	Yes
	D2	N837648	SAIL_842_F01	Yes
At1g62305	E1	N813237	SAIL_286_A08	Yes
	E2	N517871	SALK_017871	Yes
At1g68380	F1	N25148	SALK_107582	No
	F2	N65220	SALK_047206c	No
At1g68390	G1	not ordered		No
At1g73810	H1	Discussion		No
At2g19160	I1	N879626	SAIL_666_F10	Yes
At3g21310		not ordered		No
At3g52060	J1	N613224	SALK_113224	Yes
	J2	N25017	SALK_012392	Yes
	J3	N824019	SAIL_565_F07	Yes
At4g25870	K1	N613513	SALK_113513	No
	K2	N813460	SAIL_290_E10	No
At4g30060	L1	not ordered		No
At4g31350	M1	N522360	SALK_022360	Yes
	M2	N528175	SALK_028175	Yes
	M3	N643591	SALK_143591	Yes
At4g32290	N1	N25154	SALK_116524	Yes
	N2	N844989	SAIL_1229_G05	Yes
	N3	N654154	SALK_039708c	Yes
At5g11730	O1	N826474	SAIL_617_H01	Yes
At5g14550	P1	N841069	SAIL_912_D02	Yes
	P2	N828444	SAIL_655_C04	Yes
At5g16170	Q1	not ordered		No
At5g22070	R1	N816396	SAIL_353_G08	Yes
	R2	N816395	SAIL_353_G07	Yes
At5g25330	S1	not ordered		No
At5g25970	T1	not ordered		No
At5g25990	U1	not ordered		No
At5g57270	V1	N516363	SALK_016363	Yes
	V2	N819542	SAIL_423_E07	Yes
At5g28910	W1 / *ngt1-1*	N585839	SALK_085839	Yes
	W2	N585848	SALK_085848	Yes
	W3 / *ngt1-2*	N638819	SALK_138819	Yes

In the table given above 2.1, the alleles of each mutant and additional information (gene name, letter code, order, T-DNA category and T-DNA line status) are given (whether or not PCR screening of the T-DNA line was attempted is indicated).

The seeds were received from NASC and grown in *vitro*. Ten days seedlings were transferred to sterilised soil for seed amplification. The seeds thus obtained represented our F1 generation. For the selection of resistant lines for T-DNA insertion, F1 generation seeds were grown in *vitro* and checked for segregation. Approximately after one week of growth, resistant plants were scored for the expected segregation of a recessive mendelian trait (Figure 2.3). Based on segregation mutant lines were annotated homozygous, heterozygous or wild-type and further checked by PCR.

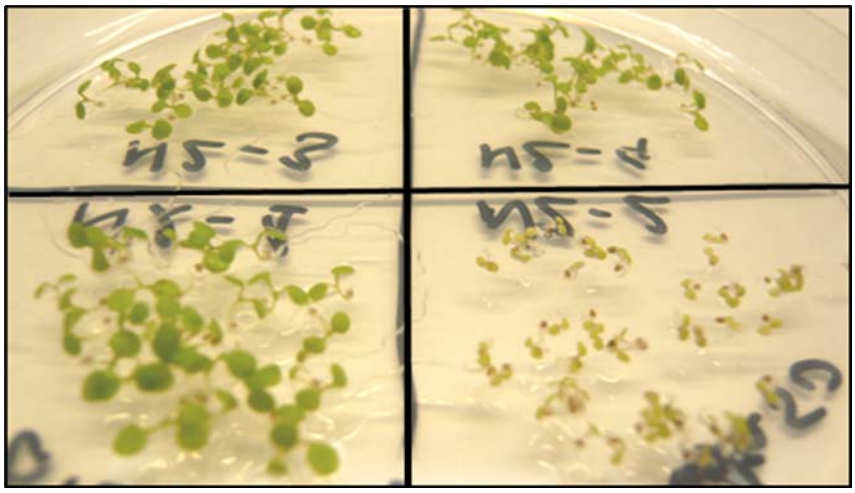


Figure 2.3: Selection of homozygous mutant plants on MS media containing kanamycin. Healthy plants were selected and genotyped by PCR

As mentioned earlier, in order to distinguish the homozygous, heterozygous and wild type lines for the T-DNA insertion we used PCR amplification technique which is explained in the figure 2.4 below. For each putative homozygous mutant line, DNA was prepared from eight individual plants and used for PCR genotyping.

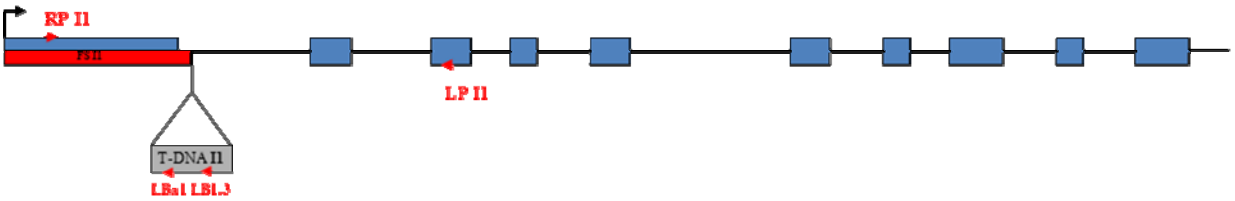


Figure 2.4: Schematic representation of the gene structure of I gene (At2g19160) and the position of insertion of T-DNA in the first intron (corresponding to "i1" mutant). The binding site of primers used in PCR reaction is indicated by arrows. Gene (I) has 10 exons represented by blue boxes.

We used 3 different pairs of primers called LB1 (LBa1 + RPI1), LB2 (LB1.3 + RPI1) and geno (LPI1 + RPI1) as shown in figure 2.4 with red arrows. The first two pairs of primers LB1 and LB2 are specific to the presence of T-DNA insertion at predicted loci and the last pair of primers (genomic) will give a product only if insertion at this predicted locus is missing. Thus homozygous lines are predicted to be LB1 (+), LB2 (+) and genomic (-), whereas heterozygous lines will be positive for all three reactions (LB1, LB2 and genomic).

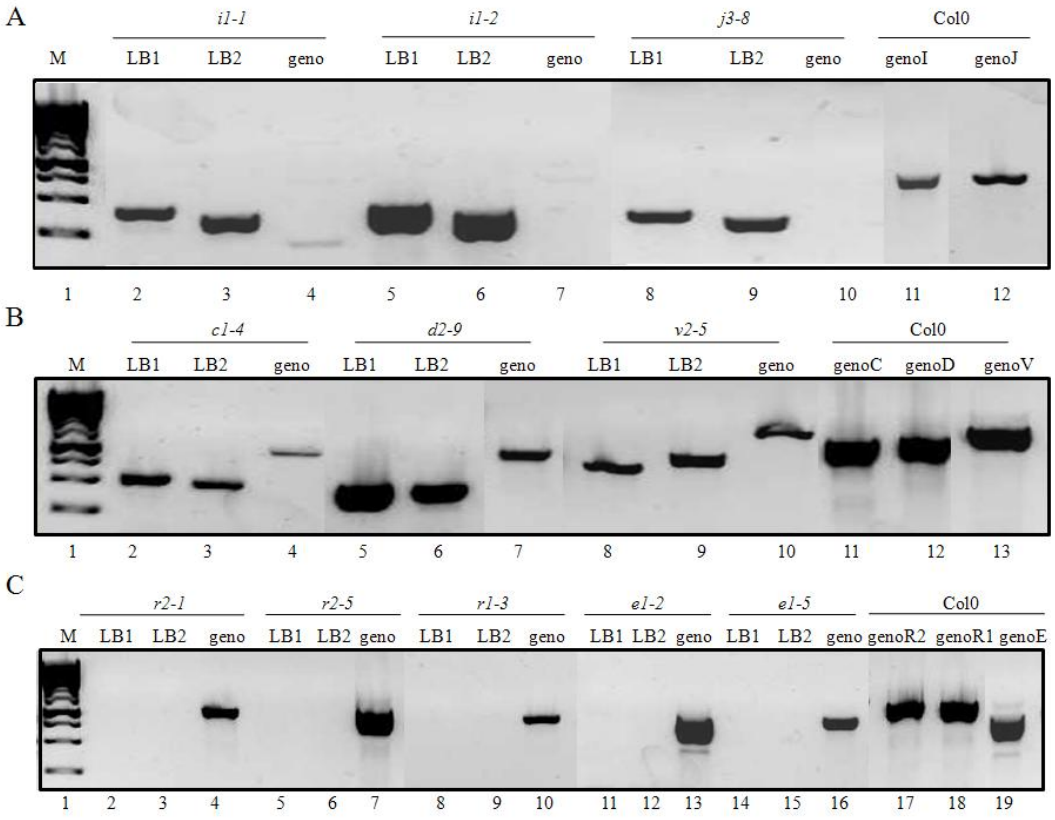


Figure 2.5: T-DNA mutant characterization through PCR. In figure A, B and C primers LB1 and LB2 are left border T-DNA specific primers while geno are genomic forward and reverse gene specific primers. Col0 DNA is used as a control. A) Homozygous plants selected by PCR for genes P, I and J. Primers genoP, genoI, genoJ represent genomic gene specific primers. B) Heterozygous plants selected by PCR for genes C, D and V. Primers

genoC, genoD and genoV represent genomic gene specific primers. C) Wild type plants found by PCR for genes R and E. Primers genoR2, genoR1 and genoE represent genomic gene specific primers.

The region corresponding to T-DNA insertion of genes of interest belonging to DUF 266 was amplified using T-DNA specific left border primer with the gene specific right primer represented in figure LB1 and LB2. Similarly the region corresponding to genomic DNA for DUF 266 genes was obtained by using gene specific primers ("geno") represented in figure 2.5 as well as left and right border primers. In the figure 2.5 A, for example *i1-1, i1-2, j3-8* and Col0 represent different DNA stocks isolated from mutant and wild type plants that were checked by PCR for T-DNA insertion using three sets of primers, two amplifying a product in the presence of T-DNA at the expected locus, one being specific to the WT version of the gene at this locus (Figure 2.5A). In the figure 2.5A, lanes 2 and 3 show a PCR product indicating insertion of a T-DNA at I locus *i1-1* in plants by the use of primers (LB1, LB2), while the lack of product in lane 4 (primers geno) from *i1-1*DNA confirmed that the Arabidopsis line *i1-1*is homozygous for T-DNA insertion at I locus. Similar results were obtained for *i1-2* (lane 5 to 7) and for *j3-8* (lane 8 to 10) indicating that these mutant lines are also homozygous. In contrast, in case of Col0 genomic DNA the presence of a PCR product in lane 11 to 13 confirmed that the DNA prepared from Col0 plants was wild type for I and J locus.

Eventually all this PCR reaction products were sequenced to confirm unambiguously their identity, but also to determine precisely the size of DNA sequence deletion (from genomic or T-DNA) that occurs in consequence of the T-DNA insertion into the Arabidopsis genome. The figure 2.5A shows that the *i1-1, i1-2* and *j3-8* mutants are homozygous. The complete list of homozygous mutant alleles is given in the table 2.2, while the figures related to other homozygous alleles are given in the Annexe figure 8.1. The Figure 2.5B shows that the *c1-4, d2-9* and *v2-5*, mutants are heterozygous as all of these lines are positive for all the sets of primers used for PCR amplification (lanes 2 to 10). The complete list of heterozygous mutant alleles is also given in the table 2.2, while the figures related to other heterozygous alleles are given in the Annexe figure 8.2.The figure 2.5C shows that the mutants lines (*r2-1,r2-5, r1-3, e1-2* and *e1-5* do not have T-DNA insertion and behaved like wild type as all the lines are negative for LB1 (lanes 2, 5, 8,11 and 14) and LB2 (lanes 3, 6, 9, 12 and 15) set of primers while all of them are positive for "geno" set of primers (lane 4, 7, 10, 13 and 16) while the

figures related to other wild type alleles are given in the Annexe figure 8.3. In the table 2.2, the results of the PCR screening are summarised.

Table 2.2: The results of PCR screening strategy

Gene name	**Letter code**	**Order**	**TDNA category**	**Ho TDNA lines**
At1g10280	A1	N613287	SALK_113287	Homozygous
	A2	N566646	SALK_066646	Homozygous
	A3	N859542	SALK_066024	Homozygous
At1g11940	C1	N801531	SAIL_31_G08	Homozygous
At1g51770	D2	N837648	SAIL_842_F01	Homozygous
At1g62305	E2	N517871	SAIL_286_A08	Wild type
At2g19160	I1	N879626	SAIL_666_F10	Homozygous
At3g52060	J2	N25017	SALK_012392	Homozygous
	J3	N824019	SAIL_565_F07	Homozygous
At4g31350	M1	N522360	SALK_022360	Wild type
	M2	N528175	SALK_028175	Homozygous
	M3	N643591	SALK_143591	Homozygous
At4g32290	N1	N25154	SALK_116524	Homozygous
	N2	N844989	SAIL_1229_G05	Wild type
	N3	N654154	SALK_039708c	Homozygous
At5g11730	O1	N826474	SAIL_617_H01	Homozygous
At5g14550	P2	N828444	SAIL_655_C04	Homozygous
At5g22070	R1	N816396	SAIL_353_G08	Wild type
	R2	N816395	SAIL_353_G07	Wild type
At5g57270	V2	N819542	SAIL_423_E07	Heterozygous
At5g28910	W1 / *ngt1-1*	At5g28910	SALK_085839	Homozygous
	W3 / *ngt1-2*		SALK_138819	Homozygous

From the above mutant characterization, 15 mutant lines with a T-DNA insertion have been characterized unambiguously homozygous, 1 heterozygous mutant line for 1 gene only and 5 lines behaved like WT.

2.2.2 Phenotypic characterization of homozygous mutant lines

In order to check that either one of these genes of interest would be responsible for any developmental or growth defect, homozygous mutants were grown (two plants for each genotype) and checked for phenotype alteration such as dwarfing or organ malformation.

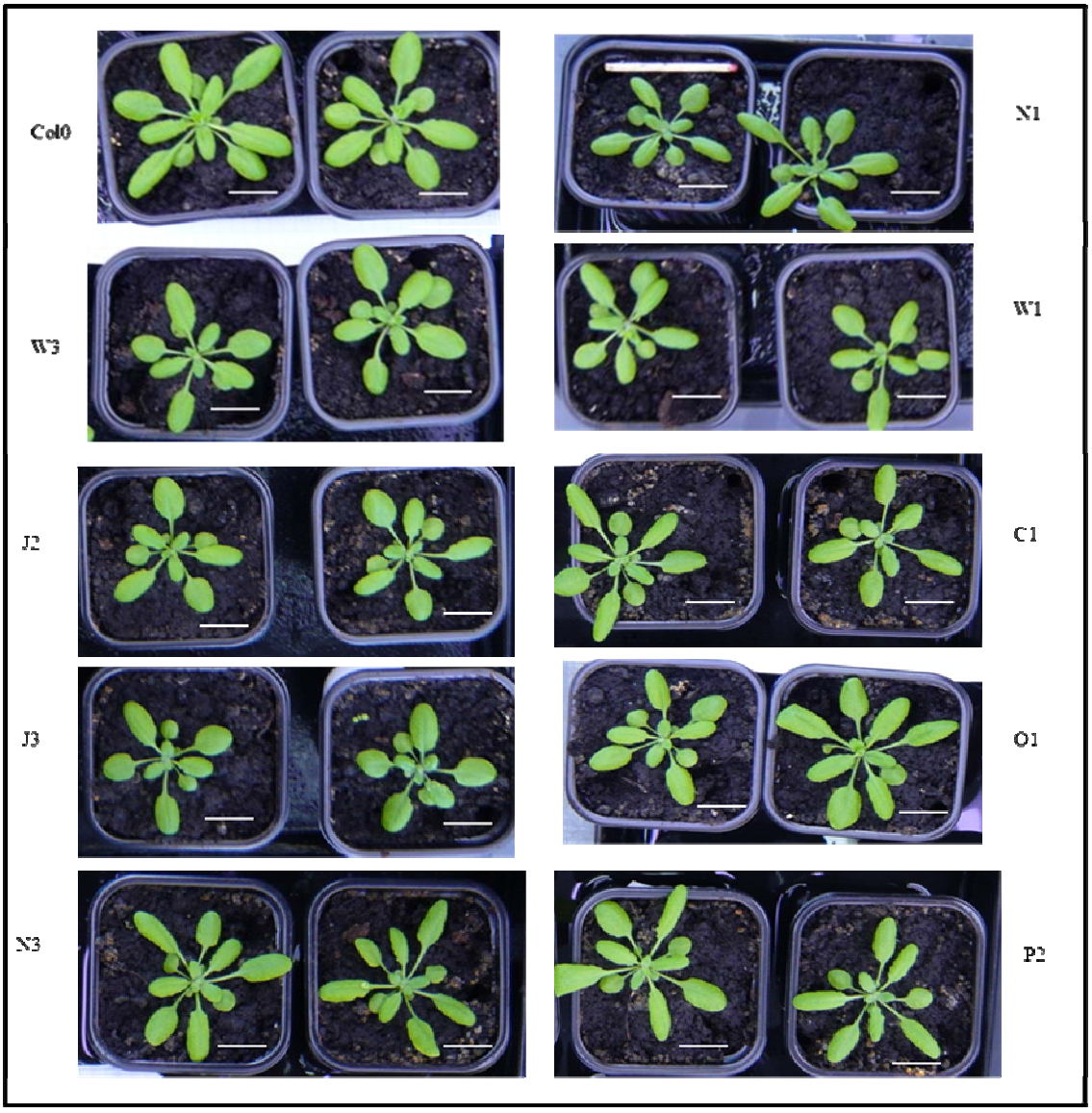


Figure 2.6: 23 days old Arabidopsis wild type and mutant plants grown on soil for phenotypic studies Mutant plants of genes N1, W3, W1, J2, C1, J3, O1, N3 and P2 have narrower leaves as compared to wild type Col0 plants. Scale bar = 1cm

We did not observe any striking effect of mutation on plant growth as observed earlier for other cell wall mutants like *qua1* and *qua2* involved in pectin HG biosynthesis (Bouton *et al.*, 2002, Mouille *et al.*, 2007). However subtle differences can be observed in 23 day-old mutant plants as compared to wild type (Col0) (Figure 2.6). These differences are more pronounced in some mutant lines (e.g. N1, W1, and J3). More accurate methods would be required to evidence growth defect phenotypes. There can be many factors which can explain the observed subtle growth phenotype. One possible reason for the lack of a clear visible phenotype for the genes belonging to DUF266 could be the redundancy of gene function

among this gene family (or from genes belonging to other families) as it has been already observed for GAUT proteins which are involved in pectin biosynthesis (Caffall & Mohnen 2009). Alternatively, it is possible that these genes may not directly be involved in cell wall biosynthesis or may have small contribution in plant growth and development. For some allelic mutants we got only heterozygous and wild type plants (e.g. E2, M1, N2, R1, R2 and V2 in table 2.2) and we were not able to get any homozygous plants which may suggest that mutants have embryo lethal phenotypes.

2.2.3 Biochemical characterization of homozygous mutant lines

The detailed and complete analysis (chemical, expression and microscopic analysis) of all the above mentioned genes could have been time consuming and an overwhelming work. Therefore I carried out the cell wall composition analysis of only 12 homozygous mutant lines in order to get an indication about possible alteration in cell wall sugar composition (A1, A3, C1, D2, J3, I1, I2, M3, O3, W1, W3 and P2.1 but the results are shown for 6 lines in figure 2.7.

For this purpose, Arabidopsis mutant seedlings A1, A3, C1, D2, J3 and I1 were grown in dark condition over 6 days, then harvested and treated with ethanol (cf materials and methods 6.6.1) to obtain an Alcohol Insoluble Residue (AIR) that would be hydrolyzed with an acid to access its sugar composition. Neutral monosaccharides including L-rhamnose (Rha), L-fucose (Fuc), L-arabinose (Ara), , D-xylose (Xyl), D-mannose (Man), and D-glucose (Glc) were quantified after hydrolysis of AIR polysaccharides to monosaccharides. Monosaccharide were then converted to alditol acetates and quantified by GC (cf materials and methods 6.6.2.1). This method offers the advantage of being efficient, quantitative, and enables the analysis of large series of samples that need to be processed at the same time to get an idea about alteration of cell wall composition in a mutant by comparison to the wild type analysed in the same conditions. Results obtained from sugar quantification of AIR in different lines are displayed in the graph below (Figure 2.7).

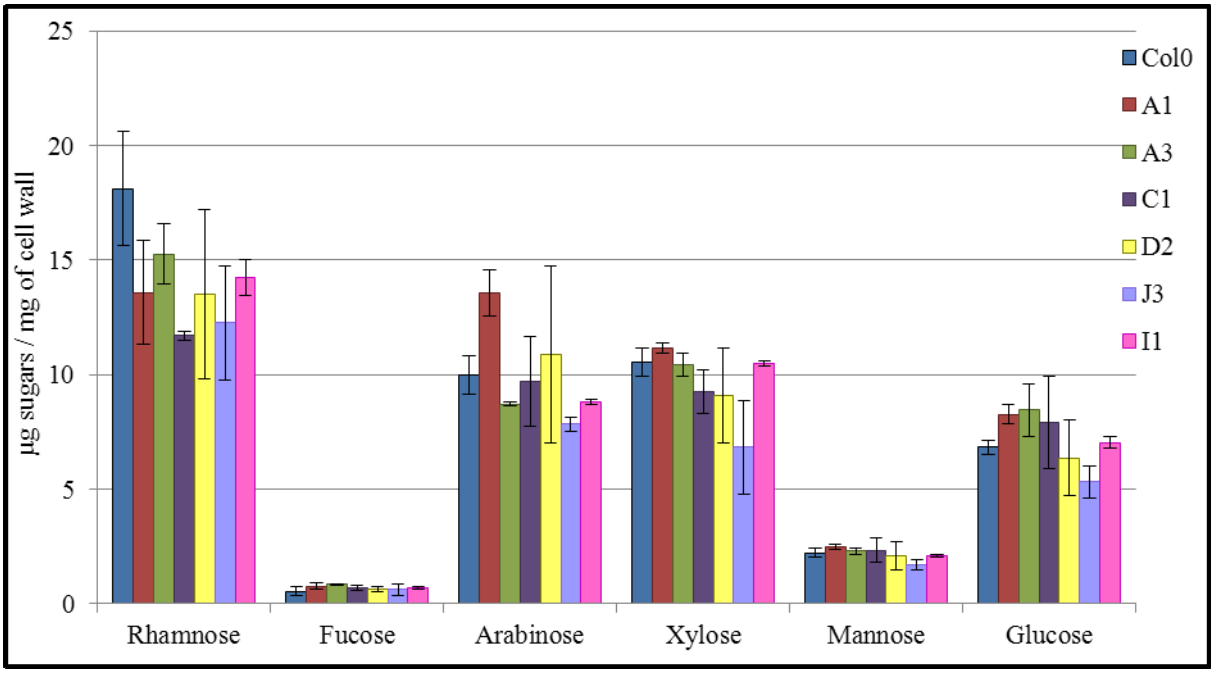


Figure 2.7: Neutral monosaccharide quantification from AIR of wild type Col0 and from different mutant lines through GC-MS. Galactose content was not integrated in order to avoid the contamination from the culture medium.

The quantification of neutral monosaccharides showed alterations in almost all the homozygous lines tested but did not provide any strong clue about the specific biochemical alteration in mutant cell wall. At this step we decided to focus our work on NGT1 (At5g28910) which harbours a fucosyltransferase signature and on At5g14550 gene (a DUF266 gene named as P, see phylogenetic tree in Figure 2.1). The latter gene was selected for the following reasons:

1- Recently Zhou and coll. have shown that BC10 rice gene which contained a domain of unknown function DUF266 is involved in cell wall biosynthesis. BC10 is a close homolog of *Arabidopsis thaliana* At5g14550 (P) and shares 82 % sequence similarity. Mutation in BC10 gene resulted in impaired cellulose and arabinogalacatan proteins (Zhou *et al.*, 2009). Moreover the mutant plant phenotype showed reduction of mechanical strength in aerial organs (sclerenchyma cells and vascular bundles), resulting in brittle culms and leaves that could be broken easily by bending compared to wild type plants (Zhou *et al.*, 2009). So it provided us evidence that P gene belonging to DUF266 family could be involved in cell wall biosynthesis in Arabidopsis. In addition, Zhou and colleagues have found that BC10 has a low but significant glycosyltransferase activity when expressed in Chinese hamster ovary (CHO)

cells. So this data supports the validity of our bioinformatics approach which was used to select the 24 new GT candidate genes in Arabidopsis (Hansen *et al.*, 2009).

2- Initial cell wall composition analysis of 10 genes out of 23 showed that mutant plants of P gene present some alteration in cell wall neutral monosaccharides contents as compared to that in wild type plants. So it would be interesting to characterize in detail for further advancement.

3- Phenotypic studies of P mutant plants have shown that the mutant leaves are smaller as compared to wild type plants which phenotype could be related to a defect in cell wall biosynthesis in this mutant.

In order to test its putative role in Arabidopsis cell wall synthesis we decided to focus our work on the At5g14550 gene (P gene). Identification and characterization of homozygous plants for At5g28910 (NGT1) which encode a putative fucosyltransferase gene will be discussed in chapter 3.

2.3 Characterization of At5g14550 (P) mutant T-DNA lines

In order to evaluate if the candidate P gene is involved in plant cell wall biosynthesis, two allelic T-DNA knock out mutant lines were ordered from Nottingham Arabidopsis Stock Centre (NASC). These two mutant lines are N841069 (SAIL_912_D02) termed as "*p1*" and, N828444 (SAIL_655_C04) termed as "*p2*," which have T-DNA insertion in 5'UTR region and in the first exon, respectively (Figure 2.8).

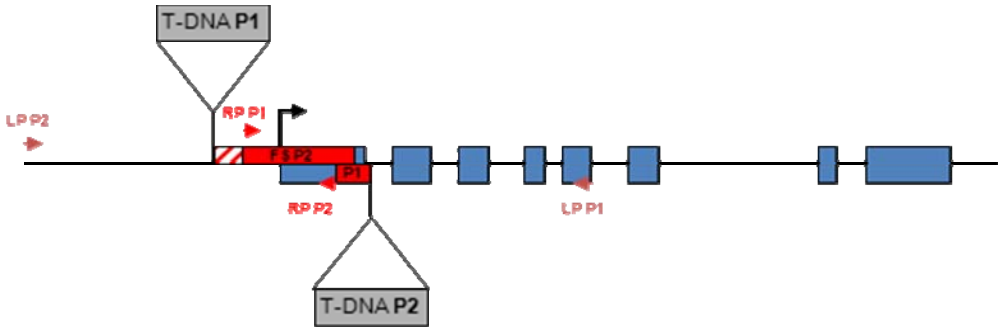


Figure 2.8: Schematic representation of the gene structure of At5g14550 (P) and the position of insertion of T-DNA for *p1* and *p2*. The binding site of primers used in PCR reaction is indicated by arrows in red color.

Arabidopsis mutants received from NASC were first amplified on soil without selection pressure and afterwards the progeny was checked for segregation of the NPT II

gene, and genotyped by PCR. For each *p1* and *p2* Arabidopsis mutant lines selected, we could identify lines showing 100% resistant plants to glufosinate, indicating that the lines were probably homozygous for the T-DNA insertion and thus have the mutation in At5g14550 (P) locus (Figure 2.9).

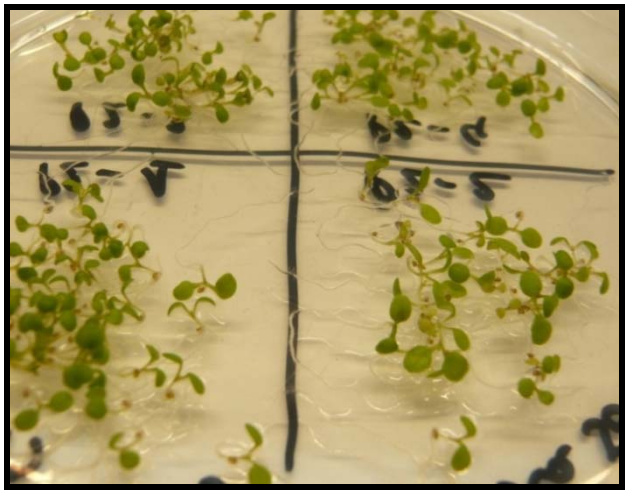


Figure 2.9: Resistant plants for *p2* were selected on MS growth medium containing glufosinate

In order to verify that the selected homozygous lines for *p1* and *p2* were definitely homozygous and altered at the locus At5g14550 (P), we performed a PCR genotyping of these two mutant lines. Using PCR it is possible to discriminate between plants genetically homozygous, heterozygous or wild type for P. DNA extracted from the leaves of selected resistant plant lines was used as template matrix in a PCR reaction. We used 3 different couples of primers LB1, LB2 and geno as described above on page 94. The first two couples of primers LB1 and LB2 are specific to the presence of T-DNA insertion at predicted loci and the last couple primer (genomic) will give a product only if insertion at this predicted locus is missing. Thus homozygous lines are predicted to be LB1 (+), LB2 (+) and genomic (-), whereas heterozygous lines will be positive for all three reactions (LB1, LB2 and genomic).

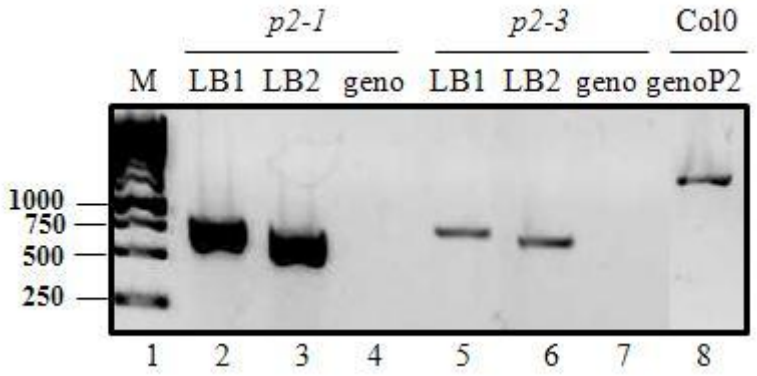


Figure 2.10: Agarose gel (0.8 %) showing the homozygous profiles observed for DNA isolated from the *p2-1 and p2-3* mutant plants. Lane 1 indicates 1 kb marker. Lanes 2 and 3 represent PCR product for T-DNA insertion and lane 4 indicates the absence of genomic product in *p2-1* mutant plant. Similarly lanes 5 and 6 represent PCR product for T-DNA insertion and lane 7 indicates the absence of genomic product in *p2-3* mutant plant.Col0 DNA was used as a control, lane 8 shows that Col0 is wild type.

PCR data in Figure 2.10 confirmed the homozygous character of mutant lines *p2-1* and *p2-3*. Unfortunately we could not find homozygous plants for the mutant line *p1* (data not shown), that is why we characterized further the two p2 plant mutant lines, i.e. *p2-1* and *p2-3*.

Then selected homozygous mutant plants were transferred onto pre-sterilized soil and allowed to grow to obtain seeds. The new generation of seeds was then again checked for the presence of resistant marker by growing the seeds on MS agar medium containing resistant marker, in which segregation was verified. Some of the seeds from this pool were grown on soil and again DNA was extracted from the leaves and used as a PCR template to differentiate homozygous, heterozygous and wild type plants, by PCR as described above.

2.3.1 Phenotypic characterization of P mutant lines *p2-1* and *p2-3*

In order to determine the effect of mutation on plant growth and development, plant growth was observed. Seeds for mutants and wild type were grown on soil and were observed for phenotypic changes. We have found that mutant plants *p2-1 and p2-3* were morphologically distinguishable from wild type Col0 plants as they have (shorter) narrow leaves. A method was developed to measure the plant leaf area in a reproducible manner.

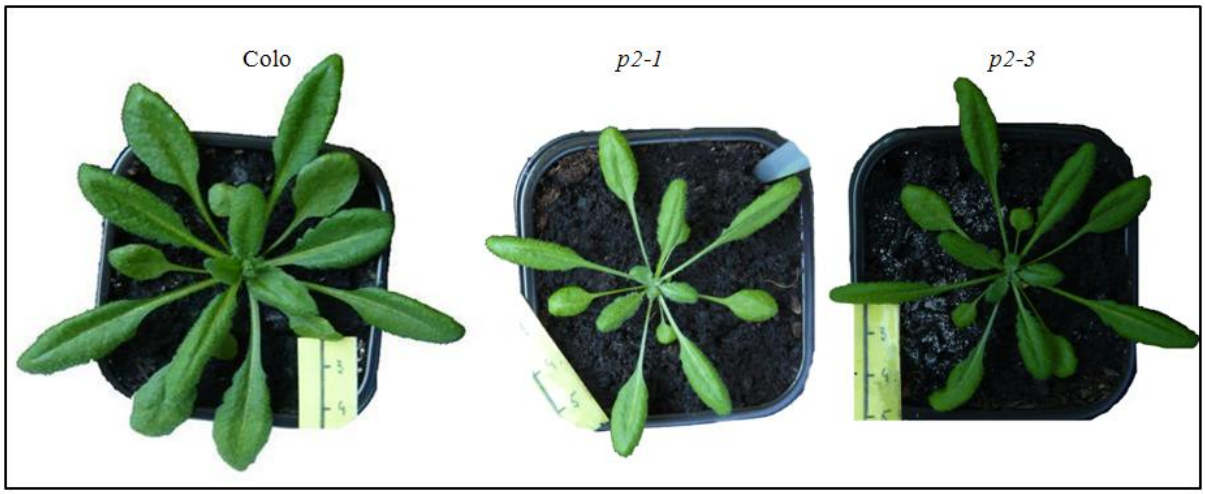


Figure 2.11 : Arabidopsis thaliana 23 days old wild type (Col0) and mutant plants (*p2-1*, *p2-3*). The leaves of mutant *p2-1*, *p2-3* are narrow than wild type plants. Scale bar = 2cm

Leaf area was measured at different developmental stages of plant growth by using publicly available software ImageJ as explained in materials and methods (cf materials and methods 6.7.1). Briefly, we measured the leaf area by taking a picture of individual plant for each *p2-1*, *p2-3* and Col0, each having a reference scale bar (Figure 2.11). Area of the leaf was calculated with the help of reference scale, and reported as histograms.

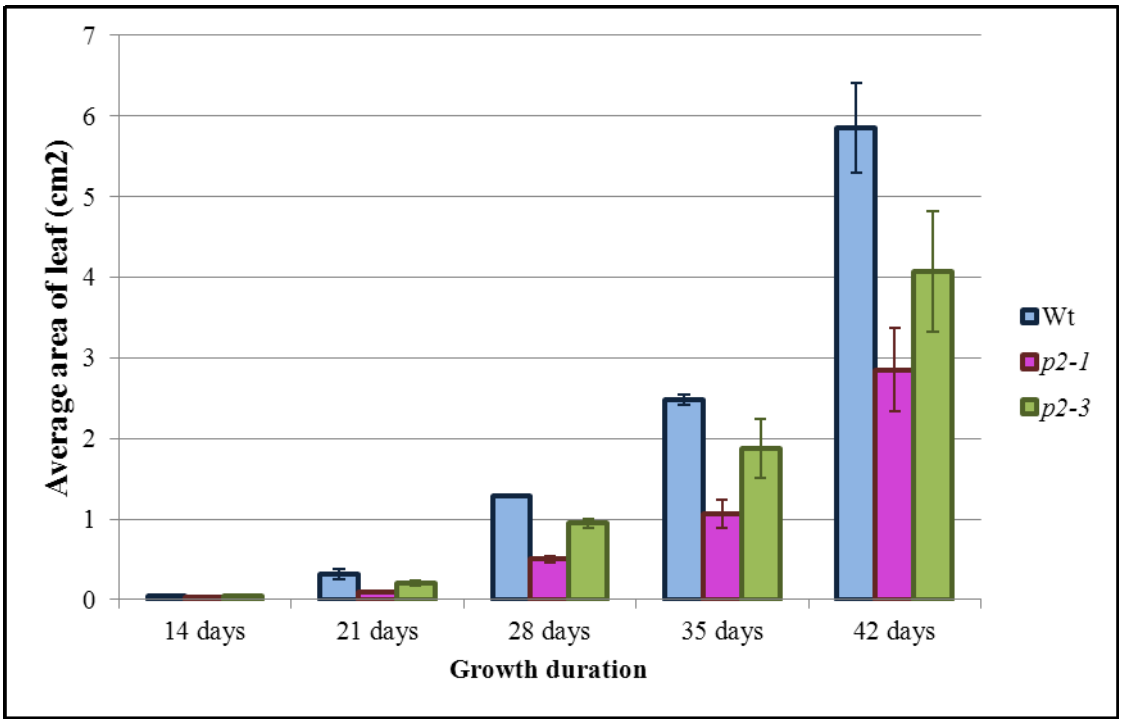


Figure 2.12: Analysis of leaf area of wild type Col0 and mutant plants *p2-1, p2-3*. Leaf area was measured quantitatively. Each value is an average determined from the measurement of leaf area of six plants in two independent experiments performed under the same conditions. The error bars represent the standard deviation calculated for all values of two experiments. At 42 day of plant growth, the leaves of the mutant plants *p2-1, p2-3* are smaller than leaves of wild Col0 plants.

This phenotypic study has shown that mutant plants of both lines have smaller leaves as compared to wild type plants but *p2-1* and *p2-3* behaved differently when compared to each other. This is little bit surprising but it could be possible because of difference in growth condition as this data is calculated from 2 leaves of 3 individual plants from *p2-1* and *p2-3* mutant plants. The results showed that mutant leaves were very smaller at 14 days of growth and this difference was remarkable even at 42 days of growth of plant with a difference of 52% for *p2-1* and 30% for *p2-3* as compared to wild type Col0 plants (Figure 2.12).

2.3.2 Neutral monosaccharide quantification of cell wall from *p2-1* mutant line through GC-MS

As plant cell wall consists of 90% carbohydrates, the quantification of neutral monosaccharide in both wild type and mutant plants can give us an idea about the implication of P gene in cell wall biosynthesis. For this purpose AIR residues were prepared from dark grown 6 days old seedlings from mutant and wild type plants. Neutral monosaccharides were quantified by first hydrolyzing the polysaccharides to their monosaccharide constituents with strong acid (TFA) and then converting them to alditol acetates by reduction with sodium borohydride to the corresponding alditol, followed by acetylation of the hydroxyls on each alditol. The resultant alditol acetates were quantified by GC-MS. Seven major monosaccharides were quantified from cell wall including rhamnose (Rha), fucose (Fuc), arabinose (Ara), galactose (Gal), xylose (Xyl), mannose (Man), and glucose (Glc).

Quantification analysis of sugars has shown that almost all the neutral sugars present in cell wall are affected and mutant line *p2-1* has significant changes in cell wall glycosyl residues as compared to wild type because of mutation in gene P as shown in figure 2.13. A reduction is observed in the quantity of sugars in mutant cell wall as compared to wild type Col0 cell wall.

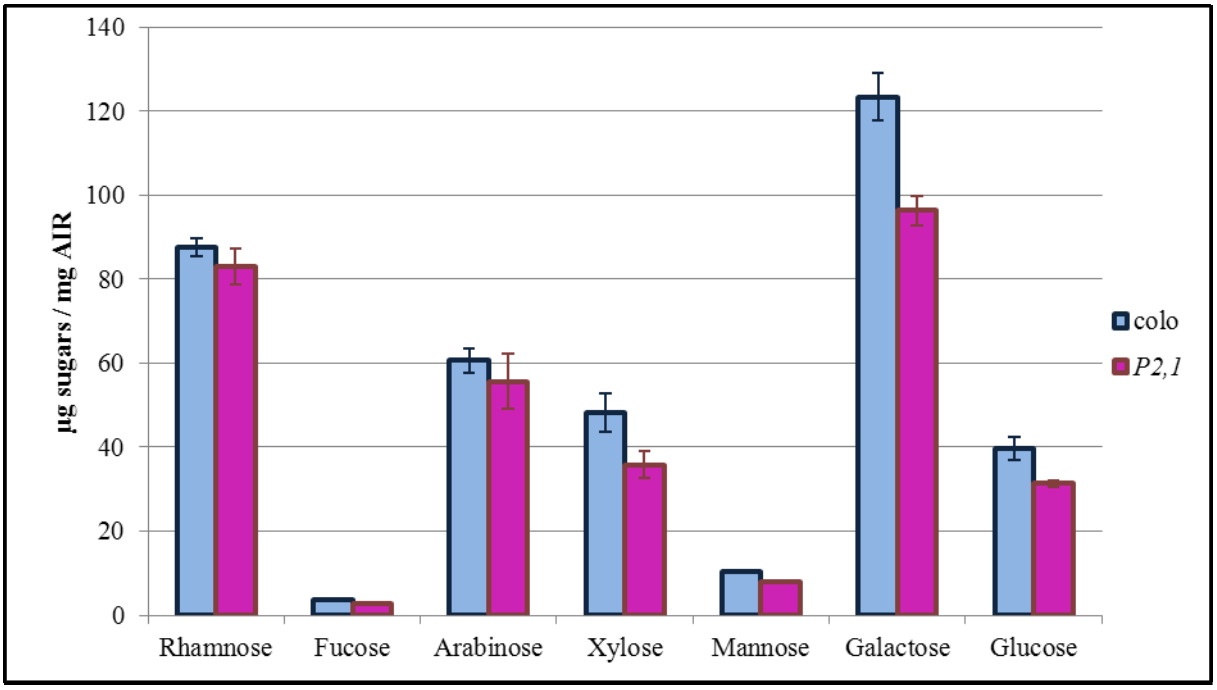


Figure 2.13: Neutral monosaccharide composition analysis of the cell wall from wild type Col0 and *p2-1*mutant plant cell wall. These values are mean of three independent assays. Each cell wall component is calculated as µg/ml of alcohol insoluble cell wall residues.

These alterations in neutral monosaccharide composition in cell wall of mutant plants have shown more convincingly that our hypothesis about the implication of P gene belonging to DUF266 family in plant cell wall biosynthesis is correct. This assay shows that there is overall reduction of total cell wall in mutant as compared to wild type. To confirm whether this reduction in all sugar components is significant or not, we calculated the relative quantity of each sugar in % in wild type and *p2-1* mutant cell walls (Table 2.3).

Table 2.3: The data are means of 3 independent assays. Relative quantity of each wall component is calculated in % age in wild type and *p2-1* mutant cell wall

Sample	Rha	Fuc	Ara	Xyl	Man	Gal	Glc
Col0	23,4 ± 3	1 ± 0	16,2 ± 4	12,9 ± 6	2,8 ± 1	33 ± 8	10,6 ± 4
p2.1	26,5 ± 4	0,9 ± 0	17,8 ± 7	11,5 ± 3	2,5 ± 0	30,8 ± 3	10 ± 1

The calculations of the data in % showed that there is a very little change in the relative quantity of each sugar in mutant line *p2-1* as compared to that in wild type Col0 plants. It means that quantity of total cell wall in mutant plants *p2-1* has decreased as

compared to wild type cell wall. As this neutral monosaccharide quantification provided us an idea about the biochemical changes in mutant cell wall but did not give us information which particular polysaccharide is modified, so in order to get this more precise information glycosidic linkage analysis after permethylation of cell wall samples and extraction of individual cell wall components is required. Zhou *et al.,* (2009) have quantified the neutral monosaccharides in rice alcohol insoluble residues and have shown that glucose is 20% decreased in mutant *bc10* as compared to wild type Col0 but all other sugars like xylose, galactose, arabinose and rhamnose are increased as shown in Table 2.4. This analysis did not provide them any strong evidence about the alteration of specific sugar and in turn about the function of BC10 gene.

Table 2.4. The data are means of five independent assays. Each wall component was calculated as mg/g of alcohol-insoluble cell-wall residue (Zhou *et al.*, 2009).

Sample	Glc	Xyl	Man	Gal	Ara	Rha
Col0	395 ± 6.5	49.2 ± 0.7	7.49 ± 0.1	14.1 ± 0.1	10.3 ± 0.2	4.74 ± 0.1
bc10	316 ± 6.3	62.8 ± 1.0	7.72 ± 0.2	16.6 ± 0.6	16.7 ± 0.3	5.58 ± 0.2

They performed immuno-labelling experiments which showed a reduction of AGPs labelling in the mutant. In order to confirm this observation, they isolated the AGPs and observed 70% reduction in AGPs content in *bc10* as compared to wild type plants. Similarly we have observed that in Arabidopsis cell wall all the neutral monosaccharides are changed quantitatively but no sugar changed dramatically in *p2-1* mutants which could be linked to its function. In order to determine its function, it needs further detailed analysis like permethylation linkage analysis and immuno-labelling assays that will provide information which particular polysaccharide is affected because of mutation in *p2-1* plants.

Because of the unavailability of the cDNAs for P gene we have not yet carry out its heterologous expression. Heterologous expression would be very useful for the functional characterization of the P gene, as well as the preliminary study of Arabidopsis cell wall characterization for P gene that we started in the lab.

2.4 Cloning of DUF 266 cDNA for heterologous expression in *Pichia pastoris*

The results of the mutant screening and biochemical analysis provided some clues about the changes in plant phenotype and cell wall composition. We decided to confirm these results by another functional genomics approach, i.e. the heterologous expression of candidate genes. Gateway Cloning Technology is a powerful methodology that greatly facilitates protein expression, cloning of PCR products and analysis of gene function with site-specific recombination. This technology provides a versatile system for transferring DNA segments between vectors. Once in the system, DNA segments can be transferred from an Entry Clone into numerous vectors (e.g., for protein expression). For the expression of our candidate genes it was an ideal system as it gives the possibility to use different expression systems.

In order to clone candidate genes, cDNAs available at that time were ordered from SALK. We ordered cDNA for 15 genes belonging to DUF 266 family. They were amplified with a 2 step PCR reaction as shown in figure 2.14. T7 N-terminal tag was added to facilitate the protein identification through western blot and purification of protein. The genes of interest were flanked by attB1 and attB2 gateway border sites which recombine with donor vector attP1 and attP2 sites to create any entry vector. These sites were added through PCR amplification of a gene (see materials and methods 6.2.1.1.1).

In order to find out the role of the proteins in the cell wall composition/biosynthesis and in the growth and development of the plant, we decided to clone the genes for which homozygous mutants were found and cell wall composition analysis was performed. Here in the figure 8, amplification has been shown for genes like A, C, I, J, N and W by two step PCR reaction (See materials and methods 6.2.1.1.1). Figure 2.14A represents the 1st PCR reaction while figure 2.14B represents the 2nd PCR reaction.

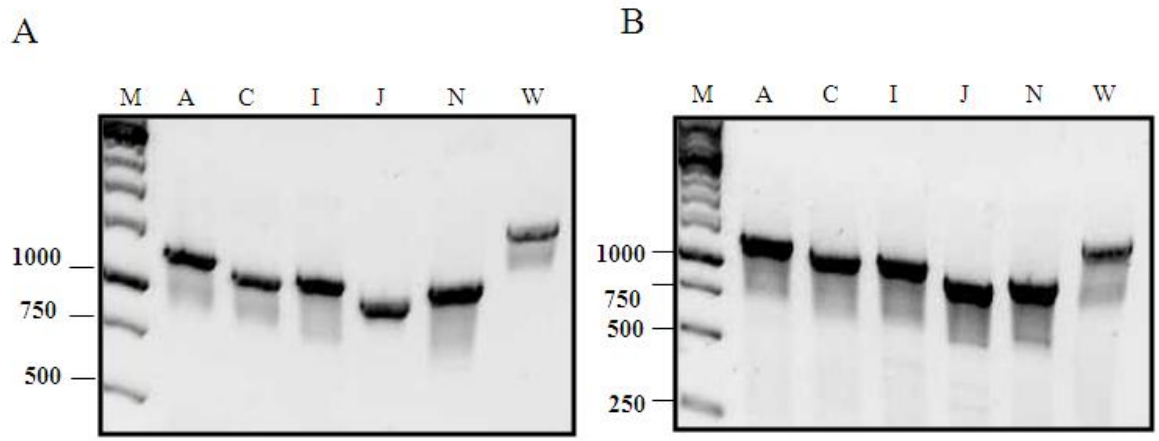


Figure 2.14: A) Agarose gel (0.8 %) showing the 1st PCR reaction for amplification of cDNA of genes A, C, I, J, N and W. B) Agarose gel (0.8 %) showing the 2nd PCR reaction for genes A, C, I, J, N and W

These PCR products were cloned into pDONR207 to create entry vectors through BP cloning reaction. Correct sequence of the reading frame was confirmed through sequencing. In an LR reaction this entry vector pENTR207 is integrated into destination vector pPICZ to make an expression clone. After LR reaction the plasmids were also sent for sequencing to confirm the correct sequence of gene of interests.

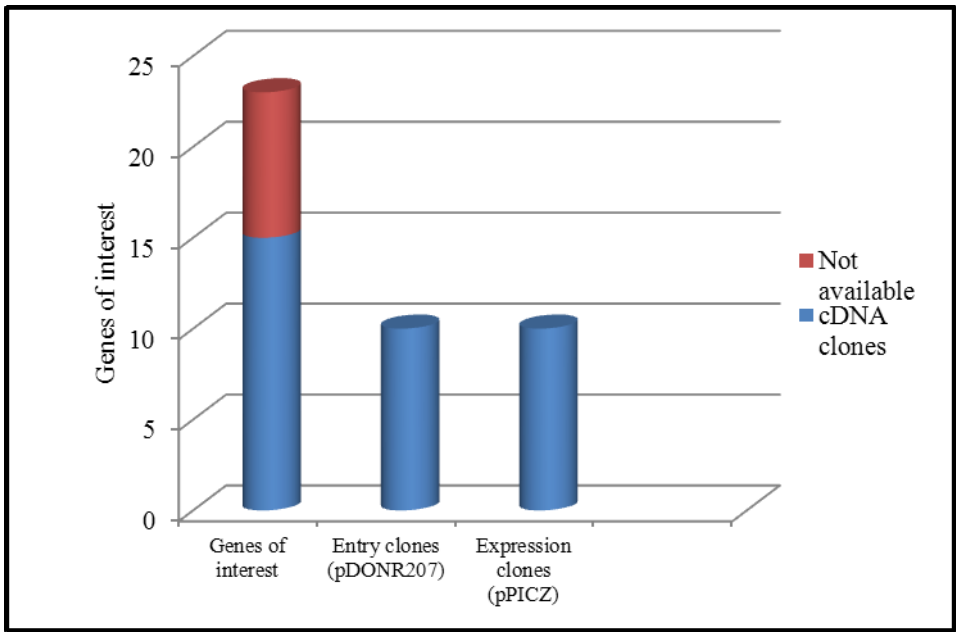


Figure 2.15: The histogram showing the number of genes whose cDNAs were cloned into entry vector pDONR207 and pPICZ expression vector

Out of total 23 genes, for 14 genes cDNAs were available at the time of ordering. I cloned 10 genes out of 14 into entry vector and expression vector through gateway cloning and sequenced (Figure 2.15). Functional characterization of all the cloned genes would have been time consuming and laborious work, so I focused only on the expression of two genes: gene E (having no fucosyltransferase signature, used as a control) which belongs to DUF266 family and gene At5g28910 (W) which has fucosyltransferase signature and will be discussed in next chapter 3.

2.5 Conclusion

The work presented in this chapter resulted in the identification of 16 homozygous mutants among the 35 allelic mutant lines for 23 genes belonging to DUFF266 family. It is a valuable work for the forthcoming researchers and students in the lab as it will save their time for antibiotic selection and mutant characterization through PCR based screening strategy.

Along with the above gene P, during my PhD I focused more on characterization and functional analysis of W (NGT1) gene because of following reasons.

1-As Hansen et al. (2009) have shown that this gene is unique in Arabidopsis genome and is distantly related to the known fucosyltransferases present in different GT families (GT11, GT23, GT37, GT65 and GT68) it could possibly be involved in cell wall biosynthesis particularly in pectin RG-II biosynthesis.

2-Allelic mutant of NGT1 gene showed developmental defects during homozygous mutant characterization (discussed in the next chapter). Preliminary data showed that mutant plants have smaller leaf area as compared to wild type plants which will be discussed in detail in chapter 3.

Chapter 3

The role of NGT1 in *Arabidopsis thaliana* cell wall biosynthesis

3 Role of NGT1 in *A.thaliana* cell wall biosynthesis

3.1 Introduction

A previous study developed in the lab uses bio-informatics to screen the Arabidopsis genome for the presence of new genes harbouring GT signatures; this study ultimately led to the identification of 24 genes without predicted functions as being putative glycosyltransferases (Hansen *et al.*, 2009). The objective of my research work was to functionally characterize one of these 24 newly identified genes, in order to validate experimentally whether or not these genes encode GTs involved in plant cell wall biosynthesis. Interestingly, among these 24 candidate genes, 23 genes belong to the DUF266 gene family and one was unique. The first part of my work (chapter 2) was mostly devoted to the molecular characterization of Arabidopsis mutants T-DNA lines from the DUF266 gene family, in order to perform a phenotypic analysis of these mutants as well as a biochemical characterization of their cell wall content, seeking for specific phenotypes related to the alteration of DUF266 genes. Accordingly, I undertook the characterization of 35 T-DNA insertion lines, phenotyped 15 mutants and analysed cell wall content for 10 mutants. Unfortunately, no specific trait could reliably be detected that would link genetic alteration in gene family DUF 266 and plant development or cell wall composition. In the present chapter, I will present data obtained for the gene At5g28910 that was not related to the DUF266 gene family. This gene was actually unique and highlighted by our bioinformatics approach as being a putative fucosyltransferase. Over the course of that work some confidence was gained that this gene (At5g28910) would be of particular interest, especially because the T-DNA lines altered for this locus appears to carry a slower growth development, but also because gene sequence showed characteristic features with known fucosyltransferases, thus providing a testable hypothesis to develop GT activity tests. This genetic locus At5g28910 was then named NGT1 for Novel GlycosylTransferase 1. Two Arabidopsis mutant T-DNA lines altered for At5g28910 locus were characterized and named *ngt1-1* and *ngt1-2*. In order to determine the function of NGT1, we used the functional genomics approaches described in chapter 2, i.e., heterologous expression and T-DNA knock out mutants characterization of candidate gene (NGT1). Heterologous expression of candidate gene shall help to assign biochemical function of NGT1 by analysing its role through activity test. While the T-DNA knock-out mutant lines will offer the opportunity to unravel the role of candidate gene NGT1 in cell wall biosynthesis through phenotypic characterization and chemical analysis of mutant cell wall.

3.2 Protein sequence analysis

There are two gene models/annotations for gene At5G28910 (NGT1) in TAIR database which are represented as At5G28910.1 and At5G28910.2. According to these models there are two possible initiation sites for the gene translation, one giving a protein of 408 amino-acids, and the other gives a 535 amino-acid protein. We characterized the gene model At5G28910.2 which will result in the synthesis of longer protein of 535 amino-acids and its nucleotide and translated sequence of the coding region is shown in figure 3.1.

Figure 3.1**.** Nucleotide sequence and translated sequence of the coding region of At5g28910.2. Predicted TMD is marked in turquoise, N-glycosylation sites with green letters underlined. The β-strands and α-helices, marked in yellow and magenta, respectively, were predicted by the consensus secondary structure prediction tool at the NPSA server (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_seccons.html).

NGT1 protein is predicted to have one transmembrane helix at its N-terminus (http://www.cbs.dtu.dk/services/TMHMM/) (Figure 3.2). Thus, NGT1 displays the typical type II membrane protein topology that is observed for most of the Golgi-resident GTs, consisting in a short cytoplasmic N-terminal region, a trans-membrane domain spanning the amino acids 26-47, a stem region and a large globular catalytic C-terminal domain facing the luminal side of the Golgi.

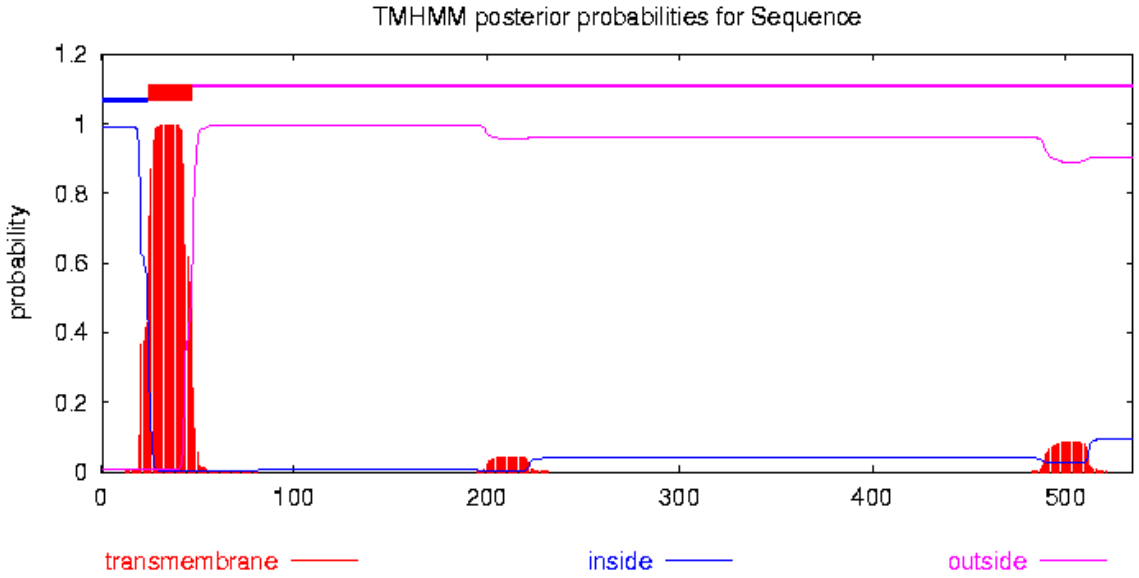


Figure 3.2: Prediction of trans-membrane domain of NGT1 protein using TMHMM. The probability of regions being transmembrane (red), inside (i.e. cytoplasmic, blue) or outside (i.e. Golgi lumen, magenta) are plotted as a function of amino acid residue number.

Seven putative *N*-glycosylation sites were predicted by the use of N-glycosylation prediction server (at http://www.cbs.dtu.dk/) at position 68, 123, 152, 188, 247, 284 and 313 of amino acids sequence (Figure 3.3).

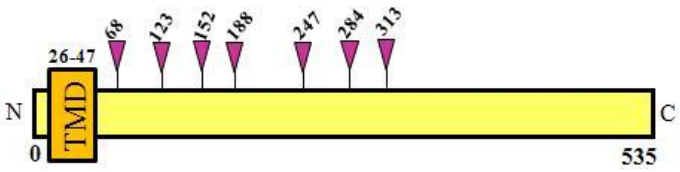


Figure 3.3: Predicted structure of NGT1 protein. NGT1contains one transmembrane domain represented by square at its N-terminal and has seven putative N-glycosylation sites shown by triangles with the positions of amino acids

3.3 Characterization of T-DNA mutants *ngt1-1* and *ngt1-2*

For this study, we hypothesized that NGT1 is a glycosyltransferase involved in plant cell wall biosynthesis, and we analyzed cell wall from NGT1 deficient Arabidopsis mutants, expecting quantitative or qualitative variation in sugar content by comparison to the wild-type cell wall. More specifically, particular attention was payed to the fucose content in the cell wall of *ngt1-1* and *ngt1-2* mutant lines. Fucose is present over various polymers in Arabidopsis, such as N-glycans in glycoproteins, arabinogalactan proteins (AGPs) and cell wall polysaccharides like pectins and xyloglucan. A few Arabidopsis genes encoding fucosyltransferase activities have already been identified and characterized. These genes are involved in the fucosylation of XG, AGPs and protein N-glycosylation and they classify into GT37 and GT10 families in CAZy database (www.cazy.org). However, no fucosyltransferase gene has been identified to date for the fucosylation of RG-II in pectins,

In order to evaluate if NGT1 is involved in pectin biosynthesis, two allelic T-DNA knock out mutant lines were ordered from Nottingham Arabidopsis Stock Centre (NASC). These two mutant lines N585839 (SALK_085839) termed as *ngt1-1* has insertion in first exon (downstream +217 bp from the translation initiation codon) and N638819 (SALK_138819) termed as *ngt1-2* has insertion in the 5' UTR (upstream -23 bp from the translation initiation start codon) of the same gene NGT1 (Figure 3.4).

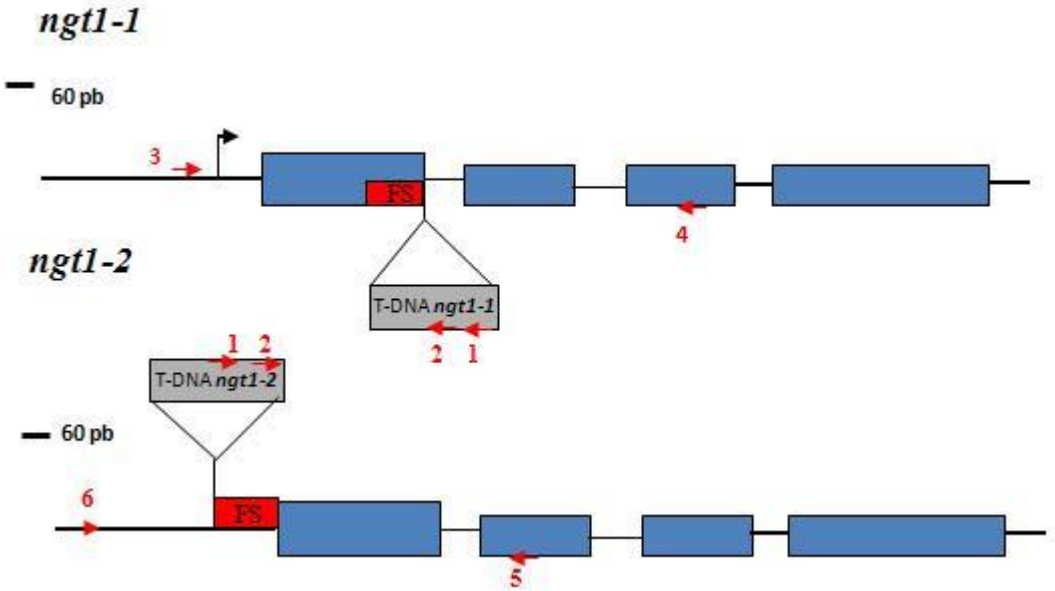


Figure 3.4: Schematic representation of the gene structure of NGT1 and the position of insertion of T-DNA for *ngt1-1* and *ngt1-2*. The binding site of primers used in PCR reaction is indicated by red arrows. Blue boxes represent exons of NGT1 gene, red boxes labelled "FS" correspond to the genomic flanking sequence of the T-DNA according to NASC seed stock center.

These two SALK lines *ngt1-1* and *ngt1-2* harbour an alteration of NGT1 that is caused by the integration of a T-DNA cassette containing the neomycin phosphotransferase II (NPT II) gene providing resistance to the antibiotic kanamycin. Arabidopsis mutants received from NASC were first amplified on soil without selection pressure and afterwards the progeny was checked for segregation of the NPT II gene, and genotyped by PCR. For each *ngt1-1* and *ngt1-2* Arabidopsis mutant lines selected, we could identify lines showing 100% resistant plants to kanamycin, indicating that the lines were probably homozygous for the T-DNA insertion and thus have the mutation in NGT1 locus (Figure 3.5). In order to verify that the selected homozygous lines for *ngt1-1* and *ngt1-2* were homozygous and altered at the NGT1 locus we performed a PCR genotyping of these two mutant lines. DNA extracted from the leaves of selected resistant plant lines was prepared and genotyped. Different sets of primers were used referenced as primer 1, 2, 3, 4, 5 and 6 in figure 3.6. Primers 1 and 2 are left border (LBa1 and LB1.3) T-DNA specific primers which will give two products of different sizes for T-DNA and genomic hybrid product while (3 + 4) and (5 + 6) are gene specific right and left primers (RPW1 and LPWI), (RPW3+ LPW3) for *ngt1-1* and *ngt1-2*, respectively.

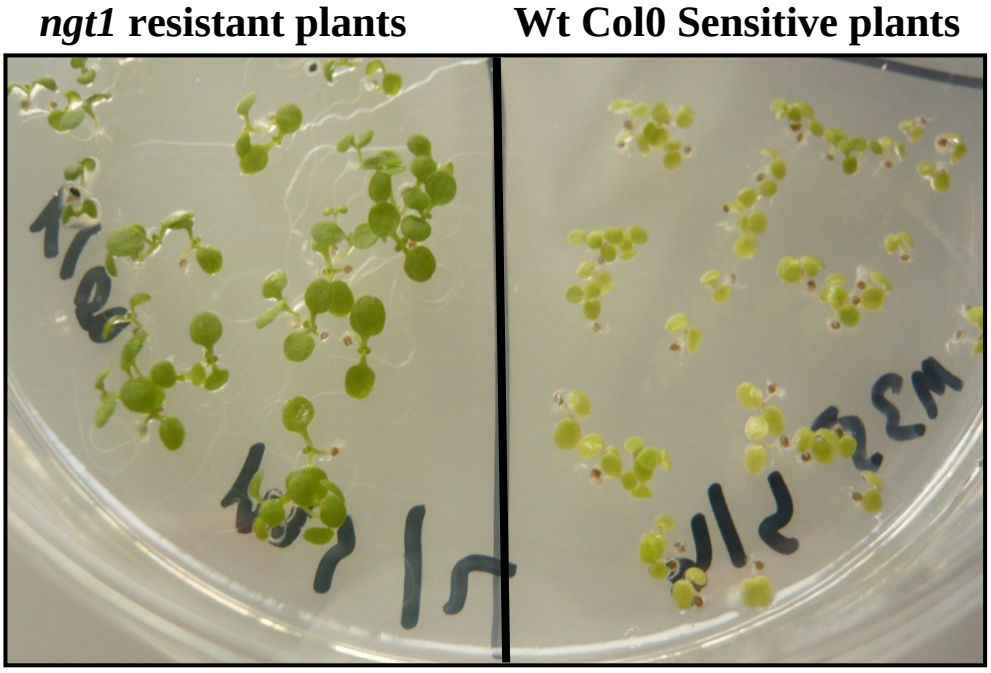


Figure 3.5: On the left, *ngt1-2* T-DNA mutant altered for At5g28910 (NGT1) gene is resistant to kanamycin whereas Wt Col0 plants are (on the right).

The region corresponding to T-DNA insertion of NGT1 gene was amplified using T-DNA specific left border primer 1 and 2 (LBa1 and LB1.3) with the gene specific primers 3 and 5 for *ngt1-1* and *ngt1-2*, respectively. Similarly the region corresponding to genomic DNA for NGT1 gene was obtained by using gene specific left border and right border primers (3+ 4) and (5+6) for *ngt1-1* and *ngt1-2*, respectively. In the figure 3.6, for example, *ngt1-1*, *ngt1-2* and Col0 represent different DNA stocks isolated from mutant and wild type plants that were checked by PCR for T-DNA insertion using three sets of primers: two sets amplifying a product in the presence of T-DNA at the expected locus, one set being specific of the WT version of the gene at this locus (Figure 3.6). Lanes 1 and 2 show a PCR product indicating insertion of a T-DNA at NGT1 locus in *ngt1-1* plants by the use of primers (1+3, 2+3), while the lack of product in lane 3 (primers 3+4) from *ngt1-1* DNA confirmed the Arabidopsis line *ngt1-1* is homozygous for T-DNA insertion at NGT1 locus. Similar results were obtained for *ngt1-2* (lanes 4 to 6) indicating that this allelic line of *ngt1-2* was also homozygous. In contrast, in case of Col0 genomic DNA the lack of product with primers (1+3, 2+3; specific of the T-DNA) in lanes 8 and 9 and the presence of a PCR product in lanes 10 and 11 confirmed that the DNA prepared from Col0 plants was wild type for NGT1 locus. All this PCR reaction products were sequenced to unambiguously confirm their identity, but also to precisely determine deletion of DNA sequences (from genomic or T-DNA) that occurs in consequence of the T-DNA insertion into the Arabidopsis genome.

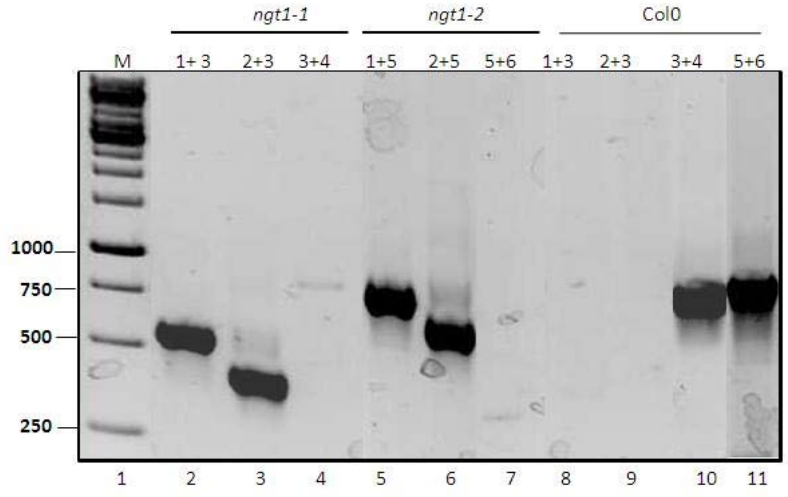


Figure 3.6: Agarose gel (0.8 %) showing the homozygous profiles observed for DNA isolated from the *ngt1-1 and ngt1-2* lines. Lane 1 indicates 1 kb marker. Lanes 2 and 3 represent PCR products for T-DNA insertion and lane 4 indicates the absence of genomic product in *ngt1-1* mutant. Similarly lanes 5 and 6 represent PCR products for T-DNA insertion and lane 7 indicates the absence of genomic product in *ngt1-2* mutant. Col0 DNA was used as a control, lanes 8 and 9 show the absence of T-DNA.

The right border of the insertion was studied using identical strategy to the left border but using right border specific primers. Despite numerous efforts we were unsuccessful to obtain a hybrid PCR product bearing sequence from the right border and the genomic near the insertion. The most likely hypothesis would be a deletion of the T-DNA right border sequence occurring during insertion into the genome of the mutants. We overcome that difficulty by carrying out an extensive amplification of the T-DNA including the genomic sequence near the left border. This PCR reaction resulted in the amplification of two products over 2kb which confirmed that at least 2 kb of T-DNA are inserted in our gene of interest for both *ngt1-1* and *ngt1-2* mutant lines. For mutant line *ngt1-1*, the size of the PCR product was 1490 bp (Figure 3.7, lane 2) with the set of primers (RPW1+OL99) while the second set of primers (RPW1+OL100) generated the product of 2049 bp (lane 3). Similarly two products of T-DNA were obtained by PCR for line *ngt1-2* of the size of 1646 bp (OL99+RPW3, lane 4) and 2205 bp (OL100+RPW3, lane 5). PCR amplicon was sequenced and showed to be a hybrid product of our gene of interest NGT1 and left border of the T-DNA by sequencing. In addition, we carried out the phenotypic studies on successive progenies which confirmed that the observed phenotype was genetically linked to the T-DNA insertion across generation. The phenotypic studies are described in the next paragraphs.

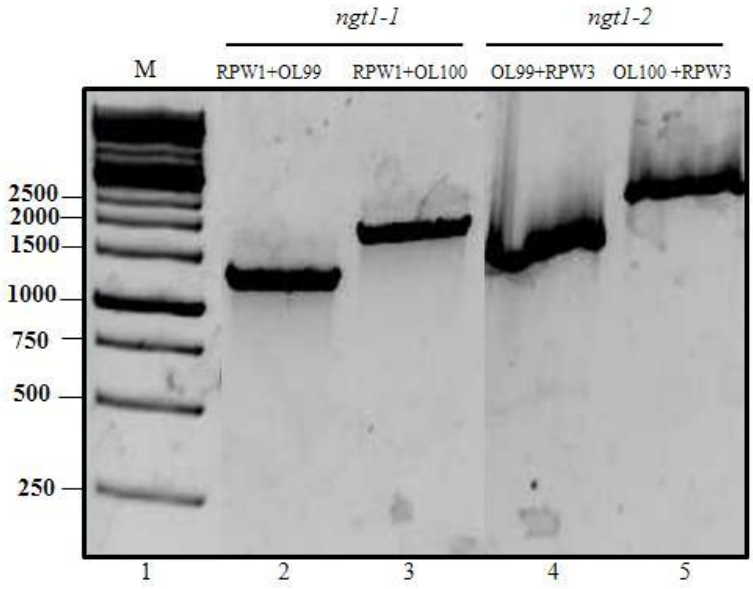


Figure 3.7: Agarose gel (0.8%) showing the left border and genomic sequence amplificon of *ngt1-1* and *ngt1-2* lines by PCR. Lane 1 shows the molecular markers. Lane 2 and 3 shows the PCR amplified product for *ngt1-1* through the use of two set of primers (RPW1+OL99) and (RPW1+OL100) respectively while lane 4 and 5 represent the left border PCR product of line ngt1-2 by the use of two set of primers (OL99+RPW3) and (OL100+RPW3), respectively.

- **Characterization of *NGT1* mutants by RT-PCR**

The T-DNA insertion sites were characterized through PCR using left border and genomic primers for both *ngt1-1* and *ngt1-2* mutant lines (cf materials and methods 6.5.6). This analysis confirmed disruption of *NGT1* in the first exon of *ngt1-1* and insertion in the 5'UTR in *ngt1-2* mutant line. Both mutant lines were then further analyzed by RT-PCR seeking the presence of NGT1 transcript (or hybrid transcript *T-DNA:NGT1*), thus in order to find out whether *ngt1-1*and *ngt1-2* mutants were null mutants (NGT1 not expressed at mRNA level). For this purpose total RNAs were extracted from 2 weeks old wild type Col0, *ngt1-1* and *ngt1-2* mutant seedlings. cDNAs were prepared and analyzed for the presence of NGT1 transcripts using PCR. Five different set of primers, namely "GS1", "GS2", "UBQ", "T-DNA *ngt1-1"*, "T-DNA *ngt1-2"* were used first for Col0 cDNA. (Ubiquitin specific primers were used as a control for determining the RT-PCR amplification efficiency among the different samples).The first two set of primers GS1 (Gene Specific primer 1) and GS2 (Gene Specific primer 2) are specific primers for NGT1 cDNA and expected to give a product of 210 bp and 389 bp, respectively. Accordingly, these two PCR products, indicative of the presence of *NGT1* cDNA, were positively amplified using Col0 cDNA library but not using cDNA libraires prepared from *ngt1-1* and *ngt1-2* mutants (figure 3.8, lanes 2 and 6). The lack of amplification for GS1 and GS2 product in both mutant lines indicate that no full length NGT1 transcript is present in any mutant lines. On the contrary, two set of primers specific for the presence of the T-DNA insertion at *ngt1-1* or *ngt1-2* loci were used in order to seek the presence of T-DNA: NGT1 hybrid product in both mutant lines. As a control Col0 cDNA library was used as matrix for such reaction and as expected did not provide any amplification as no T-DNA is inserted at *NGT1* locus in WT Col0 (figure 3.8 lanes 4 and 5). However, such T-DNA: NGT1 hybrid product would be possibly transcribed in *ngt1-1* or *ngt1-2* mutants.

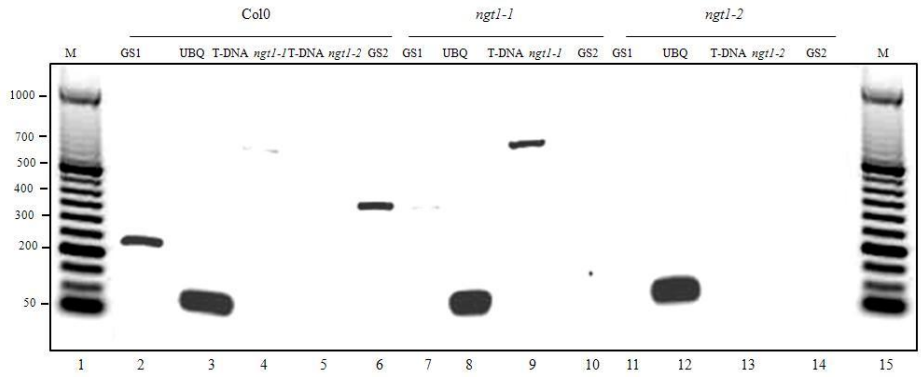


Figure 3.8: RT-PCR analysis of transcripts from the wild type Col0 and homozygous mutant lines *ngt1-1* and *ngt1-2*. NGT1 transcript is detected in Col0 cDNA library whereas no such transcript is detected from homozygous mutant lines *ngt1-1* and *ngt1-2*.

It is important to note that a hybrid product was characterized from the *ngt1-1* cDNA library. Analysis of the *T-DNA:NGT1* hybrid product (780 pb; lane 9 fig 3.8) obtained for *ngt1-1* indicates the presence of the first 219 bp from NGT1 sequence which would represent the first 73 amino acids of NGT1 protein sequence if translated. Because the first 73 amino acids at the N-ter of the sequence do not comprise the expected catalytic site for NGT1 protein, we conclude that functional NGT1 protein would be absent in *ngt1-1*, thus being a null mutant. Finally, in case of *ngt1-2* mutant, neither gene specific nor *T-DNA:NGT1* product were amplified using PCR indicating such mutant is null for *NGT1* transcript (figure 3.8, lanes 13 and 14).

3.4 Phenotypic characterization of mutant lines *ngt1-1* and *ngt1-2*

As mentioned in the introduction, normal plant development is, among other parameters, dependent of plant cell wall biosynthesis. Indeed, in order to grow properly, a plant like Arabidopsis will need to synthesize polysaccharides necessary to maintain cell wall integrity during cell elongation and expansion. Research program trying to decipher plant cell wall biogenesis actually took advantage of this tight correlation between polysaccharide biosyntheses and plant development, while developing a strategy where mutants having an altered development (i.e. short hypocotyl in dark-grown culture condition) were suspected to be altered for cellulose biosynthesis. Accordingly, as we expected NGT1 locus to be involved in cell wall biosynthesis, we observed the effect (if any) of mutation at NGT1 locus on Arabidopsis growth. Seeds for allelic *ngt1-1*, *ngt1-2* mutants and wild type Col0 were grown on soil and observed for phenotypic changes during 8 weeks. The first observation was the lack of strong phenotype altering organ development as sometimes observed for some pectin altered mutants such as *quasimodo1* and *quasimodo2* (Bouton *et al.*, 2002, Mouille *et al.*, 2007). However, during the course of their growth, we have found that *ngt1-1* and *ngt1-2* mutants were morphologically distinguishable from wild type Col0 plants as they have “narrow” leaves and a pale green color which is a classical sign of chlorosis (Fig 3.9). Although this observation of pale color appears consistent over plant cylce, and was reproducible from generation to generation of *ngt1-1* and *ngt1-2* lines, it remains difficult to quantify precisely. Thus, we focused on the developmental phenotype (narrow leaves) and sought to develop a method in order to measure the plant leaf area in a reproducible manner with the aim of shifting from a qualitative observation to a quantified trait. Leaf area was then

measured by using publically available software ImageJ (cf material and methods). Briefly, we measured the leaf area by taking a picture of individual plant for each *ngt1-1*, *ngt1-2* and Col0, each having a reference scale bar (Figure 3.9). Area of the leaf was calculated with the help of reference scale, and reported as histograms.

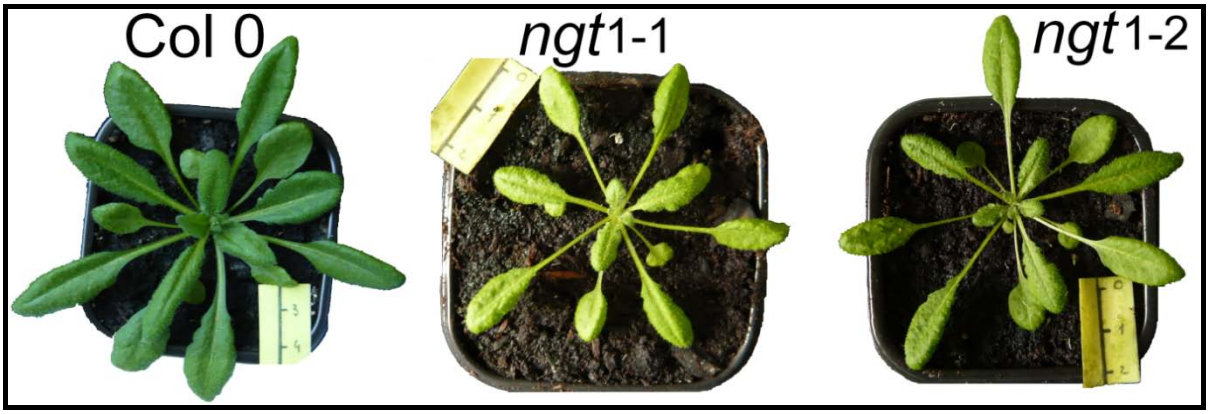


Figure 3.9 : Arabidopsis thaliana 35 days old wild type Col0 and *ngt1-1, ngt1-2* mutants. The leaves of mutant *ngt1-1, ngt1-2* are narrower than wild type plants and present a pale green color.

The quantitative analysis of the leaf surface could only be made between 14th and 42nd day of development. The leaves were too small to be measured before 14th day and too crowded to be measured correctly after 42nd day of development. This study showed the developmental defect of plants having an alteration in gene NGT1 at the beginning of the third week of growth. For example, on the 21st day of development the surface area of leaves (0.32 cm^2) of wild type plants (Col0) is significantly different from that of mutant plants *ngt1-1* (0.20 cm^2) and *ngt1-2* (0.20 cm^2) (Figure 3.10).

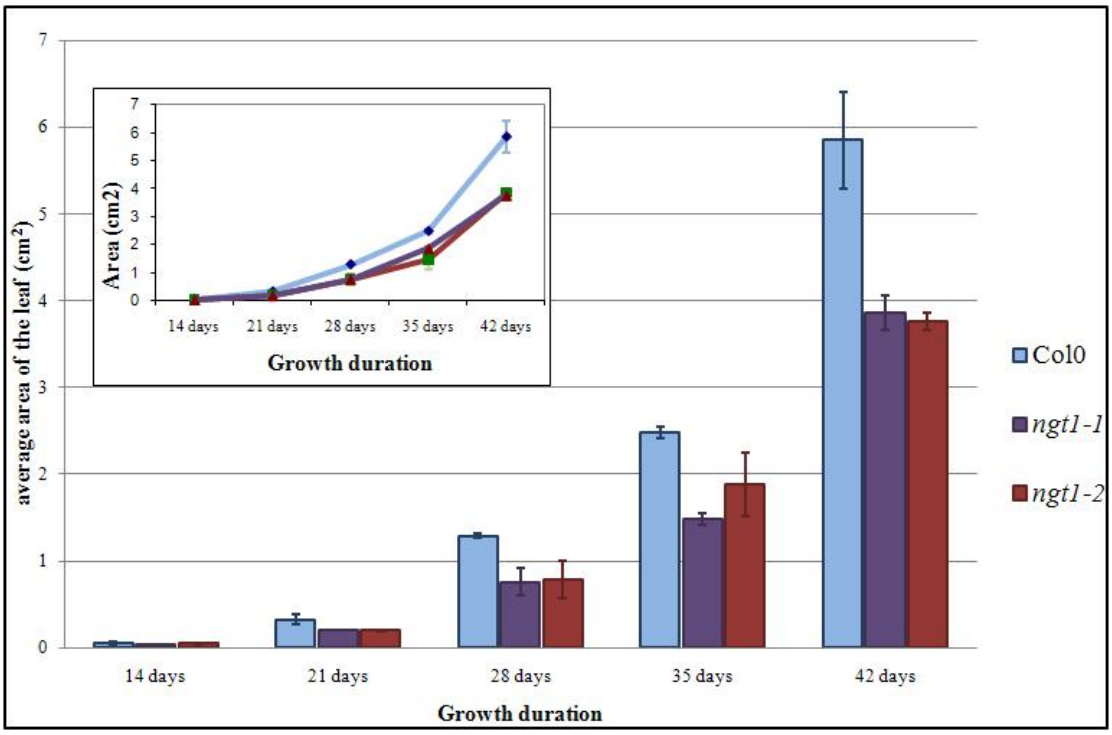


Figure 3.10: Analysis of leaf area of wild type Col0 and mutant lines *ngt1-1* and *ngt1-2*. Leaf area was measured quantitatively. Each value is an average, determined from the measurement of leaf area of six plants in two independent experiments performed under the same conditions. The error bars represent the standard deviation calculated for all values of two experiments. At 42 day of plant growth, the leaves of the mutant lines *ngt1-1* and *ngt1-2* are 34% and 36% smaller than leaves of wild Col0 plants.

The developmental difference observed among the wild type and mutant plants are more significant on 35th and 42nd days of growth. As on 42nd day, the leaves of *ngt1-1* and *ngt1-2* are 34 % and 36 % smaller as compared to that of wild type plant leaves. Although the quantitative analysis shows that leaf area of *ngt1-1* was different from that of *ngt1-2* but this difference was not relevant. On contrary, the leaf area of mutants was significantly different from that of wild type plants from the 21st day of development.

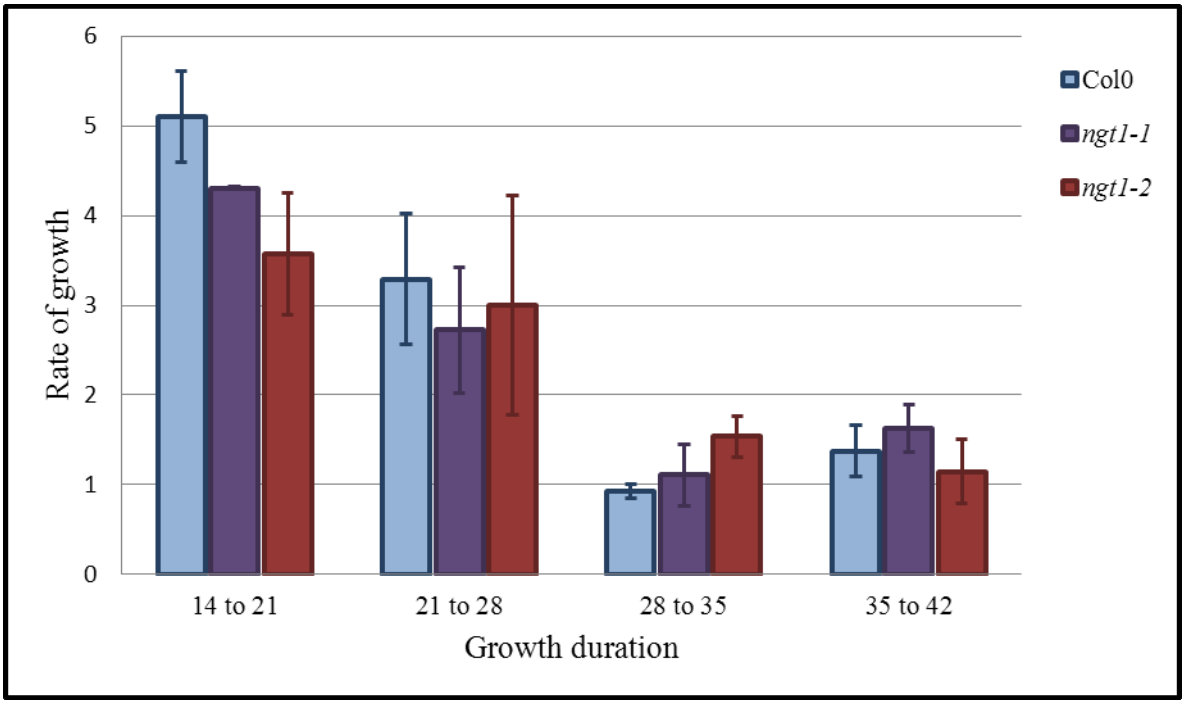


Figure 3.11: Comparative analysis of leaf growth rate of wild type (Col0) and mutant lines *ngt1-1* and *ngt1-2* followed during 4 weeks of growth. Rate of growth of *ngt1-1* and *ngt1-2* mutants is weaker than Col0 plants only before 4th week of growth

The quantification of the leaf surface area made it possible to measure the growth rate [(Leaf area of the week W+1) - (Leaf area of the week W)] / (Leaf area of the week W)] of different plants during the phenotypic studies. The growth rate profiles could only be calculated between 14th and 21st day, 21st and 28th day etc. Significant growth rate difference was observed among the wild type and mutant plants only between 14th and 21st day of development (Figure 3.11). From 21st to 35th day of development, the plants seemed to have the tendency of similar growth. This observation was confirmed from the growth rate of 35th and 42nd day. These results indicate that the mutation in NGT1 alters the growth rate only during early developmental stage and has no or little effect on the growth rate during late development.

This phenotypic difference showed that NGT1 gene plays a role in controlling leaf development and particularly slows down normal growth. The next step we envisioned was then to evaluate whether or not the polysaccharide content of the cell wall would be modified in mutants. Accordingly, we performed biochemical analysis of the cell wall from *ngt1-1* and *ngt1-2*, in order to account for its composition.

3.5 Quantification of neutral monosaccharide of cell wall from *ngt1-1* and *ngt1-2* using gas chromatography

As the plant cell wall consists of roughly 90% carbohydrates, one would expect that the alteration of a glycosyltransferase activity involved in cell wall biosynthesis would lead to a deficiency in carbohydrate that could be determined in a quantitative manner. It is noteworthy that this speculation has led researchers to screen Arabidopsis plants for changes in the neutral monosaccharide composition of mutant cell walls, and successfully identify Arabidopsis mutants altered for cell wall biosynthetic enzymes (Reiter *et al.,* 1997). Reiter and his co-workers used gas chromatography to analyze the neutral sugar content of 5,200 plants, successfully identifying eleven mutants (*mur1 to mur11)* among which 5 have been characterized (*mur1*, *mur 4, mur2*, *mur3* and *mur10;* (Burget & Reiter 1999, Pauly *et al.,* 2001, Vanzin *et al.,* 2002b, Madson *et al.,* 2003, Tamura *et al.,* 2005, Bosca *et al.,* 2006). Based on this observation we decided to perform the analysis of *ngt1-1* and *ngt1-2* cell walls by gas chromatography, quantifying sugar contents after hydrolysis of the polysaccharides to monosaccharides and their reduction to the corresponding alditol acetates (cf materiel and methods 6.6.2.1). Arabidopsis mutants *ngt1-1* and *ngt1-2* (along with Col0 WT for comparison) were grown during 6 days in dark-grown condition not only to standardize culture conditions but also to avoid starch accumulation during photosynthesis and consequently to ensure that the quantified glucose would be mostly from cell wall content. After 6 days, Arabidopsis hypocotyls, cotyledons and roots were heated several times in ethanol (70%) and seedlings were ground using a glass homogenizer. The pellet obtained after low speed centrifugation corresponded to all macromolecules insoluble in 70% ethanol and was referenced as AIR (Alcohol Insoluble Residue). Briefly AIR was analyzed in order to quantify neutral monosaccharides, after hydrolysis of polysaccharides with strong acid (TFA) and conversion of resultant monosaccharides to alditol acetates by reduction with sodium borohydride. Finally alditols were further derivatized followed by acetylation of the free hydroxyls forming alditols acetates molecules. As plant cell wall is particularly rich in seven monosaccharides, namely L-rhamnose (Rha), L-fucose (Fuc), L-arabinose (Ara), D-galactose (Gal), D-xylose (Xyl), D-mannose (Man), and D-glucose (Glc), the resultant alditol acetates for those seven neutral monosaccharides were quantified by gas chromatography.

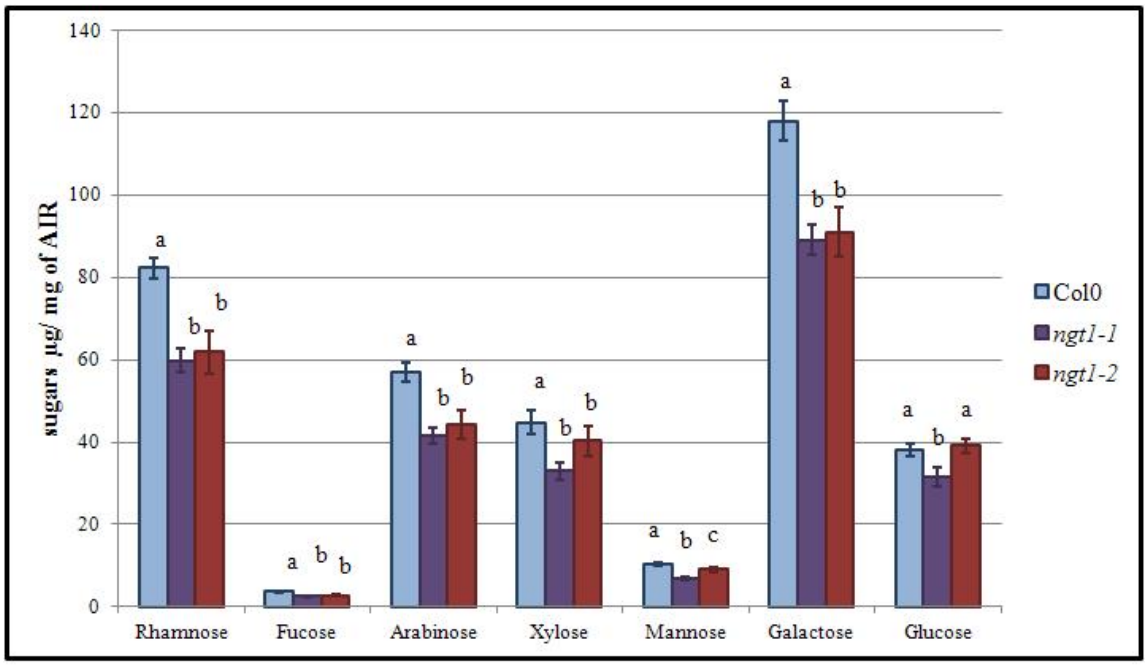


Figure 3.12: Neutral monosaccharide composition analysis of AIR isolated from *ngt1-1*, *ngt1-2* and wild type Col0 through GC-MS. Rhamnose, arabinose and galactose contents are significantly decrease in *ngt1-1*, *ngt1-2* as compared to wild type plant cell wall. The statistical differences were evaluated by ANOVA test followed by LSD test. The comparison between a/b, b/c and a/c indicate the significant difference while a/a and b/b indicate that non-significant difference among cell wall neutral sugars in wild type Col0 and *ngt1-1*, *ngt1-2* mutant plants. The mean difference is significant at the 0.05 value. Bar indicates standard errors where n = 10

These values are mean of ten independent series of samples. Results are expressed as mean of quantity of each sugar $\pm$ standard error between wild type and mutant plants. Statistical analysis of data was performed by using a Shapiro's tests for normality of data and then ANOVA. The mean comparison test was a Least Significant Difference test (LSD) carried out with 95% confidence interval. Changes in neutral sugars were detected for almost all monosaccharides present in the cell wall while the amount of arabinose was significantly reduced in both allelic mutant *ngt1-1* and *ngt1-2* (68% and 72% of wild type plants respectively) (Figure 3.12). This decrease in arabinose contents has already been reported for Arabidopsis mutant lines of ARAD1 which is a putative arabinosyltransferase involved in pectin biosynthesis (Harholt *et al.,* 2006). Similarly the amounts of galactose and rhamnose were also decreased significantly in mutant cell wall as compared to WT plants. These results have shown that mutation in NGT1 affected the cell wall polysaccharides biosynthesis: more specifically the pectin representative sugars like arabinose, rhamnose and galactose. These

defects in cell wall composition showed more convincingly that our hypothesis about the implication of NGT1 in plant cell wall biosynthesis is correct.

Arabinose and galactose sugars are mainly present in arabinogalactan proteins, arabinan and rhamnoglacturonan I while rhamnose is mainly present in pectic components, rhamnoglacturonan I and rhamnoglacturonan II. This quantification of neutral monosaccharide provided us important information about the biochemical changes in mutant cell wall but could hardly be informative about which particular polysaccharide is modified, as monosaccharides can be constitutive of various polysaccharides. In order to get more information about cell wall alteration in the mutant lines, we performed glycosidic linkage analysis after permethylation of cell wall samples.

3.6 Glycosyl linkage analysis of *ngt1-1* and *ngt1-2* mutant cell walls

As quantitative variations of the monosaccharide contents were observed in the two allelic *ngt1-1* and *ngt1-2* by comparison to the WT cell wall composition, we sought to get a comprehensive overview of the polysaccharides content in the cell wall using linkage analysis. Indeed, glycosyl linkage analysis performed onto cell wall samples permits to resolve all neutral monosaccharide from a sample with respect to the position these monosaccharides had in the polysaccharide. Firstly, the polysaccharides are derivatized to form acid-stable methyl ethers by converting free OH groups to methyl ethers, and afterwards hydrolysed with trifluoroacetic acid (TFA). Then samples are peracetylated, and analyzed by gas chromatography coupled to mass spectrometer detector (GC-MS). For each peak resolved on the chromatogram, a mass profile is generated from the fragmentation of the monosaccharide derivative forming the peak. The mass spectra generated from the molecules fragmentation, is characteristic of the type of sugar (pentose, hexose, deoxyhexose...) and the way the sugar were linked in the polysaccharide. Analysis of cell wall samples using these techniques enables to detect differences between cell wall samples that would be missed by a single composition of the monosaccharides.

First of all composition of wild type cell wall was determined through permethylation linkage analysis and 29 molecules corresponding to major monosaccharide sugars present in

Arabidopsis cell wall were identified. A typical chromatogram obtained for the Col0 cell wall by GC-MS is shown in figure 3.13.

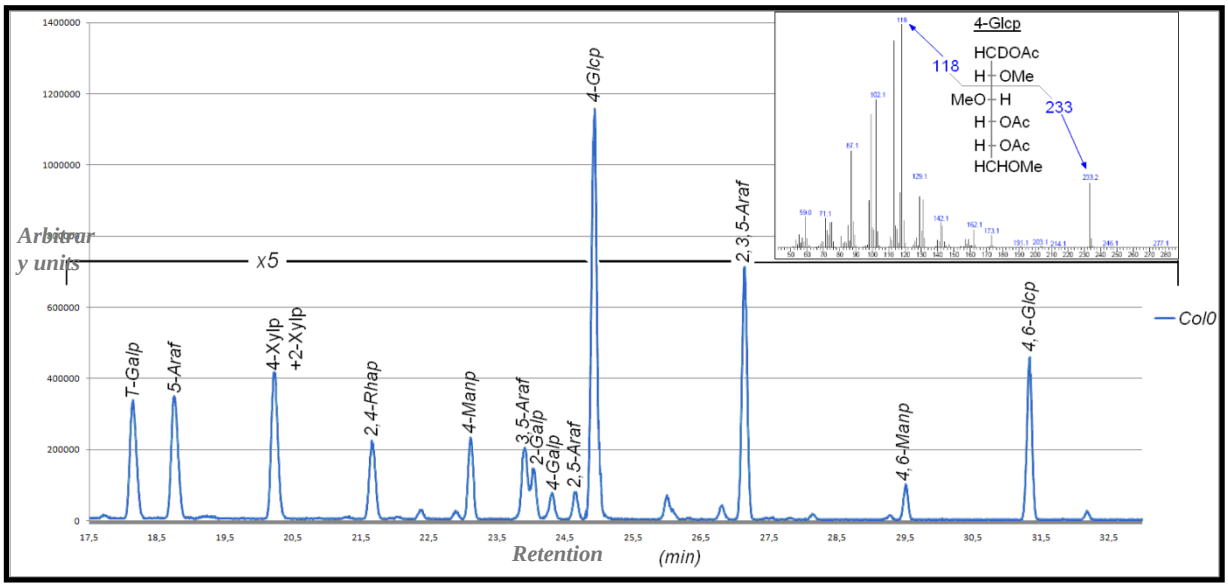


Figure 3.13: Example of glycosidiclinkage analysis of cell wall from WT Col0 with GC-MS. Chromatogram is shown with an inner box representing the mass spectra of daughter ions derived from 4-Glcp peak, where m/z at 118 and 233 mass units are characteristic fragments for a 4-linked hexose molecule.

One milligram of cell wall from 6-days old dark grown seedlings was treated to determine structural changes of the polymers in mutant *ngt1-1*, *ngt1-2* and wild type Col0. We observed that 5-Arabino*furanose* (5-Ara*f*) and 3,5-Arabino*furanose* (3,5-Ara*f*) were decreased in both allelic mutant lines *ngt1-1*, *ngt1-2* as compared to wild type. The 3,5-Ara*f* were reduced in both lines *ngt1-1*, *ngt1-2* (51% and 25%, respectively) while 5-Ara*f* was reduced 50% in both mutant lines as compared to wild type (Figure 3.14). Linkage analysis of cell wall showed the reproducible differences between the two mutants and the wild type cell wall. Interestingly we observed the increase in terminal galacto*pyranose* (T-Gal*p*) and 2,5-Arabino*furanose* in both mutant lines as compared to wild type cell wall (Figure 3.14 and table 3.5). We have also calculated the mol % of each structural molecule (Table 3.5). From these results we have concluded that mutant lines harbour alteration in their arabinan and galactan contents and thus that NGT1 locus is related to normal cell wall biosynthesis either directly or indirectly. The decrease in the amount of the molecule of 4,6-Glc*p* seems an indirect effect of the mutation in NGT1 gene. Indeed, the molecule 4,6-Glc*p* is a characteristic part of xyloglucan polymer, and the genes involved in its biosynthesis in plants have been identified (Lerouxel *et al.*, 2006, Cavalier *et al.*, 2008). This would indicate that the alteration of the NGT1 gene indirectly influence the content in other polymers of the wall through

indirect (or compensatory) mechanisms, as for many cell wall mutants (Scheible & Pauly 2004).

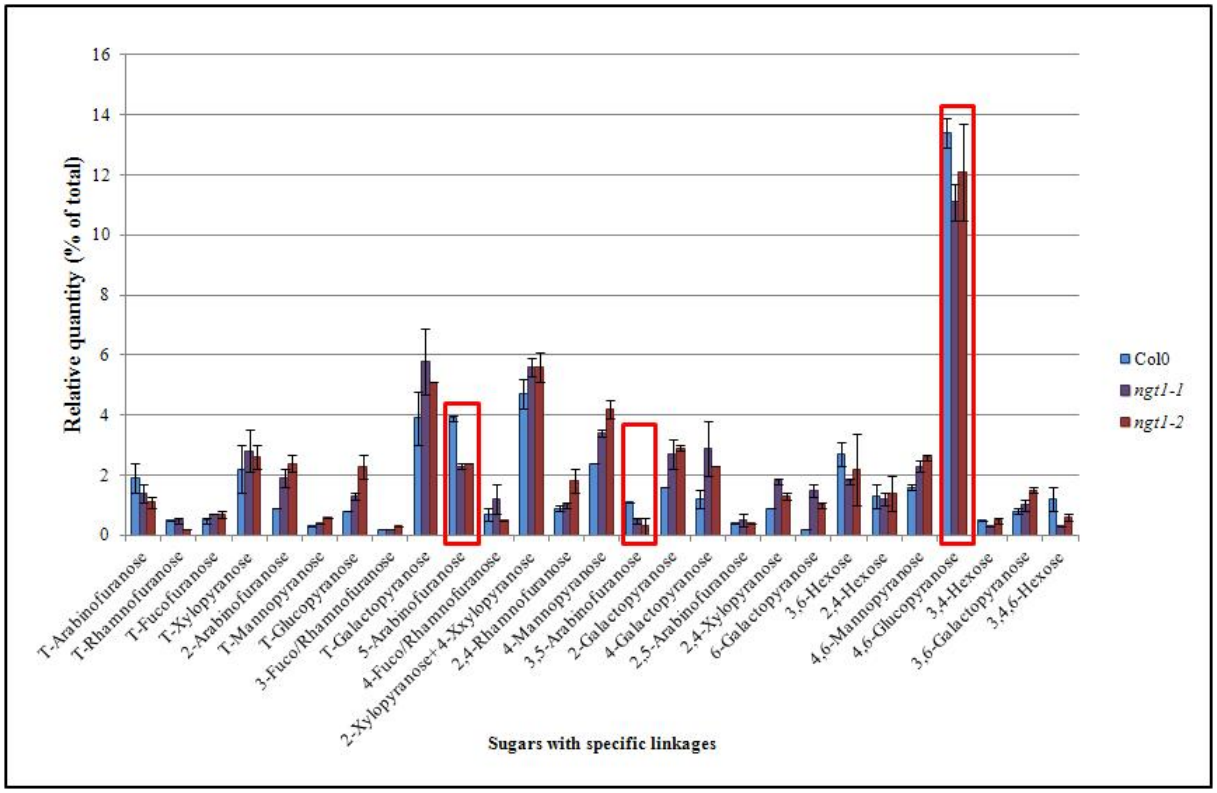


Figure 3.14: Permethylation linkage analysis of AIR of wild type Col0, *ngt1-1* and *ngt1-2* mutants through GC-MS. Three linkages which are rectangled in red colour are significantly less in both allelic mutants as compared to wild type. Histogram is made by using the data from table 3.1 which is made without integration of 4,Glucopyranose peak.

Table 3.1: Molar % of glycosydic linkages present in Col0 and *ngt1-1* and *ngt1-2* cell wall

Without 4,Glucopyranose	% Genotype			écartype/2		
composante de pic	Col0	*ngt1-1*	*ngt1-2*	Col0	*ngt1-1*	*ngt1-2*
T-Arabinofuranose	1,9	1,4	1,1	0,5	0,3	0,2
T-Rhamnofuranose	0,5	0,5	0,2	0	0,1	0
T-Fucofuranose	0,5	0,7	0,7	0,1	0	0,1
T-Xylopyranose	2,2	2,8	2,6	0,8	0,7	0,4
2-Arabinofuranose	0,9	1,9	2,4	0	0,3	0,3
T-Mannopyranose	0,3	0,4	0,6	0	0	0
T-Glucopyranose	0,8	1,3	2,3	0	0,1	0,4
3-Fuco/Rhamnofuranose	0,2	0,2	0,3	0	0	0
T-Galactopyranose	3,9	5,8	5,1	0,9	1,1	0
5-Arabinofuranose	3,9	2,3	2,4	0,1	0,1	0
4-Fuco/Rhamnofuranose	0,7	1,2	0,5	0,2	0,5	0
2-Xylopyranose+ 4-Xxylopyranose	4,7	5,6	5,6	0,5	0,3	0,5
2,4-Rhamnofuranose	0,9	1	1,8	0,1	0,1	0,4
4-Mannopyranose	2,4	3,4	4,2	0	0,1	0,3
3,5-Arabinofuranose	1,1	0,5	0,3	0	0,1	0,3
2-Galactopyranose	1,6	2,7	2,9	0	0,5	0,1
4-Galactopyranose	1,2	2,9	2,3	0,3	0,9	0
2,5-Arabinofuranose	0,4	0,5	0,4	0	0,2	0
2,4-Xylopyranose	0,9	1,8	1,3	0	0,1	0,1
6-Galactopyranose	0,2	1,5	1	0	0,2	0,1
3,6-Hexose	2,7	1,8	2,2	0,4	0,1	1,2
2,4-Hexose	1,3	1,2	1,4	0,4	0,2	0,6
4,6-Mannopyranose	1,6	2,3	2,6	0,1	0,2	0,1
4,6-Glucopyranose	13,4	11,1	12,1	0,5	0,6	1,6
3,4-Hexose	0,5	0,3	0,5	0	0	0,1
3,6-Galactopyranose	0,8	1	1,5	0,1	0,2	0,1
3,4,6-Hexose	1,2	0,3	0,6	0,4	0	0,1

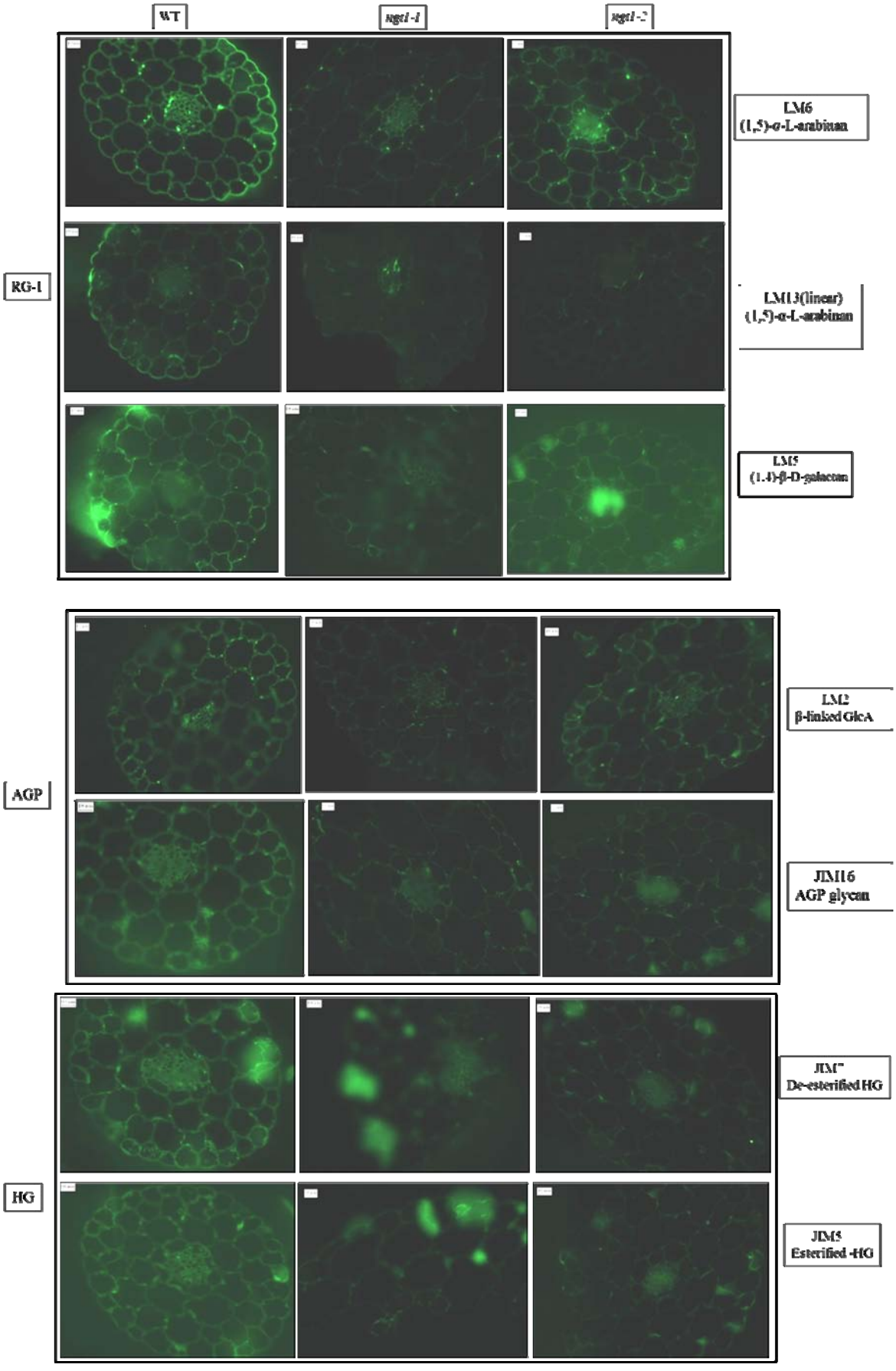
As the sequence alignment has shown that NGT1 could be a fucosyltransferase but linkage analysis data strongly provide us with an alternative hypothesis. Indeed from the analysis of glycosidic linkage analysis where arabinan-related linkages were decreased, we alternatively postulate that NGT1 could encode an arabinosyltransferase involved in pectin RG-I biosynthesis. As we did not observe any decrease in fucose content, it appears essential to decipher the NGT1 activity *in vitro*.

3.7 Immunolabeling of *ngt1-1*, *ngt1-2* and wild type hypocotyls

Monosaccharide composition and glycosyl linkage analysis demonstrated that the cell wall composition was altered in a similar way for both *ngt1-1* and *ngt1-2*, and eventually suggested that arabinan or galactan containing polymers were affected in both *ngt1-1*, *ngt1-2*

mutant lines. Several polymers related to the cell wall, like pectin and arabinogalactan proteins, contain arabinose and galactose which were quantitatively and qualitatively different in the mutant lines. Hence, in order to confirm the biochemical phenotypes observed in the cell wall from both mutants, we sought to characterize them using antibodies directed against various cell wall epitopes specific of pectins and AGPs. The immunolabelling experiments were performed on hypocotyl cross-sections prepared from 6 day-old dark grown seedlings from mutants and WT plants. Etiolated seedlings were used for these experiments, mostly because the biochemical glycosidic linkage analysis was performed on cell wall derived from such plants but also because the analysis of leaf development showed differences of the growth rate at early stages. Several different but specific antibodies against plant cell wall polymers were tested in order to get a comprehensive overview of polymer alteration in mutant lines. To assess differences in pectin RG-I related epitopes, the stem cross section of mutant and wild type seedlings were labelled with LM-6 antibody which recognizes α-(1→5)-arabinan (Willats *et al.,* 1998) as well as LM13 antibody which recognizes linearised α-(1→5)-arabinan (Moller *et al.,* 2008, Verhertbruggen *et al.*, 2009) and LM5 antibody specific for β-(1→4)-galactans associated with side chains of RG-I (Jones *et al.,* 1997).

Homogalacturonan (HG) specific antibodies were also tested, like JIM5 which recognizes relatively low esterified HG and JIM7 which recognizes highly esterified HG. As galactan and arabinan are also present in arabinogalactan proteins (AGPs), we used antibodies against AGPs: LM2 (Smallwood *et al.,* 1996, Yates *et al.,* 1996) and JIM16 (Knox *et al.,* 1991, Yates *et al.,* 1996) to test the hypocotyls cross-sections. Control incubations were performed by skipping the incubation with the primary antibodies described above, and incubating only the secondary antibody. These controls permit to assess background fluorescence due to the use of secondary antibody alone. After immunolabelling of stem cross sections, samples were observed with an epifluroscence microscope.


WT
rgt1-1
rgt1-2
LM6
(1,5)-α-L-arabinan
RG-I
LM13(linear)
(1,5)-α-L-arabinan
LM5
(1,4)-β-D-galactan
LM2
β-linked GlcA
AGP
JIM16
AGP glycan
JIM7
De-esterified HG
HG
JIM5
Esterified HG

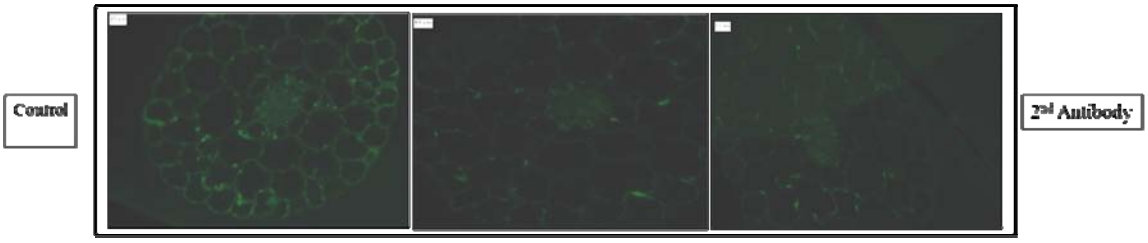


Figure 3.15: immunolabelling of stem cross sections of 6 days old etiolated Arabidopsis wild type and *ngt1-1* and *ngt1-2* mutant lines. For the detection of RG-I epitopes LM6, LM13 and LM5 antibodies were used. For the detection of AGPs, LM2 and JIM16 antibodies were used while for the detection of HG, JIM7 and JIM5 antibodies were used. The control reaction lacked any of the above primary antibody which were tested in this experiment.

Interestingly, a qualitative and reproducible reduction of α-(1→5)-L-arabinan epitope was observed in both *ngt1-1* and *ngt1-2* mutant lines, as compared to wild type plants, using LM6 and LM13 anti-arabinan labelling. Additionally, we observed that *ngt1-1* mutant line was always more affected as compared to *ngt1-2* mutant line for the arabinan labelling (Figure 3.15). This relative difference in labelling is difficult to interpret, because even though the T-DNA insertion of *ngt1-2* mutant is located upstream the start codon whereas insertion for *ngt1-1* occurs within the gene, RT-PCR analysis of the mutants concludes that no functional cDNA was synthesized in both lines. Globally, the polysaccharide content of the mutant lines using probes directed against arabinan supported an important and reproducible alteration of the arabinan structure within the mutant lines, thus confirming the biochemical phenotype that was quantified (reduction in arabinose content, specifically 5-arabinan and 3,5-arabinan). We also observed reduction in other epitopes, such as HG using JIM5 and JIM7 antibodies which recognizes partially and highly methyl esterified HGs. We observed that fluorescent signals were uniformly reduced in mutant as compared to wild type stem cross section. To a lesser extent, labelling of *ngt1-1* and *ngt1-2* mutant stem cross sections with LM2 and JIM16 also resulted in lower signals which were indicative of less AGP epitopes in both the mutant lines. But the reduction in *ngt1-1* was more important in all cases.

3.8 Complementation of *ngt1-1* and *ngt1-2* mutant lines

As mentioned earlier in this chapter, the mutant lines *ngt1-1* and *ngt1-2* shared a particular developmental phenotype as compared to wild type Col0 plants (narrow leaves) and showed characteristic modification of their cell wall composition when linkage analysis was performed (less 3,5-Ara*f* and 5-Ara*f*). These developmental and biochemical phenotypes were observed in both allelic *ngt1-1* and *ngt1-2*, with identical magnitude, thus indicating that they

were most probably genetically related to the alteration of NGT1 gene in these lines. However, in order to confirm that assumption we carried out the complementation of both *ngt1-1* and *ngt1-2* mutants with a 35S::NGT1 construct in vector pH2GW7, and transformed Col0 WT with the same construct (control). Transformation was performed using *A. tumefaciens* previously transformed with the 35S::NGT1 cassette by dipping 4-weeks-old Arabidopsis plant in the bacterial suspension, plants were allowed to recover and develop, and transformants were selected on hygromycin (see materials and methods section 6.5.7.3).

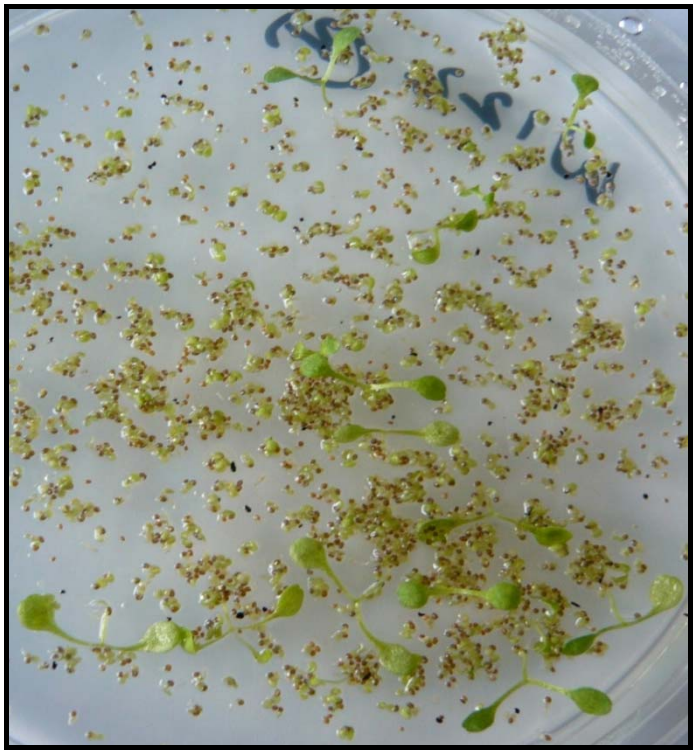


Figure 3.16: Screening of positive transformants for 35S::NGT1. Seeds harvested from Col0, *ngt1-1* and *ngt1-2* plants transformed with *Agrobacterium* carrying 35S::NGT1 were plated on MS growth medium containing 50µg/mL hygromycin. Sensitive plants harbour a stunt development (small greenish leaves, no roots) whereas 35S::NGT1 transformed at the bottom of the picture developed almost normally.

Through this selection, we could identify plants harbouring hygromycin resistance, indicative of plants complemented with 35S::NGT1 gene (Figure 3.16). These resistant plants were transferred onto soil and analysed by PCR to confirm their complementation.

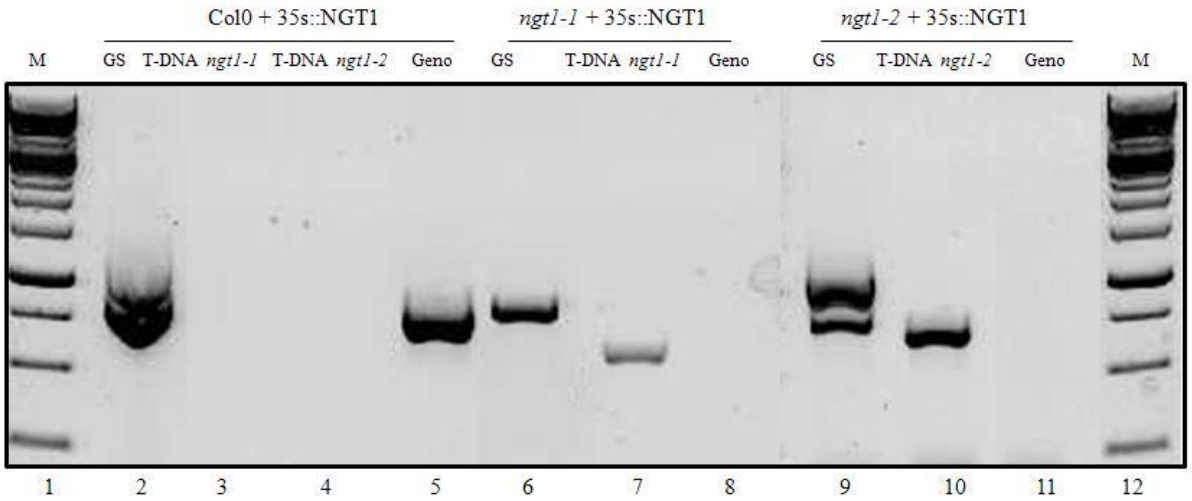


Figure 3.17: Genotyping of transformed plants through PCR. Lanes 1 and 12 represent the 1 Kb marker. The presence of product in lane 2, 6 and 9 indicates that plants are successfully transformed. Other PCR reactions were performed to check genetically each line regarding the occurrence (for *ngt1-1* and *ngt1-2*) or not (for Col0) of T-DNA insertion at specific NGT1 locus.

DNA was extracted from individual complemented plants and used as a template in a PCR reaction. Different set of primers GS (cDNA NGT1 gene specific), T-DNA *ngt1-1* (LBa1 + RPW1 for *ngt1-1*), T-DNA *ngt1-2* (LBa1 + RPW3 for *ngt1-2*) and Geno (LPW1+ RPW1 or LPW3 or RPW3 gene specific primers) were used for amplification.

A product of 981pb is positively amplified with GS primers using Col0 (lane 2), *ngt1-1*(lane 6) and *ngt1-2* (lane 9) DNA which indicates that wild type Col0 and mutant lines *ngt1-1*, *ngt1-2* are transformed with 35S::NGT1 construct (Figure 3.17). The presence of PCR product (lanes 7 and 10) using T-DNA left border primers in combination with the lack of amplification in lanes 8 and 11 in both *ngt1-1* and *ngt1-2* lines indicates that in these two lines, NGT1 gene contains a T-DNA insertion. No amplification was observed in control reactions of Wt col0 while using *ngt1-1* and *ngt1-2* T-DNA specific set of primers

After genotyping the plants, we collected the seeds from complemented plants. We grew the seeds for phenotypic studies, i.e. the measurement of leaf area between 14^{th} and 35^{th} day of development as previously done to genotype Col0 and *ngt1-1* and *ngt1-2* mutant plants. We examined the phenotype of the transformed plants of the first generation and at the same time we grew the non-transformed wild type and mutant plants (control). This analysis has shown that mutant phenotype is fully restored after the complementation with 35S::NGT1. We have observed that at 14^{th} day of development, *ngt1-1*+ 35s::NGT1 and *ngt1-*

2+35s::NGT1 have same leaf area as compared to that of Col0_35s::NGT1 plants. It indicates that the expression of NGT1 gene in the mutant background fully rescue the mutant phenotype. Whereas for non-transformed control mutant plants we confirmed our previous results of the narrow leaf phenotype for both mutant lines *ngt1-1* and *ngt1-2* (described on page 116.)

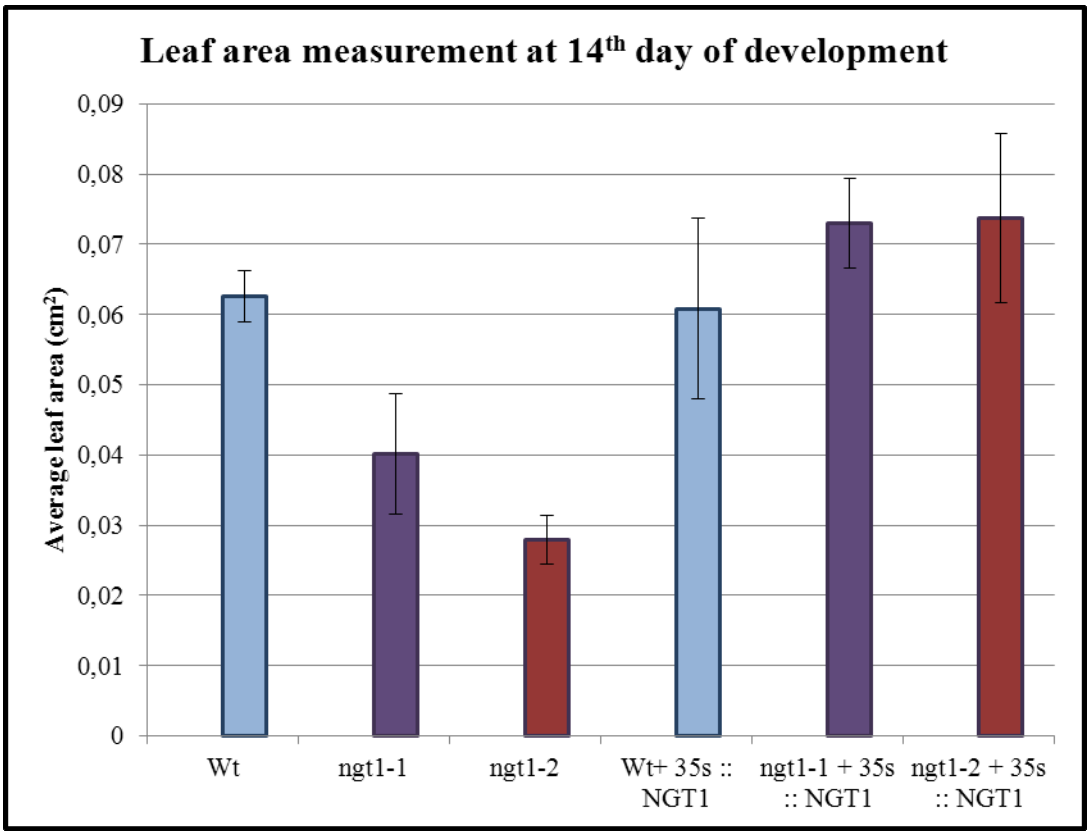


Figure 3.18: Analysis of leaf area of wild type Col0, mutant lines *ngt1-1* and *ngt1-2 and* Col0+35s:: NGT1, *ngt1-1*+ 35s::NGT1, and *ngt1-2*+35s::NGT1. Leaf area was quantified using ImageJ. Each value is an average determined from the measurement of leaf area of three individual plants. The error bars represent the standard deviation. This data shows that mutant phenotype is restored after the complementation.

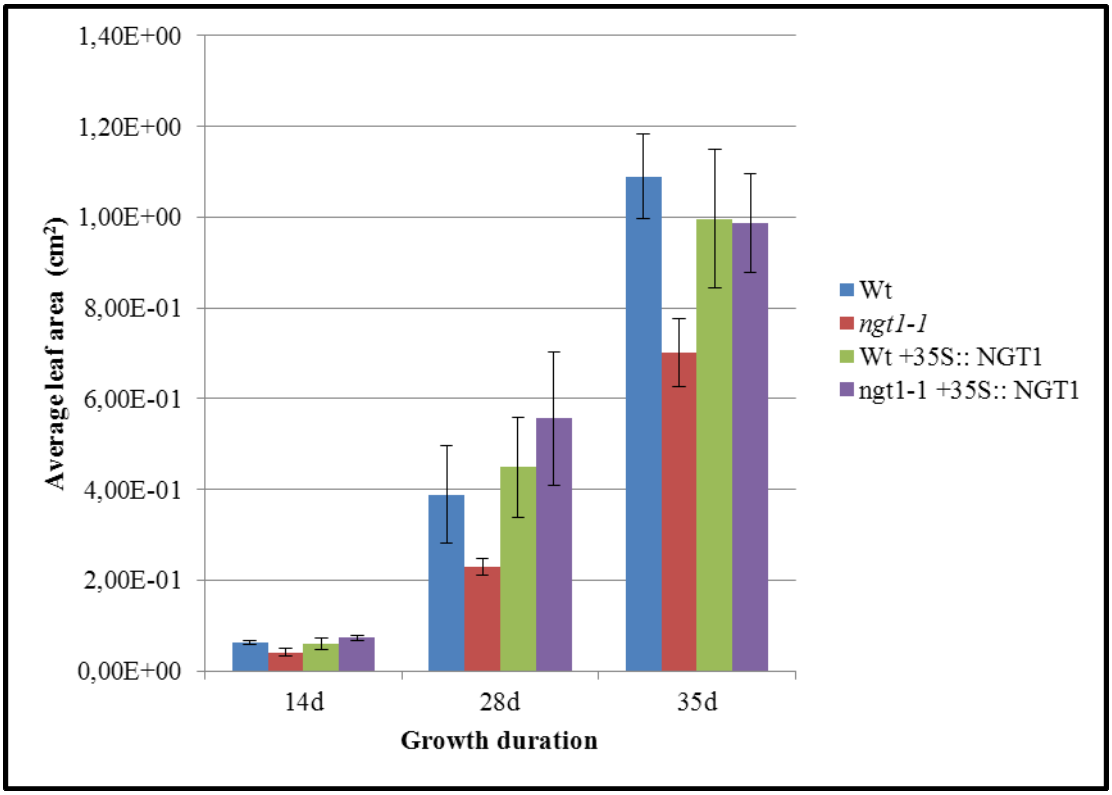


Figure 3.19: Analysis of leaf area of wild type Col0, mutant lines *ngt1-1* and *ngt1-2 and* Col0+35s::NGT1, *ngt1-1*+ 35s::NGT1 and *ngt1-2*+35s::NGT1 .Leaf area was measured quantitatively at 14TH 28TH AND 35TH DAY OF developmental. Each value is an average determined from the measurement of leaf area of three plants. The error bars represent the standard deviation. This data shows that mutant phenotype is restored even after the 35th day of growth with the complementation of NGT1.

We measured the leaf area on 28th and 35th day of growth also. Here we have presented the data about only *ngt1-1*+ 35s::NGT1 and not for *ngt1-2*+35s::NGT1 because we lost the transformed plants during manipulation. We did not reported the values for 21st day of development, because the transfer of seedlings from the Petri dishes to the soil, occurred between 14th to 21st day, and we anticipated that this set of data (21st day) would be impaired by the stress corresponding to transfer to new growth conditions. It is clear from the above figure that the mutant (not complemented) has narrow leaf area as compared to that of wild type and the rescued-mutant line, and this at all observed developmental stages.

3.9 Heterologous expression of NGT1 in *Pichia pastoris*

Heterologous expression is a key element in the assignment of function to a protein, but successful heterologous expression and demonstration of enzymatic activity of plant cell

wall glycosyltransferases are rare and difficult. In this study we used *Pichia pastoris* as a host system for the expression of NGT1 protein. As an eukaryote, *Pichia pastoris* offers many advantages for the expression of plant proteins, for protein processing, protein folding and post-translational modifications. Furthermore, it is relatively easy to manipulate and less costly compared to other expression systems like insect and mammalian cells. Original cDNA of NGT1 was ordered from SALK institute and provided subcloned into a pUNI plasmid. Overlapping PCR was then used, in order to add to NGT1 cDNA specific sequences coding for the T7 tag epitope and Gateway border sequences necessary for the recombination of the PCR product in the pDONR207 entry plasmid (BP cloning; cf materials and methods 6.2.1.1.2). Recombination of the synthesized PCR product into pDONR207 vector led to the formation of novel plasmid, named pENTR207_T7:NGT1 that was checked by sequencing. pENTR207_T7:NGT1 corresponds to an entry vector that can be used to transfer the gene sequence into various destination vectors such as pPICZα expression (vector for expression in Pichia). Such reaction was performed forming an expression vector (named pPICZ_T7:NGT1) containing the bacterial pUC origin of replication for propagation in *E. coli* and AOX1 (Alcohol Oxidase) promoter which is used to enhance transcriptional regulation of NGT1 in the presence of methanol inducer for recombinant protein expression in *Pichia pastoris.* After Pichia transformation with pPICZ_T7:NGT1 plasmid, Pichia cells were streaked on zeocin selection medium and 12 positive clones were selected. We selected 12 clones for characterization of protein expression because we anticipated this parameter may vary between clones. Checking 12 clones for protein expression will provide the opportunity to select the higher expressing and productive clone. Pichia_T7:NGT1 clones were grown (only 6 are shown in Figure 3.20A and 3.20B) on BMMY medium and protein expression was induced for 4 days by adding methanol 0.5 % final to the medium. Microsomal proteins were prepared by breaking the Pichia cells (see materials and methods 6.3), protein content was quantified through Bradford assay and first checked by SDS-PAGE (Fig 3.20A). No differential expression pattern could actually be determined between transformed Pichia_T7:NGT1 and wild-type line GS115, at the expected size of 61 kDa. This observation indicates that if T7_NGT1 is produced in our clones, it is below the threshold of Coomassie blue staining.

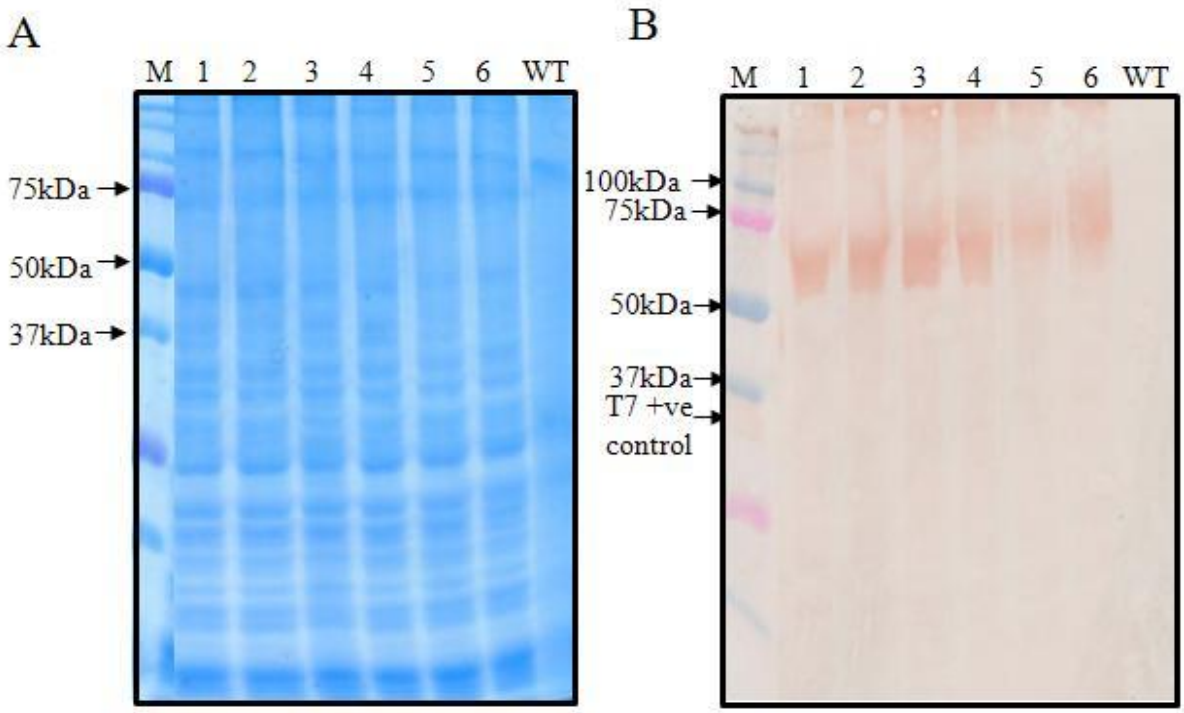


Figure 3.20:A. SDS-PAGE analysis of total proteins in six NGT1 clones (lanes 1-6) and WT Pichia cells as a control. Coomasie blue staining of 12% polyacrylamide gel. M: molecular weight markers B. Immunoblot detection of NGT1 protein in clones 1 to 6. Expression of different clones was compared to select highly productive clone for NGT1 and wild type Pichia strain (WT) used as a control. T7 was used as a positive control with the molecular weight markers (M) and its band is visible below 37 kDa.

The expression of T7:NGT1 protein was further verified using western blot analysis and the T7-tag antibody (Figure 3.20B). The lack of immunodetection in the lane of the wild-type indicates that there was no cross reactivity of the T7 antibody with microsomal proteins in Pichia GS-115 WT. For clones 1 to 6 (Figure 3.20B) a signal was picked up by the antibody indicating all this 6 clones were actually expressing a protein recognized by the antibody, most probably T7:NGT1. We observed a difference in migration between the lanes 1-6 because of migration parameters. By visual comparison, clone #2 was selected for expression optimization and large scale production in order to produce enough microsomal proteins for radioactive activity test. However, prior to large scale production of microsomes from our selected clone#2, we spent some effort to determine whether or not the band (smear) immunodetected actually corresponded to the expression of T7:NGT1 protein. Indeed, the T7:NGT1 protein was apparently produced at a higher molecular weight than expected, corresponding to a smear spread around 60-75 kDa when subjected to immunoblot analysis (Fig 3.20B). One explanation would be that T7:NGT1 protein is produced but hyper-

glycosylated by the *Pichia* system as a consequence of the yeast N-glycosylation specificity (hyper-mannosylation). The NGT1 protein sequence comprises 7 potential N-glycan sites as represented in the figure (3.21A). N-Glycosylation is one of the most common post-translational modifications of proteins in eukaryotes. It occurs when N-glycans are attached to an asparagine residue present in the consensus frame N-X-S/T where X could be any amino-acid except a proline. In order to check if the smear observed for T7:NGT1 immudetection is caused by the addition of hypermannosylated N-glycans onto the protein; we designed an experiment aiming at N-Glycans removal. T7:NGT1 protein was then subjected to enzyme mediated deglycosylation. The enzyme endoglycosidase H (Endo-β-N-acetylglucosaminidase H) is a highly specific endoglycosidase which cleaves asparagine-linked mannose rich oligosaccharides and commonly used to deglycosylate glycoproteins. After treatment with endoglycosidase H, protein size shifted to 61 kDa which was the expected size of NGT1 protein (Figure 3.21B).

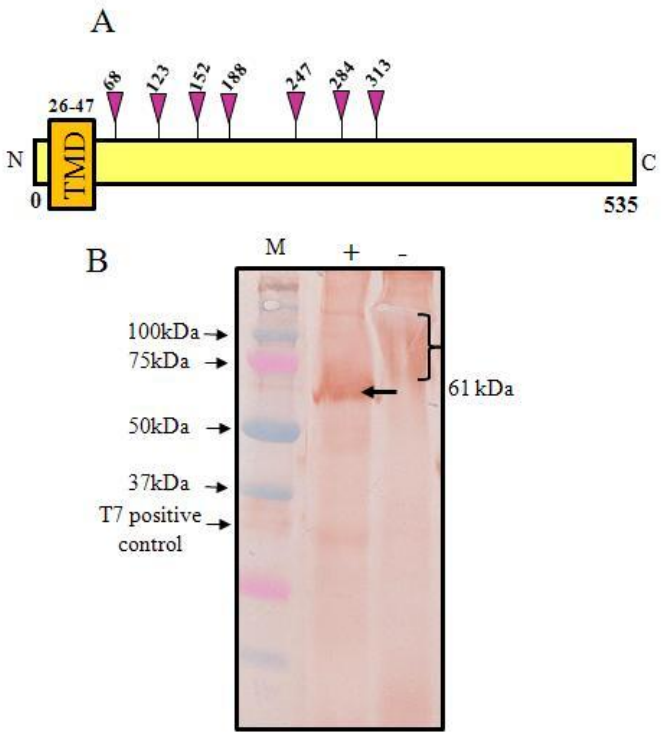


Figure 3.21: A. Predicted N-glycosylation sites of NGT1 http://www.cbs.dtu.dk/. B. Treatment of NGT1 microsomes with endo-glycosidase H to remove N-linked glycans (. Lane 1 shows molecular weight marker with T7 positive control. Lane 2 shows NGT1 microsomes treated with endo-glycosidase H and lane 3 shows untreated NGT1 microsomes.

3.10 Free sugar assay using T7:NGT1 microsomes

A T7- tag version of NGT1 protein was successfully expressed and produced in *Pichia pastoris*. We pursued the functional characterization of NGT1 gene, taking advantage of NGT1 annotation to test a Pichia line expressing NGT1 for fucosyltransferase activity. Microsomes were prepared (for details see materials and methods 6.3) from this T7:NGT1 expressing *pichia pastoris* and used for activity test, after induction of protein expression. Enzyme activity tests were developed using radioactive GDP-[^{14}C]-fucose taking advantage of the high sensitivity of radioactive assays particularly adapted to decipher glycosyltransferase functions. Indeed, using radioactive test, picomolar transfer of radiolabelled fucose onto an acceptor can be quantified with certainty. One main disadvantage in the use of radioactivity test to assess a glycosyltransferase activity, is the limited availability of both radioactive nucleotide-sugar and relevant acceptors. This is particularly true when one is studying a glycosyltransferase potentially involved in cell wall biogenesis because of the high degree of diversity and complexity that can be found throughout cell wall structure, specific acceptor of each cell wall structure can hardly be found. In an attempt to overcome that difficulty we adapt a system called “free sugar assay” where the acceptor molecules would be a monosaccharide present at high concentration (half molar).

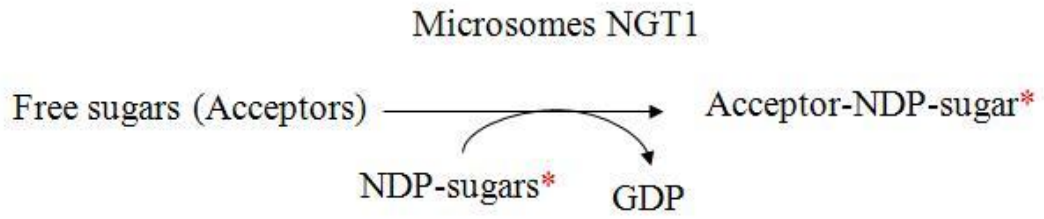


Figure 3.22: Schematic representation of principle for free sugar assay

The rationale of the assay (is based on the principle shown in figure 3.22) being to force the enzyme to transfer a radioactive sugar onto a monosaccharide even though this monosaccharide is obviously not the preferred acceptor of the enzyme, and this in order to gain knowledge about which monosaccharide the enzyme is able to work with. Then, a second step would permit to check the plant cell wall for possible acceptor molecule comprising the monosaccharide onto which the enzyme is active. Thus, in order to determine the putative activity of T7:NGT1, we used the microsomal protein fractions (enriched for Golgi membranes) prepared from *Pichia pastoris.* We checked membrane fractions for the ability of the T7:NGT1 protein to catalyze the transfer of [^{14}C]-labelled UDP donor sugars onto

acceptors. We tested 3 different types of NDP-sugars as donors taking advantage of their availability in the laboratory (UDP-Gal, UDP-Glc and GDP-Fuc) and 6 different monosaccharides as acceptors (Arabinose, Galactose, Glucose, Mannose, Rhamnose and Xylose). We provided equal quantity (50 nCi) of UDP-Glc [^{14}C] (control) and GDP-Fuc [^{14}C] (as the NGT1 protein is a putative fucosyltransferase). First we observed that whatever the acceptor the transfer of Glc from UDP-Glc appears highly stable (4420 +/-212 cpm/h) whereas the values of transfered radioactivity, even if low, were fluctuating in the case we use GDP-[^{14}C]-Fuc (8305 +/-1641cpm/h). This indicates that regarding UDP-[^{14}C]-Glc, all monosaccharides behave identically, none of them being a substrate for the enzyme. In the case of GDP-Fuc the observation that the standard deviation is high may indicate that some acceptors have value away from the mean which would be expected in case of a transfer. Noticeably, the transfer of GDP-[^{14}C]-Fuc onto different acceptor sugars is variable (Fig 3.23), and particularly high onto arabinose as an acceptor sugar. However the level of transferred observed either using UDP-[^{14}C]-Glc and GDP-[^{14}C]-Fuc as donors are just around the background level because approximately 250,000 cpm (5nCi) were provided to the assay and 4,000 to 10,000 were recovered at best. The only conclusion that could be drawn is that the enzyme would better accommodate GDP-Fuc compared to UDP-Glc in its catalytic domain, explaining why values would more vary while using GDP-Fuc compared to UDP-Glc. This last remark is consistent with NGT1 being a putative fucosyltransferase although no data collected from this free sugar assay would really show enough transfer to strengthen unambiguously this hypothesis.

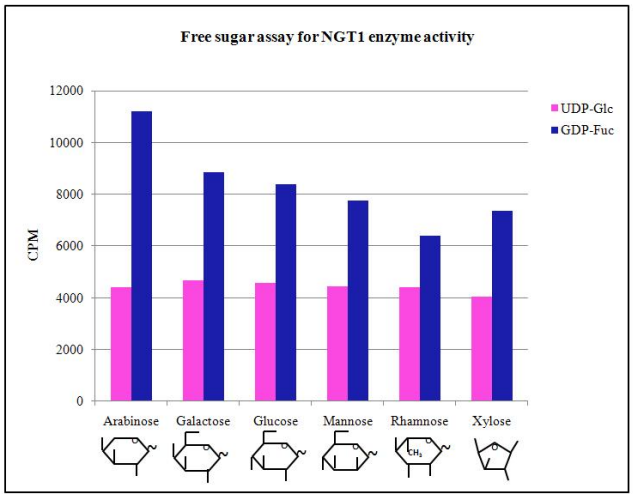


Figure 3.23: Free sugar assay for NGT1 activity with UDP-Glc and GDP-Fuc donors. Half molar concentration of different monosaccharides like arabinose, galactose, glucose, mannose, rhamnose and xylose were used as acceptor substrate.

We also tested UDP-Gal [^{14}C] as a donor but provided a smaller quantity (5 nCi). This assay demonstrates that transfer level of [^{14}C]-Gal onto monosaccharide acceptor the same on all the tested monosaccharide acceptors, no strong transfer of the donor sugar (Gal) was observed, as observed when UDP-Glc was used (Figure3.24).

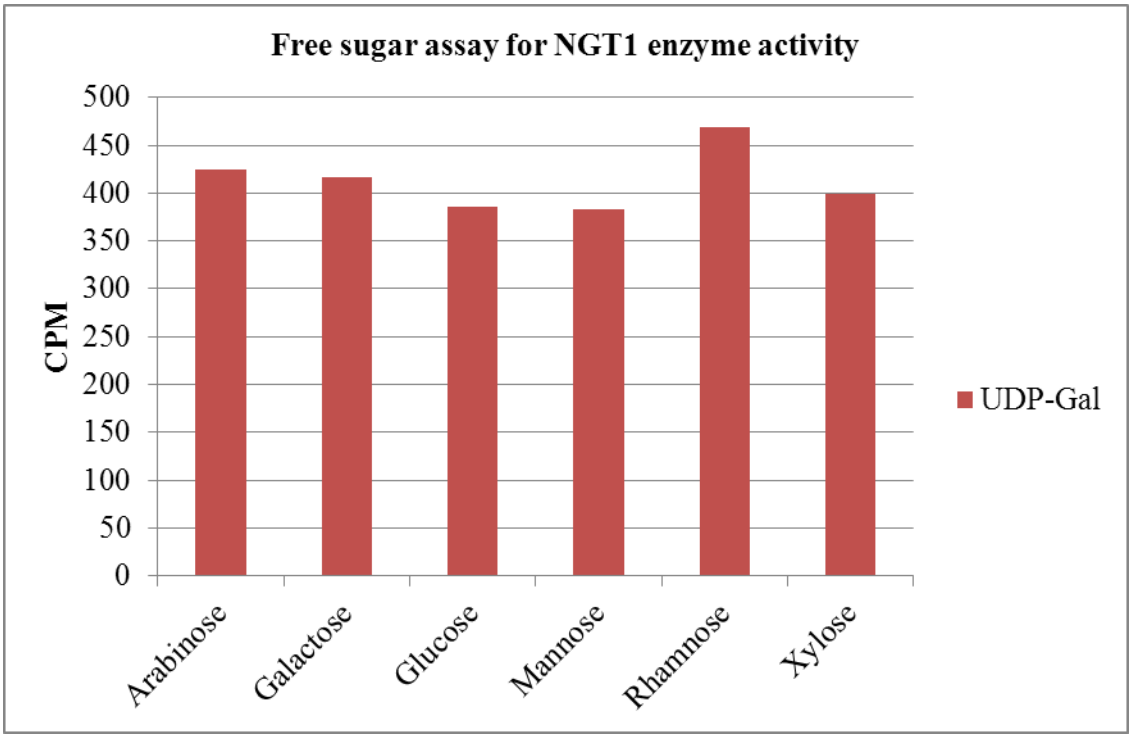


Figure 3.24: Free sugar assay for NGT1activity with UDP-Gal as donor. Half molar concentration of different monosaccharides like arabinose, galactose, glucose, mannose, rhamnose and xyloses were used an acceptor

From the above free sugar assays we have concluded that GDP-[^{14}C]-Fuc would be a better donor for T7:NGT1 protein compared to UDP-Glc and UDP-Gal, and that the level of transfer of radioactivity is higher for arabinose, and to a lesser extent galactose, as acceptors. However the level of transfer detected was too low to worth any product characterization using TLC separation. We repeated the free sugar assay using Arabinose and Galactose because these two sugars showed some ability to accept transfer of Fucose (from GDP-[^{14}C]-Fuc) in the presence of T7:NGT1. We included another sugar (Rhamnose) that did not show particular level of transfer of Fucose onto it, and was thus served as a control. Another control reaction was the assay lacking acceptor sugars, and the assay was performed using heat-inactivated microsomes. Interestingly this assay confirms that Arabinose and Galactose had a small but reproducible capability to accept transfer of [^{14}C]-Fucose whereas Rhamnose would not (Figure 3.25). Unfortunately, the level of transfer observed were again too small too worth any product characterization, most probably because the acceptor and or the donor sugars

provided to the T7:NGT1 protein were too different from the actual specificity of the reaction that the protein would catalyzed.

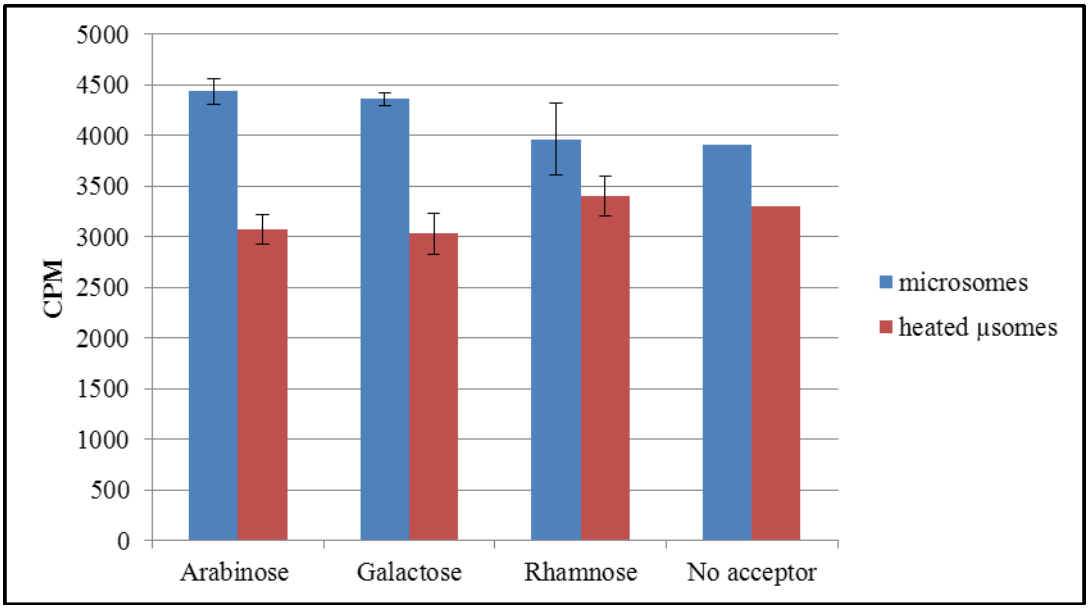


Figure3.25: Free sugar assay for NGT1activity with GDP-Fuc as donor. Different monosaccharides like arabinose, galactose and rhamnose were used as putative acceptors. Control reaction lacks any acceptor.

3.11 Fucosyltransferase assay using *ngt1-1* mutant cell wall as an acceptor

The free sugar assay experiment demonstrates that microsomal membranes expressing T7:NGT1 had preference to accommodate GDP-Fuc compared to others nucleotide-sugars tested and to transfer them, to a small extent, onto Arabinose or Galactose. In order to confirm this observation and get better understanding of the role of T7:NGT1, we carried out a fucosyltransferase activity test using mutant cell wall as the acceptor substrate. This strategy has been successfully used by Egelund and his colleagues for the characterization of RGXT1 and RGXT2 xylosyltransferases involved in pectin rhamnogalacturonan II biosynthesis (Egelund *et al.*, 2006). The rationale of the assay is based on the principle (shown in figure3.26) that the mutation in NGT1 gene will cause an alteration of the cell wall in *ngt1-1* mutant and consequently makes its cell wall deficient for specific polysaccharide structure. Adding the T7:NGT1 protein and the correct (radioactive) NDP-sugar, this cell wall structure could be used as an acceptor. As a control we would use cell wall prepared from the wild-type

Col0 for which we do not expect the structure synthesized by NGT1 to be deficient, providing a negative control to this test. Another control was provided thanks to the use of microsomal preparation of membranes expressing At1g62305 protein. This gene (named E in Figure 2.1 of chapter 2) belongs to DUF266 family and do not show any fucosyltransferase signature.

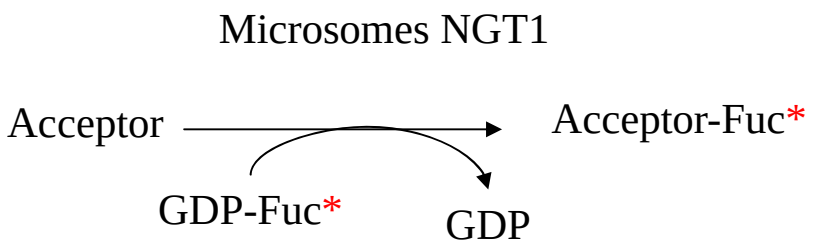


Figure 3.26: Schematic representation of mechanism of NGT1 putative fucosyltransferase

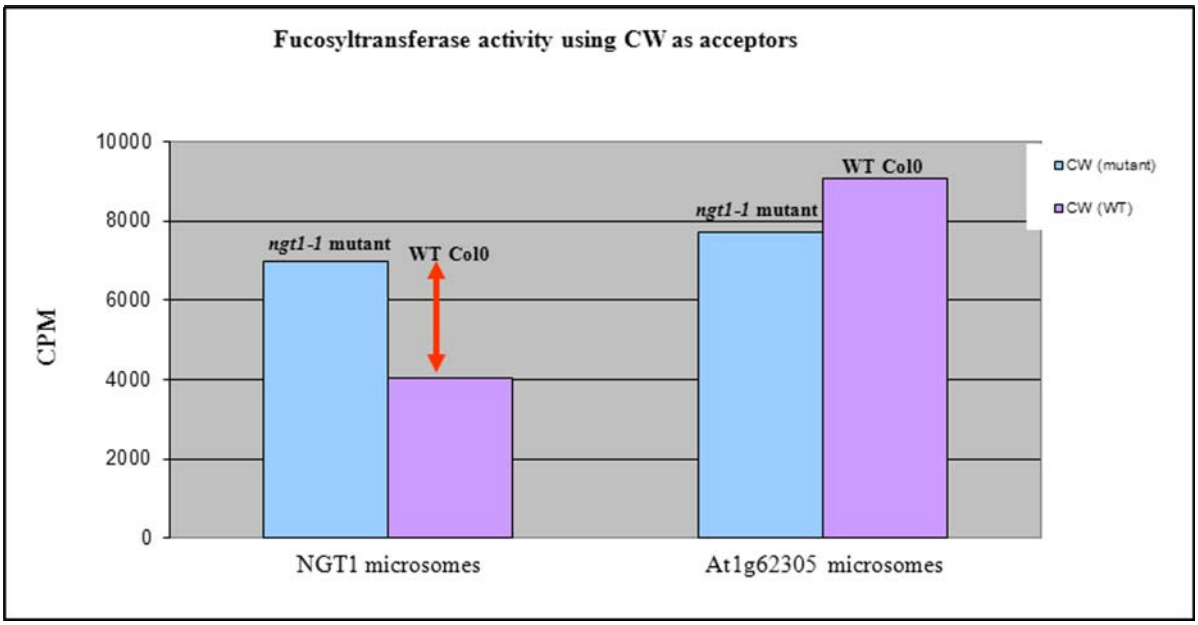


Figure 3.27: Fucosyltransferase activity assay for NGT1 activity with mutant cell wall as an acceptor. Transfer of fucose is observed onto mutant cell wall in presence of NGT1 microsomes while there is no transfer of fucose onto mutant cell wall in the presence of At1g62305 microsomes which are used as a control

This assay shows that T7:NGT1 catalyzes the transfer of [^{14}C]-Fuc to the mutant cell wall as there is more transfer of Fucose in the *ngt1-1* mutant as compared to the wild type Col0 cell wall (Figure 3.27). In case of control microsomes expressing another protein At1g62305 no significant transfer of fucose was observed onto mutant cell wall as compared to wild type. From this experiment, we concluded that cell wall from *ngt1-1* mutant appears a good acceptor of fucose as compared to the wild-type cell wall, but again the level of transfer observed was low compared to the amount of radioactivity included in the test. This indicates

that the reaction studied is probably not the actual (true) activity of the T7:NGT1 protein. Cell wall prepared from the *ngt1-1* mutant should offer a large variety of molecules to be acceptors, hence the lack of strong transfer in our assay could be interpreted as the donor sugar of the reaction (GDP-Fuc) not being the proper one, if we assume that the numerous potential acceptor of the cell wall were evenly accessible.

We did another assay by using the microsomes of NGT1 and GS-115 wild type pichia strain as a control. We observed that in the presence of NGT1 microsomes and GDP-Fuc [^{14}C] donor, there is transfer of fucose onto two allelic mutants *ngt1-1* and *ngt1-2* cell wall as compared to wild type cell wall (Figure3.28). In contrast there is less transfer of fucose in the presence of wild type Pichia GS-115 microsomes.

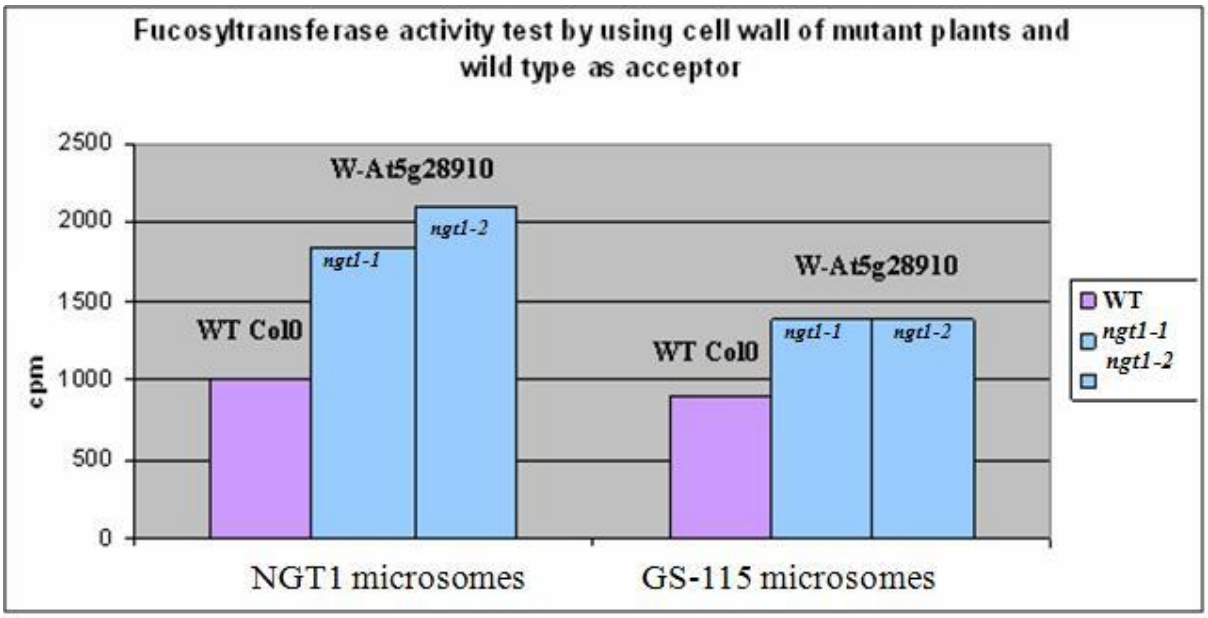


Figure 3.28: Fucosyltransferase assay from Pichia microsomes expressing NGT1. This assay used cell wall extracted from wild type Col0, *ngt1-1* and *ngt1-2* mutants as acceptors. Transfer of fucose is observed onto mutant cell wall in presence of Pichia microsomes expressing NGT1 while only a background transfer of fucose onto mutant cell wall when using WT GS-115 microsomes (negative control).

From all above assays we concluded that NGT1 protein has the ability to transfer fucose onto mutant cell wall as compared to wild type cell wall but this level of transfer is very low. We were not able to purify and characterize the reaction product in result of free sugar assay and fucosyltransferase activity tests with mutant cell wall acceptor for further chemical analysis.Radioactive fucosyltransferase assay data did not prove NGT1 fucosyltransferase enzyme activity. So we envisioned to develop an assay to alternatively test

arabinosyltransferase activity as the biochemical analysis of *ngt1-1* and *ngt1-2* mutants cell wall shown the reduction in arabinose contents.

3.12 Arabinosyltransferase assay using microsomal fraction of Pichia-NGT1 and NGT1-Δ69 produced in Hi-5 cells

In order to test putative arabinosyltransferase activity from heterologously expressed NGT1 or NGT1-Δ69 protein, we carried out this non-radioactive assay for NGT1, that is why when it was not possible to characterize NGT1 using radioactive assays because of unavailability of commercial radioactive UDP-Ara*f* (donor molecule). UDP-Ara*f* was provided by Dr Richard Daniellou (ENS Rennes). We developed an assay using arabinotetraose or arabinohexaose acceptors (4mM), UDP-Arabinofuranose (0.4mM) in buffer containing 50mM Hepes-KOH pH 7 supplemented with $MnCl_2$ 5mM. Putative transfers of Arabinofuranose onto acceptors was later evaluated by MALDI-TOF MS at it was previously done with success to characterize activity of AtFUT1-Δ68 (cf section 6.4.2). In a first assay we used NGT1 expressing Pichia microsomes and wild type strain GS-115 microsomes (as control) in the presence of arabinotetraose and arabinohexaose (not shown). We provided the cold UDP-Araf to the enzyme (for details see materials and methods section 6.4.3) and the reaction was incubated for 2h. Acceptor molecules were then labelled with a 2-aminobenzamide (2-AB) fluorophore (to increase sensitivity) and analyzed by mass spectrometry (MALDI-TOF-MS) (Figure 3.29A, 3.29B, 3.30C and 3.30D). MALDI-TOF-MS analysis of 2-AB labeled neutral oligosaccharides allowed the detection of $[M + H]^+$, $[M + Na]^+$ and $[M + K]^+$ in the positive-ion mode.

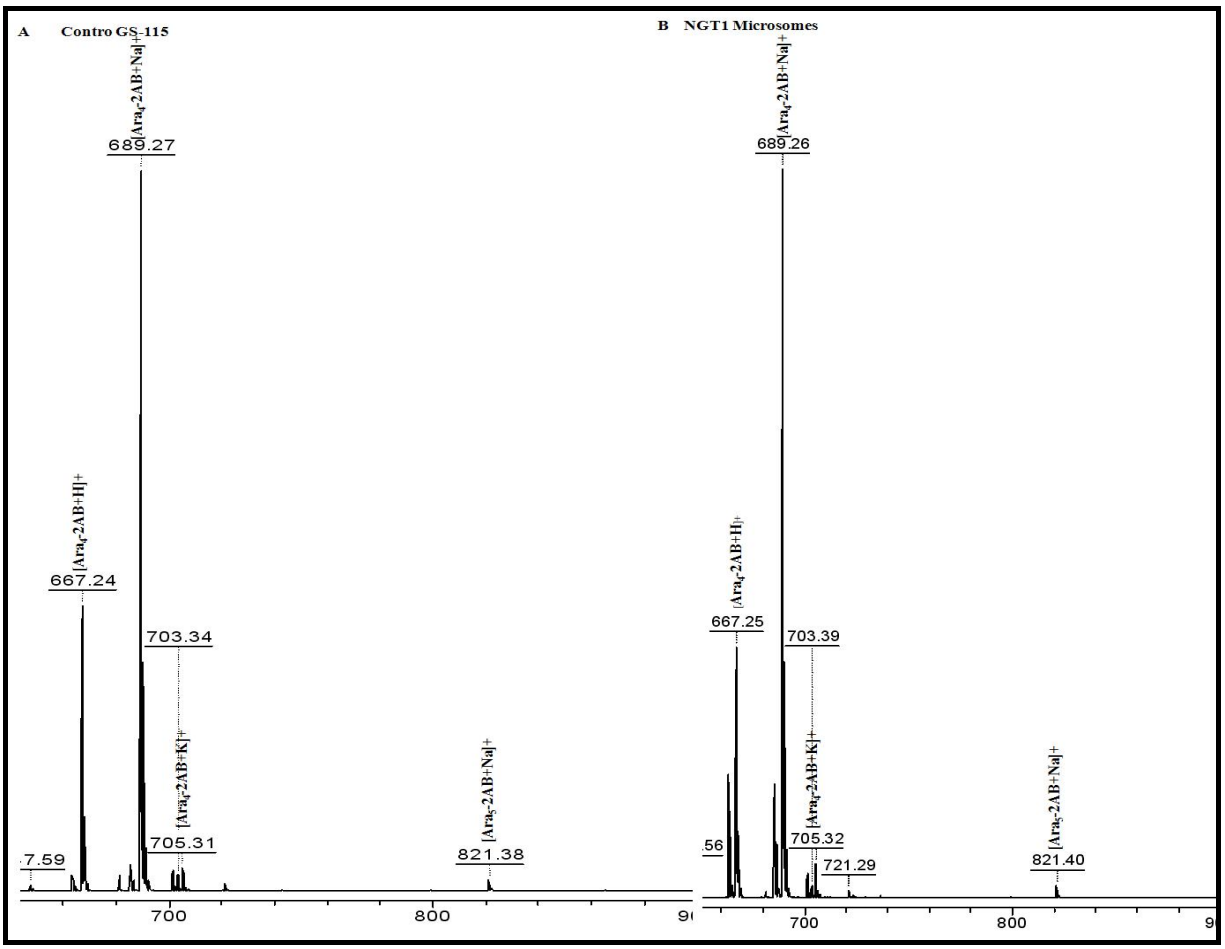


Figure 3.29: MALDI-TOF MS analysis of NGT1 for arabinosyltransferase activity. A. Mass spectra of Arabinotetraose in the presence of WT Pichia GS-115 microsomes. This control reaction (WT microsomes) shows that the assay contain a small proportion of Arabinopentaose (m/z=821), but in relatively low amount as compared to arabinotetraose (m/z= 689). B.MALDI-TOF-MS analysis of the assay including Arabinotetraose (m/z=689) and in the presence of NGT1 microsome: No addition of arabinose was observed in the presence of NGT1 microsomes as the relative quantity of Arabinopentaose peak (m/z=821) remains unchanged.

We observed one peak with a mass of 821.38 (Figure 3.29B) corresponding to mass of arabinopentaose that was expected in case of transfer of Arabinose onto acceptor, but the presence of this peak (at the same intensity) in the control reaction is indicative of the lack of arabinosyltransferase activity of microsome prepared from NGT1 expressing Pichia. Indeed the control indicates a lack of purity of the arabinotetraose acceptor that was used in the study and the fact that the arabinopentaose peak has the same intensity (relatively to the arabinotetraose peak) in both reaction confirmed the lack of transfer, and thus the lack of arabinosyl transferase activity in the assay using NGT1 microsomes.

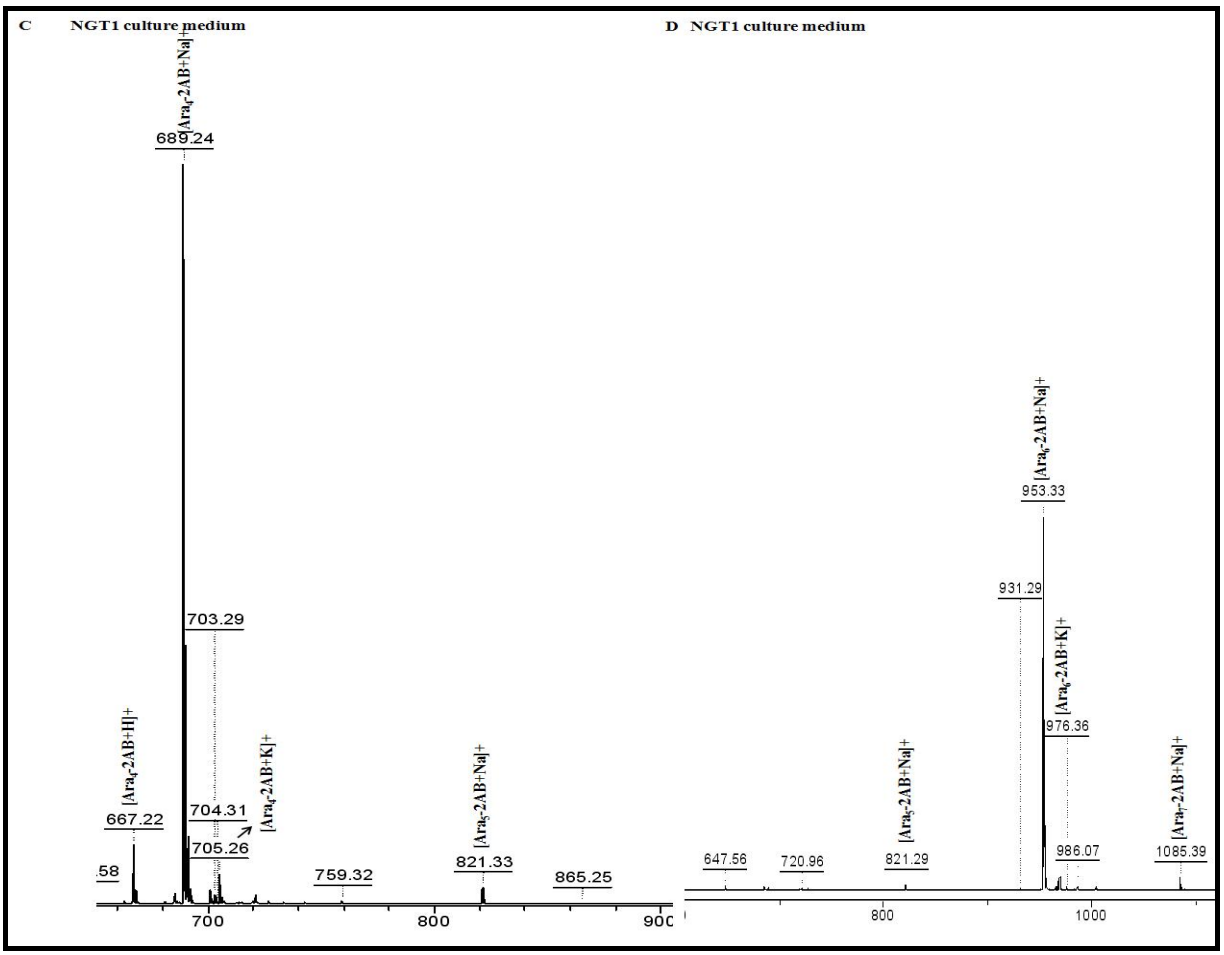


Figure 3.30: MALDI-TOF MS analysis of NGT1 for fucosyltransferase activity. C. Mass spectra of Arabino-tetraose in the presence of culture medium of NGT1. This reaction shows that the assay contain a small proportion of Arabinopentaose (m/z=821), but in relatively low amount as compared to arabinotetraose (m/z= 689). No mass increment was observed in the peaks. D. Mass spectra of Arabino-hexaose in the presence of culture medium of NGT1. This reaction shows that the assay contains a small proportion of Arabinoheptaose (m/z=1085), but in relatively low amount as compared to arabinohexaose (m/z= 953). No addition of arabinose was observed in the presence of culture medium of NGT1 enzyme.

As in the above described experiment we did not observe the NGT1 activity; this might be because of the membranous form of NGT1 protein extracted from the pichia microsomes. In order to obtain the soluble form, the truncated NGT1 protein was expressed in insect cells. In this assay we used arabinotetraose (not shown) and arabinohexaose acceptor molecules. Similarly, Arabinopentaose molecule (821.33) could be identified in both the reaction with NGT1-Δ69 and the control, and at the same relative intensity which indicates consequently a lack of arabinosyltransferase activity.

3.13 Conclusion

In conclusion, phenotypic studies have shown that both mutant lines *ngt1-1* and *ngt1-2* have narrow leaves at 14th and 21st day of development. These results indicate that the mutation in NGT1 alters the growth rate only during early developmental stage so it could be hypothesized that NGT1 gene is essential at early stages of plant development, possibly in the biosynthesis of cell wall. Neutral monosaccharide quantification has shown that mutant cell wall has less arabinose as compared to that of wild type Col0. In addition biochemical analysis of mutant cell wall through GC-MS has shown that both mutant lines *ngt1-1* and *ngt1-2* have 50% less 3,5-Ara*f* and 5-Ara*f* which indicates that NGT1 is involved directly or indirectly in arabinan chain biosynthesis. This reduction in arabinan polymer was additionally observed when immunolabelling was performed on cross section of stem labelled with LM6 and LM13 anti-arabinan antibodies.

NGT1 gene was expressed hetrologously in *Pichia pastoris* and microsomes were used to determine the catalytic activity of NGT1 protein. Free sugar assay has shown that GDP-Fuc ^{14}C would be a better donor for T7:NGT1 protein as compared to UDP-Glc and UDP-Gal. A fucosyltransferase assay was also carried out by using mutant cell wall as the acceptor substrate. This assay resulted in the transfer of ^{14}C-Fuc to the mutant cell wall in the presence of T7:NGT1 protein but the level of transfer observed was low indicating that the reaction studied was not the actual activity of the T7:NGT1 protein.

Because the mutational studies of NGT1 altered mutant lines suggested that NGT1 protein could encode an arabinan-arabinosyltransferase, this later hypothesis was tested in a non-radioactive activity test using MALDI-TOF MS detection but unfortunately no Arabinosyltransferase activity could be shown so far using Arabinotetraose and arabinohexaose acceptor molecules. Whether NGT1 protein would encode a GT activity remains unproved, so far.

Chapter 4

Heterologous expression of *Arabidopsis thaliana* xylosyltransferase (AtXT1) and fucosyltransferase (AtFUT1) for structural characterization

4 Heterologous expression of *Arabidopsis thaliana* xylosyltransferase (AtXT1) and fucosyltransferase (AtFUT1) for structural characterization

4.1 Introduction

Glycosyltransferases (GTs) are classified into over 91 families on the basis of sequence similarities in Carbohydrate Active enZyme database (CAZy). At present, the crystal structures of 104 different GTs have been solved providing structural information for 37 GT families. Despite the sequence diversity, only two main structural folds, namely GT-A and GT-B folds, have been revealed to date. Glycosyltransferases, which are involved in biosynthesis of complex polysaccharides, are located in Golgi (cf annexe review paper). Golgi located GTs are typically type II membrane proteins which consist of a short *N*-terminal cytoplasmic tail followed by a trans-membrane domain, a stem region of variable length and a large C-terminal globular catalytic domain facing the luminal side (Breton *et al.,* 2001). However for most of GTs that adopt this topology, it is possible to express only the catalytic domain in a soluble and active form thus permitting to develop a crystallographic study of the catalytic domain.

During my Ph.D, I have worked on the determination of 3D structure of two already characterized Arabidopsis enzymes involved in xyloglucan biosynthesis (Figure 4.1). The first enzyme AtFUT1 belongs to GT37 family, and catalyzes the addition of fucose onto xyloglucan (Perrin *et al.,* 1999), while the second enzyme AtXT1 belongs to GT34 and catalyzes the addition of xylose onto cellopentaose and cellohexaose acceptor substrates (Cavalier & Keegstra 2006).

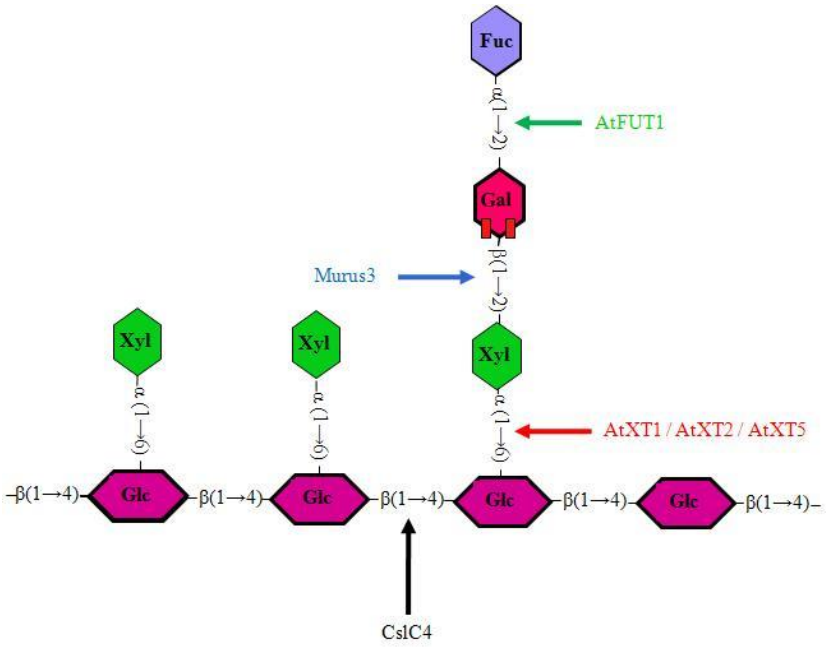


Figure 4.1: Schematic view of a typical xyloglucan structure (type XXFG). Glycosyltransferases enzymes involved in its biosynthesis are indicated with arrows.

It was demonstrated that, *in vitro*, AtFUT1 catalyzes the addition of L-fucose at the 2-position of galactose residue into XyG, using non-fucosylated xyloglucan acceptor and radiolabelled GDP-L-[^{14}C] fucose as the donor (Faik *et al.*, 2000). Afterwards, a genetic screen isolated *mur2* Arabidopsis mutants showing a decrease in fucose content in the cell wall and fine analysis of the mutant cell wall revealed a 99 % reduction in xyloglucan fucosylation for *mur2* plants. Later, mutation in *mur2* was mapped and revealed a mutation in the AtFUT1 gene, demonstrating that AtFUT1 encodes a xyloglucan specific fucosyltransferase (Vanzin *et al.*, 2002b). Moreover, the strong reduction in xyloglucan fucosylation observed in the analysis of mutants (99%) indicates that AtFUT1 is the only fucosyltransferase responsible for xyloglucan fucosylation in Arabidopsis (Reiter *et al.*, 1997, Vanzin *et al.*, 2002b, Perrin *et al.*, 2003). No crystal structure is currently available for AtFUT1 or for any GT belonging to GT37, but fold recognition studies predicted a similar fold (at least for the nucleotide binding region) to the human FUT8 and bacterial NodZ protein, which are α–(1–−>6)-fucosyltransferases belonging to family GT23 (Breton *et al.*, 2006).

The second plant GT selected for this study was the Arabidopsis xylosyltransferase (AtXT1) characterized to be involved in xyloglucan biosynthesis (Cavalier & Keegstra 2006, Cavalier *et al.*, 2008). Heterologous expression of the AtXT1 in *Pichia pastoris* showed an α-

(1→)6-Xylosyltransferase activity *in vitro*, using UDP-Xyl and cellopentaose as donor and acceptor substrates, respectively (Faik *et al.*, 2002). AtXT1 belongs to a small gene family (comprising seven members in Arabidopsis) and which classifies into family GT34. Another gene AtXT2 belonging to this family having 85 % similarity to AtXT1 has been expressed into *Pichia pastoris* and was shown to also encode a α-(1→6)-Xylosyltransferase activity (Cavalier & Keegstra 2006). Recently, an Arabidopsis double mutant KO for both AtXT1 and AtXT2 genes was named "*xxt1-xxt2"*, and demonstrated to lack detectable amount of xyloglucan within its cell wall. The double mutant "*xxt1-xxt2"* showed a severe root hair phenotype (bulging) and lacked detectable xyloglucan at the whole plant level. This study demonstrated that the two genes are partially redundant and required for xyloglucan biosynthesis in Arabidopsis. More recently, a T-DNA insertion in another gene, named AtXT5 was characterized. AtXT5 deficient mutant was consequently named "*xxt5*", and study showed that *xxt5* mutant had shorter root hairs and less xyloglucan quantity in cell wall. Accordingly, authors conclude that AtXT5 encodes another xylosytransferase activity involved in xyloglucan biosynthesis (Zabotina *et al.*, 2008b). Although fold prediction suggested that GT34 enzymes could adopt a GT-A fold, no crystal structure is currently available for enzymes belonging to this GT family. It is therefore challenging to get a 3D structure of at least one enzyme of this family. I carried out the expression of the truncated proteins of both the enzymes: AtFUT1 and AtXT1 in order to produce soluble proteins for the determination of 3D crystal structure of these enzymes.

4.2 AtXT1

4.2.1 Expression of truncated AtXT1-Δ140 in insect cells

Our aim was to express a truncated and soluble form of AtXT1. Protein sequence analysis allowed to delineate the different protein domains of AtXT1 and to define the best truncation site. The use of fold recognition programs such as Phyre (http://www.sbg.bio.ic.ac.uk/~phyre/) and the hydrophobic Cluster analysis method (Gaboriaud *et al.*, 1987) predicted that the catalytic domain would encompass the region [140-461]. The baculovirus/insect cell heterologous expression system was chosen as it was in the past demonstrated to work well with eukaryotic GTs, particularly of plant origin.

A DNA fragment lacking the first 420 bp within the coding region of AtXT1 was generated by PCR using pUNI51-AtXT1 from NASC stock center as a template, comprising the full length cDNA of *Arabidopsis thaliana* AtXT1 gene. The restriction sites *Pst1* and

Kpn1 were added to the amplified sequence through primers at the 5' and 3' end, respectively (Figure 4.2), and used for cloning into insect cells expression vector (pVT-Bac-His1) (Tessier *et al.*, 1991). Using this plasmid, the AtXT1 gene was cloned in frame with the melittin peptide signal, thus allowing the secretion of the recombinant protein in the culture supernatant. In addition, the protein harbors at its N-terminus a His-tag and X-Press tag useful for immunodetection and/or purification of the protein. As shown in Figure 4.2, the truncated protein comprises only one putative N-glycosylation site (at position 137 of amino acid sequence).

```
                atgatagagaagtgtataggagcgcatcggtttcggagattacagagattcatgcgtcaa
                 M  I  E  K  C  I  G  A  H  R  F  R  R  L  Q  R  F  M  R  Q
                gggaaagtgacgattctttgtctcgttctcaccgtcatcgtcttacgtggcacaatcgga
                 G  K  V  T  I  L  C  L  V  L  T  V  I  V  L  R  G  T  I  G
                gccggtaagtttggtacgccggagaaagatatcgaggagatccgtgagcatttcttctac
                 A  G  K  F  G  T  P  E  K  D  I  E  E  I  R  E  H  F  F  Y
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                 W  E  R  Y  K  D  Y  N  L  V  M  H  G  W  N  E  M  V  Y  D
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                 Q  K  N  W  I  G  L  N  T  G  S  F  L  L  R  N  S  Q  W  S
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                 G  K  V  L  T  R  E  L  K  D  R  P  A  F  E  A  D  D  Q  S
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                 A  M  V  Y  L  L  A  T  E  R  E  K  W  G  G  K  V  Y  L  E
                agtggttattacttgcacggttattgggggattttggtagaccggtacgaggagatgatt
                 S  G  Y  Y  L  H  G  Y  W  G  I  L  V  D  R  Y  E  E  M  I
                gagaatcataaaccgggttttggagaccatcggtggccattggttacgcatttcgtcggg
                 E  N  H  K  P  G  F  G  D  H  R  W  P  L  V  T  H  F  V  G
                tgtaaaccgtgcgggaaatttggagattatccggtggaacggtgtctacggcagatggat
                 C  K  P  C  G  K  F  G  D  Y  P  V  E  R  C  L  R  Q  M  D
                agagcgtttaatttcggagacaatcagatccttcaaatgtatggtttcacgcataaatcg
                 R  A  F  N  F  G  D  N  Q  I  L  Q  M  Y  G  F  T  H  K  S
                cttgggagccggcgcgtgaaacccacgcgcaatcagacggataggccgctcgatgccaag
                 L  G  S  R  R  V  K  P  T  R  N  Q  T  D  R  P  L  D  A  K
                gacgagtttgggctgcttcatccgccgttcaaagcggccaagcttagtacgacgacgacgtga
                 D  E  F  G  L  L  H  P  P  F  K  A  A  K  L  S  T  T  T  T  -
                                                                catgctgctgctgcactCCATGGGGC
```

Figure 4.2: Nucleotide sequence and translated sequence of the coding region of AtXT1. Predicted TMD is marked in turquoise, *N*-glycosylation sites with green letters underlined. Forward and reverse primers are added in red and the bold letters in the DNA sequences represent their respective annealing sites. DNA sequence was translated at ExPASy (http://www.expasy.ch/tools/dna.html).

Cloned sequences were further confirmed by sequencing and used for transformation. After transformation and three repeated amplifications in Sf9 insect cells, a titer of $2x10^7$ pfu/ml was estimated for the AtXT1 virus stock. This stock was used for the production of recombinant proteins in Hi-5 cells infected with the recombinant virus at MOI of 5 (5 pfu/cell). Hi-5 cells were typically grown at 27°C and supernatants collected after 4 days post infection. The collected supernatants were clarified by centrifugation and stored frozen until use.

Protein was produced in two different media of production, namely Express Five^M and Excell-405 serum-free media. The recombinant protein was trapped using either UDP-Fractogel or Ni-agarose beads. Bound proteins were analysed by SDS-PAGE and western blot. The calculated size of AtXT1- is 39 kDa. Two protein bands were observed in western blot from the protein production in Hi5-Express medium that could correspond to the non-glycosylated and glycosylated isoforms (Figure 4.3A). Protein yield was higher in Hi5 Excell-405 medium, where we observed a more diffuse band, but two bands can also be clearly seen, particularly when protein is trapped using Ni-agarose beads (as observed in figure 4.3B)..

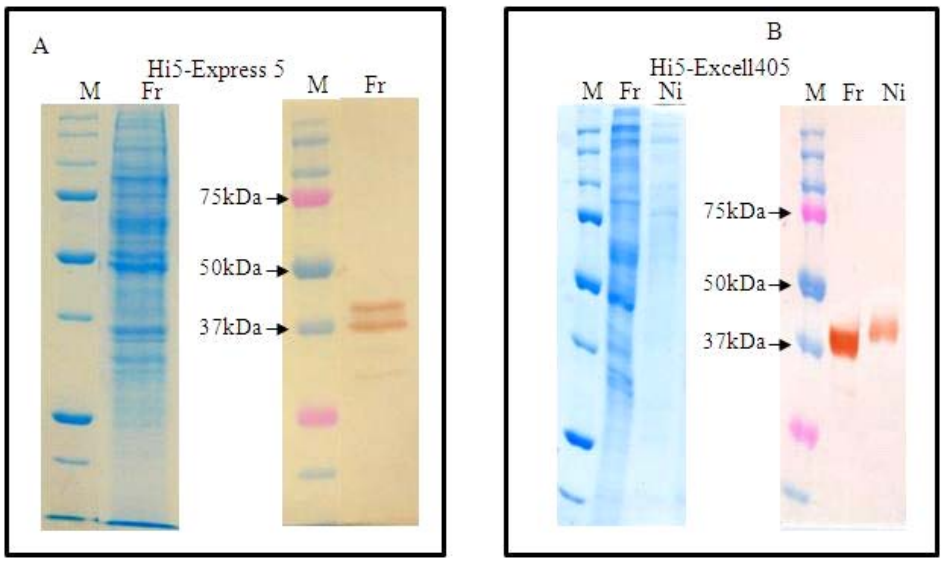

Figure 4.3: SDS-PAGE and Western blotting of recombinant AtXT1- $\Delta 140$ produced into two different serum-free media, Express-Five medium (**A**) and Excell-405 (**B**). Left panels correspond to a Coomassie blue staining of 10 % polyacrylamide gel and right panels to the detection of the recombinant protein using anti-Xpress antibodies. Lane M represents molecular weight markers, lanes Fr and Ni indicate protein bound onto UDP-Fractogel or Ni-agarose beads, respectively.

4.2.2 Xylosyltransferase assay for AtXT1-Δ140

In order to determine the activity of truncated AtXT1-Δ140, xylosyltransferase activity test was performed as described previously for full length AtXT1 (Faik *et al.*, 2002, Cavalier & Keegstra 2006). We tested cellohexaose and celloheptaose as acceptor substrates and UDP-^{14}C Xyl as donor in this assay. Typically, enzyme assays were carried out at 30°C for 1h, in presence of $MnCl_2$ 5 mM. We performed the assay using a "HEPES-Triton-X100" buffer as previously described by Cavalier and colleagues, albeit the use of Triton detergent necessary in their study (expressing a full length AtXT1with transmembrane domain) was probably not necessary in our study, while expressing a truncated version of AtXT1. We observed that heterologously expressed AtXT1-Δ140 did not catalyze the transfer of xylose onto cellohexaose and cellopentaose acceptor substrates (data not shown).

In order to check if the lack of transfer of xylose onto cellohexaose and cellopentaose was due to our experimental conditions, we tested other buffer conditions like Hepes- pH 7 buffer or replace buffer with water, but again no transfer of xylose was detected (Figure 4.4).

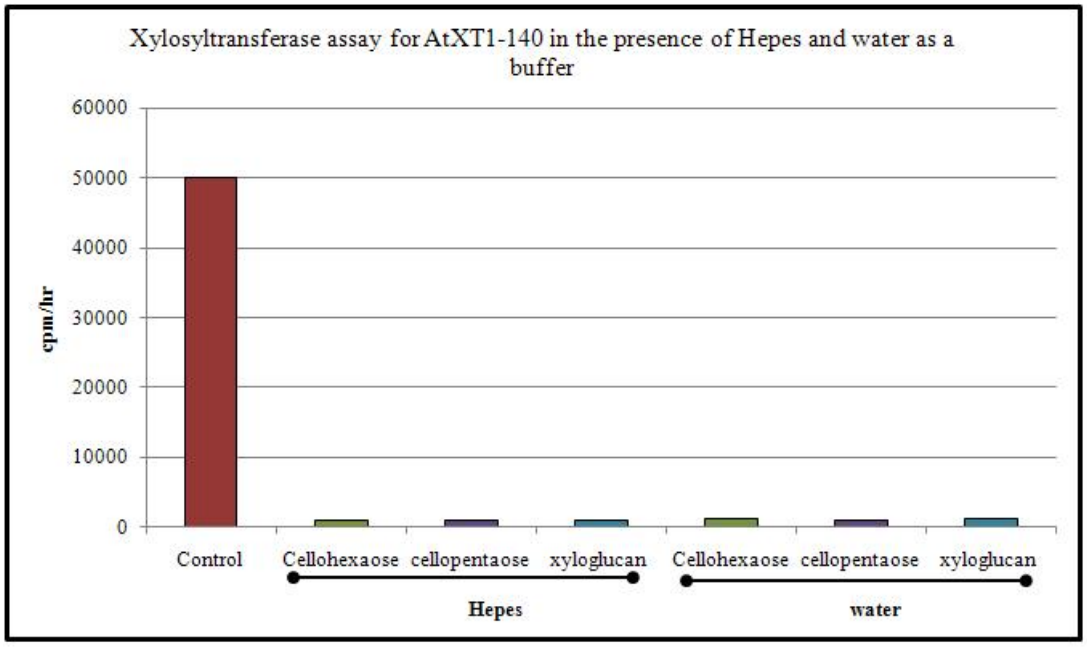

Figure 4.4: Xylosyltransferase assay for truncated AtXT1-Δ140 in the presence of Hepes buffer or water. Cellohexaose and cellopentaose are used as potential acceptors of [^{14}C]-xylose transfer whereas xyloglucan is used as a negative control. The red bar named as 'control represent the total radioactivity (CMP/hr) present in the reaction.

This assay shows that AtXT1-Δ140 is not able to transfer [^{14}C]-xylose onto cellohexaose and cellopentaose acceptor substrate whatever were the experimental conditions (Triton-X100, Hepes buffer or water) (Figure 4.4). From the above results we hypothesized that AtXT1 is not functional after the removal of 140 amino acids because this region may comprise part of the catalytic site or an additional domain necessary for the transfer reaction. In order to check our hypothesis, we analyzed the activity of a less truncated AtXT1-Δ44 protein consisting in the removal of only 44 amino acids which correspond to the cytoplasmic domain and transmembrane region.

4.2.3 Expression of AtXT1-Δ44 in insect cells

In order to generate an AtXT1- Δ44 protein, we amplified a DNA fragment lacking the first 132 bp within the coding region of AtXT1 by PCR, using pVT-Bac1-AtXT1as a template comprising the full length cDNA of *Arabidopsis thaliana* XXT1 gene. The restriction sites *Pst1* and *EcoR1* were added to the amplified sequence through primers at the 5' and 3' end during the PCR reaction. PCR product was cleaved with the restriction endonucleases *Pst1* and *EcoR1*. For further steps of cloning and expression, the same procedure was adopted as described in the section 4.4.1 of this chapter. AtXT1-Δ44 protein was produced in Excell-405 serum-free media. The recombinant protein was trapped using Ni-agarose beads. Bound proteins were analyzed by SDS-PAGE. Western blot analysis was done to check the expression of protein (Figure 4.5). Protein was detected by using the anti-histidine antibody. The calculated size for the protein is 50 kDa. Protein was produced in media of production, Excell-405 serum-free media which is shown in figure 4.5.

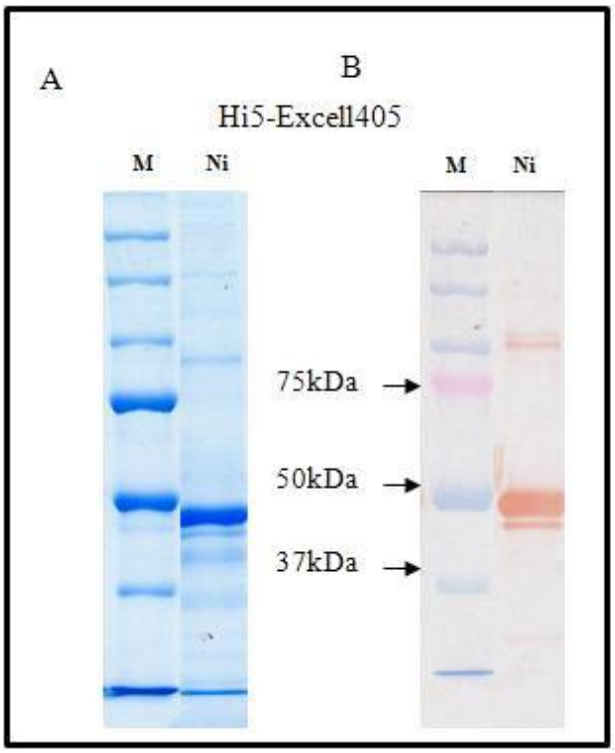

Figure 4.5: SDS-PAGE and Western blotting of recombinant AtXT1-Δ44 produced into two different serum-free media, Express-Five medium (**A**) and Excell-405 (**B**). Left panels correspond to a coomassie blue staining of 10 % polyacrylamide gel and right panels to the detection of the recombinant protein using anti-Xpress antibodies. Lane M represents molecular weight markers, lanes Fr and Ni indicate protein bound onto UDP-fractogel or Ni-agarose beads, respectively.

4.2.4 Xylosyltransferase assay for AtXT1-Δ44

In order to determine the activity of truncated AtXT1-Δ44, xylosyltransferase activity test was performed as described previously for full length AtXT1 (Faik *et al.*, 2002, Cavalier & Keegstra 2006). We tested cellohexaose and celloheptaose as acceptor substrate and UDP-[^{14}C]-Xyl as a donor in this assay. We used the Hepes buffer pH7 and $MnCl_2$ in the assay. We observed that heterologously expressed AtXT1-Δ44 did not catalyze the transfer of [^{14}C]-xylose onto cellohexaose and celloheptaose acceptor substrates (Figure 4.6). The activity level was same as compared to control reaction which lacked the acceptor. We repeated the same experiment and again no activity was observed for AtXT1-Δ44 truncated protein.

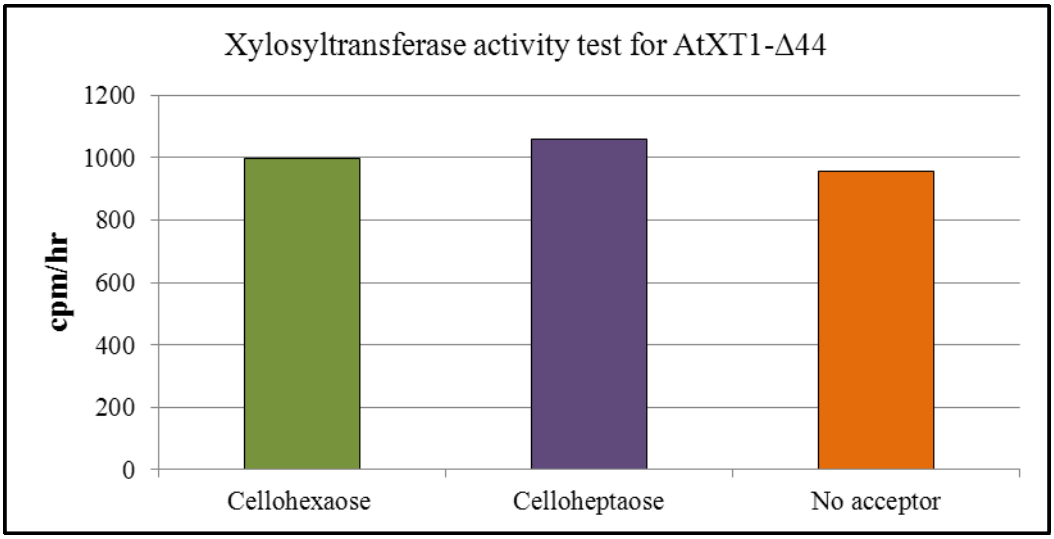

Figure 4.6: Xylosyltransferase assay for AtXT1-Δ44 in the presence of Hepes buffer. Cellohexaose and celloheptaose are used an acceptors. Control reaction lacked the acceptor substrate.

Several explanations could explain the lack of activity for AtXT1-Δ44 and AtXT1-Δ140 proteins: either the full-length form and/or a correct location in the microsomal fraction is required or alternatively a cofactor that could be necessary for activity is missing. Upon the lack of success in the obtention of an active truncated form of AtXT1, we pursue the effort of expressing a plant GT for crystallographic study by expressing truncated form of AtFUT1.

4.3 AtFUT1

4.3.1 Expression of truncated AtFUT1-Δ160 in insect cells

A soluble form of the AtFUT1 catalytic domain was produced. As for AtXT1, the truncation site was determined through protein sequence analysis. HCA, secondary structure prediction and fold recognition analysis suggested a truncation site at a position around 160. This resulted in the deletion of cytoplasmic and a trans-membrane domain as well as a large portion of what is expected to correspond to the stem region. This truncation site also eliminates two putative N-glycosylation sites.

A DNA fragment lacking the first 480 bp (160 amino acids) within the coding region of AtFUT1 was generated by PCR using pENTR-AtFUT1 as a template comprising the full length cDNA of *Arabidopsis thaliana* FUT1 gene. The restriction sites *Pst1* and *Kpn1* were added to the amplified sequence through primers at the 5’ and 3’ end during the PCR reaction (Figure 4.7). PCR product was cleaved with the restriction endonucleases *Pst1* and *Kpn1*. For

further steps of cloning and expression, the same procedure was adopted as described above for AtXT1.

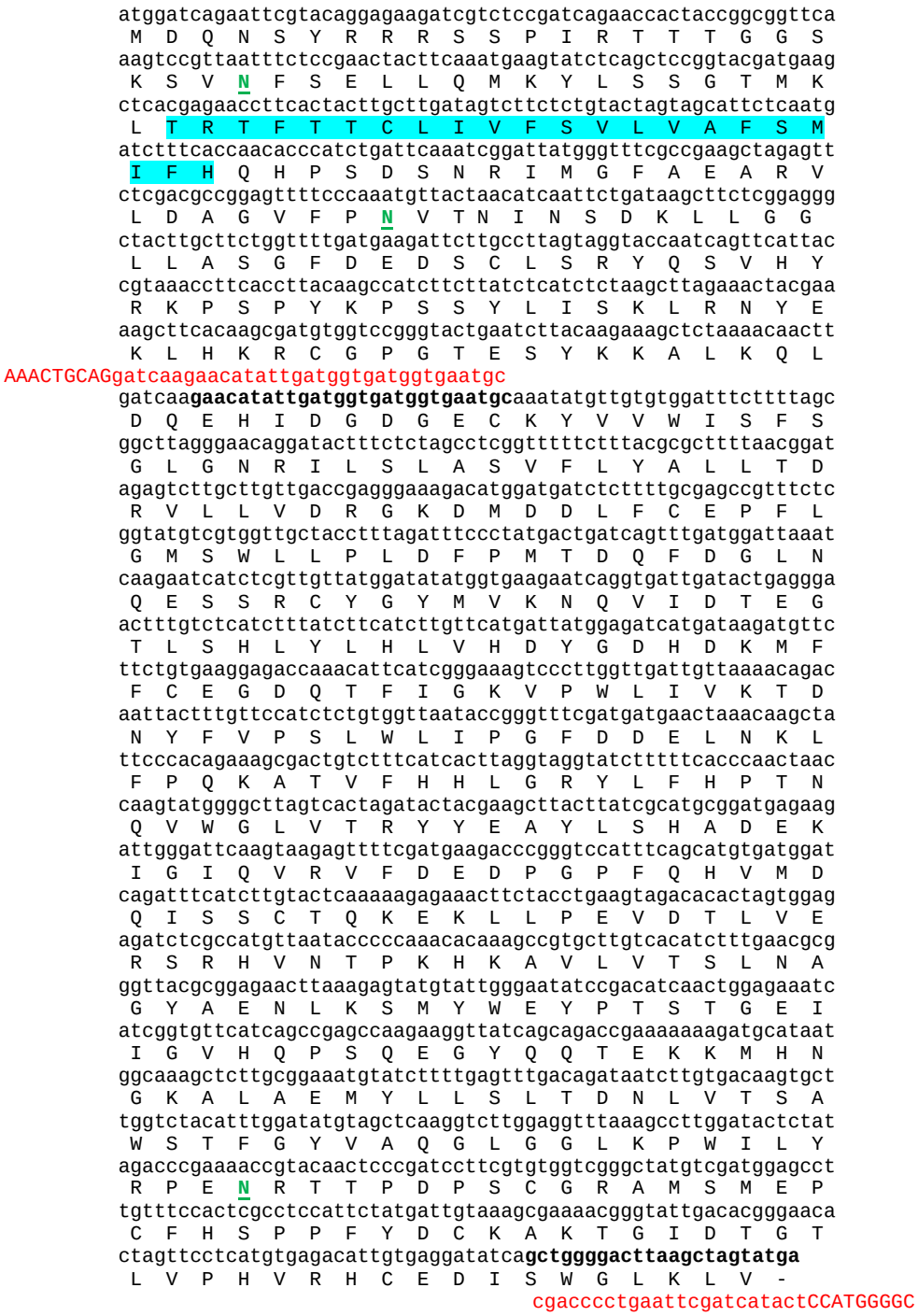

Figure 4.7: Nucleotide sequence and translated sequence of the coding region of AtFUT1. Predicted TMD is marked in turquoise, N-glycosylation sites with green letters underlined. Forward and reverse primers are added in red and the bold letters in the DNA sequences represent their respective annealing sites. DNA sequence was translated at ExPASy (http://www.expasy.ch/tools/dna.html).

The recombinant protein was trapped using either GDP-Fractogel or Ni-agarose beads. Bound proteins were analyzed by SDS-PAGE followed by western blotting. The calculated size of AtFUT1-160 is around 48 kDa. Protein was produced in two media of production, Express Five^{M} and Excell-405 serum-free media which are shown in figure 4.8.

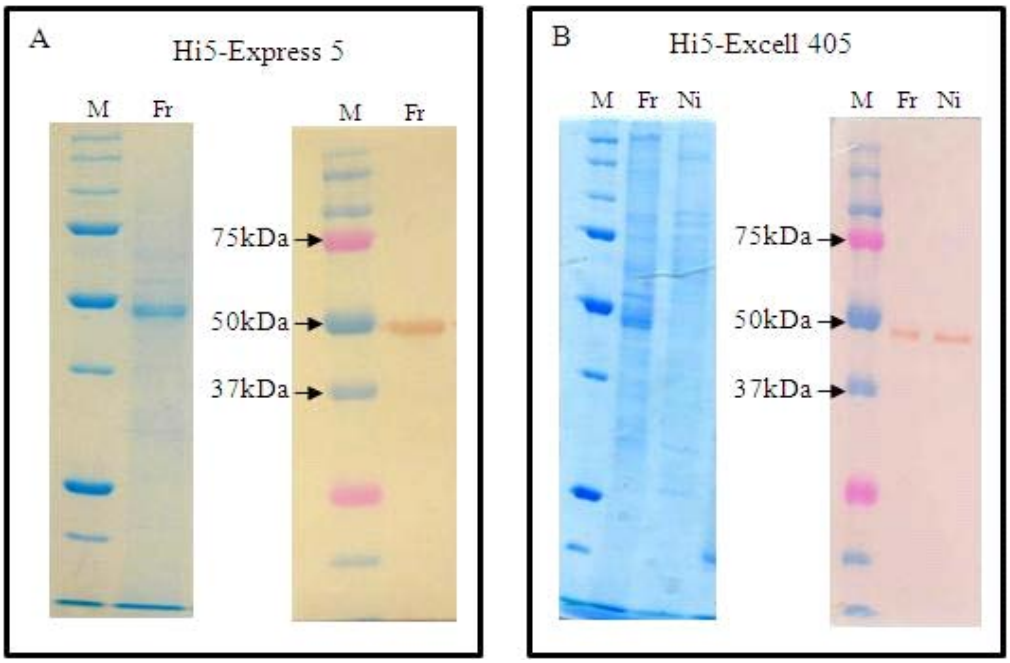

Figure 4.8: SDS-PAGE and Western blotting of recombinant AtFUT1-Δ160 produced into two different serum-free media, Express-Five medium (**A**) and Excell-405 (**B**). Left panels correspond to a coomassie blue staining of 10 % polyacrylamide gel and right panels to the detection of the recombinant protein using anti-Xpress antibody. Lane M represents molecular weight markers, lanes Fr and Ni indicate protein bound onto UDP-fractogel or Ni-agarose beads, respectively.

4.3.2 Fucosyltransferase assay for AtFUT1-Δ160

We tested enzyme activity using radioactive assays because they are highly sensitive as little transfer of radioactive labeled sugar onto acceptor sugars can be detected .To determine the activity of the truncated AtFUT1-Δ160 protein, fucosyltransferase assay for full length AtFUT1 was performed as described previously (Vanzin *et al.*, 2002). We provided the tamarind seed xyloglucan as an acceptor and labeled GDP-[^{14}C]-Fuc as a donor. We observed that in the presence of recombinant protein AtFUT1-Δ160 there is no incorporation of fucose onto xyloglucan acceptor as shown in figure 4.9. Control reaction lacked the acceptor substrate (water instead of xyloglucan).

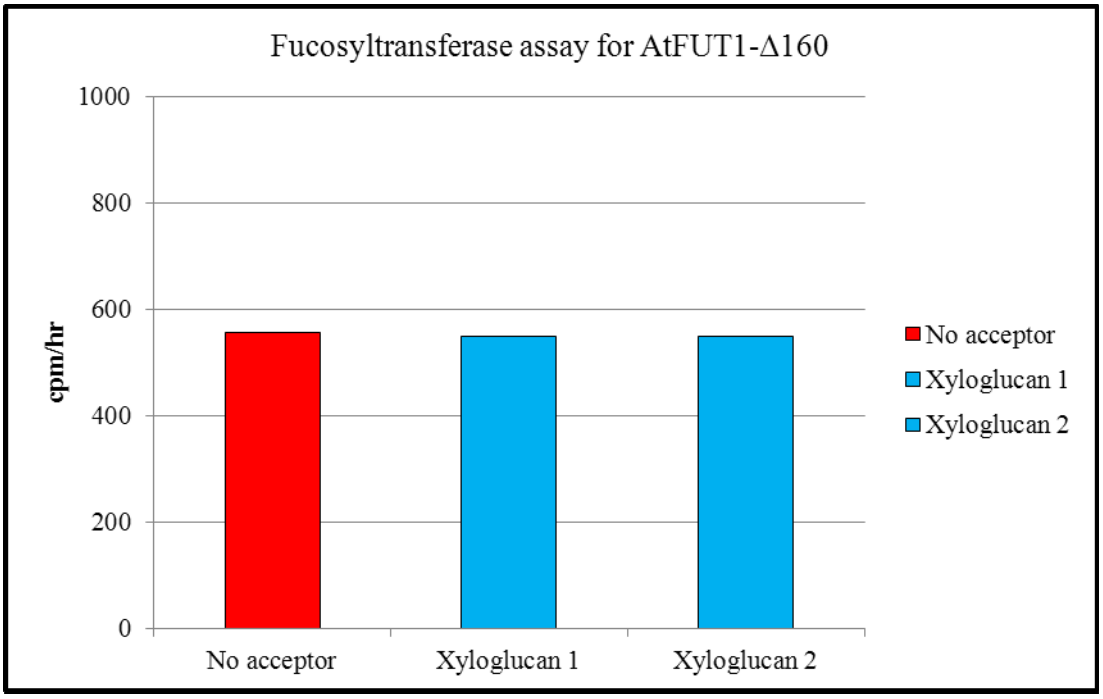

Figure 4.9: Fucosyltransferase assay using GDP-[^{14}C]-Fucose for AtFUT1-Δ160 protein produced in insect cells. Control reaction lacked the acceptor substrate, whereas xyloglucan 1 and xyloglucan 2 represent two independent reactions with the same protein production

This assay showed that truncated AtFUT1-Δ160 was not able to catalyze the transfer of GDP-[^{14}C]-Fuc onto xyloglucan acceptor substrate, hence AtFUT1-Δ160 is not active. Again, several hypotheses can be drawn to explain the lack of activity for AtFUT1-Δ160, one likely explanation would be the removal of part of catalytic site of the enzyme while designing the truncation. We hypothesize that part of the catalytic site of the AtFUT1 lies in the first 160 amino acids of the N-terminus as an explanation for why no activity was observed. In order to confirm this hypothesis, we prepared a second (less truncated) version of AtFUT1 protein which lacked only 68 amino acids from the N-terminal side (named AtFUT1-Δ68).

4.3.3 Expression of AtFUT1-Δ68 in insect cells

In order to express AtFUT1-Δ68 in insect cells, a DNA fragment lacking the first 204 bp within the coding region of AtFUT1 was generated by PCR using pVT-Bac1-AtFUT1 (as a template comprising the full length cDNA of *Arabidopsis thaliana* AtFUT1 gene. The restriction sites *Pst1* and *EcoR1* were added to the amplified sequence through primers at the 5’ and 3’ end during the PCR reaction. PCR product was cleaved with the restriction

endonucleases *Pst1* and *EcoR 1*. For the further steps of cloning and expression of truncated AtFUT1- Δ68, the same procedure was followed as described in section 2 of this chapter. The calculated size of AtFUT1-68 is around 58 kDa. Protein was produced in Excell-405 serum-free media. The recombinant protein was trapped using Ni-agarose beads. Bound proteins were analyzed by SDS-PAGE. Western blot analysis was done to check the expression of protein using the anti-histidine antibody (Figure 4.10).

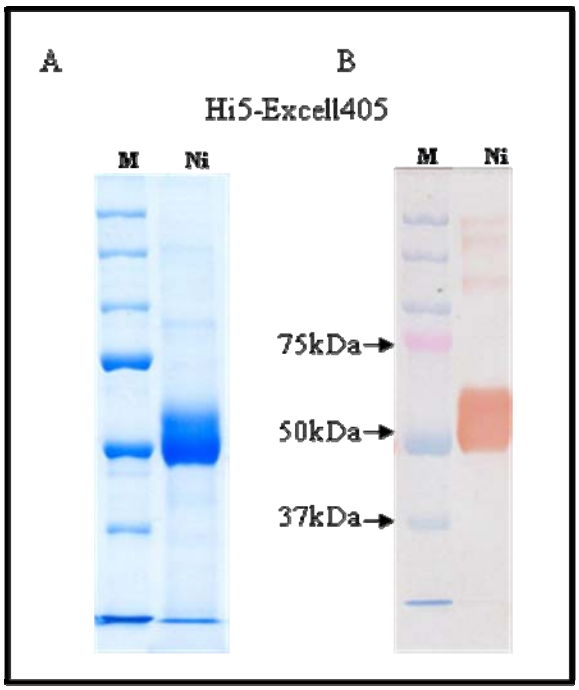

Figure 4.10: Quantitative and qualitative analysis of AtFUT1-Δ68 by PAGE and western blot. (A) Coommasie staining of PAGE, and (B) western blot analysis of truncated AtFUT1-Δ68 produced in Excell-405 media. Lane M represent molecular weight marker, lane Ni indicates protein bound onto Ni-agarose beads.

4.3.4 Fucosyltransferase activity test for AtFUT1-Δ68 protein produced in insect cells

In order to check whether truncated AtFUT1-Δ68 protein produced in insect cells is active, fucosyltransferase assay was performed as discussed above. We used the crude protein for this activity assay because the radioactive assay is very sensitive. We observed that AtFUT1-Δ68 was able to transfer fucose from GDP-[^{14}C]-Fuc onto xyloglucan acceptor (Figure 4.11). We performed two independent reactions one for xyloglucan acceptor and a second for galactoglucomannan (GGM). In control reaction which lacked the acceptor, no transfer of fucose was observed. Similarly for galactoglucomannan which is not the right

acceptor for fucosyltransferase enzyme, no activity was observed. This assay confirmed that truncated protein AtFUT1-Δ68 which lacked transmembrane domain is active.

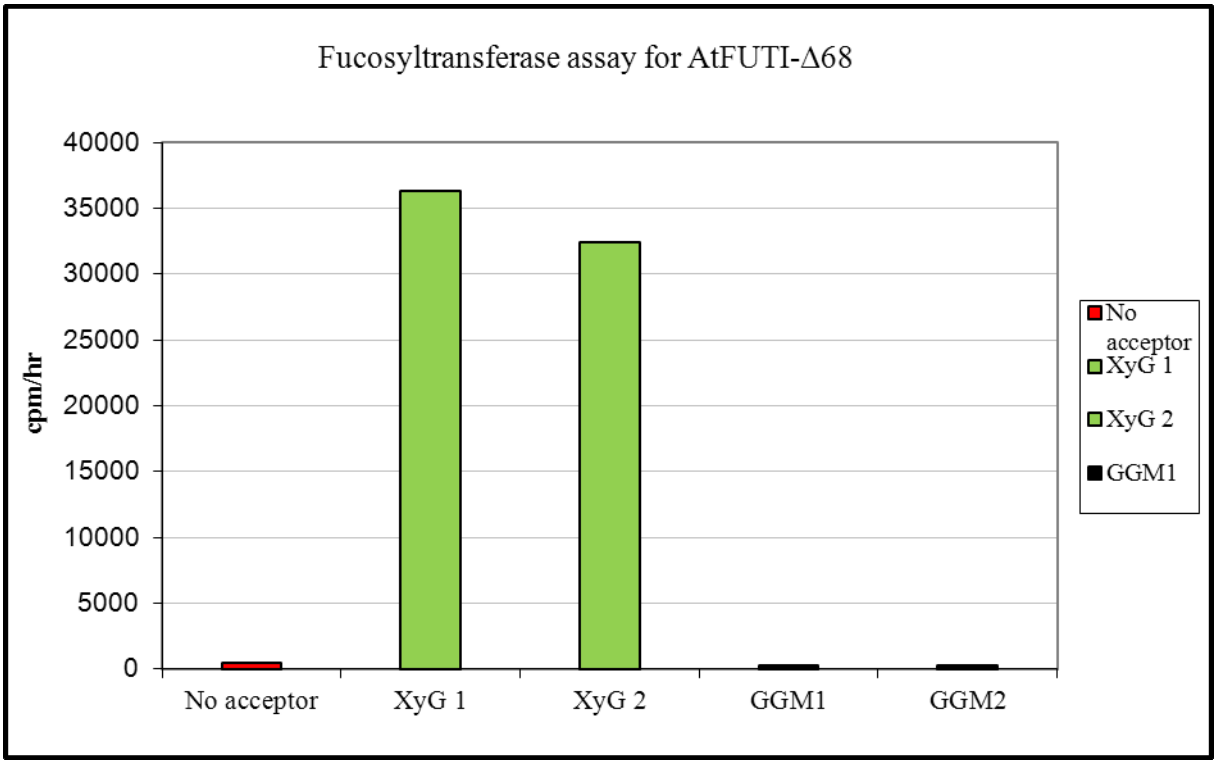

Figure 4.11: Fucosyltransferase assay for truncated AtFUT1-Δ68 produced in insect cells. xyloglucan acceptor substrate was used for this assay whereas galactoglucomannan is used as a control which is not a good acceptor for fucosyltransferase enzyme. Control reaction lacked the acceptor substrate. Two independent reactions were done with xyloglucan and galactoglucomannan acceptor substrates which are indicated by XyG1 and 2 and GGM1 and 2 respectively.

In order to confirm these results, we performed activity test of AtFUT1-Δ68 while expressing the protein in *Pichia pastoris*, another expression system which is not expensive and easy to carry out for the expression of recombinant proteins.

4.3.5 Cloning and expression of truncated AtFUT1-Δ68 in *Pichia pastoris*

Pichia pastoris revealed to be also an excellent heterologous expression system for plant GTs. We therefore decided to turn on this system that was recently set up in the laboratory. A truncated form of AtFUT1 (AtFUT1-Δ68) was produced by PCR using pENTR-AtFUT1 as a template which comprises the full length cDNA of *Arabidopsis thaliana* fucosyltransferase gene. This construct AtFUT1-Δ68 lacks the nucleotides encoding the first 68 amino acids of AtFUT1.

The 1575 bp coding region corresponding to the truncated soluble form of AtFUT1-Δ68 was obtained using the forward and reverse primers. These primers were designed to generate the restriction sites *EcoR1* and *Not1* at the 5' and 3' of the amplified sequence, respectively.PCR product was cleaved with the restriction endonucleases *EcoR1* and *Not1*. DNA was isolated and inserted by ligation into the pPICZαHis flag vector, digested with the same enzymes. Afterwards the ligated plasmid was transformed into *Pichia pastoris* GS115. Plasmid insertion was checked by PCR by using gene specific primers and was further confirmed by sequencing. Protein was produced in BMMY media and expression was induced for 5 days with methanol. Recombinant protein was secreted into media and analyzed by SDS-PAGE gel following Coomasie blue staining.

4.3.6 Fucosyltransferase activity test for AtFUT1-Δ68 protein produced in *Pichia pastoris*

AtFUT1-Δ68 protein was expressed in *Pichia pastoris*, recombinant protein was secreted into the culture media (for details see materials and methods) and collected from two AtFUT1-Δ68 expressing *Pichia pastoris* clones, named as AtFUT1-A and AtFUT1-B. Activity test was performed as previously described for full length AtFUT1 (Vanzin *et al.*, 2002). We collected the secreted protein after 3 days and 5 days of expression from *Pichia pastoris* cells. In the fucosyltransferase assay, we have observed that in the presence GDP-[^{14}C]-Fuc donor, AtFUT1-Δ68 catalyzed the transfer of fucose onto tamarind xyloglucan acceptor (Figure 4.12), whereas no transfer of fucose was observed in the presence of galactoglucomannan as an acceptor. Similarly no transfer was observed in the control reaction which lacked the acceptor.

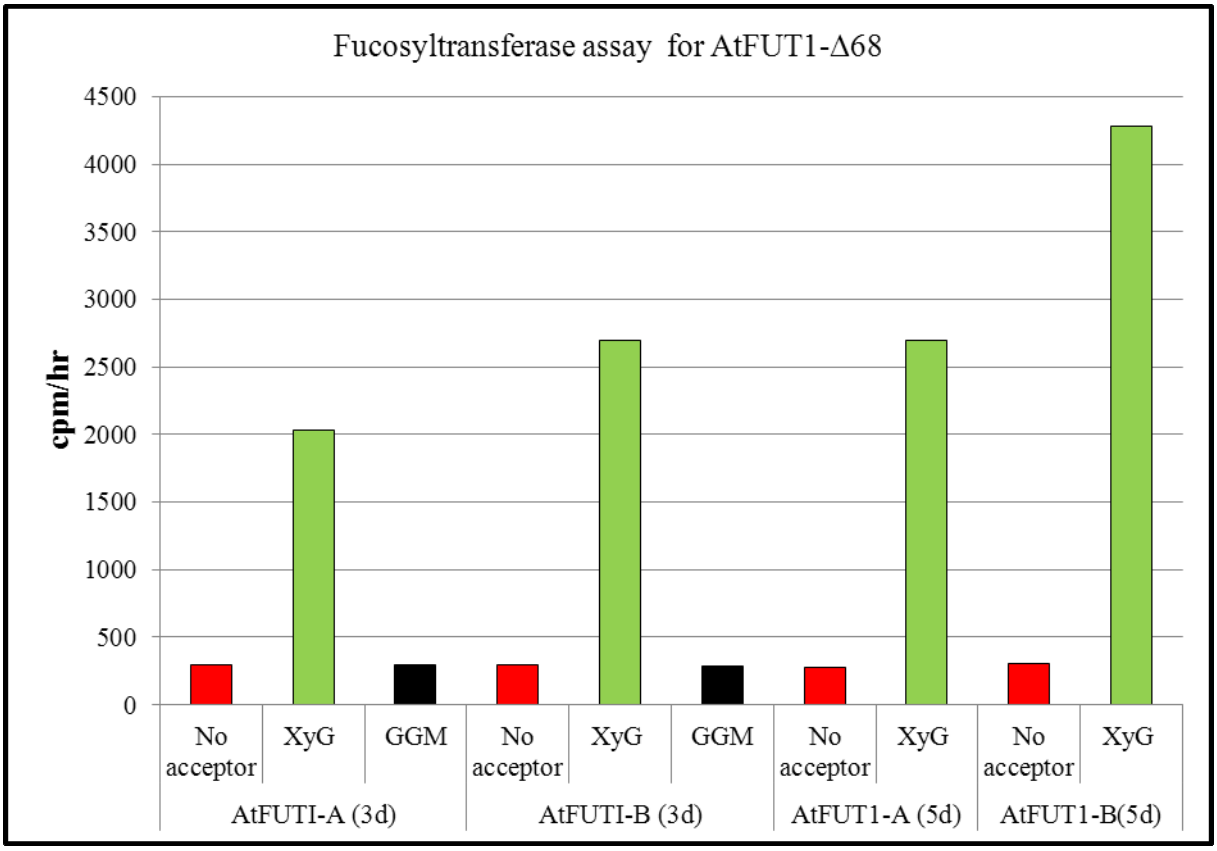

Figure 4.12: Fucosyltransferase assay for truncated AtFUT1-Δ68 in the presence of xyloglucan acceptor substrate whereas galactoglucomannan is used as a negative control for fucosyltransferase activity. A second control reaction lacking the acceptor substrate was also included. Activity was checked for two AtFUT1-Δ68 expressing Pichia clones after 3 days and 5 days of protein induction.

We observed that the extension of the induction from 3rd to 5th day may increase protein activity (Figure 4.12, clone Pichia-AtFUT1-B), possibly as a consequence of protein accumulation. It is noteworthy that AtFUT1-Δ68 protein secreted into media was present but diluted in an important volume of medium. To overcome this difficulty, we used Vivaspin column to concentrate 15 mL of medium to 1mL. This concentrated medium was then used to test activity and we observed a 10 fold increase in the activity of AtFUT1-Δ68, as expected (Figure 4.13). Accordingly, no activity was observed from the filtrate medium and no transfer of fucose was observed in the presence of galactoglucomannan acceptor (negative control). Another negative control was performed with an assay lacking the acceptor, no activity was observed. So these results confirmed that truncated AtFUT1-Δ68 catalyzed the incorporation of [^{14}C]-fucose onto xyloglucan acceptor substrate.

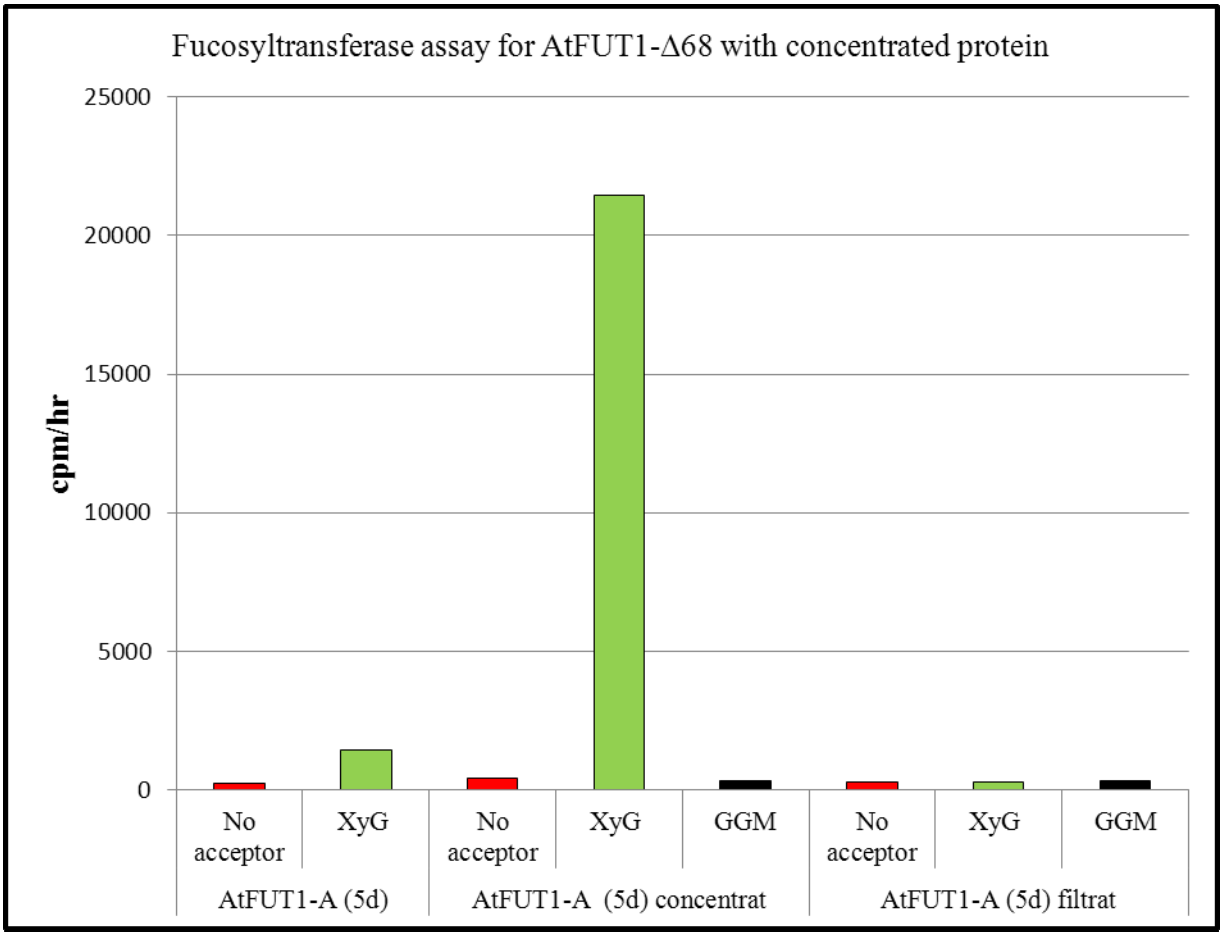

Figure 4.13: Fucosyltransferase assay for "concentrated" AtFUT1-Δ68 protein containing medium, in the presence of xyloglucan (acceptor) and galactoglucomannan is (negative control) for fucosyltransferase activity. Another control reaction lacked the acceptor (water). Protein AtFUT1-Δ68 was isolated after 5 days of expression in *Pichia pastoris* and used for activity assays.

In summary, the AtFUT1-Δ68 protein is active and able to transfer fucose onto xyloglucan acceptor substrate. However, when western blot analysis was carried out and total proteins were transferred, for unknown reasons, no signal was detected at the expected size of AtFUT1-Δ68. One possible explanation could be a degradation of the histidine tag during protein accumulation.

4.4 Enzyme kinetics of AtFUT1-Δ68

4.4.1 Initial rate analysis of AtFUT1-Δ68

After demonstrating the activity of AtFUT1-Δ68, we analyzed the kinetic parameters of fucosyltransferase regarding the xyloglucan acceptor. Biochemical characterization of AtFUT1-Δ68 activity was realized using AtFUT1-Δ68 expressed in insect cells. We determined the effects of changing time of incubation on fucose addition onto xyloglucan acceptor substrate through radioactive assays. Radioactive assays involve the incorporation of radioactivity to measure the amount of product made over time. We determined the initial rate of reaction by drawing the graph from two independent series values. We checked the effect of time on product formation each 5 minutes. We observed that the maximum product is formed after 30 minutes and is in linear range indicative of initial rate conditions. We selected the initial rate of reaction 30 minutes because the incubation time should be long enough to permit a moderate amount of product to be formed and to make the error in timing insignificant and reproducible.

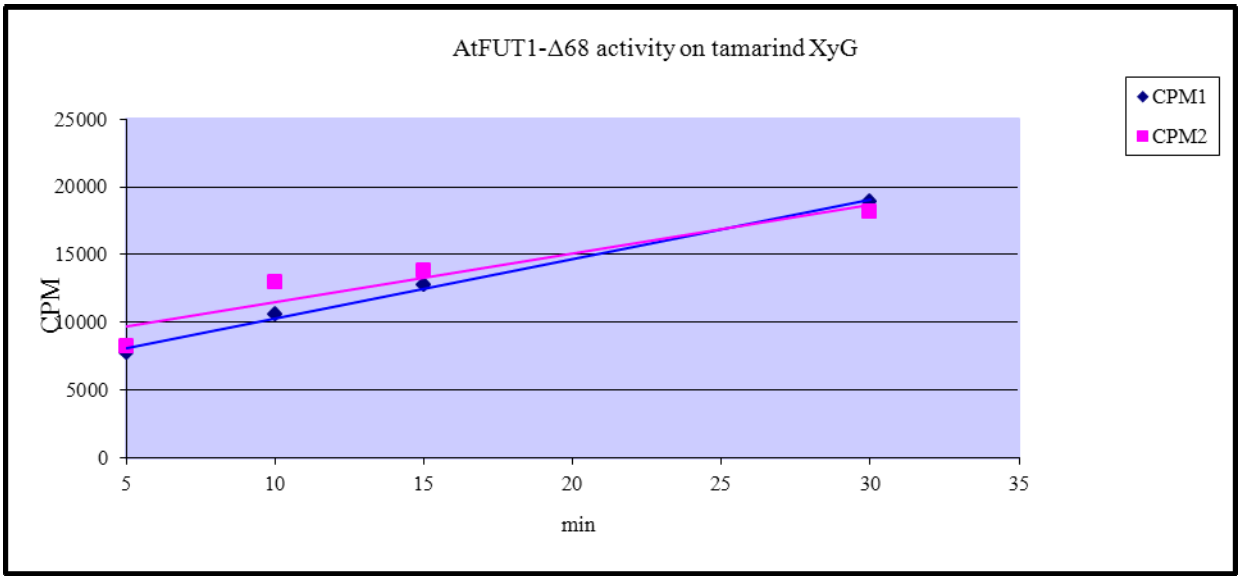

Figure 4.17: Initial rate incorporation of GDP-[^{14}C]-Fuc into tamarind XyG using culture medium from Hi5cells expressing AtFUT1-Δ68, with fixed concentration of GDP-Fuc (20µM) and XyG (1mg/mL). Data from 2 series of identical experiment were plotted.

4.4.2 Determination of K_m and V_{max} of AtFUT1-Δ68

Michaelis-Menten K_m and maximal reaction rates V_{max} were determined for AtFUT1-Δ68 express in insect cells, by varying the concentration of acceptor xyloglucan substrate with a fixed concentration of GDP-Fucose. Results showed that the AtFUT1-Δ68 has a K_m value of 0.72 mg/mL (~ 0.62 µM) in good accordance with the K_m (0.46 mg/mL) value obtained by Faik and coll. (2000) while studying activity of full length XyG fucosyltransferase from pea (PsFUT1). It is intersting to note that in case of XyG acceptor, K_m is relatively low because of numerous acceptor sites available on the acceptor molecule. V_{max} of AtFUT1-Δ68 produced in insect cells was determined at 12,2mM/h/mg of total protein, which appeared significatively lower than PsFUT1 (200mM/h/mg) but could be explained by various factors such as the protein truncation or the xyloglucan fucosyltransferase from Arabidopsis being less active than its counter-part in *Pisum sativum*. Efforts have been undertaken to purify AtFUT1-Δ68, and such kinetics study could then be completed unambiguously.

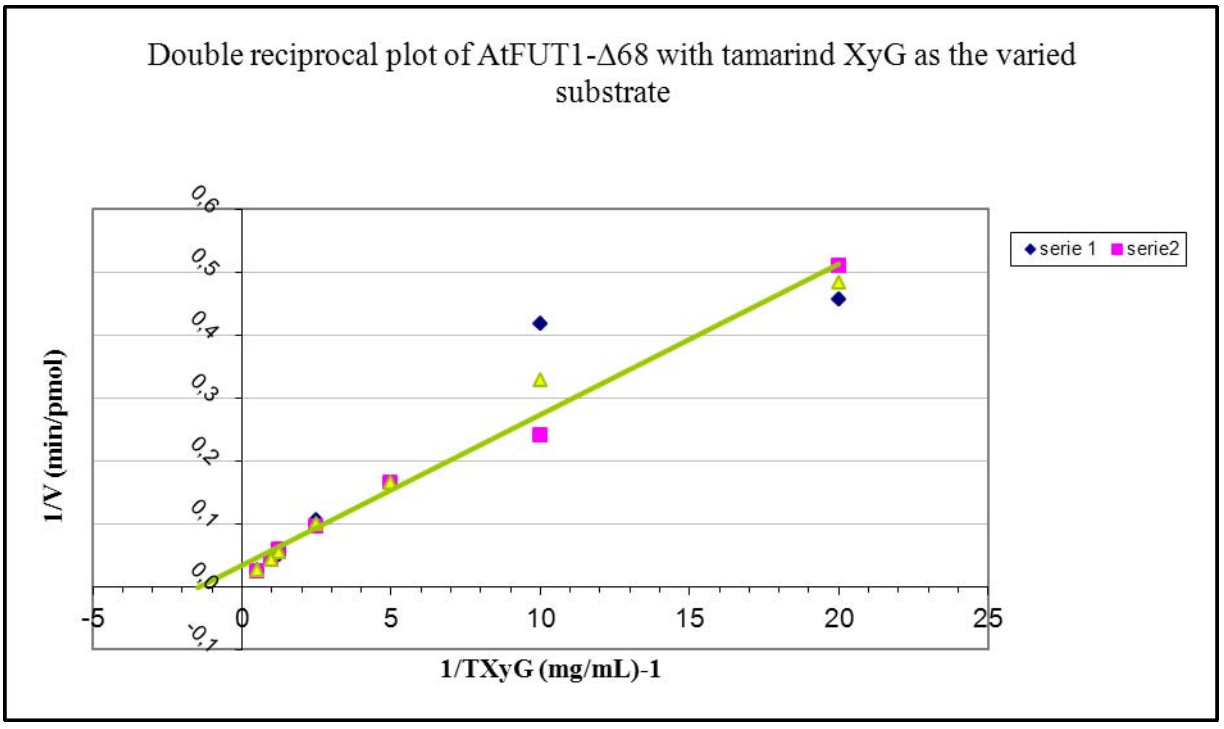

Fig 4.18: Double-reciprocal plot of the initial rate reaction with tamarind xyloglucan as the varied substrate. Data presented are 2 series of identical experiment using culture medium from Hi5cells expressing AtFUT1-Δ68 protein with fixed concentration of GDP-Fuc (100µM). XyG concentration were as followed (0.05 ; 0.1 ; 0.2 ; 0.4 ; 0.8 ; 1 and 2 mg/mL).

4.5 Development of a non-radioactive activity assay for AtFUT1-Δ68

We sought to develop non radioactive assays for the determination of activity of truncated AtFUT1-Δ68, as a proof of concept of this approach for characterizing GT activities in general. Indeed, it is not possible to characterize GTs using radioactive assays when radioactive NDP-sugars are not commercially available. In the case of AtFUT1-Δ68, the methodology is based on the enzymatical digestion of the acceptor molecule (tamarind xyloglucan) when the assay reaction is stopped, labeling with a fluorophore and the characterization of the labeled oligosaccharides using fluorophore-assisted polyacrylamide carbohydrate gel electrophoresis (FACE) or mass spectrometry (MALDI-TOF-MS) (Figure 4.14). Both methods were successfully tested for the characterization of AtFUT1-Δ68. For this experiment, acceptor xyloglucan in assay reactions was digested with commercial endo-cellulase (EG II) from *Trichoderma longibrachiatum*.

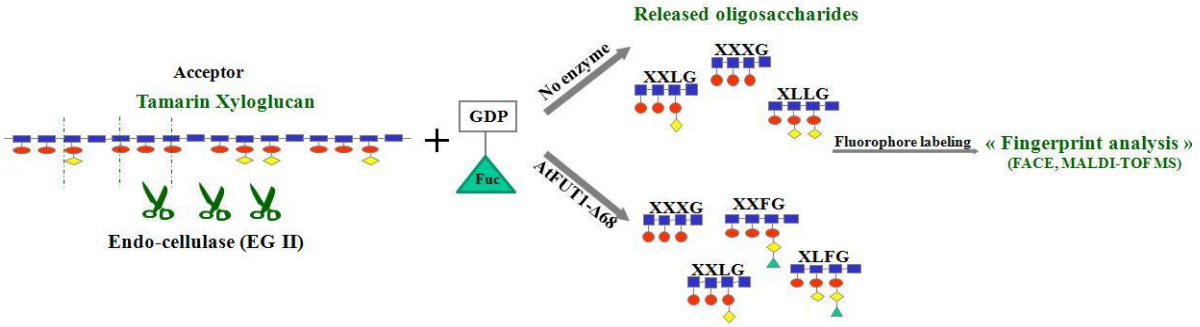

Figure 4.14: Schematic view of non-radioactive assay for AtFUT1-Δ68 activity. In a typical reaction Tamarind xyloglucan (acceptor molecule) and GDP-Fuc were incubated in the presence or absence of enzyme. Then the reaction mixture was digested with endo-cellulase (EG II). The released fragments were labeled with a fluorophore (ANTS) and analysed by FACE.

4.5.1 Fluorophore-assisted polyacrylamide carbohydrate gel electrophoresis (FACE)

Non radioactive fucosyltransferase assay was performed on using xyloglucan acceptor and GDP-L-Fuc for two recombinant protein AtFut1-Δ68 (active protein) or AtXT1-Δ44 (negative control). Another control reaction lacked the enzyme. In this assay we provided the

cold GDP-L-Fuc to the enzyme (for details see materials and methods 6.4.2). The reaction was performed in the presence of Hepes and $MnCl_2$. After 2 hours of incubation, 2µL of endoglucanase was added and put at 30°C for 1h. Samples were derivatized after concentration under N_2 (see materials and methods). Depending on labeling intensities on a first ANTS (8-Aminonaphthalene-1,3,6-TriSulfonate) gel, loading quantity was adjusted for the second gel.

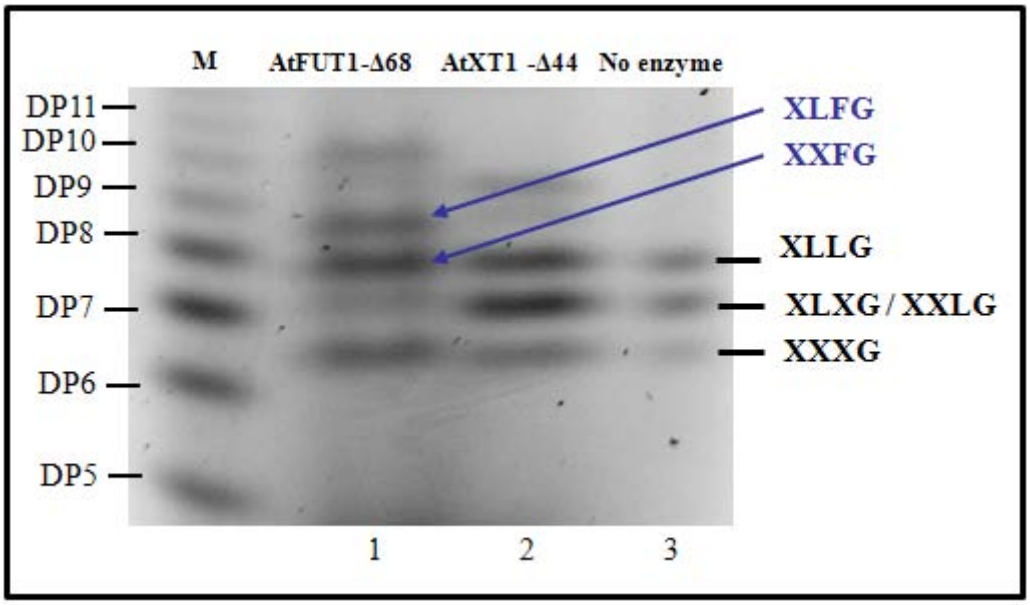

Figure 4.15: FACE profile of endoglucanase generated xyloglucan fragments. Lane M shows the maltodextrine ladder which is derivatized using ANTS. Other lanes show the XyG fragments obtained upon incubation of XyG in the presence of AtFut1-Δ68 protein, AtXT1-Δ44 protein and a negative control without the enzyme. Similarly lane 3 which lacked the AtFut1-Δ68 no activity was observed.

We use maltodextrine ladder (dp5 to dp11) which was also derivatized using ANTS. The data shows that in the presence of AtFUT1-Δ68 an extensive fucosylation of xyloglucan occurred (at the level of detection). Lanes 2 and 3 show characteristics mobility from tamarind XyG oligosaccharides whereas lane 1 shows the presence of fucosylation (higher mobility) which is shown in figure 4.15 by arrows. We observed that in the reaction containing AtFut1-Δ68 enzyme, xyloglucan fragments that were released correspond to XXXG, XLXG, and XLLG fragments characteristics of non-fucosylated XyG from tamarind seeds, but additionally two new structures with reduced electrophoretic mobility were identified which corresponds to XXFG and XLFG fragments. This assay confirmed that truncated AtFUT1-Δ68 is able to transfer fucose onto xyloglucan acceptor substrate.

4.5.2 Matrix Assisted Laser Desorption Ionization Time Of Flight (MALDI-TOF MS) analysis

MALDI-TOF MS is a fast and reproducible qualitative technique which could be used to determine the proportion of different oligosaccharides present in a mixture. The reaction mixture which contained cold GDP-Fuc (donor), xyloglucan substrate (acceptor) and AtFUT1-Δ68 enzyme was incubated for two hours for the fucosyltransferase reaction to take place. In parallel, a control reaction which lacked AtFUT1-Δ68 enzyme and another control reaction which contained AtXT1-Δ44 in place of AtFUT1-Δ68 enzyme were also performed. All the above described reactions were subjected to endoglucanase enzymatic digestion. Endoglucanase cleaves the glucosidic bond of the xyloglucan and results in the production of oligosacharides. After digestion, the released oligosaccharide fragments were labeled with 2-aminobenzamide (2-AB, see material and methods) easier to desorb and easily detectable by MALDI-TOF-MS. Mass spectra of the labeled xyloglucan fucosylated and unfucosylated (control reaction) oligosacharides were performed. As MALDI-TOF-MS analysis of 2-AB labeled neutral oligosaccharides allowed the detection of $[M + Na]^+$ in the positive-ion mode.

As expected from FACE characterization of AtFut1-Δ68 enzyme assay, MS analysis showed two new peaks, one with a mass of 1513.63 where fucose is added onto XXLG then producing XXFG fragment, and a second new peak with the mass of 1675.68 where fucose is added onto XLLG, then producing XLFG fragment in the presence of AtFut1-Δ68 enzyme (Figure 4.16A). This mass exactly corresponds to the mass which is calculated after the addition of fucose. This assay showed us that AtFut1-Δ68 is able to transfer fucose onto xyloglucan acceptor substrate. This is the definitive proof of its activity. While in the absence of enzyme, only peaks for XXXG, XLXG and XLLG were observed and no peaks were observed for fucosylated xyloglucan fragments (Figure 4.16B). Similarly no peaks for fucosylated xyloglucan fragments were observed in the control reaction in the presence of AtXT1-Δ44 (Figure 4.16C).

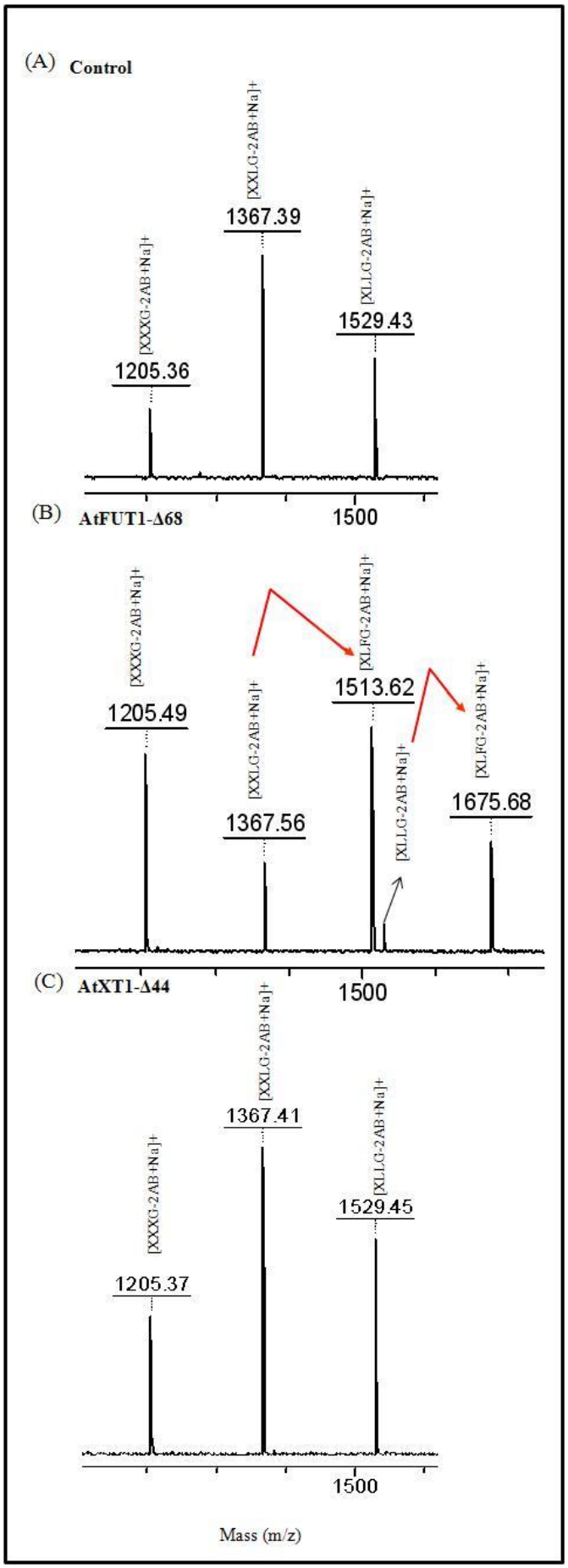
(A) Control
[XXXG-2AB+Na]+
1205.36
[XXLG-2AB+Na]+
1367.39
[XLLG-2AB+Na]+
1529.43
(B) AtFUT1-Δ68
[XXXG-2AB+Na]+
1205.49
[XXLG-2AB+Na]+
1367.56
[XLFG-2AB+Na]+
1513.62
[XLLG-2AB+Na]+
[XLFG-2AB+Na]+
1675.68
(C) AtXT1-Δ44
[XXXG-2AB+Na]+
1205.37
[XXLG-2AB+Na]+
1367.41
[XLLG-2AB+Na]+
1529.45
1500
Mass (m/z)

Figure 4.16: MALDI-TOF MS analysis of endoglucanase digested fragments of xyloglucan for fucosyltransferase activity. A. Mass spectra of xyloglucan in the presence of AtFUT1-Δ68 enzyme. The mass increment was observed in the peaks due to the addition of fucose. B. Mass spectra of xyloglucan in the absence of AtFUT1-68 enzyme. No addition of fucose was observed when the reaction lacked the enzyme. C. Mass spectra of xyloglucan in the presence of AtXT1-Δ44 which is used as a control. Similarly in the control reaction, the addition of fucose was not observed.

4.5.3 Conclusion

In this chapter we have carried out the expression of truncated versions of both AtFUT1 and AtXT1 proteins in order to determine the 3D-structure of these GTs using x- ray crystallography. Unfortunately, none of the truncated version of AtXT1 proteins (both AtXT1-Δ140 and AtXT1-Δ44) that were expressed in insect cells happened to be active. The lack of activity for AtXT1-Δ44 and AtXT1-Δ140 could have numerous reasons; from the lack of the transmembrame domain important for protein folding and activity or the lack of a necessary cofactor that would be missing in our assays.

On the other hand, AtFUT1-Δ68 was successfully expressed either in insect cells or *Pichia pastoris*, and was shown active in both systems. Thus, it was possible to determine the kinetic parameter of AtFUT1-Δ68, and to compare these parameters with the one previously obtain for the characterization of the pea xyloglucan fucosyltransferase (PsFUT1). AtFUT1-Δ68 was additionally demonstrated functional over non-fucosylated xyloglugan oligosaccharides using a non-radioactive assay (FACE). Analysis of the Fluorophore-assisted polyacrylamide carbohydrate gel electrophoresis (FACE) experiments by mass spectrometry (MALDI-TOF-MS) offer the opportunity to do product characterization of the fucosylated XyG oligosaccharides, and thus confirm the protein activity. Actually, efforts are underway to purify the AtFUT1-Δ68 protein using affinity chromatography on nickel column, in order to crystallize the protein and perform a 3D-structure study of a first plant GT.

Chapter 5

General discussion and perspectives

5 General Discussion and perspectives

Cell walls play many important roles in defining the unique biology of plants; they also have practical applications as a feedstock, for biomaterials and for the production of biofuels. Cell walls also have strong economical interests: i.e. 40,000 tons of pectins are produced every year to be used in food industry, and some pectic polymers are studied as pharmaceuticals for prostate cancer treatment (Jackson et al., 2007). Despite the fundamental and practical importance of cell walls, far too little is known about the biosynthesis of the macromolecular components that comprise them. Unfortunately, this lack of knowledge prevents any manipulation of the plant biosynthetic pathway responsible for cell wall elaboration by plant, and thus any optimization of the biomass products for human applications. However as stated recently by Pauly and Keegstra (2008): “the natural variability in wall compositional quantity and quality suggests that there is an opportunity for altering the abundance of specific wall components without compromising the life cycle of a plant”. Thus a comprehensive understanding of polysaccharides biosyntheses by glycosyltransferases (GTs) is required for the development of plant with optimized biomass contents for human uses.

My Ph.D. work at CERMAV fits this goal, as we aim to understand how polysaccharides are synthesized by GTs, delivered to the cell surface, incorporated into the plant cell wall matrix, and how these processes are regulated. We postulated that one likely hypothesis for the lack of success in the identification of pectin biosynthetic enzymes relates to the fact that all plant glycosyltransferases have not been annotated yet in plant genome, and thus could not be studied. Accordingly, Hansen and Coll. (2009) developed a novel method where they have used bioinformatics, seeking Arabidopsis genome for novel glycosyltransferases candidate genes and identified a new family of genes called “NGT” (Novel Glycosyltransferase) by using bio-informatics strategy (Hansen et al., 2009). This method led to the identification of more than 150 candidate genes. Among them, 24 were considered as strong candidates that should be further investigated since known GT signatures were clearly identified. More specifically, one putative GT identified displayed a fucosyltransferase signature (and was named NGT1 for “Novel GlycosylTransferase 1”),

whereas the 23 other gene were related and belong to the "Domain of Unknown Function 266" (DUF 266) family (Hansen *et al.*, 2009).

The first work I took over in the lab was the characterization of T-DNA Arabidopsis mutants from the NGT family that were ordered. The genetic characterization of 35 mutant lines was carried out and permit after PCR screening the isolation of 16 homozygous mutant lines from NGT family. These homozygous mutant lines have been used in a developmental study seeking abnormal growth characteristics or unusual organ development that could be indicative of defect in the way cell wall was laid down in these mutants. Peculiar but tenuous phenotype could be identified for some of the lines such as narrow leaves during early development for *ngt1-1* and *ngt1-2* mutants (or mutants altered for At5g14550 gene; coded "P" in this manuscript). However, many homozygous mutants that have been characterized during the first part of my work have not been analysed specifically for this trait which open perspectives for other students in the lab as the methodology developed for leaf area quantification can easily be duplicated to all the NGT mutant lines.

The NGT family comprises 24 genes that are considered strong candidates to encode GT activity on the basis of clear GT signatures that have been identified in their sequences with high similarity; but, 24 candidate genes appeared an overwhelming number of genes to characterize. As this project aim was mainly to determine whether or not some (or none) of the gene identified using bioinformatics encode a GT activity, and thus validate our strategy of GTs identification, I focused on the characterization of one gene At5g28910 (NGT1).

The identification of homozygous mutants for the DUF266 gene family was not carried out with much detail during the course of my Ph.D. but it would be a valuable work for the forthcoming researchers and students in the lab. Noticely, the extensive characterization of T-DNA mutant lines that I carried out should save time in the future for such work to be achieved. Heterologous expression would also be very useful for the functional characterization of the At5g14550 gene but it was not carried as we focused on NGT1 activity characterization and because the unavailability of At5g14550 cDNA would have delayed such study. However, At5g14550 cDNA was recently added to the NASC, and heterologous expression of this DUF266 gene should be carried out to test GT activity for the protein.

After, the characterization of T-DNA lines for NGT family, we decided to focus our functional genomic study on NGT1 gene for which a putative function was assigned, providing a testable hypothesis for protein activity tests. Moreover, as two homozygous mutant lines *ngt1-1* and *ngt1-2* were characterized, we were able for this locus to study the impact of NGT1 alteration on cell wall polysaccharides content. During the course of our study, several lines of evidences have shown that NGT1 gene was either directly or indirectly involved in cell wall biosynthesis. Phenotypic studies have shown that leaves of mutant plants *ngt1-1* and *ngt1-2* are smaller than WT plants. This observation is interesting as cell wall mutant plants have often shown various developmental growth defects (Scheible and Pauly, 2004). In order to check the growth defects in our mutant lines, leaf area of both lines were measured. This analysis has shown that mutant lines have narrow leaves at the beginning of the third week of growth which remained significant till 6^{TH} week of growth. This reduction in leaf area of mutant plants has shown that mutant plants have faced some difficulty to grow and have changed properties like elasticity and rigidity of cell wall. Interestingly, significant growth rate difference was observed among the wild type and mutant plants only between 14^{th} and 21^{st} day of development (Figure 3.11). From 21^{st} to 35^{th} day of development, the plants seemed to have the tendency of similar growth. These results indicate that the mutation in NGT1 alters the growth rate only during early developmental stage and has no or little effect on the growth rate during late development.

In order to check this hypothesis, expression profile of NGT1 during different developmental stages was studied by using Genevestigator (www.genevestigator.com). It showed that NGT1 is highly expressed at germination stage, in young leaves and in mature silics but their expression is decreased in mature leaves and at inflorescence stage. In conclusion, finally this phenotypic growth defect in *ngt1-1* and *ngt1-2* mutant plants have shown that NGT1 gene is involved at early stages of plant development in the biosynthesis of cell wall.

Second evidence for the involvement of NGT1 gene in cell wall biosynthesis came from the biochemical analysis of cell wall of mutant plants. Neutral monosaccharide quantification through GC-MS has shown that *ngt1* mutant plant cell wall has significantly less arabinose, galactose and rhamnose as compared to that in wild type col0 cell wall (Figure 3.12). This data confirmed the link between the biochemical composition and phenotype which is due to the alteration in NGT1 gene. These results have shown that mutation in NGT1

affected the cell wall polysaccharides biosynthesis: more specifically the pectin representative sugars like arabinose, rhamnose and galactose. These defects in cell wall composition showed more convincingly that our hypothesis about the implication of NGT1 in plant cell wall biosynthesis is correct but in order to get detailed idea, which specific polymer is modified, permethylation linkage analysis was performed.

This biochemical analysis has shown that cell wall from both *ngt1-1 ngt1-2* mutant cell wall was significatively impaired for 3 molecules namely 3,5-Ara*f*, 5-Ara*f* and 4,6-Glc*p* as compared to wild type content(Figure 3.14). The reduction of two linkages 3,5-Ara*f*, 5-Ara*f* (50%) constitutive of arabinan was notice, as the neutral monosaccharide quantification results showed the decrease in total arabinose content in mutant cell wall (as compared to WT). Arabinan pectic polymer actually contains these 2 linkages (3,5-Ara*f*, 5-Ara*f*), and this observation suggested us to study the possibility that NGT1 would encode a GT activity responsible for the biosynthesis of arabinan, as an alternative to the putative fucosyltransferase activity predicted through gene sequence homology. The decrease in the amount of the molecule of 4,6-Glc*p* was ruled out as a direct effect of NGT1 activity as this linkage is present in xyloglucan due to xylostransferase activities, and because such GTs have been already characterized in GT family 34. We then hypothesized that decrease in 4,6-Glc*p* was an indirect effect of the NGT1 as a compensatory mechanisms responding to the alteration of other polysaccharide of the cell wall, such as arabinan. Interesting information were gathered while analysing cell wall of etiolated seedling with permethylation to unravel glycosidic linkages between polysaccharides; however this study could be completed by a sequential extraction of plant cell wall polysaccharides in order to confirm and strengthen observation that were made. Indeed, a detailed analysis of the pectin fraction would be informative of the quality of arabinan in *ngt1-1* and *ngt1-2* mutants. Interestingly, we performed immunolabelling of stem cross section of etiolated seedlings, showed a qualitative and reproducible reduction of -α-(1→5)-L-arabinan epitope in *ngt1-1* and *ngt1-2* mutant lines, using LM6 and LM13 anti-arabinan antibodies (Fig 32). Thus these results confirmed the biochemical phenotype identified for both mutants, even though subtle differences in labelling intensity were found between *ngt1-1* and *ngt1-2*. It is noteworthy that results obtained from *ngt1* Arabidopsis mutants characterization directly challenge our hypothesis of a fucosyltransferase activity supported by the analysis of the gene sequence homologies. This indicates how careful one should be over the course of a functional genomic study, especially in the case of the functional anaylysis of a new gene family for which no activity has been

characterized. This ambiguity in possible function for NGT1could be definitely unravelled by demonstrating the activity *in vitro* of NGT1 protein. Accordingly, heterologous expression of NGT1 protein was undertaken firstly trying to demonstrate a putative fucosyltransferase activity (hypothesis from gene sequence prediction) and secondly testing putative arabinosyltransferase activity (hypothesis from mutants characterization). In order to narrow down the range of donors and acceptors, free sugar assay was carried out with NGT1 microsomes. This assay showed that GDP-Fuc ^{14}C would be a better donor for NGT1 protein as compared to UDP-Glc and UDP-Gal. As putative sugar acceptor, arabinose and galactose appeared slightly better compared to rhamnose, xylose, glucose and mannose but efficiency of transfer was too low above background to be quantified. Another study using cell wall from WT plant or mutant *ngt1-1* as potential acceptor was carried out. This study showed a two-fold increase of [^{14}C]-Fuc from GDP-[^{14}C]-Fuc while cell wall from the mutant was used, but again the total amount of radioactivity transferred was too low to envision any product characterization.

Later, based on the study of *ngt1* mutants cell wall, biochemical results, we evaluated the hypothesis of NGT1 protein encoding an α-(1→3)-arabinosyltransferase activity. Indeed, the biochemical data from *ngt1* mutants has shown that there is significant reduction of 5-Ara*f* and 3,5 Ara*f* glycosidic linkages, which defects could be modeled by a deficiency in an arabinan α-(1→3)-Arabinosytransferase activity (fig 5.1).

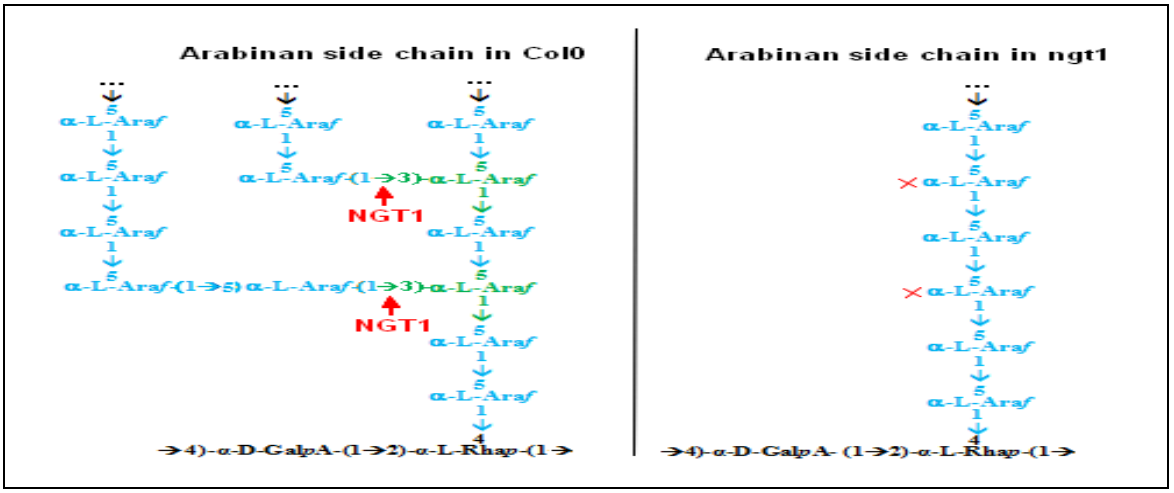

Figure 5.1: Hypothesis of function of NGT1 protein in Arabidopsis cell wall. In green: the residue 3,5-Araf; in blue: the residue 5-Araf; in red: linkage made by NGT1

We then develop a non-radioactive arabinosyltransferase activity assay with the heterlogously produced NGT1 protein. We used the arabinotetraose, arabinohexaose and arabinan as acceptors in the presence of UDP-Ara*f* sugar donor in an assay, but no transfer of arabinose onto these two putative acceptors was monitored after analysis of the acceptor oligosaccharide by MALDI-TOF MS. Meanwhile, advance further in understanding the role of NGT1, an activity test in *in-vitro* is required. One possibility for future work would be to use purified α-(1→5)-arabinan polysaccharides free of α-(1→3)-arabinose side chain and to use them as acceptor in an assay. We envisioned that if NGT1 activity is to create branching in arabinan polymer, then the α-(1→5)-arabinan provided in the assay would be modified and that an increase in Terminal-Arabinose could be quantified using GC-MS as a proof of NGT1 α-(1→3)-arabinosyltransferase activity.

Finally, another study that would require attention in future work is the subcellular localization of NGT1 within the plant cell. Indeed, It has been well studied that non-cellulosic and pectic polysaccharides are synthesized within the Golgi apparatus (Fincher & Stone, 1981; Gibeaut & Carpita, 1994). Using protein features software, it has been predicted that NGT1 adopt a type II membrane protein topology, typical of Golgi located GTs but it would be of importance to confirm experimentally this prediction. For this purpose Arabidopsis plants have been transformed with a GFP version of NGT1 protein, where GFP was fused N-terminal to NGT1. Generating plant harbouring stable expression of GFP:NGT1 should enable the characterization of protein subcellular localization using YFP marker available for various plant cell compartment, and thus provide support for NGT1 to be involved in polysaccharide biosynthesis if Golgi-located.

Chapter 6
MATERIALS AND METHODS

6 Material and methods

6.1 Materials

- **Biological materials**

T-DNA knockout mutant lines were ordered from Nottingham Arabidopsis Stock Centre (NASC). *Pichia pastoris* strain GS115 was purchased from Invitrogen and E.Coli (XLI Blue) strain was obtained from Agilent. Agrobacterium strain C58C1 was kindly provided by Dr Gabrielle Tichtinsky (LPCV, CEA Grenoble).

- **Antibodies used**

For western blotting of NGT1 protein, T7 monoclonal antibody was ordered from Novagen, Penta-Histidine N-ter antibody for the detection of AtFUT1-Δ68 was obtained from QIAGEN and secondary Anti-Xpress antibody was purchased from Invitrogen.

Antibodies used for the immunolabelling of plant stem cross section, LM13 monoclonal anti- (1→5)-α-L-arabinan was purchased from Plant Probes Leeds, UK. Other antibodies like LM6, LM2, LM5, JIM7, JIM5 and JIM16 was provided by Azeddine Driouich (Glycomev lab, University of Rouen). Secondary antibody for immunolabeling of stem cross section, Anti-rat IgG-FITC produced in goat was purchased from Sigma.

- **Specific materials or reagents used for biochemistry**

Reagents 2,5-Dihydroxybenzoic acid (2,5-DHB), trifluoroacetic acid (TFA), trimethylsylil reagent (TMS), methanolic-HCl, $NaBD_4$, 2-aminobenzamide (2-AB), 8-amino-1,3,6-naphtalene trisulfonic acid (ANTS) were purchased from Sigma. AEC 101 staining kit used for developing the western blot membrane was purchased from Sigma. Endocellulase was obtained from Megazyme. Endo-H and protease inhibitor tablets were provided by Roche. Radioactive sugar donors GDP-Fuc ^{14}C (specific activity: 925Bq/µL) and UDP-Xyl ^{14}C (specific activity: 925Bq/µL) were purchased from GE Healthcare. Protease inhibitor tablet (EDTA-free) were from Roche (11836153001). GDP-Fucose, UDP-Galactose, UDP-Glucose were from Kyowa Hakko Kyoga LTD (Japan). UDP-Xylose was from Carbosources (ww.ccrc.uga.edu).

- **Specific material for molecular biology**

Hot Star high fidelity polymerase and MiniElute PCR purification kit were obtained from QIAGEN. Go Taq DNA polymerase and DNase treatment kit of RNAs were purchased from Promega. For RT-PCR, cDNAs were made by using the iScript kit obtained from Bio-Rad.

BP Cloning kit, LR Cloning kit and Plasmids pPICZ gatewayable, pPICZ α, pDONR207, pDONR221 and LB culture media were ordered from Invitrogen. Nucleospin Plasmid kit extraction and Nucleotrap kit were purchased from Macherey-Nagel. Gel red for DNA gel staining was purchased from Interchim. Silwet-L77 was ordered from Lehle Seeds. Plant gateway vectors pH2GW7 and ph7WGF2 for Agrobacterium transformation were obtained from the department of Plant Systems Biology (http://gateway.psb.ugent.be/). Nitrocellulose membrane used for western blot was ordered from PALL membranes, BioTrace™ NT.

- **Specific materials for microscopy**

Osmium tetraoxide was purchased from Sigma. LR white, CFM-1 and Eukitt mounting medium were ordered from Electron Microscopy Science (EMS).

6.2 Methods

6.2.1 Methods for heterologous expression of proteins

6.2.1.1 Gateway cloning for heterologous expression in Pichia pastoris

Gateway Cloning Technology is a powerful method that allows the subcloning of a gene of interest into a pDONR vector to produce a versatile pENTR from which gene can easily transferred into various destination vector depending on the system selected for protein expression in the course of the study. It basically consists of two reactions i.e. BP reaction and LR reaction. BP reaction transfers the gene of interest (between *att*B sites) into the pDONR vector (containing *att*P sites) to produce a new Entry vector (*att*L sites). This reaction is catalyzed by the BP Clonase mix of two enzymes integrase (Int) and integration host factor (IHF) necessary for recombination. The LR Reaction is a recombination reaction between an Entry Clone and a Destination Vector mediated by the LR Clonase mix containing three different enzymes integrase (Int), excisionase (Xis) and integration host factor (IHF). This reaction transfers DNA segments (e.g. cDNA, genomic DNA, or gene sequences) from the Entry vector to the Destination vector to create an expression vector (Figure 6.1).

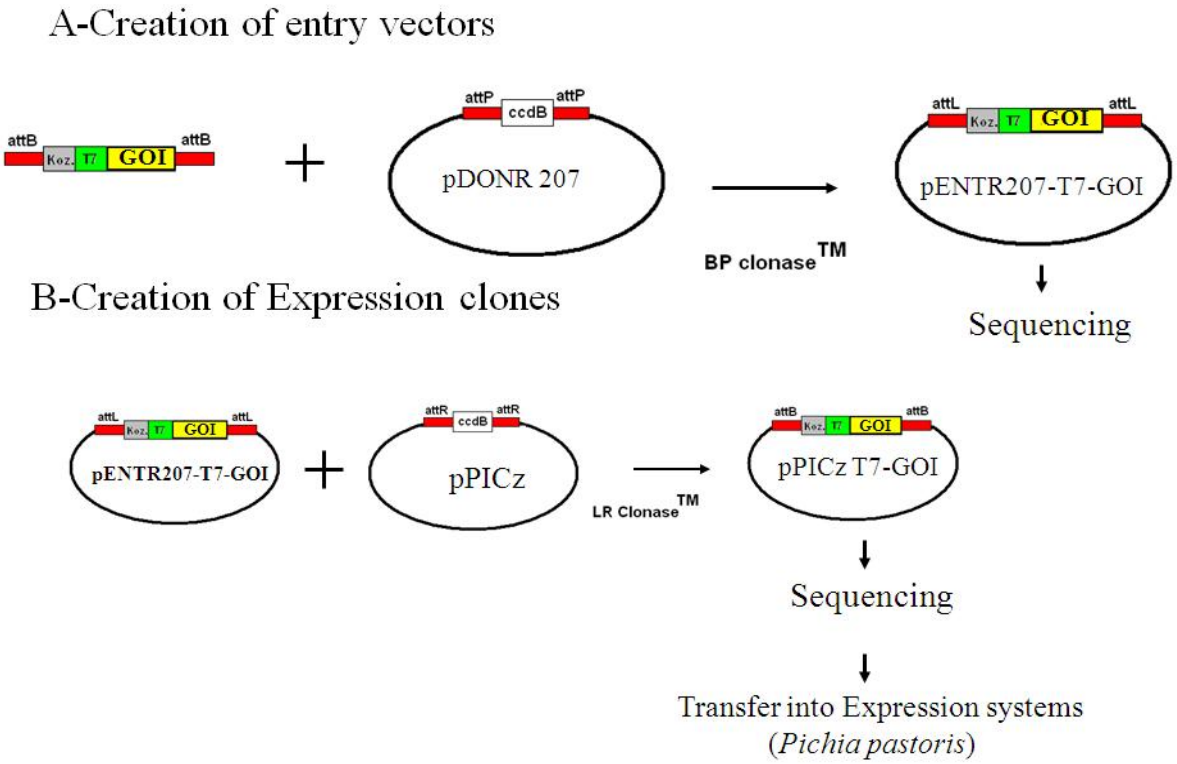

Figure 6.1: Schematic representation of the two steps gateway cloning recombination system. A) First step, the PCR product carrying appropriate flanking sequences is integrated into the pDONR207 vector by recombination forming a new vector named pENTR207-T7-GOI in our study (GOI: Gene Of Interest). B) Second step, the gene of interest (T7-GOI) is transferred from the pENTR207-T7-GOI to a gatewayable version of pPICz destination vector, forming pPICz-T7-GOI used for Pichia transformation.

6.2.1.1.1 PCR amplification of gene of interests

Amplification of our gene of interest required a two-round PCR reaction in order to add at 5' and 3' of the gene sequence the coding sequence of the T7 tag and the gateway border (Figure 6.2). Each PCR reaction was carried out in a final volume of 25 µl containing 1 µl of DNA, and following final concentration of 1X hot star PCR buffer (containing dNTPs and $MgSO_4$), 0.4 µM of each (forward and reverse) primer and 0.1 units of hot star high fidelity Polymerase. DNA template was amplified with gene specific primers in the first PCR reaction and then T7 tag and gateway border sequences were added by the second PCR reaction. The sequence of primers are given in table 6.1.

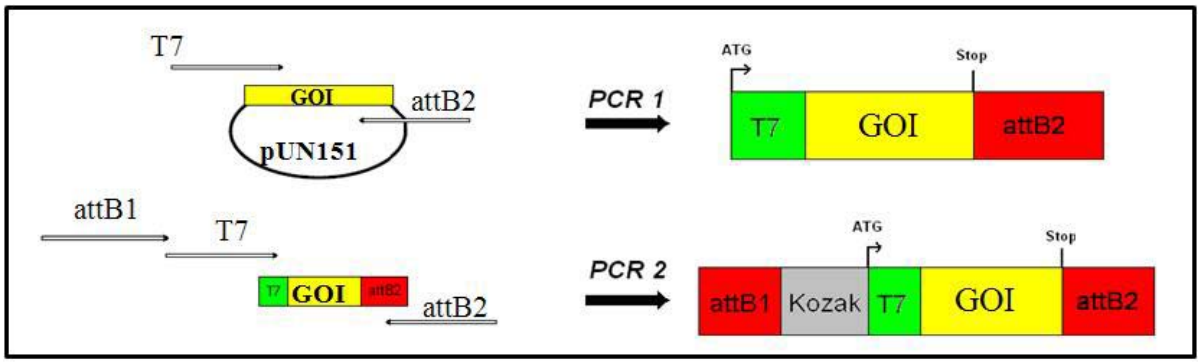

Figure 6.2: Schematic presentation of the procedure of PCR for the addition of gateway borders and T7 tag. attB1 and attB2 represent gateway border recombination sites. GOI (Gene of Interest).

Table 6.1: Primers used for amplification of the indicated genes for gateway cloning

Gene	Primer	Name	Sequence (5'→3')
At1g10280 (A)	**Forward**	T7_A_for	GGTGGACAGCAAATGGGTATGGCGAGAGGAGGGAAA
	Reverse	B2_A_rev	GTACAAGAAAGCTGGGTTTCAAAACCCCAGGACAGT
At1g11940 (C)	**Forward**	T7_C_for	GGTGGACAGCAAATGGGTATGACGAAGAAATCACAG
	Reverse	B2_C_rev	GTACAAGAAAGCTGGGTTTTATAAGGTTTTGTTTGC
At1g62305 (E)	**Forward**	T7_E_for	GGTGGACAGCAAATGGGTATGCCGCGGCTTCCGTCG
	Reverse	B2_E_rev	GTACAAGAAAGCTGGGTTCTAAAAGGTTGTTGTATC
At1g68380 (F)	**Forward**	T7_F_for	GGTGGACAGCAAATGGGTATGCCGACCCAAAAGGAC
	Reverse	B2_F_rev	GTACAAGAAAGCTGGGTTTCAGAAATACATGACGAT
At2g19160 (I)	**Forward**	T7_I_for	GGTGGACAGCAAATGGGTATGAAGGCAATTAAGGGA
	Reverse	B2_I_rev	GTACAAGAAAGCTGGGTTCTACACGATGCTTGTGTA
At3g52060 (J)	**Forward**	T7_J_for	GGTGGACAGCAAATGGGTATGTTCTCATCTTCTACA
	Reverse	B2_J_rev	GTACAAGAAAGCTGGGTTTCAATCCCTAAAGATCAC
At4g30060 (L)	**Forward**	T7_L_for	GGTGGACAGCAAATGGGTATGAAGGCAGTTAAAAGA
	Reverse	B2_L_rev	GTACAAGAAAGCTGGGTTTCACAAATTCCAAGTGAC
At4g32290 (N)	**Forward**	T7_N_for	GGTGGACAGCAAATGGGTATGGCGAACCCCAACGAG
	Reverse	B2_N_rev	GTACAAGAAAGCTGGGTTCTATGATTTCTCTTGGGT
At5g11730 (O)	**Forward**	T7_O_for	GGTGGACAGCAAATGGGTATGCAGAATAGGATTGTA
	Reverse	B2_O_rev	GTACAAGAAAGCTGGGTTCTATGATTTCTCTTGGGT
At5g22070 (R)	**Forward**	T7_R_for	GGTGGACAGCAAATGGGTATGTTCTCGAATCAGTTT
	Reverse	B2_R_rev	GTACAAGAAAGCTGGGTTTTAATCTCTGAAAATCAC
At5g25330 (S)	**Forward**	T7_S_for	GGTGGACAGCAAATGGGTATGTTGGCCTTATATTTC
	Reverse	B2_S_rev	GTACAAGAAAGCTGGGTTTTACACAAGAGCCGAATC
At5g57270 (V)	**Forward**	T7_V_for	GGTGGACAGCAAATGGGTATGGGAGAGAATCTGCAT
	Reverse	B2_V_rev	GTACAAGAAAGCTGGGTTAATGACCACTGTGCTTGT
At5g28910 (W)	**Forward**	T7_W_for	GGTGGACAGCAAATGGGTATGAGCATGAAGTCATTA
	Reverse	B2_W_rev	GTACAAGAAAGCTGGGTTTTACCAGAACCGATCTTT

6.2.1.1.2 BP cloning

*Att*B-flanked PCR product after second PCR reaction was used to create entry clone. Following components; *att*B-PCR product (=10 ng/µl; final amount ~15-150 ng) 1-7 µl, Donor vector pDONR207 (150 ng/µl), 1 µl TE buffer (pH 8.0) to final volume of 8 µl were added to a 1.5 ml tube at room temperature and mixed. The BP Clonase™ II enzyme mix was thawed on ice for about 2 min. The BP Clonase™ II enzyme mix was vortexd briefly twice (2 seconds each time). 2 µl of BP Clonase™ II enzyme mix was added to the reaction and gently mixed with two brief vortex pulses. Reaction mixture was incubated at 25°C for 1h. Then

reaction was stopped by adding 1 µl of the Proteinase K solution, vortexed briefly and samples were incubated at 37°C for 10 min.

6.2.1.1.3 Transformation and plasmid extraction

One microliter of BP reaction was used to transform 50 µL of XL1 blue competent cells transformation. Cells were incubated on ice for 30 min with BP reaction, heat-shocked for 45s at 42°C and put back on ice before 250 µl of S.O.C medium is added. Cells were then allowed to recover 1 h at 37°C with shaking, plated on LB agar (1% tryptophane; 0.5% yeast extract, 0.5% NaCl and 1.5% bacto-agar) supplemented with kanamycin 50 µg/ml and placed overnight in an incubator at 37°C.

Plasmid extraction from bacteria was carried out using Nucleospin Plasmid kit (Macherey-Nagel). First, single colony was precultured over night at 37°C in 2 ml LB supplemented with appropriate antibiotic. Preculture was then centrifuged at 11,000 g for 1 min at room temperature (RT) and processed for plasmid DNA extraction following manufacturer protocol. This includes briefly cells lysis, neutralisation of the first buffer, DNA binding onto a silica membrane, wash and elution of DNA with deionised water or TE buffer. Obtention of Entry vector carrying gene of interest was confirmed by PCR using gene specific primers and sequencing.

6.2.1.1.4 LR cloning into pPICZ

Gene of interest was transferred from a Gateway pDONR207 entry clone to pPICZ destination vector by the following protocol. Following components; Entry clone (~15-150 ng) 1-7 µl, Destination vector pPICZ (150 ng/µl), 1 µl TE buffer (pH 8.0) to final volume of 8 µl were added to a 1.5 ml tube at room temperature and mixed. Then the LR Clonase II enzyme mix was thawed on ice for about 2 min. The LR Clonase II enzyme mix was vortexed briefly twice (2 sec each time). To each sample (Step 1, above), 2 µl of LR Clonase™ II enzyme mix was added to the reaction and was mixed well by vortexing briefly twice following brief table-top centrifugation. Reaction mixture was incubated at RT for 1h, then stopped by adding 1 µl of the Proteinase K solution, vortexed briefly and incubated at 37°C for 10 min. Plasmid DNA was extracted as described above in paragraph 6.2.1.1.3. Obtention of destination vector carrying gene of interest was confirmed by sequencing PCR products.

6.2.1.1.5 Preparation of Pichia competent cells and transformation

Five milliters of WT Pichia were grown in YPD (table 6.2) at 30°C overnight. 150 ml YPD (1 liter flask) was inoculated with 2 ml of the previous culture. The flask was incubated at 28-30°C and the next day OD_{600} was checked. To prepare competent cells, Pichia cells were collected when the OD_{600} reached log phase (between 1.3 and 1.5 OD). The Pichia cells were then centrifuged (Beckman JLA-8000, 4000rpm) and re-suspended in 50 ml YPD. Ten milliters of 1M HEPES pH8 and 1.25 ml 1M DTT (both filter-sterilized and freshly made) was added to the 50 ml culture, and then incubated at 30°C with shaking (200 rpm). After 15 min, ice cold ddH_2O was added up to 250 ml final volume. Cells were recovered by centrifugation (4000x*g* at 4°C) and washed once with 125 ml of ice cold ddH_2O water. The cells were finally centrifuged (4000xg, 10min at 4°C) and re-suspended in 10 ml of ice cold 1M sorbitol. The cells were collected by centrifugation (Sigma 3K30C, 4000x*g*, and 10min) and re-suspended in a minimal volume (~0.5 mL) of ice cold 1M sorbitol (thick semi-liquid cell suspension). The competent cells were kept on ice and used on the same day. Linearized plasmid DNA 11 µg (in 30 µL) was added to 80 µL of the cell suspension and the mixture transferred to a pre-cooled electroporation cuvette incubated on ice for 5 min. The cells were electroporated (1500V, 40µF, 200 Ohm) and immediately 1 ml of 1M sorbitol was added. The culture was transferred to a sterile 10 ml tube and incubated at 28-30°C for 1h and then spread on solid medium YPDS (1 % yeast extract, 2% peptone, 2% dextrose, 1M sorbitol, histidine 0.004%and 2% agarose), complemented with zeocin 50 µg/mL for selection. Composition of all the mediums used for Pichia expression is given in the table 6.2.

Table 6.2: Mediums used for culture of *Pichia pastoris*

	YPD	YPDS	BMMY	BMGY
Yeast Extract	1 %	1 %	1 %	1 %
Peptone	2 %	2 %	2 %	2 %
Dextrose	2 %	2 %		
Glycerol				1 %
Sorbitol		1 M		
Methanol			0.5%	
YNB			1.34 %	1.34 %
Biotin			4 x 10-5 %	4 x 10-5 %
KPO_4; PH 6.5			100 mM	100 mM

6.2.1.1.6 Screening of transformants

Transformants were selected on YPDS plates complemented with zeocin 50 µg/ml; fifty transformants have been patched on a new plate using sterilized toothpicks. Plates were incubated at 28-30°C for two days and putative transformants were selected for PCR screening.

- **Yeast DNA miniprep**

To determine whether the gene of interest has been integrated into *Pichia* genome, genomic DNA was isolated from 12 transformants by using the following protocol: Cells were grown in 2 ml of YPD culture medium overnight at 28-30°C. One milliter of cells was centrifuged at 11,000xg for 1 min and medium was discarded. Cells were washed with 1 ml of water and centrifuged again. Cells were re-suspended in 200 µl of lysis buffer and then boiled for 5 min in a water bath. Centrifugation was done and liquid phase was recovered into another tube. 100 µl of 7 M ammonium acetate pH 7.0 was added and incubated at 65°C for 5 min and then 5 min on ice. 200 µl of chloroform was added, vortexed and centrifuged for 2 min. Supernatant was taken into a new tube and DNA was precipitated with 250 µl isopropanol. It was incubated for 5 min at RT and then centrifugation was done for pelleting the DNA at 13,000 g for 10 min. The pellet was washed with 70 % EtOH, dried under vacuum and dissolved in 40 µl sterile water.

- **PCR analysis of integrants**

After isolation of genomic DNA, PCR was done to amplify the gene of interest either using gene specific primers or 5' *AOX1* forward primer (5'-ATGTTGAGTTGTCCTTGC TTC-3') paired with 3' *AOX1* reverses primer (5'-GATAAGACCAACTTTCAAGGAG-3'). The reaction was carried out in a final volume of 25 µl containing 2 µl of DNA, and following final concentration of 1X Go Taq DNA polymerase buffer, 2 mM MgSO4 , 0.2 µM of dNTPs mix, 0.2 µM of each (forward and reverse) primer and 0.03 units of Taq DNA Polymerase enzyme. Denaturation of double stranded DNA was done at 94°C for 2 min and it was followed by 25 cycles of amplification which included 10 sec at 94°C, 50 sec at Ta (Tm-5°C) and 1min45s at 72°C. Samples were analyzed on a 1X TBE, 0.8 % agarose gel. The integration of gene of interest in Pichia was further confirmed by purifying and sequencing PCR product.

6.2.1.2 Heterologous expression of proteins into *Pichia pastoris*

- **Expression of full length NGT1**

Pre-culture was started from a single colony in 5 ml YPD complemented with Zeocin (50 µg/ml) overnight at 28-30°C. 40 ml of BMGY (Buffered Glycerol-complex Medium) were inoculated with 50 µl of pre-culture and cells were grown at 28-30°C in a shaking incubator until culture reached an OD_{600} = 2-6 (~16-18h). The cells would be in log-phase growth. The cells were harvested by centrifugation at 3000 x g for 5 min at RT. Supernatant was removed and cell pellet re-suspended to an OD_{600} of 1.0 in BMMY (Buffered Methanol-complex Medium to induce expression (approximately 100-200 ml). Culture was placed in a 1 litre baffled flask and the flask was covered with 2 layers of sterile gauze or cheesecloth and returned to incubator to continue growth. 100 % methanol was added to a final concentration of 0.5 % methanol every 24h to maintain induction. The expression of clones was stopped after 4 days. Cells were centrifuged at 5000xg for 5 min, and pellet was stored at -20°C to check the protein expression by Coomassie stained SDS-PAGE and western blot.

6.2.2 Cloning of AtFUT1-Δ68 for heterologous expression in *Pichia pastoris*

6.2.2.1 Obtention of AtFUT1-Δ68 PCR product

A truncated form of gene AtFUT1-Δ68 was designed and amplified from the plasmid pENTR207 harbouring the full length cDNA of AtFUT1 with gene specific primers and primers include *EcoR1* and *Not1* restriction endonucleases sites. PCR reaction was carried out in 25 µl with final concentration of 50 ng of DNA, 1X hot star PCR buffer (containing dNTPs and MgSO4), 0.4 µM of each (forward and reverse) primer and 0.1units of hot star high fidelity Polymerase. The volume was made to 25 µl by adding water. The PCR programme consisted of initial denaturation at 95°C for 5 min, followed by 30 cycles of 94°C for 15 sec, annealing for 1 min at a 45°C temperature specific to the primer pairs and elongation at 72°C for 1min40sec. The programme ended with an extension at 72°C for 10 min. The PCR products were visualized on a 0.8 % agarose gel stained with Gel red. PCR product was purified by using MiniElute PCR purification kit following manufacturer protocol and DNA was eluted by adding 40 µl ddH_2O.

6.2.2.2 Cloning AtFUT1-Δ68 into PpicZαhis flag

PCR product (1730 ng) and plasmid pPICZαhis flag (2.5 ng) was digested and linearized using 1.5 µl of *EcoR1* and 1.5 µl of *Not1* in the presence of buffer React 3 and water was added up to 34 µL final volume. Reaction mix was incubated for 3h at 37°C, and digested PCR product was purified using SureClean kit. For this purpose, an equal volume of SureClean solution was added to the digestion and mixed thoroughly. Mixture was incubated for 10 min at RT. Centrifugation was done at 14,000xg for 10 min and supernatant was removed. Precipitation was done by the addition of 2 vol of 70 % ethanol and then vortexed for 10 sec. Centrifugation was done again, removed supernatant and the pellet was air dried to ensure complete removal of ethanol. Pellet was re-suspended in 6 µl of sterilized water.

6.2.2.3 Gel extraction and purification of plasmid

After DNA electrophoretical separation on an agarose gel, plasmid purification from the gel was carried out. First of all the DNA fragment with the correct size was identified under UV light. DNA fragment was cut with the use of clean scalpel and purified by using Nucleotrap kit. DNA gel slice was weighed and for 100 mg of agarose 300 µl of buffer NT1 was added. Nucleotrap suspension was vortexed thoroughly, and incubated at 50°C until the gel slices were dissolved. Centrifuged for 30 sec at 10,000 *g* and supernatant was removed. Silica matrix was washed by adding 500 µl of buffer NT2, centrifuged again for 30s at 10,000 *g* and supernatant was removed completely. Then the silica matrix was washed a second time by adding 500 µl of buffer NT3, vortexed, centrifuged and supernatant was removed and third wash was repeated with NT3 buffer. After washing, silica matrix was dried at RT and DNA was eluted with 50 µl of water by adding to the silica matrix, pellet was resuspended through vortexing and then centrifugation was done for 30 sec at 10,000x*g* and DNA was transferred to clean tube.

- **Ligation**

Ligation of digested vector and insert was done by using the standard protocol. Two tubes were prepared: the first tube for the ligation of insert into the vector contained 1µl of digested insert (117ng/µl), 4 µl of vector (45ng/µl) and 5 µl of ligation mixture whereas the second tube used water instead of insert to control the vector for self-ligation. Tubes were incubated for 5 min at 25°C. 2 µl of reaction was used for transformation of XL1-blue cells, plasmid was extracted and insertion checked after digestion with EcoR1 and Not1 endonucleases.

6.2.3 Cloning of AtXT1-Δ140, AtXT1-Δ44 and AtFUT1-Δ68 for heterologous expression in insect cells

6.2.3.1 PCR for cloning of AtXT1-Δ140, AtXT1-Δ44 and AtFUT1-Δ68 in insect cells

A truncated form of gene AtXT1 was designed and named AtXT1-Δ140. This gene sequence encodes the AtXT1-Δ140 protein lacking 140 aminoacids at the N-terminus of AtXT1 (including the transmembrane domain) but conserving the predictive catalytic sequence at the C-ter of the protein. AtXT1-Δ140 gene sequence was amplified from the pUN51-XT1 plasmid (U14458) template using forward primer (5'-AAACTGCAGCCTAATTTCGTGGCGCCAAAC-3') and the reverse primer (5'-CGGGGTACCTCACGTCGTCGTCGTAC-3'). These primers were designed to include the cloning restriction sites *Pst*I and *Kpn*I at the 5'end and 3'end, respectively of the amplified sequence.

Similarly, a truncated form AtXT1-Δ44 was amplified using the forward primer (5'-GGTACGCCGGAGAAAGATATCG-3') and reverse primer (5'-GCTTAGTACGACGACGACG-3'). These primers were designed to include the restriction sites *Pst*I and *EcoR*I at the 5'end and 3'end, respectively of the amplified sequence.

A truncated form AtFUT1-Δ160 was amplified from pENTR-AtFUT1 as template comprising the full length cDNA of the *A. thaliana* AtFUT1 gene. The 1197 bp coding region corresponding to the desired truncated soluble form of AtFUT1 was obtained using the forward primer (5'-AAACTGCAGGATCAAGAACATATTGATGGTGATGG-TGAATGC-3') and the reverse primer (5'-CGGGGTACCTCATACTAGCTTAAGTCCCCAGC-3'). These primers were designed to include the restriction sites *Pst*I and *Kpn*I at the 5'end and 3'end, respectively of the amplified sequence

A truncated form of gene AtFUT1-Δ68 was amplified from the plasmid pENTR 207 harbouring the full length cDNA of AtFUT1. The 1473 bp coding region corresponding to the desired truncated soluble form of AtFUT1 was obtained using the forward primer (5'-AAACTGCAGTCAAATCGGATTATGGGTTTCG-3') and the reverse primer (5'-CCGGAATTCTCATACTAGCTTAAGTCCCC-3'). These primers were designed to include the restriction sites *Pst*I and *EcoR*I at the 5'end and 3'end, respectively of the amplified sequence. For PCR reaction and cloning of all these constructs, same steps were followed as explained in sections 6.2.2 .1 to 6.2.2.2.

6.2.3.2 Transformation and expression of AtXT1-Δ140, AtXT1-Δ44 and AtFUT1-Δ68 in insect cells

- **Transformation and amplification in Sf9 cells**

Spodoptera frugiperda cells (Sf9 cells from Invitrogen) derived from ovarian tissue were used for the production and amplification of recombinant baculoviruses. Sf9 cells were grown in monolayer culture at 27°C in Grace's medium (Invitrogen) supplemented with 10% fetal bovine serum (Sigma) and 50 µg/ml gentamycin (Sigma). Cotransfection of the cells with baculovirus DNA (7 ng BaculoGold linear DNA) and pVT-Bac-His1 containing the insert (30 ng) was done according to the manufacturer's instructions (Pharmingen BD Dickinson, see annex Protocol 1). High-titer viral stocks around 10^7 to 10^8 plaque- forming units/ml (pfu) of recombinant viruses were obtained by three repeated transfection procedures of Sf9 cells (annex protocol 2). After the third amplification, culture medium containing the recombinant virus was collected by centrifugation of the infected cell culture and was stored at 4°C. A titration of the virus stock was done to estimate the pfu/ml.

- **Protein Expression in hi-5 cells**

The production of recombinant proteins was done in *Trichoplusia ni* Hi-5 (High Five™, Invitrogen) cells, cultivated in serum-free medium (Express Five™ from Invitrogen or Excell-405 from Sigma), and supplemented with 16mM L-glutamine and 50 µg/ml gentamycin (Sigma). Cells were seeded at 70% confluency and allowed to attach for 30 min to the plastic culture flasks before addition of the virus. Cells were typically infected with the recombinant virus at a MOI (multiplicity of infection) of 5 (5 pfu/cell), and then grown at 27°C for 4 days. The culture supernatants (containing the secreted recombinant protein) were collected, clarified by centrifugation (8000xg for 5 min), and stored at -70°C or used directly for the protein isolation (For details see annex protocol 3).

- **Protein Isolation and Visualization**

Commercial or homemade affinity matrices (Nucleotide-Fractogel beads, Nucleotide-agarose beads, Ni-agarose beads) were used to concentrate the secreted recombinant protein. Samples of crude hi5 supernatants were incubated for 1h at 4°C, under stirring, with affinity adsorbents (50 µl of bead suspension for 2 ml of supernatant) to trap the recombinant protein (annex protocol 4). The resin matrix was afterwards centrifuged, washed twice with 1 ml PBS, and then resuspended in Laemmli denaturing buffer. Samples were heated at 100°C for 5 min, sonicated for 2 min, and proteins were then separated on SDS-PAGE gels (annex

protocol 5). The gels were either stained using Coomassie Brilliant blue (Protocol 6), or electro-transferred to a nitrocellulose membrane (annex protocol 7), using a semi-dry system (BioRad) for Western blotting. The nitrocellulose membrane was probed by incubation with mouse anti-Xpress antibody (1:4000, Invitrogen) directed against the Xpress epitope, followed by peroxidase-conjugated goat anti-mouse secondary antibody (1:2000, Sigma), and developed using the AEC staining kit (Sigma). (See annex protocol 8).

6.3 Methods for protein separation and identification

6.3.1 Microsomes preparation from *Pichia pastoris* to test activity

Cells were broken by using cell disruptor. Samples were re-suspended in a volume of 2 ml to 7 ml of cold Extraction buffer (50 mM Hepes-KOH pH 7.5, 0.4 M Sucrose, 10 mM $MgCl_2$ and 1/20 ml protease inhibitor cocktail), and broken at 2.5 kbar pressure.

6.3.2 Protein extraction from *Pichia pastoris*

After breaking the cells for NGT1, extraction buffer was added up to final volume of 20 ml. For the preparation of microsomes, samples were centrifuged at 9’600 *g* for 15 min at 4°C and supernatant was taken into ultracentrifuge tubes. Tubes were balanced by adding extraction buffer. Ultracentrifugation was performed at 100,000x*g* for 1h at 4°C. The pellet was re-suspended in 250 µl of extraction buffer using a 2 ml homogeneiser (all steps were performed at 4°C). After the 3rd and 5th days of expression of AtFUT1-Δ68 protein was collected from the supernatant as it was secreted into the medium instead of breaking the Pichia cells.

6.3.3 Protein quantification

The protein quantification was carried out using the Bradford assay. 200 µl of Bradford reagent was added to 800 µL of standards and samples containing from 0 to 10µg proteins. Tubes were mixed gently by inversion, covered with aluminum paper and reacted for 15 min before reading absorbance at 595 nm. Data were plotted between absorbance of the standards vs. their concentration, and extinction coefficient was computed to determine the concentrations of the unknown samples.

6.3.4 Protein analysis by electrophoresis

6.3.4.1 SDS-PAGE

The gel apparatus was set, resolving gel was poured and let to polymerize for 30 min before stacking gel was poured in gel cassette. The composition of resolving and stacking gels are given in the below table 6.3. Resolving gel was 12 % and run for 1-2h at 150 V using migration buffer (0.12 M Tris base, 1.25 M Glycine 10 % SDS and water).

Table 6.3: Composition of SDS-PAGE resolving 12 % and stacking gel 5 %

Sloutions	Resolving gel (12 %) for 10 ml	Stacking gel (5 %) for 3 ml
Acrylamide//bis-acrylamide (30/1; p/v)	3.3 ml	500 µl
Tris-HCl 1.5 M pH 8.8	2.5 ml	
Tris-HCl 1.0 M pH 6.8		380 µl
10 % SDS	100 µl	30 µl
10 % ammonium persulfate	100 µl	30 µl
TEMED	4 µl	3 µl
Water	4 ml	2.1 ml

6.3.4.2 Coomassie blue staining

Classically, two SDS-PAGE gels were run in parallel. One for Coomassie blue staining and verification of the protein contents of samples and a second for western blot. Gel was stained in coomassie blue solution for 15-30 min, before de-staining using solution of 7.5% acetic acid and 5% ethanol in water.

6.3.4.3 Western blot

For electric transfer of the proteins, the nitrocellulose membrane and 6 whatman papers at the same size of the respective gel were cut. Two buffer solutions namely cathode buffer (25 mM Tris-HCl, pH 9.4, Glycine 30 mM) and anode buffer (Tris 0.3 M pH 10.4) were prepared. Membrane was dipped in anode buffer for 5 min. Three whatman papers were dipped in cathode buffer and three in anode buffer. Three whatman papers soaked in anode buffer were stacked on graphite electrode. A clean plastic rod was rolled over the stack to remove any bubbles trapped between them and soaked nitrocellulose membrane was put on these papers. Polyacrylamide gel was placed on the top of the transfer stack. Three whatman

papers soaked in cathode buffer were put on the gel. A clean plastic rod was rolled again over the stack to remove any bubbles trapped between them. The current flows from cathode to anode, so the proteins being negatively charged due to SDS move from the gel (cathode side) to the membrane (anode side) and are trapped in the membrane. Transfer was done for 2h at 0.8 mA/cm^2.

The membrane was stained in "Red Ponceau" to confirm protein transfer efficiency. After transfer, the nitrocellulose membrane was washed in 20 ml TBS (10 mM Tris-HCl (pH 8), 150 mM NaCl) for 5 min. Saturation of non-specific sites was carried out with a solution of 1 % gelatin in a total of 20 ml TBS for 45 min with agitation at room temperature. Membrane was washed twice for 5 min in 20 ml TBS-T (10 mM Tris-HCl (pH 8), 150 mM NaCl and 0.05 % Tween20). The membrane was incubated with a mouse primary antibody directed against T7 tag (1:4000) in TBS-T for 90 min. Membrane was washed three times for 5 min in 20 ml TBST. The membrane was again incubated with secondary antibody Gam-per (1:2000, Sigma, goat anti-mouse conjugated to peroxidase) for 90 min at RT in TBS-T. The membrane was washed four times for 5 min in 20 ml TBST and once with 20 ml TBS for 5 min. Blot was revealed using the AEC 101 kit (Sigma).

6.3.4.4 Enzymatic removal of N-glycans using Endo-H

Reaction was carried out in 40 µl containing 100 µg of protein extract which was mixed with 1% SDS (2 µl), 1.5 M (1.2 µl) 2-mercaptoethanol, 10 µl of Extraction buffer (50 mM Hepes-KOH pH 7.5, 0.4 M Sucrose, 10 mM $MgCl_2$ and 1/20 ml protease inhibitor) and volume was made up to 40 µl with distilled water. Reaction mixture was heated at 50°C for 15 min. 300 mM (3.5 µl) of sodium acetate (pH 5.8) was added. Samples were put at 37°C for 15 min. 5 mU (1.5µl) of EndoH was added and incubated at 37°C for 18h. 15 µl of sample containing (50 µg of protein) was mixed with DB 5X buffer (3 µl) for western blotting.

6.4 Methods to test protein activities

6.4.1 Radioactivity test

- **Free sugar assay for NGT1 microsomes**

Free sugar radioactive assay was carried out by using NGT1 microsomes from *Pichia pastoris* cells. Reaction was carried out in final volume of 50 µl which containing 100µg of microsomes, 10 mM $MnCl_2$, 10 mM $MgCl_2$, 30 µM of Cold GDP-Fuc, 3 µM (50 nci) of GDP-[^{14}C]-Fuc, and 0.5 M of each monosaccharide acceptor substrate like arabinose,

galactose, glucose, mannose, rhamnose, xylose and mannitol, 25 mM of buffer Hepes-KOH (pH 6.5). Reactions were incubated at 30°C for 1h and stopped by addition of 500 µL of AG 1-X8 ion-exchange resin resuspended in water (1g: 4mL resin in ddH_2O). Resin has been prepared at least 1h in advance to equilibrate in water. Free NDP-sugars will bind to resin which quench the reaction. Tubes were spun in a microcentrifuge at 5000*g* for 3 min. 600µL of supernatant is transferred into scintillation counter vials and resin is then washed again with 600 µL of ddH_2O, centrifuged and supernatant is pooled to the same vial. 4.0 ml of scintillation counting liquid (BCS scintillation cocktail; GE Health Care) is added and radioactivity is measured on a Beckman Tricarb 1600 TR scintillation counter.

- **Fucosyltransferase activity test for NGT1 using cell wall as acceptor**

Fucosyltransferase activity test was carried out by using NGT1 microsomes from *Pichia pastoris* cells. Reaction was carried out in final volume of 50 µl containing 100µg of microsomes, 10 mM $MnCl_2$, 10 mM $MgCl_2$, 30 µM of Cold GDP-Fuc, 3 µM (50 nci, 44'000cpm) of GDP-[^{14}C]-Fuc, and 500µg of cell wall prepared from either WT or *ngt1-1* mutant (acceptor), 25 mM of buffer Hepes-KOH (pH 6.5). Reactions were incubated at 37°C for 1h, terminated, processed and counted as described above.

- **Xylosyltransferase activity test for AtXT1-Δ44 and AtXT1-Δ140**

The assay was performed as described previously by Cavalier and coll. (2006). Xylosyltransferase radioactive assay was carried out by using cell extracts from Hi 5 cells expressing AtXT1-Δ140 or AtXT1-Δ44. For each assay 40 µl of culture medium was mixed with 1 mM UDP-xylose, 50,000 cpm UDP-[^{14}C]-xyl, and 4 mM acceptor substrate (cellopentaose or celloheptaose), 50 mM Hepes-triton 1% (pH 7) buffer and 5mM $MnCl_2$. In some experiments, we also used the Hepes buffer without Triton-X100 or water. Reactions were incubated at 30°C for 1h, terminated, processed and counted as described above.

- **Fucosyltransferase activity test for AtFUT1-Δ160 and AtFUT1-Δ68**

The assay was performed as described previously by Vanzin and Coll. (2002). Fucosyltransferase radioactive assay was carried out using either cell extracts /supernatants from Hi 5 cells or *Pichia pastoris* cells expressing AtFUTI-Δ160 and AtFUT1-Δ68. Each reaction contained 40µl of cell extract mixed with 1µl of 20 µM GDP-Fuc, 2µl of GDP-[^{14}C]-Fuc (45,000 cpm), and 2µl of (0.4µg/µl) tamarind xyloglucan acceptor substrate, 5 µl of 0.5 M Hepes-KOH buffer, pH 7, and 50 mM $MnCl_2$. Reactions were incubated at 30°C for 1h, terminated, processed and counted as described above.

6.4.2 Non-radioactive activity test for AtFUT1-Δ68 protein

- **AtFUT1-Δ68 Fucosyltransferase activity test**

60 µl of total reaction mixture containing 50 mM Hepes-KOH 0.5 M, pH7, 50 mM $MnCl_2$, 166 µM cold GDP-Fucose, 0.83 µg/µl of xyloglucan tamarind and 39 µl of culture medium or microsomal protein of AtFUT1-Δ68 was added. The mixture was incubated for reaction during 2h at 30°C. Then 2 µl of endoglucanase was added to the mixture to cleave xyloglucan in fragments and reaction was incubated at 30°C for 1h. 700µl of AG 1x8 resin was added to bind NDP-sugar and thus stopped the reaction. Reaction mix was centrifuged and 400 µl of supernatant (avoiding resin) was uptaken and concentrated using evaporator. Endoglucanase digested xyloglucan fragments (up to 500 µg) were lyophilized in a microreaction tube with heating temperature not exceeding 45°C.

- **Non-radioactive analysis of AtFUT1-Δ68 activity using ANTS labeling of Xyloglucan oligosaccharides**

ANTS labelling of oligosaccharides enables easy and sensitive detection (less than 25 nmol) of derived oligosaccharides. Labelled molecules can be analyzed on acrylamide gel after electrophoresis, chromatography with fluorescence detector or alternatively using mass spectrometry (Lerouxel *et al.*, 2002). In this study, endoglucanase digested xyloglucan fragments were labelled using addition of 5 µl of ANTS (0.15M ANTS in acetic acid: water 3:17, v/v) solution and 5 µl of 1M sodium cyanoborohydride in dimethylsulfoxide and incubating at 37°C for 16h. The reaction mixture was dried from 1h to 4h at 45°C, dissolved in 100 µl of glycerol:ddH_2O (1:4, v/v) and analysed by fluorophore-assisted carbohydrate electrophoresis (FACE). For FACE analysis, the gel system consisted of two discontinuous gel, a resolving gel (8 ml acrylamide/bis-acrylamide (37.5/1), 1.94 ml stock gel buffer solution 10 µl Temed and 50 µl APS 10 %) and a stacking gel (1 ml acryl/bis-acryl (37.5/1; v/v), 1.9 ml stock gel buffer solution 10 µl TEMED, 100 µl APS 10 % and 7 ml water . 10 ml of resolving gel was prepared and poured into the gel cassette. 1 ml of water (to make 1 cm layer) was added to it to keep the surface levelled and let it polymerized. After 30 min water was removed with the help of whatman paper and 10 ml of stacking gel solution was pipetted between to glass plates on the top of the solid resolving gel. The comb of required size was placed on it. After 30 min the comb was removed and the samples and molecular weight marker were loaded to it. The gel was run in a Tris-Glycine migration buffer (25 mm Tris-HCl

and 192 mm Gly, pH 8.5) for 1-2h at 15 mA until excess of unreacted ANTS reagent run out of the gel and then fluorogramme was analyzed by using UV.

- **Non-radioactive analysis of AtFUT1-Δ68 activity using MALDI-TOF MS**

For MALDI-TOF analysis, Endo-glucanase digested xyloglucan fragments were lyophilized and then labelled by 2-aminobenzamide. Assay was resuspended in 100 µl of freshly made solution prepared by mixing 64 mg of sodium cyanoborohydride, 41 mg of 2-AB, 700 µl of DMSO and 300 µl of acetic acid. Labeling was done overnight (16h) at 37°C and samples were dried using evaporator at 50°C under N_2 stream. Excess of reagent was removed using successive resuspension and evaporation of 75 % methanol and 85 % methanol. Samples were resuspended in 300 µl of water and basicity was adjusted by adding 300 µl of 2 M NH_4OH, pH 10. Then excess of reagent was extracted with 500 µl of dicholoromethane (8X) and aqueous phase was loaded on a conditioned Sep-pak C18 column. C18 column was conditioned as following: 4 ml methanol, then 4 ml water, and then 4 ml acetonitrile and 4 ml water were successively added. Aqueous phase was loaded and allowed to pass through the column. Column was washed with 5 ml of water and oligosaccharides were eluted using 80 % acetonitrile (in water). Acetonitrile was evaporated using N_2 and resulting sample was freeze dried. For analyzing the samples by MALDI –TOF, oligosaccharides were resuspended in 300 µl of MeOH and 0.1 % formic acid. They were analyzed by MALDI-TOF MS (Autoflex Bruker). Mass spectra were obtained in the reflectron mode using 2,5-dihydroxybenzoic acid (50 mg / mL) of matrix mixed with the solubilized sugars 1:1 (v/v).

6.4.3 Arabinosyltransferase activity of microsomal protein from Pichia expressing NGT1 using MALDI-TOF MS

50 µl of total reaction mixture containing 50 mM HEPES-KOH 0.5 M, pH 7, 50 mM $MnCl_2$, 0.4 mM cold UDP-Araf, 4 mM of acceptor (arabinotetraose, arabinohexasoe or arabinan) and 39 µl of microsomes containing NGT1 protein were added. The mixture was incubated for 1h at 30°C. Tubes were dried using evaporator and labelled with 2-AB reagent, as described above.

6.5 Methods for T-DNA mutants identification

6.5.1 Sterilization of seeds

T-DNA knockout mutant lines were ordered from Nottingham Arabidopsis Stock Centre (NASC). The *Arabidopsis* seeds of Wild type Columbia (Wt), and *ngt1-1* (N585839) and *ngt1-2* (N638819) mutants were surface sterilised before *in vitro* cultivation. Surface sterilisation of the seeds was carried out in a 50 ml solution of 0.75 % hypochlorite and 95 % Ethanol. Seeds were dipped into the solution for 5 min and continuously agitated by inverting the 50 ml Falcon tube manually. The seeds were washed twice with absolute Ethanol and kept in a hood for at least 10h or overnight until complete drying.

6.5.2 Plant culture in Petri dishes

The sterilized seeds were cultivated in Petri dishes on 25 ml of a growth medium MS/2 containing 2.2 g MS salt (SIGMA); 8 g of bacto agar per liter without sucrose. Stratification of the seeds was done at 4°C during 48h in darkness in order to remove dormancy and to have a synchronised germination. Then the seeds were transferred to a growth chamber with a cycle of 16h light and 8h darkness for germination and growth. The light intensity in the growth chamber was 10,000 lumens/cm^2 and temperature was set at 22°C and 16°C for light and dark period, respectively. Petri dish lids were scotched with urgopore scotch to avoid contamination but to ensure passage of air for respiration of growing seedlings. Seeds were exposed 4h to light in growth chamber, wrapped in aluminium foil and cultured during 5 days in dark conditions. Etiolated seedlings were used for cell wall extraction. If required, antibiotics were added to the medium providing selection.

6.5.3 Plant culture on soil

To measure the plant leaf area, the seeds were spread over the surface of the autoclaved soil, and covered with a plastic film to avoid dryness. Plastic film was removed after the first pair of leaves was detectable. Seeds were stratified for two days at 4°C in darkness. The plants were grown in a growth room (22°C, light intensity 10’000 lumens/cm^2, 16h light/8h dark photoperiod).

6.5.4 Plant genotyping through PCR

Genomic DNA isolated from seedlings was further genotyped through PCR to discriminate for each locus of interest whether the plant line was WT, heterozygous or homozygous for the T-DNA insertion. A specific pair of primers was used in such PCR reaction for each locus to be characterized. The sequence of genomic and T-DNA specific primers are given in table 6.4 and 6.5.

The PCR programme consisted of initial denaturation at 95°C for 5 min, followed by a first step at 94°C for 30s, a second step for annealing for 30s at a temperature specific to the primer pairs used for each gene, and a third step of elongation at 72°C for 1 min. This cycle was repeated 29 times. The programme ended with an extension at 72°C for 10 min. The PCR products were analysed on a 1% agarose gel. List of primers, gene specific and T-DNA specific which were used for the characterization of mutant lines of DUFF 266 gene family and At5g28910 (NGT1) are given in table I and table II respectively.

Table 6.4: Primers used to verify T-DNA insertion

Gene name	**Letter code**	**Name**	**Right border primer (5'→3')**	**Name**	**Left border primer (5'→3')**
At1g10280	A1	RPA1	AGCTGTGATCTGCTTAGCTGTG	LPA1	TAGATTAGCTCTGCAGGTCGG
	A2	RPA2	TGGAATCCAAGTTACACTGGC	LPA2	AGGCAGAAGGAGAGAACTTGC
	A3	RPA3	GGCTTGACCATTACGATCATC	LPA3	AGCTGTGATCTGCTTAGCTGTG
At1g10880	B1	RPB1	TGCTCACATGAAACTTCCTCC	LPB1	CCGATACATTCACATCCTTGG
	B2	RPB2	CGAAATCTCACGTAGAGTCGC	LPB2	GGTAATCCTCAATCGGTCTCC
At1g11940	C1	RPC1	TCACCGGAGTATTTGTGAAGC	LPC1	TGCTAGATCGCAGCTAAAAGC
At1g51770	D2	RPD2	TCAGGTTCCATTTCTGTCCTG	LPD2	ATTCGACGCATAAACAAATGC
At1g62305	E1	RPE1	AATCAAGTGGCAAATCACGTC	LPE1	TTCTCGGACTTAGCTTCCCTC
	E2	RPE2	TCTTTTCTGGTGTCAATCATGG	LPE2	ATCATTGATCAGAAGCCATGC
At1g68380	F1	RPF1	GACCCAAAAGGACCAAAACTC	LPF1	ATCAGAGACTACCTCGAGGGC
	F2	RPF2	ATCAGAGACTACCTCGAGGGC	LPF2	GACCCAAAAGGACCAAAACTC
At2g19160	I1	RPI1	ATGGATCATTGCAGTGCTTTC	LPI1	TAGCTGACATTGCTGTGCATC
At3g21310	J2	RPJ2	GGATTTGGAGAGAGGGAGATG	LPJ2	GGCTTCAGATTCTTCCCAATC
	J3	RPJ3	ACGTTTACATCCACGCAGATC	LPJ3	ACTTTTGCCATTCCCTCTCTC
At4g25870	K1	RPK1	GGAATAGAGGCCGACAAGAAC	LPK1	CTGTCAAGAATCTGCAGGAGG
	K2	RPK2	CAGTAACCGCTTCTCAGCATC	LPK2	CTCTTTTGACCTCACATTCGC
At4g31350	M1	RPM1	GCTACAATCTCAATGGGCATC	LPM1	GCTTGGGGTCAGATATCAATG
	M2	RPM2	CGAGATTCAAAAACAGAACCAC	LPM2	TCTTGCCGTTTATACATTCGG
	M3	RPM3	ATAGCTCTTGCTTTTGGTGGG	LPM3	CTTCCAGCCTCAGAGGAAATC
At4g32290	N2	RPN2	CGGGTTAACCCGAGAAGATAG	LPN2	AATCAAATAAACCGATTGTCCG
	N3	RPN3	TGGACCACCGTACAGAGAAAC	LPN3	GAAGCGCGTGTGCTAGTAGTC
At5g11730	O1	RPO1	AAGGATTTCCATCGAAACTGC	LPO1	AACTTCAGGTTCCATGTTCCC
At5g14550	P1	RPP1	GAGACTCGAAGCTGCGTGTAC	LPP1	TTGGAACTTCACCTGTGATCC
	P2	RPP2	GAAGGCAATCTGTGGTCTCTG	LPP2	TCCTTCAAATCTGCTGAGAGC
At5g22070	R1	RPR1	GAAAACAAAAATCGGAAACCC	LPR1	CGCCTACCACGAAGTATTGAC
At5g57270	V1	RPV1	AGCGGTTAGAGCAGGAGGTAG	LPV1	TTACGGAGAAGAAAAGCCTCC
At5g28910	W1 / *ngt1-1*	RPW1	CAAAAGAAAGCATTTTCCGAG	LPW1	ACCTTTGCAACCATCATGATC
	W3 / *ngt1-2*	RPW3	CCATTTGGTACATCGGATTTG	LPW3	TCTTTGTGGGTGGATTCTTTG

Table 6.5 : T-DNA insertion primers

Lines	**Name**	**Left border primer of the T-DNA insertion (5'→3')**
SALK	LBb1	GCGTGGACCGCTTGCTGCAACT
SALK	LBb1.3	ATTTTGCCGATTTCGGAAC
SALK	LBa1	TGGTTCACGTAGTGGGCCATCG
SAIL	LB1	GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC
SAIL	LB2	GCTTCCTATTATATCTTCCCAAATTACCAATACA
SAIL	LB3	TAGCATCTGAATTTCATAACCAATCTCGATACAC

6.5.5 PCR product sequencing

PCR products were purified and send for sequencing to GATC for determining the exact position of insertion in T-DNA mutants.

6.5.6 RT PCR analysis of *ngt1-1* and *ngt1-2*

6.5.6.1 RNA preparation

Plants were grown *in vitro* for 2 weeks (500 mg fresh weight) for Wild type Col0, *ngt1-1* and *ngt1-2* mutant lines were collected and frozen into liquid nitrogen which ensures inactivation of ribonucleases. Then the entire plants (roots, stems and leaves) were ground into a fine powder in liquid nitrogen with the help of pestle and mortar. All the necessary measures were taken to avoid melting of frozen powder as it activates ribonucleases which in turn cause degradation of RNAs. 2.5 ml of Plant RNA reagent extraction solution (from Fisher) was added and mixture was vortexed until the sample is thoroughly resuspended. Tubes were incubated for 5 min at room temperature. Centrifugation was carried out at 4°C for 15 min at 26,000xg. Aqueous phase was taken into a new microreaction tube and 400 µl of 5 M NaCl and 1.2 ml of chloroform were added. Centrifugation was carried out at 4°C for 10 min at 12,000xg. Aqueous phase was transferred to an RNase-free tube and its volume was measured. 0.9 volume of isopropyl alcohol was added, mixed well and kept at room temperature for 10 min. Centrifugation was carried out at 4°C at 12,000xg for 10 min and supernatant was removed. 5ml of 75% EtOH was added to the pellet and centrifuged at 4°C for 5 min at 12,000xg. Supernatant was removed and pelleted RNAs were dissolved in RNase-free water. RNAs were quantified by a spectrophotometer and was run on a 1% agarose gel to check the quality of RNAs. RNA aliquots were stored at –20°C.

- **Treatment of RNAs with DNase**

In order to efficiently remove DNA (as required for RT-PCR reaction) Promega kit was used. Reaction was carried out in a final total volume of 10 µl containing 2 µg of RNA mixed with (1-8 µl of water), 1 µl of RQ1 RNase-free DNase 10X reaction buffer and 1U of RQ1 RNase-free DNase. Reaction was incubated at 37°C for 30 min. 1 µl of RQ1 DNase stop solution was added to terminate the reaction and incubated at 65°C to inactivate the DNase. Sterile water was added to increase the volume to 100 µl and phenol extraction was done with one volume of phenol followed by chloroform extraction. The aqueous phase was removed into a new tube and precipitated with isopropanol overnight at -20°C followed by centrifugation at 13000 rpm at 4°C for 25 min. The pellet was washed by 70% ethanol and dried using vacuum dryer. RNAs were quantified by spectrophotometer and gel electrophoresis.

- **RT-PCR detection of a "T-DNA:NGT1" hybrid mRNA**

After DNase treatment, cDNAs were synthesized using the iScript kit. The reaction was carried out in a final volume of 30 µl containing 2 µg of RNAs (11 µl of above reaction), 8 µl of 5x iScript reaction mix, 2 µl of iScript reverse transcriptase and 9 µl of Nuclease-free water. The reaction mix was incubated at 25°C for 5 min, at 42°C for 30 min and 85°C for 5 min. 3 µl of cDNA is utilized to carry out the PCR reaction. Five different set of primers, namely "GS1 (RT1FUTs + RT2FUTs)", "GS2 (ATG-FUTs + RPW3)", "UBQ (UBQ10- For + UBQ10-Rev)", "T-DNA *ngt1-1* (LBa1 + RPW1)", "T-DNA *ngt1-2* (LBa1 + RPW3)" were used. Ubiquitin specific primers were used as a control. Sequences of primers are given in table 6.6.

Table 6.6: sequence of primers used for RT-PCR

Set of primers	**Name**	**Forward primer (5'→3')**	**Name**	**Reverse primer (5'→3')**
GS1	RT1-FUTs	CAAGTTCCTTGTAGCTGATTGGG	RT2-FUTs	CAGGAAACGTCAGAAGAGTGTCG
GS2	ATG-FUTs	GGCTTGACCATTACGATCATC	RPW3	CCATTTGGTACATCGGATTTG
UBQ	UBQ10-For	GGCCTTGTATAATCCCTGATGAATAAG	UBQ10-Rev	AAAGAGATAACAGGAACGGAAACATAGT
T-DNA *ngt1-1*	LBa1	TGGTTCACGTAGTGGGCCATCG	RPW1	CAAAAGAAAGCATTTTCCGAG
T-DNA *ngt1-2*	LBa1	TGGTTCACGTAGTGGGCCATCG	RPW3	CCATTTGGTACATCGGATTTG

The reaction was carried out in a final volume of 25 µl containing 3 µl of cDNA and following final concentration of 1 X Go Taq buffer, 0.34 µM of dNTP, 0.4 µM of each forward and reverse primer, 1 µM of $MgSO_4$ and 0.03 units of Go Taq DNA polymerase and water was added to a final volume of 25 µl. Denaturation of double stranded DNA was done at 94°C for 2 min and it was followed by 29 cycles of amplification which included 30 sec at 94°C, 50 sec at Ta (Tm-5°C) and 1 min at 72°C. Samples were analyzed on a 1X TBE, 0.8 % agarose gel.

6.5.7 *ngt1-1* and *ngt1-2* mutants complementation with 35S::NGT1

6.5.7.1 Cloning 35S::NGT1 construct for complementation

Entry vector containing NGT1 gene as made in section 6.2.2.1.2 was used in a LR reaction using either pH2GW7 (to complement the mutant phenotype) or pH7WGF2 (with the GFP construct for localization experiment) as followed: Entry clone (~15-150 ng) 1-7 µl, destination vector pH2GW7 and pH7WGF2 (150 ng/µl), 1 µl TE buffer (pH 8.0) to final volume of 8 µl were added to a 1.5 ml tube at RT and mixed. Then the LR Clonase II enzyme mix was thawed on ice for about 2 min. The LR Clonase II enzyme mix was vortexed briefly twice (2s each time). To each sample (Step 1, above), 2 µl of LR Clonase™ II enzyme mix was added to the reaction and was mixed well by vortexing briefly twice following brief table-top centrifugation. Reaction mixture was incubated at RT for 1h, then stopped by adding 1 µl of the Proteinase K solution, vortexed briefly and incubated at 37°C for 10 min. Procedure for plasmidic DNA extract is the same as decribed above in paragraph 6.2.1.1.3. Integration of gene into destination vectors was confirmed by PCR product sequencing.

6.5.7.2 *ngt1-1* and *ngt1-2* mutants transformation with pH2GW7_NGT1 and pH7GWF2_NGT1

- Preparation of Agro-bacterium competent cells

Agro-bacterium strain C58C1 was grown in 2 ml YEP medium containing rifampicin (50 µg/ml) and gentamycin (50 µg/ml) overnight at 30°C. Next day it was transferred to 200 ml YEP flask and shaked at 250 rpm until the OD600 is 0.3 (4-5hrs). Cells were centrifuged at 5000 rpm for 10 min, supernatant was removed and pellet was re-suspended in 20 ml ice cold 1 mM Hepes buffer (pH 7) and centrifuged again. This step was repeated twice and pellet was re-suspended in 2 ml ice cold 10 % sterile glycerol and then immediately

distributed in aliquots of 40 µl in pre-chilled sterile microreaction tubes, frozen in liquid nitrogen and stored at -80°C.

- **Agrobacterium electroporation**

1µl of DNA from plasmid pH2GW7-NGT1 and pH7GWF2-NGT1 with a concentration of 10-50 ng/µl was added into completely thawed agrobacterium competent cells strain C58C1, kept on ice for 60 seconds and then quickly transferred in chilled 2 mm cuvette. Cells were electroporated at a voltage of 1800 V, 500 µl of LB was added and then the mixture was transferred in 1.5 ml microtubes and incubated with shaking at 30°C for 2h. The bacteria were evenly spread on LB plates with appropriate antibiotics (spectinomycin, rifampicin and gentamycin at 50 µg/ml). Plates were incubated at 30°C overnight or till the colonies appear (one more day).

- **Agrobacterium DNA miniprep**

As plant transformation and transformant selection is time consuming, Agrobacterium positive clones were checked for the presence of gene construct prior to plant transformation. For that matter, a culture was started with a single colony in 5 ml LB with antibiotics (see above), and incubated overnight at 30°C with shaking. 2 ml of cells were centrifuged, supernatant was removed and 2 ml of cells were added again to the tubes and centrifuged again. The composition of buffers used in this prep MPS1, MPS2 and MPS3 is given in the table 6.7 below. The supernatant was removed and cells were re-suspended in 200 µl MPS1 solution, and tubes were incubated at room temperature for 5 min. 20 µl of 20 mg/ml zymolase solution was added, briefly vortexed and incubated at 37°C for 15 min. 300 µl of freshly prepared MPS2 solution. The mix was agitated gently by inverting the tubes 3-4 times and tubes were incubated on ice for 5 min. Then 200 µl of MPS3 solution were added. Tubes were vortexed at least for 10 seconds and incubated for 5 min on ice. Centrifuged for 5 min and supernatant was removed to new tubes. 400 µl of phenol/chloroform/isoapropanol (25:24:1) was added, vortexed, centrifugation was done for 5 min at 15000 rpm and supernatant was removed to new tubes. This step was repeated again and then this step was repeated with chloroform alone. 300 µl of isopropanol was added and incubated on ice for 10 min. centrifugation was done for 5 min and then pellet was washed with 70 % EtOH, dried and re-dissolved in 40 µl water. 2 ml of DNA was used for PCR.

Table 6.7: composition of buffers used for agrobacterium DNA miniprep

MPS1 for 5 ml (Final concentration)		MPS2 for 10 ml		MPS3 for 100 ml	
Glucose (1M)	2.5 ml (50 mM)	NaOH (10N)	200 µl (0.2 N)	Potassium acetate 5M	60 ml
EDTA (0.5 M)	1 ml (10 mM)	SDS 10 %	1 ml (1 %)	Glacial acetic acid	11.5 ml
Tris pH 8.0 (1M)	1.25 ml (25mM)	H_2O	8.8 ml	H_2O	28.5 ml

- **Agrobacterium-mediated transformation of *Arabidopsis thaliana* using floral dip method**

Sterilized seeds of Col0, *ngt1-1* and *ngt1-2* mutants were sown on soil and kept in darkness for 2 days to optimize seed germination. Plants were grown in a growth chamber until the plants begin to bolt and produce floral inflorescence and siliques. Agrobacterium strain transformed with pH2GW7_NGT1 or pH7GWF2-NGT1 were grown by inoculating a single colony into 5 ml liquid LB medium containing the appropriate antibiotics (see above) for binary vector selection and the culture was incubated at 28°C overnight. This pre-culture was used to inoculate 500 ml liquid LB medium (with antibiotics) and cultured at 28°C for 16-24h (till 1.5<OD<2). Transformation of Agrobacterium with plasmids was confirmed by PCR with a gene specific and a primer specific of plasmid antibiotic resistance gene. Cells were collected by centrifugation at 4,000xg for 10 min at room temperature and cells were gently re-suspended in 1 volume of freshly made 5 % (w/v) sucrose solution with a stirring bar. Silwet-L77 detergent was added to a final concentration of 0.02 % (100 µl per 500 ml of solution) and mixed well, immediately before dipping and then transferred to a 500 ml beaker. Plants were covered with plastic film to avoid contamination from the soil. Plants were inverted to dip the aerial parts of the plant including the inflorescence and the rosettes to soak shorter secondary inflorescence in the Agrobacterium cell suspension for 10s with gentle agitation. Dipped plants were removed from the solution and drained the treated plants for 3-5 sec to remove the excessive solution to avoid damage to the flower buds which can occur if they are soaked in solution for too long. Plants were covered with plastic film and laid down the treated plants on their sides for 16-24h to maintain high humidity. Next day the cover was

removed and plants were put back to the growth chamber and grew then normally. Seeds were harvested after siliques maturation.

6.5.7.3 Plant transformants selection

Surface sterilisation of the seeds was carried out in a 50 ml solution of 0.75 % hypochlorite and 95 % ethanol. Seeds were dipped into the solution for five min and continuously agitated by inverting the 50 ml Falcon tube. They were washed twice with absolute Ethanol. They were kept in a hood for at least 10h or overnight for drying. The sterilized seeds were cultivated in Petri dishes on 25 ml of a growth medium MS/2 containing 2.2 g MS salt (Sigma); 8 g of bacto agar per liter without sucrose and appropriate antibiotics (cefotaxime 250 µg/ml and hygromycin 50 µg/ml). Stratification of the seeds was done at 4°C for 48h in darkness in order to remove dormancy and to have a synchronised germination. Then the seeds were transferred to a growth chamber with a cycle of 16h light and 8h darkness for germination and growth. The light intensity in the growth chamber was 10,000 lumens/cm^2 and temperature was 22°C. Petri dishes were wrapped with urgopore scotch to avoid contamination but to ensure passage of air for respiration of growing seedlings. After 7-10 days, transformants were readily distinguished as seedlings have healthy green cotyledons and true leaves. Then potential transformants were transferred to water saturated soil and tray was covered with plastic film to maintain high humidity. Tray was put in growth chamber and plants were grown for seed collection.

6.6 Methods for cell wall analysis

6.6.1 Cell wall preparation

For the preparation of alcohol insoluble residues (AIR), cell wall was extracted from 5 days old dark grown seedlings and for sub-fractionation of polysaccharides cell wall was prepared from 8 weeks old light grown plants to get sufficient quantity of material.

6.6.1.1 Alcohol insoluble residues preparation

Cell wall was prepared from 5 day-old dark-grown seedlings, culture on top of a nylon mesh membrane. Seedlings were harvested with the spatula by sliding over nylon mesh. Seedlings were washed with 15 ml of 70 % EtOH and placed at 70°C for 15min for inactivation of enzymes and to wash intracellular contents (Fry, 1988). Centrifugation was done for 10 min at 5,000xg to remove ethanol, organic phase and seedlings were transferred into tissue homogeniser for grinding. They were ground in 5 ml of 70 % EtOH. Wash two

times seedlings with 5 ml EtOH 70 %, 15 min at 70°C, followed by centrifugation as did previously. Supernatant was removed and the pellet was dried at room temperature or at 40°C with nitrogen evaporator.

Cell wall material was weighted and re-suspended in water (containing 0.02 % NaN_3 to avoid contamination) up to the required dilution (i.e. 50µg/ml).

6.6.1.2 Cell wall preparation for sub-fractionation

Leaves were harvested from 35 days old *Arabidopsis thaliana* plants from Col0 (WT) and *ngt1-1* and *ngt1-2* mutants. Ten grams of plant material was ground by mortar and pestle using liquid N_2 for each cell wall preparation. Plant material was extracted once during 4h with 50 mL of buffer (Hepes 20mM, pH7.5, SDS 0.5%, sodium metabisulfite 3 mM, one drop of octanol), centrifuged to pellet insoluble material and pellet is then rinsed twice with 50 mL ddH2O and once with 50 mL EtOH 70%. All supernatants (230 mL for each sample) were pooled and stored at 4°C overnight to precipitate most SDS, and then centrifuged 30 min at 13,000xg at 4°C. As all materials did not totally pellet down, the suspension was further filtrated on nylon cloth to obtain clear supernatants. Supernatants are then mixed with EtOH 96 % (1 / 4; v/v) in order to precipitate polymer contents and centrifuged 45 min at 12,000xg. Pellets (*ngt1-1* and *ngt1-2*) from each SDS extraction were resuspended in 30 mL phosphate buffer containing α-amylase (phosphate buffer 100 mM, pH6, 0.02% NaN3, 2500 units α-amylase) and let overnight at 30°C. Then 15mL of EtOH absolute was added to quench the reaction and suspension was spun 8,000xg for 30min at 10°C. Pellets were resuspended and washed twice EtOH 70% spun 20 min at 8'000g and at 10°C. All supernatants were combined. Remaining pellets were washed once again with acetone, spun 20 min at 8,000xg at 10°C and dried. After drying, pellet was ground using mortar and pestle and weight. Pellets were considered cell wall material and ready for further extraction.Typical yields for Col0 and mutants cell wall material were around 100-130 mg Col0 (112 mg), NGT1-1 (110 mg), NGT1-2 (129 mg).

Cell wall from Col0, *ngt1-1* and *ngt1-2* was extracted with 15+5 mL ammonium oxalate 0.5% pH 4.5 (2h, 90°C) with a previous homogenization using glass potter, then centrifuged 9,000xg for 15 min at 15°C. Supernatants were saved and pellets were extracted once more with 20 mL ammonium oxalate 0.5% pH 4.5 (1h, 90°C). Suspension was centrifuged (swinging, 15 min, 15°C, 4,000xg). Supernatants were combined to the first one, transferred to dialysis tube (MWCO 3500Da, snakeskin), dialysed 48h against ddH2O (5L) at 4°C with water changes each 12h. Pellet was washed once with EtOH 70 % (15 mL), centrifuged and

considered ready for hemicelluloses extraction. Cell wall remaining from each oxalate fraction were extracted overnight with 20 mL KOH 1N containing 0.1% $NaBH_4$ at RT, centrifuged (swinging) 10 min at 15°C 4,000xg, and pellet was extracted once again for 4h with 20mL KOH 1N containing 0.1% NaBH4 at RT then centrifuged 10 min, 15°C 4,000xg. Supernatants were pooled and neutralised (using concentrated CH3COOH 17N) previously to dialysis. Sample volumes were measured before dialysis (Col0 KOH 1N extract 40mL, *ngt1-1* KOH 1N extraction was 42mL and *ngt1-2* KOH 1N extraction was 38mL). Supernatants KOH 1N were combined to the first one, transferred to dialysis tube (MWCO 3500Da, snakeskin), dialysed 48h against ddH2O (5L) at 4°C with water changes each 12h. After dialysis sample was freeze-dried. Cell wall remaining from each KOH 1N fraction were extracted overnight with 20 mL KOH 4N containing 0.1% NaBH4 at RT. Centrifuged 10 min at 15°C 4,000xg. Pellets were extracted once again for 4h with 20 mL KOH 4N containing 0.1% NaBH4 at RT, then centrifuged 10 min, 15°C 4,000xg and supernatants were pooled and neutralised previous to dialysis, using concentrated CH3COOH. Supernatants KOH 4N were combined to the first one, transfer to dialysis tube (MWCO 3500 Da, snakeskin), dialysed 48h against ddH2O (5L) at 4°C with water changes each 12h. After dialysis sample was freeze-dried. Pellet was rinsed once with 10mL ddH2O, combined to KOH 4N supernatant, and once with 20 mL acetone. Acetone is discarded after centrifugation 10min, 15°C 4,000g. Residual pellet is dried and weighted. All fractions were analysed using alditol acetates procedure for sugar content quantification.

6.6.2 Monosaccharide composition

6.6.2.1 Alditol acetates

This method is used to obtain a quantification of the neutral sugar composition from a cell wall sample. In order to decompose sugar polymers to monomers, sample is hydrolyzed with trifluoroacetic acid (TFA), then sample is reduced to open the ring structure of the sugar monomer and in final step sample is acetylated. The derivative molecules are volatile and can be separated on Gas Chromatography (GC)

- **TFA hydrolysis**

2.5 mg of cell wall material was placed and dried in a clean glass tube. 500 µl of TFA 2N was added to each tube and tubes were placed at 121 ºC (250 ºF) for 2h and tubes were let 10 min on the bench to cool down. 25 µg of inositol was added in each tube, and tubes were dried using nitrogen evaporator. 250 µl of NH_4OH 2M were added to neutralize the residues

and tube contents were dried (Heating Block could be setup around 50ºC). 250 µl of NH_4OH 0.2M were added to neutralize the residues and tube content was dried. Now the samples were ready for reduction step.

- **Derivatization of sugar into corresponding alditol acetates**

For reduction of samples, residues were re-suspended in 200 µl of NH_4OH 2M (could be vortexed or sonicate to help dissolution). 200 µL of $NaBH_4$ 50 mg/ml in NH_4OH 2M were added (this solution has to be made fresh). Tube was quickly vortexed for homogenisation and placed for 90 min at 40 ºC. 10 µl of acetic acid was added (check for the freezing), to destroy excess of reductive agent and tube content was dried under N2. If no feezing, the reduction step was done once again. 200 µl of a MeOH/Acetic acid (9/1) were added and samples were dried under N_2. 500 µl of MeOH were added, residues re-suspended and dried again. This was repeated two times again and samples were reduced and were ready for acetylation

300 µl of 1-Methyl-imidazole (catalyst) was added to residues and residues were re-suspended in the catalyst (by strong vortexing). 2 ml of anhydride acetic was added and tubes were vortexed quickly for homogenisation with catalyst and then let stand 10 min at room temperature. 4 ml of ddH_2O were added to destroy the excess of anhydride acetic acid. 1 ml of dichloromethane was added and tubes were vortexed. Centrifugation was done for 1 min at 1000 rpm to separate the two phases. Organic phase was washed with 4 ml ddH_2O, recovered (at least 500 µl) into GC auto sampler vials, and injected on GC-MS.

6.6.2.2 Trimethylsilyl derivative (TMS)

- **Methanolysis**

Tubes containing 1 mg of dried cell wall (with 20 µg of inositol as an internal standard) and dried standard mixture (100 µg of each sugar with 20 µg of inositol) were taken. 500 µl of 1M HCL in MeOH was added and tubes were heated at 80°C for about 16h. Tubes were checked after 10 min to ensure the caps are tightly closed. Methanolic HCl was evaporated at 40°C with the stream of N_2. 250 µl of MeOH was added and dried. This step is repeated once.

- **TMS derivatization**

200 µl of Silylation reagent was added, tubes were sealed or closed and heated at 80°C for 20 min. Samples were allowed to cool, and reagent was evaporated with the N_2 until dried but not too long. 1 ml of cyclohexane was added, vortexed and centrifuged. Cyclohexane was transferred to GC vials and samples were analyzed by GC.

6.6.3 Glycosyl linkage composition analysis

This method is used to get linkage information about the polysaccharides present in a sample, by comparison of retention time with a standard library. In this method polysaccharides or cell wall is treated for methylation then sample is hydrolyzed with TFA, in order to decompose sugar polymers to monomers. Sample is reduced to open the ring structure of sugar monomer. Sample is acetylated, and then the derivative molecules are volatile and can be separated on gas chromatography (Figure 6.3).

- **Methylation**

1 mg of cell wall material was placed and dried in clean glass tube. Sample was re-suspended in 300 µl of DMSO-NaOH (1g of NaOH ground in 4 ml DMSO), vortexed and stirred at least 2h (RT) with a small magnetic stirrer in the tube. Tubes were placed on ice and 500 µL of CH_3I were added. Tubes were put back to RT and stirred for 1h. 500 µl of CH_3I were added again, and the slurry was stirred for 1h. 2 ml of ddH_2O were added and tubes were stirred. 2 ml of dichloromethane were added; tubes were vortexed and centrifuged 2 min at 1,500 rpm to separate the phases. Aqueous phase was transferred to a glass tube and washed with 1 ml of CH_2Cl_2; this organic wash was pooled to organic phase. Organic phase was recovered, washed twice with 5 ml of ddH_2O and tubes were vortexed. Centrifugation was done again for 2 min at 1,500 rpm to separate the phases. Aqueous phase was discarded and organic phase was dried under N_2 (with heating 40°C). The partially methylated polysaccharides were treated with TFA for hydrolysis, reduced with $NaBD_4$ and acetylation was performed as in section 2.5.2.1.

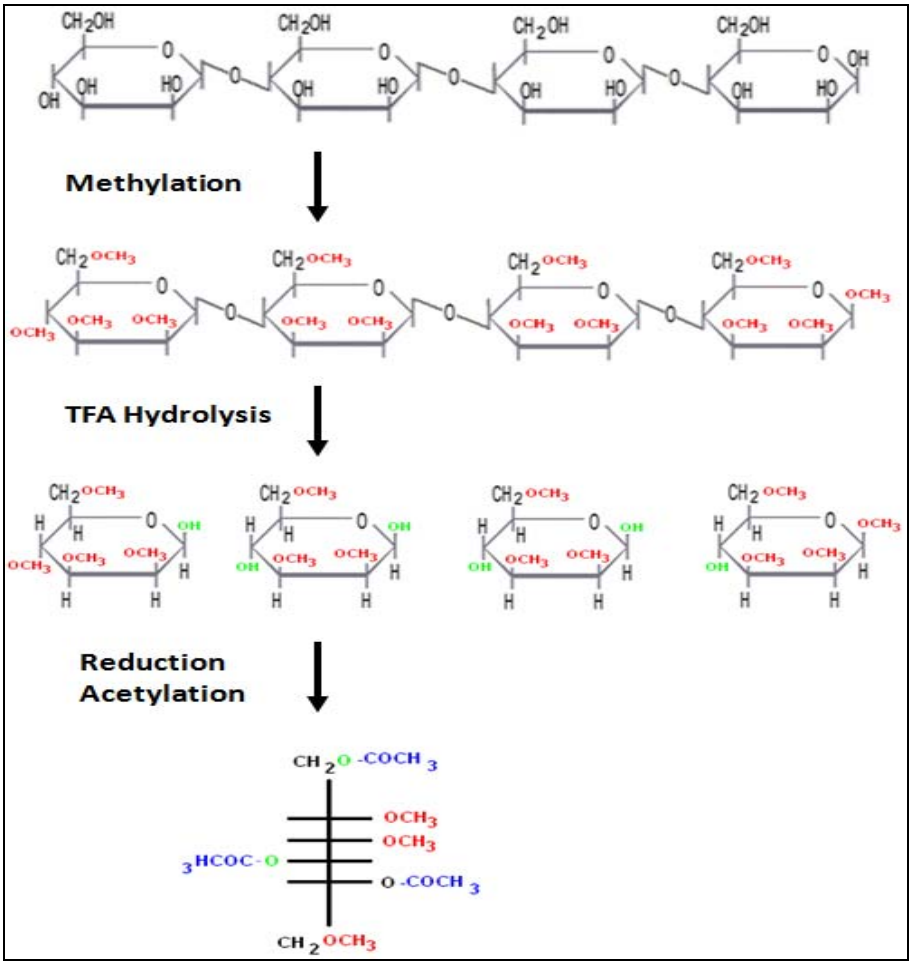

Figure 6.3: Scheme explaining the different steps of per-methylation and per-acetylation of polysaccharides. In red: O-methylation; in blue: O-acetylation and green colour indicates the free hydroxyl groups due to TFA

- **Analysis by GC-MS**

Gas chromatography-mass spectrometry (GC-MS) is a method that combines the features of gas chromatography and mass spectrometry to identify different substances within a test sample. Derivatized monosaccharide resulting from treatment of cell wall polysaccharides are separated using a gas chromatograph (Agilent 6850) equipped with a capillary column SP-2380 (length 30 m, internal diameter 0.25 mm, 0.20 µm phase thickness, Sigma-Aldrich) and analyzed by mass spectrometry with Electron Ionization detector. Injector was heated at 240°C, solvent delay was set at 4 min and MS transfer line was set at 150°C. Oven program for alditol acetates started at 160°C with a 2.5°C/min ramp till 220°C, then 5°C/min till 240°C. Oven program for partially methylated alditols acetates was a linear ramp from 140°C to 220°C at 1.5°C min, then 5°C/min till 240°C.

6.7 Phenotypic characterization of T-DNA lines

6.7.1 Leaf area measurement using image J

To measure the area of leaves in a precise and reproducible manner, a method has been developed in the lab. The photo of each pot was taken, including the plant to be measured and a reference (a mark of 2 cm on paper) at different stages of development. These photos were then analyzed using the free software ImageJ (http://rsbweb.nih.gov/ij/), as follows: a line is drawn over the mark of 2 cm to calculate the pixel/cm. Ellipses are then drawn around two or three leaves (depending on the stage of development) and leaf area of outermost leaves are measured. With over pixel/cm, the leaf area in cm^2 can be calculated. The data are then analyzed using Excel software (Microsoft ©).

6.7.2 Sample preparation for microscopy

Tissue fixation was done in 2.5 % glutaraldehyde in 0.1 M sodium cacodylate buffer at room temperature (pH 7.2) during 2h and then washed in 0.1 M sodium cacodylate buffer pH 7.2. Post fixation was done in 1 % osmium tetraoxide at 4°C in 0.1M sodium cacodylate buffer during 1h. For embedding in LR white, dehydration of tissues was done with 25 %, 50%, 70% and 80% ethanol during 20 min for 2 times with each concentration of ethanol. Afterwards for impregnation for embedding in LR White, tissues were dipped into a mixture of 1vol of LR white and 1vol of 80 % alcohol for 20 min. They were dipped into a mixture of 2vol of LR white and 1vol of 80 % alcohol for 20 min. Dipping was done again into pure LR White two times for 30 min. Incubation was done overnight into pure LR White at 4°C and they were embedded in closed, labelled, gelatine capsules with fresh resin and let them polymerised at 60^oC for 72h. Several sections were cut at a thickness of 1 micron using diamond knife and an ultramicrotome. The sections were deposited onto a glass slide and dried on a hot plate at 60°C. The sections were then stained with 1 % toluidine blue in 1 % borax solution for 1 min at 60°C. The slides were rinsed off with distilled water, dried and the sections were covered with a glass coverslip using a Eukitt mounting medium.

- **Immunolabeling of stem cross sections**

For immunofluorescence staining technique, we used the semi-thin sections of sample without post-fixation by osmium tetraoxide. The slides were incubated in PBS buffer containing 5 % BSA for 1h to block the non-specific sites and to avoid background. Sections

were incubated with specific primary antibody at 1/5 dilution in PBS-B (PBS with 1 % BSA), at 4°C overnight. The sections were washed three times with PBS-T (PBS with 0.1 % Tween 20) for 5 min, then incubated with secondary antibody at 1/50 dilution in PBS-B for 1h at RT in dark. Sections were again washed three times with PBS-T and finally washed twice with ddH_2O for 5 min. Sections were allow to dry and covered with a glass coverslip using glycerol-phosphate buffered saline based mounting solution (CFM-1). After immunolabelling of stem cross sections, they were observed with epifluorescence microscope at 5030 ms acquisition time.

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Annexes

8 Annexes

Protocol 1: Transfection of insect cells

For transfection of insect cells the following protocol was used (Sf9 cells and Grace medium were provided from Invitrogen; Baculovirus DNA, Buffer A and B from Pharmingen BD Dickinson; fetal bovine serum and gentamycin from Sigma)

1. Resuspend Sf9 cells from a 3 days culture 25cm2 flask in 4ml of supplemented Grace medium.
2. Transfer the supernatant to a 50ml tube.
3. Create a 1/4 cell dilution (25µl cells + 65 µl PBS + 10µl trypan).
4. Transfer the dilution to a Malassez cell with a cover glass.
5. Count living and dead cells (dead coloured blue by trypan), on a 2x25 square (4x25= 1mm3=1µl)

   Cells pr. ml are counted as (no. of living cells x dilutionx100 squaresx103 (cm3))

   % of dead cells are counted as ((dead cells/total cells) x100)

   6. Prepare in a 50 ml sterile tube a dilution of 4x104 Sf9 cells/ml in complete Grace medium.
7. Add 100 µl of 4x105 Sf9 cells in 12 wells (3x4) of a 96 well plate (4x104 cells per well)

   Four wells for transfection, four for buffer control, and four for negative control (cells only).
8. Filled with water the surrounding wells to prevent drying.
9. Incubate the plate 30 min at 27°C. (If the number of dead cells is higher than 30%, remove the medium and add new medium at this after step 9)
10. While cells are attaching to the plate, prepare, in 4 sterile 0,5 ml tubes, a dilutions of the Baculogold DNA (1µl + 13,3µl TE, final 7ng/µl).
11. Add 1µl of the recombinant vector pVT-BAC-His with insert (in TE; final 30ng/µl) to each of the four tubes with 30 seconds intervals. Timing starts after the addition in the first tube. Let in contact exactly 5 min at room temperature.

12. During the 5 minutes, remove medium from 4 wells (for transfection) within the 96 well plates and replace it with 16µl of buffer A.
13. At the end of the timing, add 15µl of buffer B to the DNA mix within each PCR tube and transfer the 17µl to each of the 4 wells. Stir gently the plate.
14. Remove medium from 4 wells (for buffer control) within the 96 well plate and replace it with 16µl of buffer A and 15 µl of buffer B.
15. Wrap parafilm around the plate and incubate 4h at 27°C.
16. Remove supernatants from the 12 wells with a pipette and replace it by 100µl fresh supplemented Grace medium.
17. Incubate for 72h at 27°C.

Protocol 2: Amplification of recombinant virus

3 days after transfection of insect cells the 1st amplification was carried out.

1. Resuspend Sf9 cells from a 3 days culture 25cm^2 flask in 4ml of supplemented Grace medium.
2. Transfer the supernatant to a 50ml tube.
3. Create a 1/4 cell dilution (25µl cells + 65 µl PBS + 10µl trypan).
4. Transfer the dilution to a Malassez cell with a cover glass.
5. Count living and dead cells (dead coloured blue by trypan), on a 2x25 square (4x25= 1mm^3=1µl)
   Cells pr. ml are counted as (no. of living cellsxdilutionx100 squaresx10^3 (cm^3))
   % of dead cells are counted as ((dead cells/total cells)x100)
6. Add 2 ml diluted (10^6) Sf9 cells in 4 wells of a 6 well plate.
7. Incubate the plate 30 min at 27°C.
8. Transfer 100 µl of supernatants from transfection of insect cells (transfection wells from protocol 6).
9. Put parafilm around the plate and incubate 3 days at 27°C.

3 to 4 days after 1st amplification of insect cells, a 2nd amplification was carried out.

10. Transfer medium from the 4 wells including the infected cells by pipeting to 2 ml sterile tubes.
11. Centrifuge at 5000 rpm for 5 minutes.
12. Transfer the supernatants to screw cap sterile tubes and store at 4°C for one night.

13. Resuspend Sf9 cells from a 3 days culture 25cm^2 flask in 4ml of supplemented Grace medium.
14. Transfer the supernatant to a 50ml tube.
15. Create a 1/4 cell dilution (25µl cells + 65 µl PBS + 10µl trypan).
16. Transfer the dilution to a Malassez cell with a cover glass.
17. Count living and dead cells (dead coloured blue by trypan), on a 2x25 square (4x25= 1mm^3=1µl)
    Cells pr. ml are counted as (no. of living cellsxdilutionx100 squaresx10^3 (cm^3))
    % of dead cells are counted as ((dead cells/total cells)x100)
18. Split 3.10^6 Sf9 cells/5ml of Grace complete medium in a 25cm^2 flask.
19. Add 500µl of the 1rst amplification to the cells.
20. Incubate the flask for 3 days at 27°C.

3 to 4 days after 2nd amplification of insect cells, a 3rd amplification was carried out.

21. Transfer medium from the 2nd amplification including the infected cells by pipeting to a 50 ml tubes.
22. Centrifuge at 2000 rpm for 5 minutes in a swing bucket rotor.
23. Transfer the supernatant to 3 screw cap tubes (2 to conserved at -70°C and 1 at 4°C for 3nd amplification and titration)
24. Resuspend Sf9 cells from a 3 days culture 25cm^2 flask in 4ml of supplemented Grace medium.
25. Transfer the supernatant to a 50ml tube.
26. Create a 1/4 cell dilution (25µl cells + 65 µl PBS + 10µl trypan).
27. Transfer the dilution to a Malassez cell with a cover glass.
28. Count living and dead cells (dead coloured blue by trypan), on a 2x25 square (4x25= 1mm^3=1µl)
    Cells pr. ml are counted as (no. of living cells x dilution x 100 squares x 10^3 (cm^3))
    % of dead cells are counted as ((dead cells/total cells) x100)
29. Split 9.10^6 Sf9 cells in 12ml of Grace complete medium in a 75cm^2 flask.
30. Add 100µl of the 2nd amplification to the cells.
31. Incubate the flask for 3 days at 27°C.
32. Transfer medium from the 3nd amplification including the infected cells to a 50 ml tube.
33. Centrifuge at 2000 rpm for 5 minutes in a swing bucket rotor.

34. Transfer 1 ml of the supernatant to a 1.5ml screw cap tube (to conserve at -70°C) and the resting to a 50 ml skirt tube, aluminium foiled, plus parafilm the cap and store at 4°C.

Protocol 3: Protein expression in insect cells

Production of recombinant protein in Hi5 insect cells was carried out as follow.
(Express Five™ and Hi5 cells were provided by Invitrogen; Glutamine and gentamycin from Sigma)

1. Resuspend Hi5 cells in 4ml from a 3 days culture 25cm^2 flask of supplemented Express Five medium.
2. Transfer the supernatant to a 50ml tube.
3. Create a 1/4 cell dilution (25µl cells + 65 µl PBS + 10µl trypan).
4. Transfer the dilution to a Malassez cell with a cover glass.
5. Count living and dead cells (dead coloured blue by trypan), on a 2x25 square (4x25= 1mm^3=1µl)
   Cells pr. ml are counted as (no. of living cells x dilution x100 squares x10^3 (cm^3))
   % of dead cells are counted as ((dead cells/total cells) x100)
6. Prepare in a 50 ml sterile tube a dilution of 9x10^6 Hi5 cells/ml in Express Five medium.
7. Split 9x10^6 diluted Hi5 cells in 12 ml in a 75 cm^2 flask.
8. Incubate the plate 30 min at 27°C.
9. Infect the cells with the 3rd amplification at MOI = 5 (5 particles per cell) according the titre of the virus.
10. Incubate the flask for 4 days at 27°C.
11. Recover the expressed protein by centrifugation of the supernatant at 2.000 rpm for 5 minutes, in a 50ml tube.
12. Transfer and divide the supernatant in less sample fraction and store at -70°C.

    In some experiments the Express Five medium was replaced with Excell-405 (a serum-free medium from Sigma)

Protocol 4: Protein concentration

To concentrate the proteins collected after expression in insect cells the following protocol was used.
(The different resin was provided by; Fractogel by Calbiochem, Agarose from Sigma, His-Bind (Ni-trap) by Novagen)

1. Resuspend the resin suspension (Fractogel, Agarose or Ni-trap).
2. Pipette (with a cut tip) 25µl resin/ml of supernatant to concentrate and transfer to a 2ml tube.
3. Add 500µl H_2O mQ; mix the tube by inverting it.
4. Centrifuge 2 min at 6000 rpm; and let resin settle to the bottom of the tube before removing the supernatant with a pipette.
5. Repeat this washing step 2 times.
6. Remove as much of H_2O as possible after the 3rd washing step.
7. Add 1-2ml cultured supernatant of expressed protein (from protocol 8, last step)
8. Add a magnet to each of the tube.
9. Incubate the tube for 2 hours at 4°C (with magnetic stirring).
10. Remove the magnet.
11. Centrifuge 2 min at 6000 rpm; and let resin settle to the bottom of the tube before removing the supernatant with a pipette.
12. Add 1 ml of PBS to resin.
13. Mix well with a pipette and transfer the entire suspension to a 1.5ml tube.
14. Centrifuge 2 min at 6000 rpm; and let resin settle to the bottom of the tube before removing as much as possible of the supernatant with a pipette.
15. Resuspended in denaturing buffer (25-50µl Blue 2x, see p171).
16. Boil the tube for 5 minutes in a preheated water bath.
17. Sonicate the tubes for 2 minutes.
18. Analyze the samples by electrophoresis or store the samples at -20°C.

Protocol 5: Protein electrophoreses

Electrophoresis of proteins was carried out on SDS-PAGE (polyacrylamide gel) after concentration of expressed protein. See p171 for composition of buffers.

1. Prepare a resolving gel (10-12%) depending on the expected protein size desired visualized.
2. Pipette between to glass plates, connected like a sandwich. Let polymerize.
3. Prepare a staking gel (5%).
4. Pipette between to glass plates on top of the solid resolving gel and insert a comb. Let polymerize.
5. Place the gel (in the glass sandwich) in an electrophoresis chamber filled with migration buffer. Load between 10- 20 µl of protein sample to each well in polyacrylamide gel. (A positive control and molecular weight markers should be included)
6. Proceed to electrophoretic separation for approximately 1 hour at 200 mA.

9 Protocol 6: Protein staining

Coomassie staining was preformed to see the protein pattern after protein electrophoresis. All steps are performed on a shaking table at room temperature. See p171 for composition of buffers.

1. Fix the gel with 40 % (v/v) ethanol, 10 % (v/v) acetic acid for 1 hour.
2. Wash the gel with water for 10 minutes.
3. Stain the gel in coomassie solution for 1 hour.
4. Remove the coomassie solution and replace with 1 % (v/v) acetic acid and 50% (v/v) to distain.

Protocol 7: Protein transfer

Protein can be transferred from a gel to a nitrocellulose membrane following the protocol state below

A positive control is used to demonstrate that the protocol is efficient and correct and that the antibody recognizes the target protein which may not be present in the experimental samples. (Nitrocellulose membrane was provided by PALL, Whatman 3MM filter paper by VWR). See p171 for composition of buffers

1. Prepare cathode and anode buffers.
2. Cut 6 pieces of Whatman 3M filter paper for every gel to be transferred (at the size of the respective gel).
3. Soak 3 pieces of Whatman filter paper in the cathode buffer and 3 pieces of Whatman filter paper in the anode buffer.
4. Soak a nitrocellulose membrane (at the same size of the respective gel) in anode buffer.
5. Stack on to the anode the 3 pieces of Whatman 3MM filter paper soaked in anode buffer.
6. Roll a clean plastic rod over the stack to remove any bubbles trapped between them.
7. Add on top the soaked nitrocellulose membrane.
8. Roll a clean plastic rod over the stack to remove any bubbles trapped between them.
9. Place the polyacrylamide gel (protocol 12) on top of the transfer stack.
10. Stack the 1 pieces of Whatman filter paper soaked in cathode buffer.
11. Roll a clean plastic rod over the stack to remove any bubbles trapped between them.
12. Repeat steps 10 and 11 for the 2 resting Whatman filter paper soaked in cathode buffer.
13. Proceed to electrophoretic transfer (0.8 mA /cm^2) for 2 hours, in a Semi-Dry Electrophoresis Transfer Cell (BioRad).

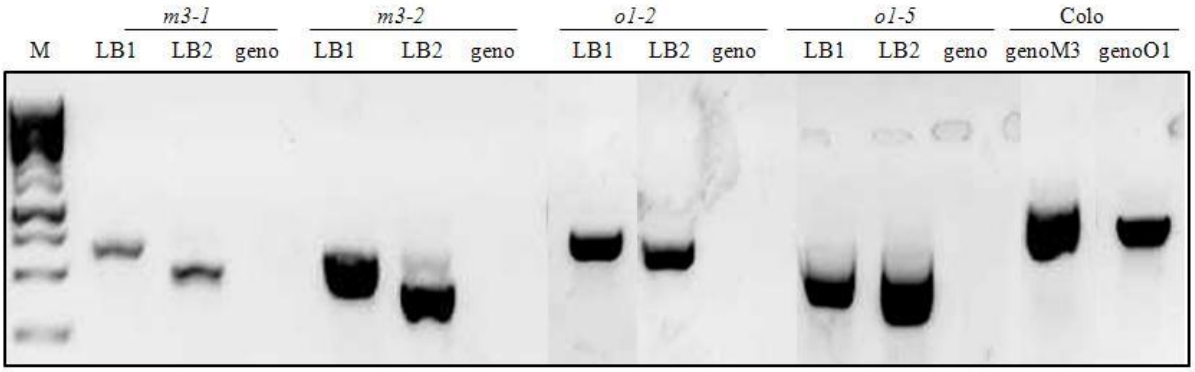
Protocol 8: Visualization of proteins on membranes by immuno-blotting

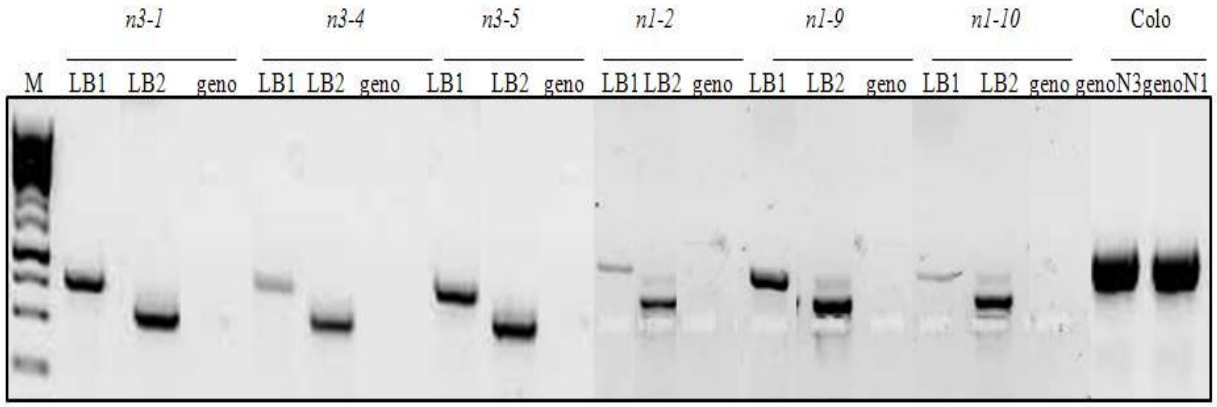
After the protein transfer to nitrocellulose membrane, a saturation step of non-specific sites, immune blotting could take place. See p171 for composition of buffers.

1. The nitrocellulose membrane (after transfer) is washed 5 minutes in 20 ml TBS.
2. Saturation of non-specific sites was carried out with a solution of 1% gelatin in a total of 20 ml TBS for 45 minutes.
3. Wash the membrane twice for 5 minutes in 20ml TBST.
4. Incubate the membrane for 1 hour in TBST and Anti Xpress (1:4000, Invitrogen).
5. Wash the membrane three times for 5 minutes in 20 ml TBST.
6. Incubate the membrane for 1 hour in TBST and secondary antibody GRAM-Per (1:2000, Sigma, goat anti mouse peroxidase).

7. Wash the membrane four times for 5 minutes in 20 ml TBST.
8. Wash the membrane one time for 5 minutes in 20 ml TBS.
9. Color development using the AEC 101 kit (Sigma).

Homozygous mutant lines





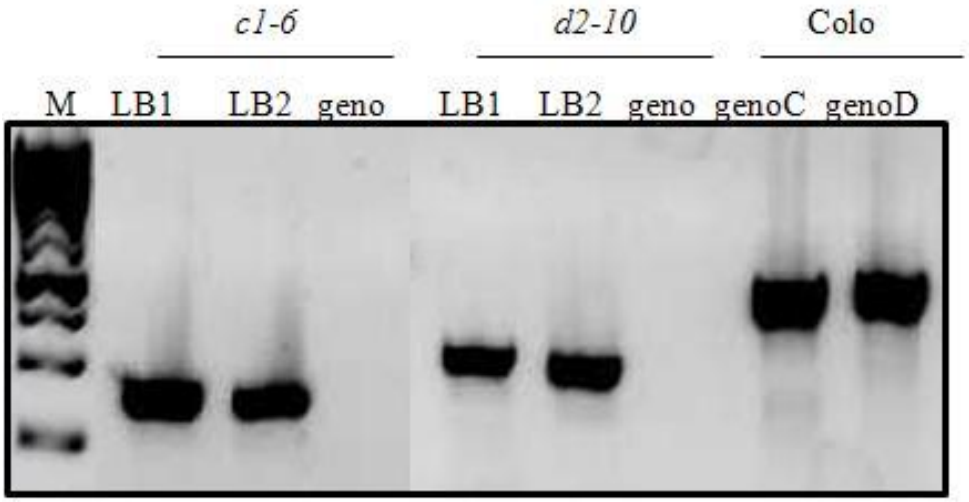


Figure 8.1: T-DNA mutant characterization through PCR. In figure LB1 and LB2 are left border T-DNA specific primers while geno are genomic forward and reverse gene specific primers. Col0 DNA is used as a control. A) Homozygous plants selected by PCR for genes M, O, N, C and D. Primers genoM, genoO, genoN, genoC and genoD represent genomic gene specific primers. B) Heterozygous plants selected by PCR for genes C, D and V. Primers genoC, genoD and genoV represent genomic gene specific primers. C) Wild type plants found by PCR for genes R and E. Primers genoR2, genoR1 and genoE represent genomic gene specific primers.

Heterozygous mutant lines

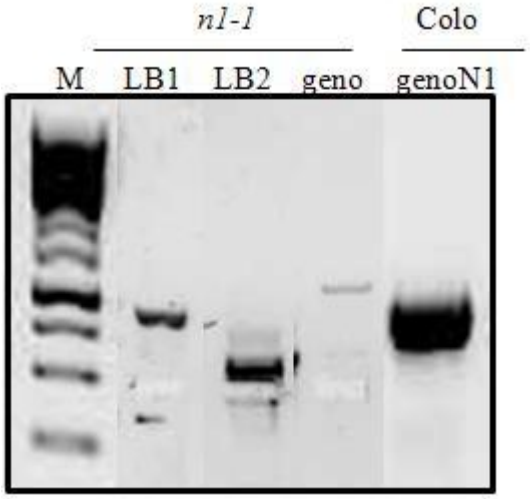


Figure 8.2: T-DNA mutant characterization through PCR. In figure LB1 and LB2 are left border T-DNA specific primers while geno are genomic forward and reverse gene specific primers. Col0 DNA is used as a control.) Heterozygous plants selected by PCR for genes N (allele N1). Primers genoN1 represent genomic gene specific primer.

Wild type mutant lines

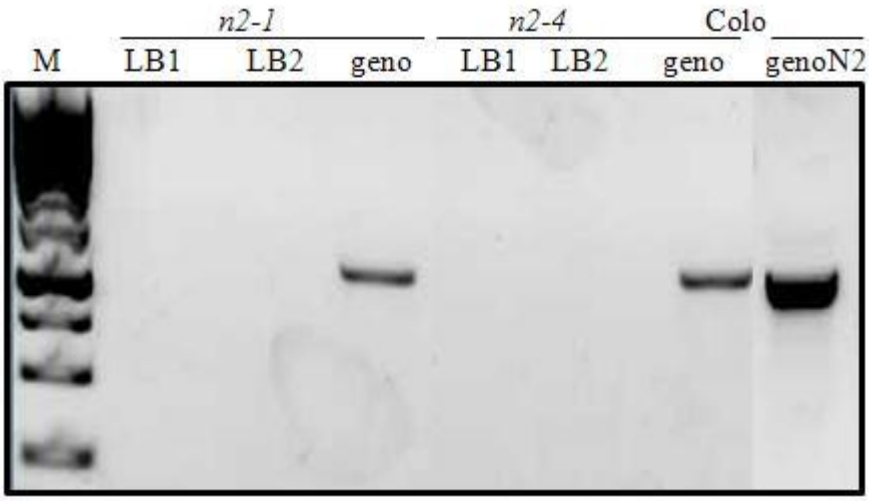


Figure 8.3: T-DNA mutant characterization through PCR. In figure LB1 and LB2 are left border T-DNA specific primers while geno are genomic forward and reverse gene specific primers. Col0 DNA is used as a control. Wild type plants found by PCR for geneN (allele N2). Primers genoN2 represent genomic gene specific primer.

Golgi-mediated synthesis and secretion of matrix polysaccharides of the primary cell wall of higher plants.

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1- Introduction:

What makes the plant Golgi apparatus unique is its ability to synthesize complex matrix polysaccharides of the cell wall. Unlike cellulose which is synthesized at the plasma membrane and glycoproteins whose protein backbones are generated in the endoplasmic reticulum, the cell wall matrix polysaccharides (pectin and hemicelluloses) are assembled exclusively in the Golgi cisternae and transported to the cell surface within Golgi-derived vesicles (Driouich *et al*., 1993).

The synthesis of cell wall matrix polysaccharides occurs through the concerted action of hundreds of glycosyltransferases. These enzymes catalyze the transfer of a sugar residue from an activated nucleotide-sugar onto a specific acceptor. The activity of these enzymes depends, in turn upon nucleotide-sugars synthesizing/interconverting enzymes in the cytosol, and also on the nucleotide-sugar transporters necessary for sugar transport into the lumen of Golgi stacks and subsequent polymerization (see chapter by Simon Turner).

Because cell wall matrix polysaccharides exhibit an important structural complexity, their biosynthesis must be adequately organized and a certain degree of spatial organization/coordination must prevail within Golgi compartments, not only between glycosyltransferases themselves, but also between glycosyltransferases and nucleotide-sugar transporters (Seifert, 2004).

Cell wall matrix polysaccharides confer important functions to the cell wall in relation with many aspects of plant life including cell growth, morphogenesis and responses to abiotic and biotic stresses. Plant cell walls are also an important source of raw materials for textiles, pulping and, potentially, for renewable biofuels as well as for food production for humans and animals.

In this chapter, we focus on the biosynthesis of complex polysaccharides of the primary cell wall. We present and discuss the compartmental organization of the Golgi stacks with regards to complex polysaccharides assembly and secretion using immuno-electron microscopy and specific antibodies recognizing various sugar epitopes. We also discuss the significance of the recently identified Golgi-localized glycosyltransferases and sugar interconverting enzymes that are responsible for the biosynthesis of complex polysaccharides of the primary cell wall matrix.

2- The primary cell wall is composed of complex carbohydrates

Plants invest a large proportion of their genes (more than 10%) in the biosynthesis and remodelling of the cell wall (Arabidopsis genome initiative, 2000; International Rice genome sequencing project, 2005; Tuskan *et al*., 2006)

Cell walls are composed of a diversity of complex carbohydrates whose structure and function vary at the level of cell type and the stage of development (Roberts, 1990). The primary wall of dicotyledonous plants comprises cellulose microfibrils and a xyloglucan network embedded within a matrix of non-cellulosic polysaccharides and proteins (i.e. glycoproteins and proteoglycans). Four major types of non-cellulosic polysaccharides are found in the primary walls of plant cells (in taxa outside the graminae), namely the neutral hemicellulosic polysaccharide xyloglucan (XyG), and three main pectic polysaccharides, homogalacturonan (HG), rhamnogalacturonan I and II (RG-I and RG-II) (Carpita and Gibeaut, 1993).

XyG consists of a β-D-(1→4)-glucan backbone to which are attached side chains containing xylosyl, galactosyl-xylosyl or fucosyl-galactosyl-xylosyl residues. In dicotyledonous and non-grass monocotyledonous plants, XyG is the principal polysaccharide that cross-links the cellulose microfibrils. It is able to bind cellulose tightly because its β-D-(1→4)-glucan cellulose-like backbone can form numerous hydrogen bonds with the microfibrils, whereas the side chains give rise to regions where microfibril binding is interrupted. A single XyG molecule can therefore interconnect separated cellulose microfibrils. This XyG-cellulose network forms a major load-bearing structure that contributes to the structural integrity of the wall and the control of cell expansion (Cosgrove, 1999).

The pectic matrix is structurally complex and heterogeneous. HG domains consist of α-D-(1→4)-galacturonic acid (GalA) residues, which can be methyl-esterified, acetylated, and/or substituted with xylose to form xylogalacturonan (Willats *et al*., 2001, Vincken *et al*., 2003). De-esterified blocks of HG can be cross-linked by calcium resulting in the formation of a gel

which is believed to be essential for cell adhesion (Jarvis, 1984). RG-I domains contain repeats of the disaccharide (4-α-D-GalA-(1,2)-α-L-Rha-(1) in which rhamnosyl residues can carry oligosaccharide side chains consisting predominantly of β-D-(1,4)-galactosyl- and/or α-L-(1,5)-arabinosyl-linked residues (McNeil *et al*., 1982). Side chains of RG-I can also contain α-L-fucosyl, β-D-glucuronosyl and 4-*O*-methyl β-D-glucuronosyl residues and vary in length depending on the plant source (O'Neill *et al*., 1990). These chains are believed to decrease the ability of pectic molecules to cross-link and form a stable gel network, and are thereby able to influence the mechanical properties of the cell wall (Hwang and Kokini, 1991). In addition, the structure and tissue distribution of arabinan- or galactan-rich side chains of RG-I have been shown to be regulated during cell growth and development of many species (for review see Willats *et al*., 2001).

RG-II is the most structurally complex pectic polysaccharide discovered so far in plants and is of a realtively low molecular mass (5 - 10Kda) (Ridley *et al*., 2001). It occurs in the cell walls of all higher plants as a dimer (dRG-II-B) that is cross-linked by borate di-esters (Matoh *et al*., 1993; Kobayashi *et al*., 1996; Ishii and Matsunaga, 1996; O'Neill *et al*., 1996). The backbone of RG-II is composed of a HG-like structure containing at least eight α-D-(1,4)-GalA-linked residues to which four structurally different oligosaccharide chains, denoted A, B, C and D, are attached. The C and D side chains are attached to C-3 of the backbone whereas A and B are attached to C-2 of the backbone (O'Neill *et al*., 2004). The C chain corresponds to a disaccharide that contains rhamnose and 2-keto-3-deoxy-D-*manno*-octulosonic acid (Kdo), whereas the D chain is a disaccharide of 2-keto-3-deoxy-D-lyxo-heptulosaric acid (Dha) and arabinose (O'Neill *et al*., 2004). The A and B oligosaccharide chains are both composed of eight to ten monosaccharides and are attached by a β-D-apiose residue to O-2 of the backbone. A D-galactosyl residue (D-Gal) occurs on the B chain.

3-The role of Golgi stacks in complex polysaccharide biosynthesis

a- Golgi structure and implication in constructing the cell wall.

In higher plants, the Golgi apparatus plays a fundamental role in "the birth" of the cell wall. Every new cell wall is formed during cytokinesis and starts to assemble with the transport of Golgi-derived secretory vesicles to the centre of a dividing cell. Fusion of these vesicles gives rise to a thin membrane-bound structure, the cell plate, which undergoes an elaborate process of maturation leading to a fully functional cell wall (Staehelin and Hepler, 1996; Segui-Simarro et al., 2004) Cutler and Ehrhardt, 2002).

The Golgi apparatus of plant cells is a dynamic and organized organelle consisting of a large number of small independent Golgi stacks that are randomly dispersed throughout the cytoplasm (*e.g.* several hundreds in root tip cells). At the confocal microscopy level, individual green fluorescent protein (GFP)-tagged Golgi stacks (around 1 µm in diameter) appear as round discs, small rings or short lines depending on their orientation and status (Nebenführ *et al*., 1999). At the level of transmission electron microscopy in high pressure frozen/freeze-substituted cells, each stack appears to consist of three types of cisternae, designated *cis*, *medial* and *trans* that were defined based on their position within a stack and their unique morphological features (Staehelin *et al*., 1990, Staehelin and Byung-Ho, 2008). The *trans* Golgi network (TGN) is a branched tubulo-vesicular structure that is frequently, but not systematically, located close to *trans* cisternae. The TGN can detach from the trans most cisternae as an independent compartment. Two types of TGN compartments have been described recently and referred to as an early and a late TGN (see Staehelin and Byung-Ho, 2008).

Generally, Golgi stacks displays a morphological polarity from the *cis* to *trans* faces that reflects different functional properties of Golgi compartments (Figure 1) (Staehelin *et al*., 1990; Driouich and Staehelin, 1997). The number of stacks per cell, as well as the number of cisternae within an individual stack, varies with the cell type, the developmental stage of the cell and the plant species (Staehelin *et al*., 1990; Zhang and Staehelin, 1992). In contrast to the Golgi complex in mammalian cells that has a fixed location near the centrosomes; Golgi stacks in plants appear to move actively throughout the cytoplasm (Boevink et al., 1998, Nebenfuhr *et al.*, 1999). GFP-fusions have allowed the study of Golgi stack dynamics in vivo and shown that each Golgi unit can move at a slow or high speed (up tp 5µm/s) without loosing structural integrity (Boevink et al., 1998, Nebenfuhr *et al.*, 1999, Brandizzi et al., 2002). In addition, cytoskeletal depolymerisation studies have indicated that the movement of Golgi stacks depends more on actin filaments than on microtubules (Nebenfuhr *et al.*, 1999). Indeed, it is now established that the movement of Golgi stacks in plant cells occurs along actin filaments driven by myosin motors (Staehelin and Byung-Ho, 2008). In the context of this chapter, it is worth noting that actin filaments interacts with Golgi stacks via an actin-binding protein, KATAMARI 1/
MURUS3 - that is also known as a glycosyltransferase required for cell wall biosynthesis (see below)- (Tamura et al., 2005). KATAMARI was shown to be involved in maintaining the organization and dynamics of Golgi membranes.

As in animal cells (Rabouille et *al.*, 1995), plant Golgi stacks function in the processing of N-linked glycoproteins (Driouich et *al*., 1994; see chapter by Bardor *et al.* in this book); but the bulk of the synthetic activity is devoted to the assembly of different subtypes of complex, non-cellulosic polysaccharides of the cell wall including pectin and hemicelluloses. The first studies implicating plant Golgi stacks in cell wall biogenesis date from the sixties and seventies and have used cytochemical staining as well as autoradiographic experiments involving radiolabeled sugars (Pickett-Heaps, 1966, 1968; Harris and Northcote, 1971; Dauwalder and Whaley, 1974). These investigations showed that Golgi cisternae and Golgi-derived vesicles are rich in carbohydrates and that a similar carbohydrate content is found in the cell plate, the cell wall and in Golgi-enriched fractions. Additionally, biochemical evidence for the role of the Golgi apparatus in the assembly of cell wall polysaccharides was obtained from fractionation experiments in which several glycosyltransferase activities (*e.g.* xylosyltransferase, arabinosyltransferase, fucosyltransferase) were detected in Golgi membranes (Gardiner and Chrispeels, 1975; Green and Northcote, 1978; Ray, 1980). Further biochemical investigations, reported in the eighties and the nineties, allowed the identification and partial characterization of Golgi-associated enzymes specifically involved in the synthesis of XyG and pectic polysaccharides (Camirand *et al*., 1987, Brummell *et al*., 1990, Gibeaut and Carpita, 1994). However, due to the inability of subfractionating plant Golgi stacks into *cis*, *medial* and *trans* cisternae (Camirand *et al*., 1987), and difficulties of purifying the enzymes, it was not possible to determine how the enzymes are spatially organized and how complex polysaccharides are assembled within Golgi subcompartments.

b- Spatial organization of the complex polysaccharide assembly pathway in Golgi stacks: insights from immuno-electron microscopy

Progress towards understanding the compartmentalization of matrix cell wall polysaccharide biosynthesis has come from immuno-electron microscopical analyses with antibodies directed against specific sugar epitopes. In most cases, these immunolabeling studies have been performed on cells prepared by high pressure freezing, a cryofixation technique that has been shown to provide excellent preservation of Golgi stacks thereby allowing different cisternal subtypes to be easily distinguished (Staehelin *et al*., 1990, Zhang and Staehelin, 1992; Driouich *et al*., 1993). Quantitative immunolabeling experiments using antibodies recognizing either the XyG backbone (anti-XG antibodies: Moore et *al*., 1986; Lynch and Staehelin, 1992) or an α-L-Fuc*p*-(1,2)-β-D-Gal*p* epitope of XyG side chains in

sycamore cultured cells have shown that the epitopes localize to *trans* cisternae and the TGN (Zhang and Staehelin, 1992). These data suggested that the synthesis of XyG occurs exclusively in late compartments of the Golgi and that no precursor forms of XyG are made in *cis* and medial cisternae. The use of these antibodies on clover and arabidopsis root tip cells have also suggested that the synthesis of XyG takes place in *trans* Golgi cisternae and the TGN (Moore *et al*., 1991, Driouich *et al*., 1994). Nevertheless, it could be argued that the sugar epitopes recognized by both antibodies are not accessible until they reach the *trans* and TGN compartments, or that the antibodies do not bind XyG precursor forms in *cis* and *medial* cisternae. Therefore, until the localization of XyG-synthesizing enzymes can be precisely determined within Golgi cisternae (as is the case for their sugar products), it will not be possible to know wether XyG synthesis is exclusively limited to *trans* and TGN cisternae. Although no antibody against any XyG-synthesizing enzyme is currently available, one possible approach of addressing this issue is to produce transgenic plants expressing GFP-tagged glycosyltransferases, followed by localization with anti-GFP antibodies. Such a strategy has been successfully used to study the compartmentation of enzymes involved in the processing of N-linked glycoproteins, including a β-1,2-xylosyltransferase responsible for the addition of β-1,2 xylose residues and an α-1,2-mannosidase responsible for the removal of α-1,2 mannose residues in tobacco suspension-cultured cells (Pagny *et al*., 2003; Follet-Gueye *et al*., 2003; Saint Jore-Dupas *et al*., 2006).

As for XyG synthesis, similar immunocytochemical studies using antibodies raised against pectin epitopes (including JIM7, anti-PGA/RG-I and CCRCM2) has allowed a partial characterization of the assembly pathway of polygalacturonic acid (PGA) and RG-I within Golgi cisternae (Zhang and Staehelin, 1992). The polyclonal anti-PGA/RG-I antibodies (recognizing un-esterified PGA) were shown to label mostly *cis* and *medial* cisternae in suspension-cultured sycamore cells as well as in clover root cortical cells (Zhang and Staehelin, 1992; Moore *et al*., 1991). In contrast, JIM7 (specific for methyl-esterified PGA) labeling was mostly confined to *medial* and *trans* cisternae. In addition, the mAb CCRCM-2 which is believed, but not proven, to bind RG-I side chains was found to label *trans* cisternae in sycamore cultured cells. These data suggest that PGA is synthesized in its un-esterified form in *cis* and *medial* Golgi cisternae and, that i) the methylesterification occurs in both *medial* and *trans* compartments, and ii) that side chains of RG-I are added in *trans* cisternae. However, as discussed above for XyG labeling, the absence of labeling in *trans* cisternae and the TGN by anti-PGA/RG-I antibodies, might be due to the non-accessibilty of the recognized epitopes in these compartments (because of the methylesterification for instance). This idea is

supported by the fact that the same epitopes are localized predominantly in *trans* Golgi cisternae and the TGN in another cell type, namely epidermal cells of clover. Thus, it is not surprising that the compartmentation of cell wall matrix polysaccharides within Golgi cisternae varies in a cell-type specific manner. The distribution of XyG and PGA in Golgi membranes has also been investigated immunocytochemically in root hair cells of *Vicia villosa* preserved by high pressure freezing (Sherrier and VandenBosh, 1994). Although no quantitative analyses were performed, methylesterified PGA epitopes recognized by JIM7 were detected within *medial* and *trans* cisternae, whereas the fucose-containing epitope of XyG (recognized by CCRCM1) was found over *trans* Golgi cisternae. These observations are consistent with those made in sycamore suspension-cultured cells and clover root cortical cells using the same antibodies (Zhang and Staehelin, 1992; Moore *et al.*, 1991). However, the *Vicia villosa* study did not address the issue of RG-I side chain distribution within Golgi stacks using the mAb CCRCM2. Therefore, the use of the more recently produced monoclonal antibodies LM5 and LM6, recognizing -1,4-D-galactan and -1,5-D-arabinans, respectively (Jones *et al.*, 1997, Willats *et al*., 1998) should prove very useful for extending the " current map" of the pectin assembly pathway within the Golgi cisternae of sycamore cultured cells and *Vicia villosa* root hairs. Both antibodies have been widely used to study the distribution of galactan and arabinan epitopes within the cell walls, but relatively very little is known concerning their localization within the endomembrane system. In flax root cells, LM5-containing epitopes have been shown to be present mostly in *trans* cisternae and the TGN (Vicré *et al*.,1998). Similarly, epitopes recognized by LM5 and LM6 have been quantitatively localized to *trans* cisternae, TGN and secretory vesicles in arabidopsis root cells and tobacco (BY2) cultures (Bernard *et al.*, 2006**,** See also Figure 2**).** Therefore, it appears that galactan- and arabinan-containing side chains of RG-I are assembled in the *trans* cisternae and TGN. Whether the enzymes responsible for the addition of these residues are confined to the same Golgi compartments remains to be determined by future studies. One of the genes involved in the synthesis of RG-I side chains, namely *ARAD1* (encoding a putative -1,5-D- arabinosyltransferase) has been recently identified and cloned (see below). The generation of specific antibodies against this glycosyltransferase, or the generation of a GFP-tagged protein should help us to understand more about its specific localization within Golgi compartments. In contrast to RG-I, nothing is known about the localization and assembly of RG-II within the endomembrane system. This polysaccharide has a complex structure consisting of a HG-like backbone and four side chains that contain specific and unusual sugars including Kdo and apiose (see above). It would certainly be interesting to find out

whether the backbone is assembled in the same compartments as the side chains and whether different side chains are assembled in similar or distinct compartments. The elucidation of RG-II assembly within Golgi stacks requires the generation of antibodies specific for the sugar epitopes of the backbone and for the epitopes associated with different side chains, as well as the associated immuno-electron microscopy studies - hopefully, this will not take too long !

It is generally accepted that the transport of Golgi products, including glycoproteins and complex polysaccharides, to the cell surface occurs by bulk flow and default (Hadlington and Denecke, 2000). To date, no specific signals responsible for targeting and transport of such products to the cell wall have been found associated with any protein or polysaccharide. It has been shown that Golgi-derived secretory vesicles mediating such a transport vary in size and are capable of carrying mixed classes of polysaccharides (Sherrier and VandenBosch, 1994) or polysaccharide and glycoproteins (see Driouich *et al*., 1994). It is possible that the processing of Golgi products such as XyG may continue to occur within the vesicles during their transport to the cell surface (Zhang *et al.*, 1996).

4- Glycosyltransferases and sugar-converting enzymes involved in the assembly of complex polysaccharides

The Golgi-mediated assembly of complex polysaccharides requires the action of a set of Golgi glycosyltransferases, in addition to nucleotide sugar transporters and nucleotide sugar interconversion enzymes (Keegstra and Raikhel, 2001; Seifert, 2004). It has been postulated that these partners could interact physically to form complexes within Golgi membranes that would coordinate sugar supply and polymer synthesis (Seifert, 2004).

a- General considerations supporting the hypothesis of protein complexes dedicated to cell wall biosynthesis

It has been observed that certain genes encoding glycosyltransferases involved in specific polysaccharide biosynthesis events are highly co-regulated at the transcriptional level (Burton *et al*., 2004). This observation was successfully exploited to detect new candidate genes responsible for primary and secondary cell wall biosynthesis through global analyses of microarray data sets (Persson *et al.*, 2005; Brown *et al.*, 2005). These studies supported the idea that genes encoding glycosyltransferases are temporally regulated during polysaccharide

biosynthesis, and –in addition- raised the question of spatial organization of glycosyltransferase activities within Golgi membranes.

Although still conceptual in plants, it is interesting to discuss a few observations that support the existence of glycosyltransferase complexes. First, in biological chemistry, a common characteristic of enzymes involved in a complex biosynthetic event is a physical "lining-up" for efficient assembly of the product. Interestingly, some glycosyltransferases involved in cell wall biosynthesis in gram-negative bacteria have evolved a combination of distinct catalytic domains in a single enzyme, thereby forming bifunctional proteins with improved capacities, and providing evolutionary advantages (Ciocchini *et al*, 2007; Lovering *et al*, 2007). A second factor supporting the possible existence of a supramolecular organization of glycosyltransferases into complexes in plants is provided by the fact that such complexes occur in mammals for enzymes responsible for glycosaminoglycan biosynthesis (Pinhal *et al*, 2001; Izumikawa *et al*, 2007). Interestingly, in plants bifunctional enzymes have also been characterized in the nucleotide-sugar interconversion pathways (Bonin and Reiter, 2000; Watt *et al*., 2004). Moreover, the existence of tight partnerships between certain biosynthetic enzymes involved in plant cell wall biogenesis has already been described. One of the best known examples concerns cotton fibers undergoing high-rate cellulose synthesis, where it has been demonstrated that more than half of the soluble sucrose synthase is associated with the plasma membrane, possibly through interaction with cellulose or callose synthase. This suggests a direct carbon channelling from sucrose *via* UDP-glucose to cellulose synthase (Amor *et al*., 1995).

b- Xyloglucan biosynthesis: glycosyltransferases and other proteins

XyG biosynthesis has long been an interesting, but challenging area of investigation. Biosynthesis of the XyG core is expected to require two different catalytic activities, a glucan synthase activity for the backbone and a xylosyltransferase activity adding xylosyl substitutions.

Interestingly, in 1980, Peter Ray suggested a "cooperative action of β-glucan synthase and UDP-Xylose xylosyltransferase in Golgi membranes for the synthesis of a XyG-like polysaccharide" (Ray, 1980). In that study, a UDP-xylose xylosyltransferase activity was measured in Golgi membranes isolated from pea, and the incorporation of xylose was shown to be stimulated by the addition of UDP-glucose. Furthermore, the stimulating effect of UDP-glucose on xylosyltransferase activity was shown to occur only in a pH range where β-glucan synthase is active, suggesting that UDP-glucose stimulates UDP-xylose incorporation by

promoting β-glucan synthase activity. Here, the β-glucan synthase produces the required β-1,4-glucan substrate molecule necessary for XyG xylosyltransferase activity. At about the same time, Takahisa Hayashi and Kazuo Matsuda, performed a detailed characterization of XyG synthase activity in soybean suspension-cultured cells and demonstrated that XyG synthesis requires the cooperation of XyG β-1,4-glucan synthase and the XyG xylosyltransferase (Hayashi and Matsuda, 1981a,b). The authors not only demonstrated that the incorporation of one sugar (xylose or glucose) depended on the presence of the other, but also that xylose was not transferred to a preformed β-1,4-glucan. This observation strongly supports the existence of a multienzyme complex responsible for XyG biosynthesis where glucan synthase and xylosyltransferase activities cooperate tightly (Hayashi, 1989). Since then considerable efforts have been devoted to the characterization of XyG biosynthesis at the molecular level using functional genomics and the model plant *Arabidopsis thaliana* (see Lerouxel *et al*, 2006).

We have currently obtained a better picture of XyG biosynthesis by identifying and characterizing some of the genes involved (Table 1), although without much (if any) understanding of how these enzymes could potentially cooperate to achieve the biosynthesis. For example the XyG fucosyltransferase AtFUT1 (CAZy GT37) was the first type-II glycosyltransferase characterized at the biochemical level (Perrin *et al*., 1999). AtFUT1 has been identified as one member of a large family containing nine putative glycosyltransferases. However, further analyses of the enzymes have shown that three members of this family are not involved in the fucosylation of XyG (Sarria *et al*., 2001). Later, the identification and characterization of the *mur2* mutant of arabidopsis provided the unequivocal proof that the *AtFUT1* gene encodes the unique α-1,2-fucosyltransferase activity responsible for XyG fucosylation (Vanzin *et al*., 2002). Likewise, one XyG β-1,2-galactosyltransferase (AtMUR3; CAZy GT47) activity was successfully characterized using both mutant analysis (*mur3*) and heterologous expression of the enzyme (Madson *et al*, 2003). Nevertheless, as the galactose residue of the XyG molecule can be found in two different positions, it seems that at least one XyG galactosyltransferase remains to be identified and characterized. An important contribution to understanding XyG biosynthesis was also made by the characterization of two α-1,6-xylosyltransferases activities required for XyG xylosylation (CAZy GT34). First, one xylosyltransferase activity (named AtXT1) was identified based on sequence homology with a previously identified α-1,6-galactosyltransferase from fenugreek (Edwards *et al*., 1999), and characterized as a α-1,6-xylosyltransferase using heterologous expression in *Pichia pastoris* (Faik *et al*., 2002). Recently, Cavalier and Keegstra, (2006) extended this work by the

characterization of a second xylosyltransferase activity (named AtXT2), encoded by a gene closely related to AtXT1, that promotes an identical reaction to the one catalyzed by AtXT1. Interestingly, the authors also demonstrated that both AtXT1 and AtXT2 are able to catalyze multiple addition of xylosyl residues onto contiguous glucosyl residues of a cellohexaose acceptor *in vitro* (even though the β-linkage introduce a 180° rotation from one glucosyl residue to the other), but non-xylosylated cellohexaose was the preferred acceptor. The observation that both AtXT1 and AtXT2 xylosyltransferases activities were able to perform multiple xylosylation might only indicate a reduced substrate specificity of these enzymes as compared to the high specificity of the XyG fucosyl- or galactosyltransferase. Nevertheless, these results raise the intriguing possibility that AtXT1 and AtXT2 would be fully redundant *in planta* and that both are able to perform multiple xylosylation. Such a hypothesis is consistent with the recent characterization of an Arabidopsis double mutant KO for AtXT1 and AtXT2 genes (named *xxt1 xxt2*; xyloglugan xylosyltransferase 1 and 2), which is lacking detectable amount of XyG *in planta* (Cavalier *et al*., 2008).

While XyG glucan synthase activity has long been studied biochemically (discussed above), efforts to purify and ultimately characterize this enzyme have not been successful. Recently, a gene from the Cellulose Synthase-Like C family (*AtCSLC4*; CAZy GT2) was shown to encode a Golgi localized β-1,4-glucan synthase activity providing a strong candidate for the, as yet, unidentified XyG β-glucan synthase (Cocuron *et al*., 2007). The candidate gene was identified using a transcriptional profiling strategy taking advantage of nasturtium's capacity to undergo high-rate XyG biosynthesis during seed development (Desveaux *et al*., 1998). This observation supports the hypothesis that the β-1,4-glucan synthase activity identified was involved in XyG biosynthesis. Interestingly, this study also indicated that *AtCSLC4* is co-regulated with *AtXT1* at the transcriptional level and that some degree of interaction occurring at the protein level could possibly alter the length of the β-1,4-glucan synthesized by the AtCSLC4 protein (Cocuron *et al*., 2007). The results of this study are similar to earlier reports showing that *in vitro* synthesis of XyG was shown to involve a cooperative action between the glucan synthase and the xylosyltransferase activities (Hayashi, 1989).

XyG biosynthesis does not only depend upon the cooperation between glycosyltransferase activities, but might also require close interaction between glycosyltransferases and nucleotide sugar interconversion enzymes. The recent study on the *reb1*/*rhd1* mutant of arabidopsis, deficient in one of the five UDP-glucose 4-epimerase isoforms (UGE4) involved in the synthesis of UDP-D-galactose, provided indirect evidence

for a possible interaction between UGE4 and XyG-galactosyltransferase (Nguema-Ona et al., 2006). The study showed that the galactosylation of XyG, unlike that of pectins (RG-I and RG-II), was absent in specific cells of the mutant and that UGE4 and XyG-galactosyltransferase are co-expressed at the transcriptional level in the root. Thus, it was postulated that the two enzymes might be associated in specific protein complexes involved in the galactosylation of XyG within Golgi membranes. Such an association could be required for an efficient galactosylation of XyG, where UGE4 would channel UDP-Gal to XyG-galactosyltransferases. The existence of such a hypothetical association was supported by the demonstration that UGE4 was not only present in the cytoplasm but also found associated with Golgi membranes (Barber *et al*., 2006). UGE4 is not the only, cytosolic nucleotide-sugar interconversion enzyme found to be associated with Golgi membranes. Recently, a UDP-arabinose mutase (UAM) that converts UDP-arabinopyranose to UDP-arabinofuranose was characterized in rice (Konishi *et al*., 2007) and found to share more than 80% identity with the reversibly glycosylated proteins (RGPs) of arabidopsis that have long been known to be cytosolic proteins capable of associating with Golgi membranes (Dhugga *et al*., 1991,1997; Delgado *et al*., 1998). The rice UDP-arabinose mutase was shown to possess reversible glycosylating properties and the high level (80%) of amino acid sequence identity with arabidopsis RGPs supports the fact that RGPs and UAMs might be the same proteins (Konishi et al., 2007). Five isoforms of the reversibly glycosylated proteins have been identified in the arabidopsis genome and a double mutant *rgp1/rgp2* was shown to harbor pollen development defects, thereby leading the authors to hypothesize that RGPs might be involved in cell wall polysaccharide biosynthesis (Drakakaki *et al*., 2006). If indeed RGPs are UAMs, the cell wall polysaccharide biosynthesis defect hypothesized in *rgp1/rgp2* double mutant might be due to a defect in UDP-arabinofuranose supply critical for pollen development. It is also noteworthy that, even though the existence of complexes associating nucleotide-sugar conversion enzymes, nucleotide transporters and glycosyltransferases remain shypothetical, enzyme complexes involved in nucleotide-sugar conversion have already been described. Indeed, UAMs associate together in complexes involving several isoforms (Langeveld *et al*., 2002; Konishi *et al*., 2007). Data also suggest that a UDP-glucuronic acid 4-epimerase exists as a dimer (Gu and Bar-Peled, 2004). In addition, a bifunctional epimerase, the GDP-4-keto-6-deoxymannose-3,5-epimerase-4-reductase (AtGER1; Bonin and Reiter, 2000) and the GDP-mannose-4,6-dehydratase (AtMUR1) catalyzing the conversion of GDP-mannose into GDP-fucose have been shown to stably interact, and this interaction is required for MUR1 activity and stability (Nakayama *et al*., 2003).

c- Pectin biosynthesis: towards identification of the glycosyltransferases involved

Because there is a considerable diversity of monosaccharide units and glycosidic linkages making up pectic polysaccharides, it has been proposed that a minimum of 53 glycosyltranferases would be needed for pectin biosynthesis (Mohnen, 1999). In addition, the complexity of pectic polysaccharides has made the identification and characterization of such glycosyltranferases difficult and, consequently, only a handful have been assigned precise biochemical functions (Sterling *et al*., 2006; Egelund *et al*., 2006; Jensen *et al*., 2008) or suggested to have such functions (Iwai *et al*., 2001; Bouton *et al*., 2002; Iwai *et al*., 2002; Harholt *et al*., 2006; Egelund *et al*., 2007). An additional degree of complexity in pectin synthesis is related to the methylesterification of certain pectin molecules which requires specific methyltransferase activities.

As for XyG biosynthesis, many biochemical studies have been devoted to the characterization of the enzymes involved in HG biosynthesis (Doong and Mohnen, 1998; Scheller *et al*., 1999). These studies showed that α-1,4-galacturonosyltransferase (GalAT) and HG methyltransferase activities are located in the lumen of isolated Golgi membranes (Goubet *et al*., 1999; Sterling *et al*., 2001). Recently, a HG-GalAT activity was partially purified from solubilized membrane proteins isolated from arabidopsis suspension-cultured cells and trypsin-digested peptides sequencing led to the identification of a candidate gene, named *AtGAUT1* (Sterling *et al*., 2006). Heterologous expression of AtGAUT1 (CAZy GT8) in human embryonic kidney cells, showed that *AtGAUT1* cDNA encodes a galacturonosyltransferase activity able to elongate α-1,4-oligogalacturonides. However, whether the AtGAUT1 activity is responsible for the biosynthesis of the backbone of HG or RG-II polysaccharides has not been determined. Further investigations, on AtGAUT1-deficient arabidopsis mutants for instance, should help to elucidate this point. Homogalacturonan backbone in arabidopsis can also be decorated with β-1,3-xylose residues thus forming a xylogalacturonan domain (Zandleven *et al.*, 2007). Recently, the characterization of the *xylogalacturonan deficient1* (*xgd1*) arabidopsis mutant along with the heterologous expression of XGD1 protein in *Nicotiana benthamiana* led to the demonstration that the *XGD1* gene is involved in XGA biosynthesis (Jensen *et al.*, 2008). Further studies of *xgd1* mutant are expected to provide a better understanding of the role of XGA in the plant cell wall. Two other genes involved in pectin biosynthesis, named *AtRGXT1* and *AtRGXT2* (CAZy GT77), have been described recently based on the characterization of arabidopsis mutants and expression of enzyme activities (Egelund *et al*., 2006). The authors convincingly

demonstrated that *AtRGXT1* and *AtRGXT2* encode α-1,3-xylosyltransferase activities that are involved in the synthesis of the pectic polysaccharide RG-II. Unfortunately, the two genes are likely to be paralogs (only 6.2 kb apart) thereby preventing the generation and study of a double KO mutant potentially characterized by the production of RG-II molecules devoid of 2-O-methyl-xylose residues. Such a mutant would obviously help us to unravel the function of this polysaccharide. Other genes have also been proposed to be involved in pectin biosynthesis although their implication has not been definitely established as yet. Mutant lines altered for pectin biosynthesis have been screened out based on cell adhesion defects - a process that involves pectin (Iwai *et al*., 2001; Bouton *et al*., 2002; Iwai *et al*., 2002; Mouille *et al*., 2007). A gene named *NpGUT1,* for *Nicotiana plubaginifolia* glucuronyltransferase 1, has been identified using *nolac-H18,* a tobacco callus mutant line that exhibits a "loosely attached cells" phenotype (Iwai *et al*., 2002). It was proposed that *NpGUT1* encodes a putative glucuronyltransferase involved in RG-II biosynthesis based on the analysis of *nolac-H18* mutant cell walls. However, because the overall sugar composition of the cell wall and not just RG-II glucuronosyl content was altered, conclusive evidence for NpGUT1 being a RG-II glucuronyltransferase will require protein expression analysis. (the genes in AT Atgut1 et 2…..I need to say that the function is not the same).

The arabidopsis mutant *quasimodo1* (*qua1*) is characterized by a reduced cell adhesion phenotype combined with a 25% decrease in cell wall galacturonic acid content, supporting the hypothesis that the *AtQUA1* gene encodes a putative glycosyltransferase activity involved in pectin biosynthesis (Bouton *et al*., 2002). AtQUA1 (also named AtGAUT8) belongs to CAZy GT 8 family and shows 77% similarity to AtGAUT1 which has a characterized HG-galacturonosyltransferase activity (Sterling *et al*., 2006). Moreover, α-1,4-galacturonosyltransferase activity was measured in *qua1* and shown to be significantly reduced in comparison with the wild type, providing further support for AtQUA1 involvement in pectin biosynthesis (Orfila *et al*., 2005). More recently, another arabidopsis mutant *quasimodo2* (*qua2),* having a 50% decrease in HG content has been described (Mouille *et al*., 2007). Interestingly, AtQUA2 does not show any similarity with glycosyltransferases but is a Golgi-localized protein that contains a putative S-adenosyl methionine dependent methyltransferase domain, and appears strongly co-regulated with AtQUA1. This study supports the hypothesis that AtQUA2 is a pectin methyltransferase required for proper HG biosynthesis. This suggests that galacturonosyltransferase activity is highly dependant upon methyltransferase activity in HG synthesis. The observation that an alteration in a putative methyltransferase can impair HG synthesis led the authors to suggest the existence of a

protein complex containing galacturonosyltransferase and methyltransferase where the latter enzyme would be essential for the functioning of the protein complex (Mouille *et al*., 2007). Finally, a third arabidopsis mutant - *ectopically parting cells 1* (*epc1*) - was characterized on the basis of cell adhesion defects, however cell wall analyses did not support the idea that pectin biosynthesis was specifically altered in *epc1* (Singh *et al*., 2005).

As compared to HG and RG-II biosynthesis, relatively little is known about the glycosyltransferases involved in RG-I biosynthesis and only one glycosyltransferase has been characterized so far. A reverse genetic approach with putative glycosyltransferases from the CAZY GT47 family led to the identification of the *arabinan deficient 1 (arad1)* arabidopsis mutant showing a reduced arabinose content in the cell wall (Harholt *et al*., 2006). Characterization of the *arad1* cell wall demonstrated that the *ARAD1* gene probably encodes an arabinan α-1,5-arabinosyltransferase activity important for RG-I biosynthesis. It is interesting to note that although arabinofuranose is normally incorporated in the cell wall during RG-I biosynthesis *in planta*, it is arabinopyranose that is actually incorporated during *in vitro* assays, using detergent-solubilized membranes. To explain this discrepancy between *in planta* and *in vitro* observations, it has been hypothesized that a mutase activity responsible for the conversion is lost upon solubilization (Nunan and Scheller, 2003). Recent characterization of an UAM, as being identical to the well characterized Golgi membrane-associated RGPs, supports this observation and also provides the exciting opportunity to study interaction between the arabinosyltransferase and the mutase.

5-Conclusions and outlook.

Cell wall biosynthesis in general, but XyG and pectin biosynthesis in particular, have progressed significantly over the past ten years with respect to the identification of the enzyme activities involved in their biosynthesis, using functional genomics. The challenge now is, to determine how those players (and more partners) cooperate, in a timely and probably spatially-resolved manner, in order to achieve the coordinated and efficient syntheses of these polymers.

In plants, the only approach that has so far provided evidence for the compartmental organization of the Golgi with regards to complex polysaccharide biosynthesis is immunogold microscopy using antibodies raised against specific sugars of different polysaccharides. Interestingly all glycosyltransferases involved in XyG and pectin synthesis characterized so far are integral membrane proteins and most have been shown to be located in the Golgi using GFP-fusions and confocal microscopy. Now we need to move forward with studies

addressing how these synthetic pathways are organized and how the enzymes are distributed within plant Golgi stacks, using electron microscopy. It is of special interest to determine whether distinct glycosyltransferases are preferentially compartmentalized within distinct Golgi cisternae, or if they are always localized in all Golgi compartments. Likewise, it is important to determine whether the distribution of these glycosyltransferases in the Golgi subcompartments is dependant on the cell type and/or on the stage of development. Finally, determining how glycosyltransferases are spatially organized to perform non-cellulosic polysaccharides biosynthesis is also of specific interest. Many strategies exist to investigate whether glycosyltransferases and other protein partners occur in complexes. Recently, the use of co-immunoprecipitation and Bimolecular Fluorescence Complementation (BiFC), allowed the characterization of protein-protein interactions between three cellulose synthases involved in primary cell wall biosynthesis (Desprez et al., 2007). Such approaches are also likely to be useful in unravelling the functional organization of enzymes responsible for the assembly of non-cellulosic polysaccharides of the cell wall matrix.

Acknowledgements. Very special thanks are due to Pr. A. Staehelin (on this year of his retirement) for having passionately launched AD onto the path of plant Golgi research. The authors wish to thank Pr. S. Hawkins and Dr. J. Moore for their critical reading of the manuscript as well as and Pr. K. Byung-Ho for providing Figure 1 D. Work in AD laboratory is supported by the University of Rouen, the CNRS and le Conseil Régional de Haute Normandie

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Table 1: List and characteristic of the glycosyltransferase and nucleotide-sugar interconversion enzymes mentioned in this chapter.
Abbrevations: *arad1, arabinose deficient1*; *At, Arabidopsis thaliana*; *epc1, ectopically parting cells1*; *nolac-H18, non-organogenic callus with loosely attached cells-H18*; *Np, Nicotiana plumbaginifolia*; *qua1, quasimodo1*; *qua2, quasimodo2*; *reb1, root epidermal bulging1*;*rgp1, reversibly glycosylated protein1*; *rgp2, reversibly glycosylated protein2; rhd1, root hair deficent1*; *rgxt1, rhamnogalacturonane II xylosyltransferase1*; *rgxt2, rhamnogalacturonane II xylosyltransferase2; xgd1, xylogalacturonan deficient1; xxt1, xyloglucan xylosyltr*

Gene		Enzyme characteristics			Arabidopsis mutant		Notes	
Designation	Code	CAZy	Activity	Localization	Designation	phenotypes	category	References
AtCSLC4	At3g28180	GT 2	-1,4-glucan synthase	Golgi	None	None	Xyloglucan	Cocuron et al., 2007
AtXT1	At3g62720	GT 34	-1,6-xylosyltransferase	Golgi	*xxt1*	10% reduction in XyG; xxt1 xxt2 double mutant lacks detectable amount of XyG		Faik et al., 2002; Cavalier et al., 2006 and 2008
AtXT2	At4g02500	GT 34	-1,6-xylosyltransferase	Golgi	*xxt2*	20% reduction in XyG; xxt1 xxt2 double mutant lacks detectable amount of XyG		Cavalier et al., 2006 and 2008
AtMUR3	At2g20370	GT47	-1,2-galactosyltransferase	Golgi	*mur3*	Hypocotyls wall strength is 50% reduced, xyloglucan lacks fucogalactosyl sidechain		Madson et al., 2003; Pena et al., 2004
AtFUT1	At2g03220	GT37	-1,2-fucosyltransferase	Golgi	*mur2*	Hypocotyls wall strength is slightly reduced, xyloglucan lacks fucose substitution		Perrin et al., 1999; Vanzin et al., 2002; Pena et al., 2004
AtQUA1	At3g25140	GT 8	putative -1,4-galacturonosyltransferase	Predicted Golgi	*qua1*	Dwarf, reduced cell adhesion, 25% reduction in cell wall GalA content	Pectins	Bouton et al., 2002
AtGAUT1	At3g61130	GT 8	-1,4-galacturonosyltransferase	Golgi	None	None		Sterling et al., 2006; Dunkley et al., 2004
AtRGXT1	At4g01770	GT 77	-1,3-xylosyltransferase	Golgi	*rgxt1*	RGII from mutant (but not from WT) is an acceptor for a-1,3-xylosyltransferase acitvity		Egelund et al., 2006
AtRGXT2	At4g01750	GT 77	-1,3-xylosyltransferase	Golgi	*rgxt2*	RGII from mutant (but not from WT) is an acceptor for a-1,3-xylosyltransferase acitvity		Egelund et al., 2006
AtARAD1	At2g35100	GT47	-1,5-arabinosyltransferase	Predicted Golgi	*arad1*	Cell wall composition altered, decrease in RGI arabinose content		Harholt et al., 2006
AtXGD1	At5g33290	GT47	-1,3-xylosyltransferase	Golgi	*xgd1*	*xgd1* mutant lacks detectable XGA in pectin enriched fraction		Jensen et al., 2008
NpGUT1	-	GT47	putative glucuronytransferase	Predicted Golgi	*nolac-H18*	callus harbored a cell-cell adhesion defect, and a reduced RGII dimerisation ability		Iwai et al., 2002
AtEPC1	At3g55830	GT64	unknown	Predicted Golgi	*epc1*	Reduced growth habit, defects in vascular formation and reduced cell-cell adhesion in hypocotyls		Singh et al., 2005
AtQUA2	At1g78240	-	putative methyltransferase	Golgi	*qua2*	Dwarf, reduced cell adhesion, 50% reduction in HG content		Mouille et al., 2007

AtUGE4	At1g64440	-	UDP-Glucose epimerase	Cytosolic and Golgi associated	*rhd1 (uge4); reb1*	Reduced root elongation rate, bulging of trichoblast cells, xyloglucan and arabinogalactan galactosylation defects in roots	Interconversion enzymes	Andeme-Onzighi et al., 2002; Seifert et al., 2002; Barber et al., 2006; NGuema-Ona et al., 2006
AtRGP1	At3g02230	GT75	putative UDP-Arabinopyranose mutase	Cytosolic and Golgi associated	*rgp1*	None, but *rgp1 rgp2* double mutant is lethal (pollen development altered)		Drakakaki et al., 2006; Konishi et al., 2007
AtRGP2	At5g15650	GT75	putative UDP-Arabinopyranose mutase	Cytosolic and Golgi associated	*rgp2*	None, but *rgp1 rgp2* double mutant is lethal (pollen development altered)		Drakakaki et al., 2006; Konishi et al., 2007

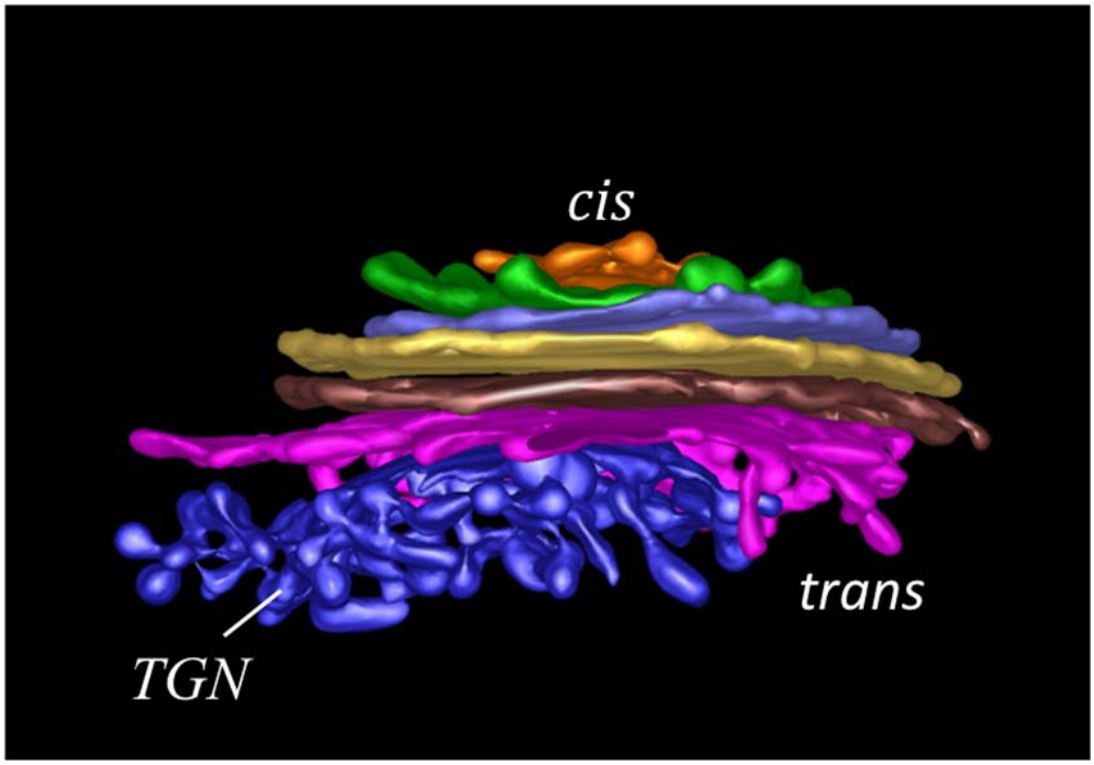

Figure 1. (A) Confocal microscopy image showing distribution of Golgi stacks in suspension-cultured tobacco cells. Golgi stacks -expressing the Golgi marker sialyltransferase fused to mRFP- are visible as bright red spots. The bar represents 8µm. (B) Electron micrograph of suspension-cultured tobacco cells preserved by high pressure-freezing showing the random distribution of Golgi stacks throughout the cytoplasm. The bar represents 0.5µm nm. (C) A Golgi stack and associated trans-Golgi network (TGN) in an epidermal root cell of *Arabidopsis thaliana. Cis*-, medial and *trans* type of cisternae as well as the TGN are indicated. The bar represents 0.5µm. D) Electron tomographic model of a Golgi stack in a columella root cell of Arabidopsis.

CV, COP-coated vesicles; CW, cell wall; ER, endoplasmic reticulum; G, Golgi stack; M, mitochondria; N, nucleus; P, plast; TGN, trans golgi network; V, vacuole

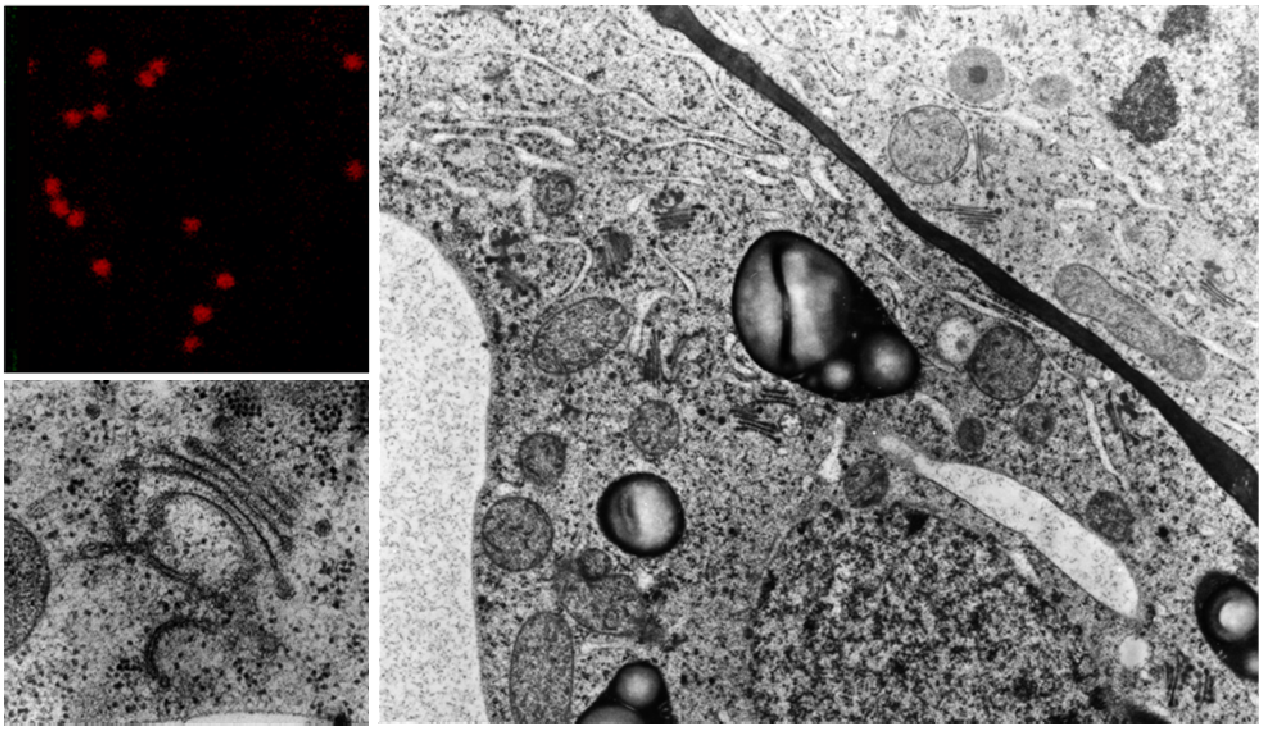

Figure 2. Electron micograph illustrating typical immunogold labeling of Golgi stacks in arabidopsis root cells with the mAbs LM5 (A) and LM6 (B). Scale bar represents 200 nm. Arrowheads point to gold particles. G, Golgi stack (Bernard et al., unpublished)