Hemicellulose biosynthesis and degradation in tobacco cell walls

Monique Compier

Promotor:

Prof. dr. R.G.F. Visser
Hoogleraar in de Plantenveredeling

Co-promotor:

Dr. ir. J-P. Vincken
Universitair docent, Laboratorium voor Plantenveredeling

Promotiecommissie:

Prof. G. Paul Bolwell (Royal Holloway, University of London)
Prof. dr. ir. E. Jacobsen (Wageningen Universiteit)
Dr. H.A. Schols (Wageningen Universiteit)
Dr. ir. G. Smant (Wageningen Universiteit)

Dit onderzoek is uitgevoerd binnen de onderzoeksschool 'Experimental Plant Sciences'.

Hemicellulose biosynthesis and degradation in tobacco cell walls

Monique Compier

Proefschrift

ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
Prof. dr. M.J. Kropff,
in het openbaar te verdedigen
op maandag 7 november 2005
des namiddags te vier uur in de Aula.

CIP-DATA Koninklijke Bibliotheek, Den Haag

Hemicellulose biosynthesis and degradation in tobacco cell walls
Monique Compier

PhD thesis, Wageningen University, The Netherlands
With references - with summaries in Dutch and English

ISBN 90-8504-278-x

Contents

Chapter 1

General introduction

GENERAL INTRODUCTION

Practical and industrial applications of plant fibres

Plant fibres consist of generally long, slender cells that commonly occur in strands or bundles. Characteristic for fibres are the thick secondary cell walls, which consist of helical wound cellulose microfibrils in a matrix of lignin and hemicelluloses. The characteristics of the cell wall make fibre cells important strengthening and supporting elements in plant parts that have ceased elongation (Raven *et al*., 1992). Agricultural crops like cotton (*Gossypium hirsutum*), hemp (*Cannabis sativa*), flax (*Linum usitatissimum*), agave (*Agave Americana*), and tree species, including birch (*Betula papyrifera*), pine (*Pinus* spp.) and poplar (*Populus* spp.) are important sources for plant fibres. Depending on the part of the plant from which they are derived, the fibres can be classified as leaf fibres, bast fibres, seed fibres and wood fibres (Table 1). Natural fibres have a wide range of technological applications. Fibrous materials derived from trees have found particular application in pulp and paper industries, whereas cotton, flax, hemp and sisal (agave derived) fibres are generally used for the production of (technical) textiles (Table 1). With the growing concern for the environment and sustainable growth, the application of flax, hemp and sisal fibres is expanding. The fibres are increasingly used in industrial composites, which can replace amongst others glass fibre, polystyrene and plastics. Flax fibre is already replacing glass fibre in some applications; an example is the production of biodegradable door panels in the car industry (IENICA report, 2000, 2003).

In terms of fibrous biomass, the production of flax and hemp is running far behind the cotton production (Table 1). This implies that almost all textiles are derived from cotton or synthetic fibres. Cotton growth is however restricted to sub-tropical climates and is dependent on the use of high amounts of pesticides to ensure good yields. In contrast to cotton, flax and hemp can be grown in a more moderate climate and need less input to give high yields (Van Roekel, 1994; Ebskamp, 2002). An increased market share for flax and hemp in textile industry would therefore be a great benefit. The major problem concerning the processing of flax and hemp is the separation of fibre bundles from the non-fibre tissues in the stem (Akin *et al*., 2001). Improvement of either the quality and properties of the plant starting material or the technical processing of fibres could result in an increased applicability of flax and hemp fibres (Pallesen, 1996; Akin *et al*., 2001).

Conversion of wood into paper pulp via the kraft and chemical pulping processes requires substantial chemical and energy expenditures (Clarke *et al*., 1997). During the pulping process, the non-cellulosic polymers and lignins are dissolved, without significantly degrading the cellulose fibres. Paper pulp produced by this process contains residual lignins that affect paper-making and also the quality of the paper. These lignins are removed by means of a multi-stage bleaching process, which consumes a lot of energy

Table 1. Overview of different fibre crops, their main application and production rates in 2003 in metric tons (Mt) fibre material in Europe and North/Central America. Textile applications include for instance apparel, diapers, fabrics, denim and handbags. Examples of technical textiles are twine, rope, canvas bags, and carpets. Source: FAOSTAT (http://faostat.fao.org).

Crop	Fibre source	Main application	Production 2003 (Mt) Europe	Production 2003 (Mt) North/Central America
cotton	seed (-hairs)	textile	1.382.012	10.217.149
flax	bast (phloem)	textile	269.229	-
hemp	bast (phloem)	textile	25.356	1.100
agave	leaf	technical textile	-	28.390
forest trees	wood (xylem)	pulp and paper	48.148.752	78.922.013
maize	bast, leaf, seed	cattle feed	76.557.930	289.668.483

and generates numerous chlorinated organic compounds, some of which are toxic, mutagenic, or bioaccumulative (Onysko, 1993; Clarke *et al.*, 1997). Biological improvement of the properties and quality of the tree raw material is an economically attractive strategy to obtain higher quality paper and to make the paper manufacturing process more environmentally benign.

A completely different use of plant fibres is related to forage digestibility (Table 1). Forage maize (*Zea mays*) is excellent roughage for ruminants because of its high energy content. The maize forage digestibility is however severely affected by the association of lignins and hemicelluloses. These components have been implicated to be clearly anti-nutritional, by creating a physical and chemical barrier (Jung and Buxton, 1994; Fontaine *et al.*, 2003). Improvement of the complex interactions between cell wall components could result in an increased forage digestibility, thereby developing the potential of maize as a forage crop.

The basic properties of plant fibres are determined by the composition of the plant cell wall. An enhanced use of plant fibres and improvement of their quality therefore requires knowledge of the molecular processes that underlie cell wall metabolism. In the following sections we will focus on the general characteristics and composition of the cell wall, and explain how its modification could influence the quality of fibres.

Introduction to the plant cell wall

The plant cell wall, which is exterior to the plasma membrane, serves multiple purposes. Its primary function is to prevent membrane rupture, and besides this it is also involved in determining cell size and shape, and in controlling the rate and direction of cell growth. By providing rigidity to the plant cells, the cell walls function as the skeleton of the plant. In

addition, cell walls are also involved in cell adhesion, cell-cell communication and defence responses (Raven *et al.*, 1992; Carpita and Gibeaut, 1993; Gibeaut and Carpita, 1994, Dhugga, 2001). The plant cell wall has been shown to be a complex composite of many polysaccharides and proteins, whose exact composition relates to size, shape, strength and function of the various plant tissues (Carpita and McCann, 2000). Morphologically, the cell wall can be divided into three distinct zones, the middle lamella, primary wall, and secondary wall. The middle lamella is shared by two contiguous cells and is composed almost entirely of pectic substances (Raven *et al*., 1992). In most cell types, the primary wall consists of three structurally independent but interacting networks: cellulose microfibrils coated with branched non-cellulosic polysaccharides (>50% dry weight), embedded in a pectin matrix (25-40% dry weight), locked into shape by glycoproteins (1-10% dry weight) (Carpita and Gibeaut, 1993). After cell growth has ceased, cross-linking may occur between cell wall constituents and, a secondary wall is being formed. The transition form primary to secondary wall synthesis is marked by cessation of pectin deposition and a notable increase in the synthesis of cellulose, hemicellulose and lignin (Raven *et al*., 1992). Lignin is thought to be bound to polysaccharides, by both covalent and non-covalent interactions to form a lignin-polysaccharide complex (Sarkanen, 1998). This leads to a maximum wall strength and rigidity for the plant body.

Plant cell wall composition

Comprehension of the structure and composition of the cell wall components is a prerequisite for the manipulation of plant fibres. Next some general features of the cell wall composition that have emerged from the cumulative results of studies over the last 40 years will be reviewed.

Cellulose

Cellulose is the major load-bearing and omnipresent polysaccharide in the plant cell wall. In general, primary walls contain 10-40% cellulose, whereas secondary walls contain 40-60% cellulose (Bacic *et al*., 1988). Cellulose is composed of linear polymer chains of β-1,4-linked D-glucosyl (Glc) residues (Figure 1). In the β-1,4-linked backbone, every Glc residue is rotated or inverted 180° with respect to its neighboring residue. This implies that the repeating unit in the backbone is cellobiose and the glucan chain itself is relatively straight. In nature, most cellulose chains are assembled into crystalline microfibrils by hydrogen bonding (Delmer and Amor, 1995; Delmer, 1999). Each microfibril is thought to consist most often of 36 glucan chains, whose arrangement determines the crystalline state and the mechanical properties (Emons and Mulder, 2000; Emons *et al*., 2002).

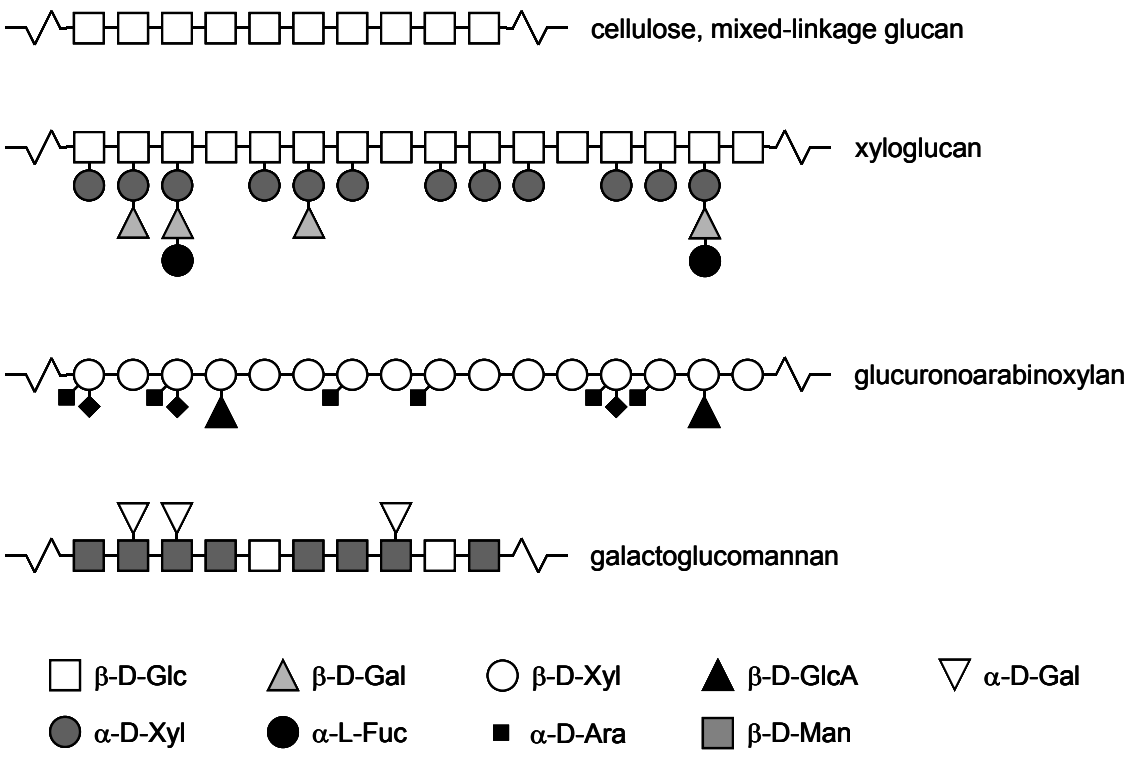


Figure 1. Schematic structures of cellulose and hemicelluloses. The cellulose and most hemicellulose backbones are composed of β-1,4-linked-D-pyranosyl residues, such as glucose, mannose and xylose. An exception is the mixed-linkage glucan backbone, which is composed of β-D-Glc residues, of which about 30% are 1,3-linked and the remaining 70% are 1,4-linked. The glucuronoarabinoxylan structure is hypothetical; it combines some of the characteristics described in the text. For further details see section *Plant cell wall composition*.

Hemicellulose

Hemicelluloses are a diverse group of polysaccharides whose function is to cross-link cellulose microfibrils. In the primary wall of dicotyledons and non-graminaceous monocotyledons, the most common hemicellulose is xyloglucan. Xyloglucans make up about 20% of the primary cell walls of dicots and 1-5% of the primary walls of grasses. They appear to be absent from most secondary walls, but are the major components of the thick storage walls of some seeds (Fry, 1988). Xyloglucan is believed to coat the surfaces of nascent microfibrils, and cross-link two microfibrils by hydrogen bonding between the glucan backbone of the xyloglucan and the cellulose chain. In this way xyloglucans are involved in the regulation of the mechanical properties of the cell wall (Whitney *et al.*, 1995, 1999). Xyloglucan has a 'cellulosic' backbone consisting of β-1,4-linked Glc residues. Unlike the unbranched cellulose, up to 75% of the backbone residues are branched, carrying a α-D-xylosyl (Xyl) residue at the 6-O-position (Figure 1). Additional β-D-galactosyl (Gal) or L-arabinosyl (Ara) groups can be added at the 2-O-position of some Xyl units, thereby extending the side chain. A α-L-fucosyl (Fuc) residue can be added at

the 2-O-position of a subtending Gal unit, providing a trisaccharide side chain (Fry, 1988; Carpita and Gibeaut, 1993; Fry *et al.*, 1993).

Xylans make up roughly 5% of the primary wall in dicotyledons, 20% of the primary walls of grasses and 20% of the secondary walls in both dicots and grasses. The linear xylan is involved in cross-linking of cellulose microfibrils (Awano *et al.*, 2001) and lignin (Hatfield *et al*., 1999). Xylans are composed of β-1,4-D-xylosyl chains, which are substituted to varying degree at the 2-O- or 3-O-position with 4-O-methyl-glucuronyl, glucuronyl (GlcA), acetyl and Ara groups (Figure 1) (Fry, 1988; Gruppen *et al.*, 1992; Ebringerova and Heinze, 2000; O'Neill and York, 2003). The nature and extent of substitution depends on the source of the xylan, with softwood and cereals containing high levels of Ara substituents, whereas hardwood contains mainly GlcA groups (Coughlan and Hazlewood, 1993).

Mannans occur in moderate amounts in certain secondary cell walls. These polysaccharides serve as carbohydrate reserves in a variety of plant species. Mannose-containing polysaccharides include (galacto)mannans and (galacto)glucomannans. (Galacto)mannans have a backbone consisting of β-1,4-linked D-mannosyl (Man) residues; in case of galactomannan, Gal residues are attached as single-unit side chains to the 6-O-position of certain Man residues (Stephen, 1982). Glucomannans, which are abundant in secondary cell walls of woody species, have a backbone that contains both β-1,4-linked Man and β-1,4-linked Glc residues. Galactoglucomannans, which are especially abundant in the primary cell walls of solanaceous species, have a similar backbone but some of the backbone Man residues bear single-unit Gal side chains at the 6-O-position (Figure 1) (Stephen, 1982).

Mixed-linkage glucans (β-(1-3),β-(1-4)-glucans), are abundant polysaccharides of the cell walls of grasses. These polymers are suggested to cross-link the cellulose microfibrils in the monocot cell wall analogous to the xyloglucans from the dicotyledons walls (Carpita and Gibeaut, 1993). Mixed-linkage glucans appear to be composed of an unbranched chain of Glc residues (Figure 1); about 30% of the linkages are β-1,3, whereas the other being β-1-4 (Fry, 1988).

Pectins

Pectin is a main component of the middle lamella, where it cements adjacent cells, and it determines the cell porosity in the primary wall (Moore *et al.*, 1986). The cellulose / xyloglucan network of the primary cell wall is embedded in a pectic matrix (Carpita and Gibeaut, 1993). The primary structure of the individual pectic polysaccharides is well established. They comprise mainly homogalacturonan (HGA) and rhamnogalacturonan I and II (RGI and RGII). HGA (the smooth region) is consisting of α-1,4-linked galacturonic acid (GalA) residues, which may be methyl-esterified and/or O-acetylated (Carpita and

Gibeaut, 1993). RGI has a backbone composed of repeating α-1,2-Rha-α-1,4-GalA disaccharide units. Also in the RGI backbone the GalA residues may be O-acetylated (Carpita and Gibeaut, 1993). Side-chains, mainly consisting of arabinan and (arabino)galactan, may be attached to the RGI backbone at the 4-O position of the Rha residues (Carpita and Gibeaut, 1993; O'Neill *et al.*, 1990; Schols and Voragen, 1994). The macromolecular structure composed of RGI, arabinan and (arabino)galactan is often referred to as hairy regions (de Vries *et al.*, 1981), with arabinan and (arabino)galactan comprising the hairs. RGII shows by far the richest diversity in sugar and linkages of all known polysaccharides, but will not be further discussed.

Lignins

Lignin makes up 20-30% of the dry weight of wood. It is laid down initially in the middle lamella and primary walls of certain cells (a.o. fibre cells) and later it later also accumulates in the secondary walls of these cells (Fry, 1988; Raven *et al.*, 1992; Terashima *et al.*, 1993). Lignins are polymers composed of phenylpropanoid alcohol units, which are derived from coniferyl- (C), sinapyl- (S) and p-coumaryl (H)-alcohol, depending on the plant source. Linking occurs by a variety of chemical bonds, including ester, ether, and carbon-carbon-bonds. The result is a three dimensional network of highly hydrophobic polymers. In addition, lignin may also be covalently or non-covalently linked to hemicelluloses (in particular xylan) and cellulose (Whetten *et al.*, 1998; Boudet, 1998, 2003; Anterola and Lewis, 2002; Boerjan *et al.*, 2003).

Callose

Callose is widely distributed within plant species, but in much lower quantities than cellulose. Callose is composed of a triple helix of a linear homopolymer of β-1,3-linked Glc residues with occasional β-1,6-linked branches (Stone and Clarke, 1992). The different linkage between the Glc residues gives callose quite different physico-chemical properties when compared to cellulose. Callose is deposited at the cell plate of dividing cells, at plasmodesmata and sieve plates, and in pollen mother cells and tubes. In higher plants, callose deposition is also induced under certain physiological conditions in which the plasma membrane is perturbed. These conditions include mechanical wounding, chemical treatment and pathogen attack. The capacity for deposition of wound callose is thought to be a protection mechanism, creating a physical barrier at the plasma membrane (Stone and Clarke, 1992).

Other components

Other cell wall components include structural glycoproteins, arabinogalactan proteins (AGPs), and enzymes. The main structural glycoprotein in the cell wall of dicots is

extensin, which is present in widely varying quantities, making up 1-10% of the wall (Fry, 1988). Extensin adds rigidity and strength to the wall by cross-linking with themselves (Brady *et al.*, 1996) or with other cell wall components, like pectins (Brady *et al.*, 1996; MacDougall *et al.*, 2001). AGPs are a family of structurally complex water-soluble proteoglycans (Gaspar *et al.*, 2001), which are found in the apoplastic fluid of many plant species. The AGPs are no structural components of the cell wall, however they may have a role in cell expansion and cell differentiation. Cell walls contain numerous enzymes, including those involved in cell wall metabolism (glucanases, xylanases, methyl and acetyl esterases, transglucosidases) and enzymes that may generate cross-links between wall components (e.g. peroxidases). Walls also contain proteins referred to as expansins that have been proposed to break hydrogen bonds between xyloglucan and cellulose and thus are believed to regulate cell expansion (Cosgrove, 1999).

Factors influencing fibre quality

Whereas cotton fibre cells contain a thick secondary wall consisting of highly crystalline, nearly pure cellulose, the secondary walls of flax, sisal, hemp and poplar fibre cells contain significant amounts of hemicellulose, lignin and pectin. Residual lignins as well as pectins and hemicellulosic polysaccharides have a negative effect on the softness and fineness of the textile fibres after processing (McDougall *et al.*, 1993; Sharma *et al.*, 1999). Lignin also has a negative effect in the pulp and paper industry where it gives pulp a characteristic brown colour. In order to eliminate this brown colour, lignin has to be extracted by a multistage bleaching process, which is costly, energy consuming as well as environmentally hazardous (Clarke *et al.*, 1997; Grima-Pettenati and Goffner, 1999). Similarly, the degree of lignification of the cell wall is one of the limiting factors in the digestibility of forage crops (Jung *et al.*, 1994; Fontaine *et al.*, 2003). It is clear that the development of plants, through genetic engineering, with a modified lignin content or with lignin of different compositions, suited for specific agricultural and industrial uses, is of great value. Reduction of lignin by down-regulating the key enzymes involved in its biosynthesis was extensively studied by others (reviewed by Anterola and Lewis, 2002; Boerjan *et al.*, 2003; Boudet *et al.*, 2003). For example, genetically modified tobacco (*Nicotiana tabacum*) plants with suppressed cinnamyl alcohol dehydrogenease (CAD) or cinnamoyl CoA reductase (CCR) levels, the two final enzymes in the biosynthesis of lignin monomers, resulted in lignin with altered structure (Halpin *et al.*, 1994; Boudet *et al.*, 1998; Lapierre *et al.*, 1999). Pulping experiments with CAD or CCR depleted plants revealed an improved extractability of lignins with an increase in pulp yield (Halpin *et al.*, 1994; Boudet *et al.*, 1998; Lapierre *et al.*, 1999).

In order to engineer the fibre characteristics, understanding of hemicellulose biosynthesis as well as their contribution to fibre properties and effects on cellulose extractability, is also important. Our approach therefore focuses on modification of the hemicellulose (in particular xylan) content and thereby also the attachment of lignin in the plant cell wall. *In planta* modification of xylan levels may alter the fibre characteristics resulting in an enhanced suitability for industrial applications. Different strategies can be applied to decrease the xylan content in the cell wall. A first strategy is the modification of the nucleotide sugar conversion pathway. Increasing knowledge on the biosynthetic pathway of the different nucleotide-diphosphate (NDP) sugars involved in cell wall biosynthesis enables the regulation of NDP-sugar levels, including UDP-Xyl. Conversion of UDP-Glc to UDP-Xyl involves two enzymatic steps, the oxidation of UDP-Glc to UDP-GlcA and the subsequent decarboxylation to UDP-Xyl (Figure 2) (Gibeaut, 2000; Seifert, 2004). Down-regulation of UGD or UGAD, the enzymes involved in the conversion of UDP-Glc to UDP-Xyl, could result in a reduction of the UDP-Xyl pool size. This could consequently affect the biosynthesis rate and composition of xylan and xyloglucan, which contain xylosyl residues. Since the UDP-GlcA and UDP-Xyl pools are directly linked to that of other UDP-

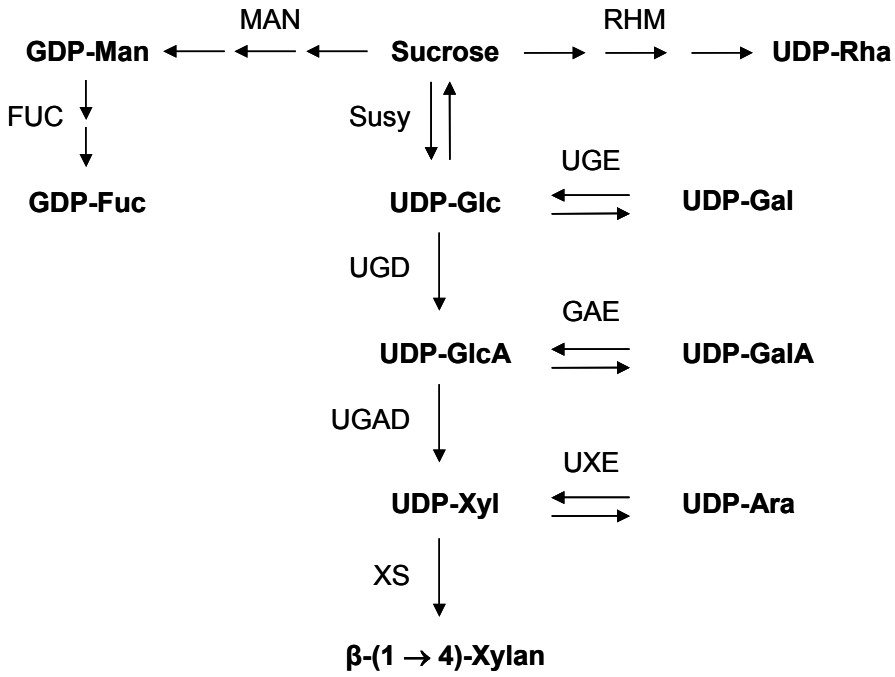


Figure 2. Carbohydrate metabolism of UDP- and GDP-sugars involved in plant cell wall biosynthesis. Abbreviations: RHM: UDP-Rha biosynthetic enzymes; MAN: GDP-Man biosynthetic enzymes; FUC: GDP-Fuc biosynthetic enzymes; Susy: sucrose synthase; UGE: UDP-D-glucose 4-epimerase; UGD: UDP-D-glucose dehydrogenase; GAE: UDP-D-glucuronate 4-epimerase; UGAD: UDP-D-glucuronate decarboxylase; UXE: UDP-D-xylose 4-epimerase; XS: xylan synthase.

sugars, the effects of modulating pool sizes are difficult to predict, and therefore we want another approach.

We focused on two more direct strategies to alter the xylan level in the cell wall: (i) Down regulation of the xylan backbone synthesising enzyme, the xylan synthase; (ii) Degradation of already deposited xylan polymers by the heterologous expression of xylan degrading enzymes (xylanases). Similar strategies to modify cell wall composition have been successfully used by others. A first example is the down regulation of a cellulose synthase (*CesA*) gene in *Nicotiana benthamiana* by using the virus-induced gene silencing (VIGS) system (Burton *et al.*, 2000). The *CesA* gene expression was inhibited upon infection with the virus, resulting in decreased cellulose levels (25% reduction). Another example is the heterologous expression of a fungal endo-β-1,4-galactanase (*e*GAL) in the apoplast of potato (*Solanum tuberosum*). Transgenic tubers expressing this enzyme, showed a reduction of cell wall galactose to 30% of the wild type (Sørensen *et al.*, 2000). Additionally, the introduction of a fungal rhamnogalacturonan lyase (*e*RGL) in potato resulted in a fragmentation of RG I, and a decrease in the levels of galactan and arabinan, which occur as side chains of RG I (Oomen *et al.*, 2002).

HEMICELLULOSE BIOSYNTHESIS

General features of plant (hemi)cellulose biosynthesis

It is clear that the cell wall polysaccharides form coextensive networks and have a tremendous structural complexity. To assemble the polysaccharides, the plant cell needs a vast biosynthetic machinery. It has been estimated that over 2,000 gene products are involved in making and maintaining the cell wall (Carpita *et al*., 2001; Perrin *et al.*, 2001).

The central process of polysaccharide biosynthesis is the action of glycosyltransferases (GTs). These enzymes form glycosidic bonds by attaching a sugar moiety of an appropriate donor substrate, mainly a nucleotide sugar, to a specific acceptor substrate. The nucleotide sugars are made in the cytoplasm. Initial reactions in the pathway of sugar nucleotide synthesis and interconversion produce UDP-Glc from sucrose and UTP / Glc-1-P, and GDP-Man from GTP / Man-1-P. Further modifications of the Glc and Man sugar units then result in the formation of all the substrates needed by the various GTs (Reiter and Vanzin, 2001). At the time of writing, GTs have been classified into 78 families (see http://afmb.cnrs-mrs.fr/CAZY/) based on the derived amino acid sequence similarities and the stereochemical outcome of the reaction that they catalyse (Coutinho *et al.*, 2003). The biological activity of only a few of these putative GTs has been demonstrated. Even fewer have been shown to be involved in wall polysaccharide biosynthesis or to affect wall structure (see below).

Cellulose is synthesised at the surface of the plasma membrane by cellulose synthase (CesA) proteins seen as terminal complexes or rosettes in freeze-fractured cell membrane surfaces (Brown, 1996; Delmer, 1999; Kimura *et al*., 1999). The rosettes have a six-fold symmetry and each particle in the rosette is believed to contain six CesA sub-units, allowing for the synthesis of 6 * 6 glucan chains that are present in a cellulose microfibril. The CesAs are processive enzymes that belong to GT Family 2. The enzymes are defined as processive because they repeatedly transfer sugar residues to the same growing glucan chain.

In contrast to cellulose, hemicellulosic and pectic polysaccharides are synthesized in the Golgi apparatus (Gibeaut and Carpita, 1994; Scheible and Pauly, 2004). Polysaccharide synthesis in the Golgi apparatus implies that the nucleotide sugars have to be transported into the lumen of the Golgi by transporters (Keegstra and Raikhel, 2001) before they can be used for biosynthesis of the wall polysaccharides. Inside the Golgi, processive GTs repeatedly transfer single sugar residues to form the backbone of the polysaccharide, and a number of distributive GTs decorate the backbone by each transferring a single sugar residue onto the backbone or the growing branch. The completed complex polysaccharides are transported to the cell surface via secretory vesicles and emptied into the cell wall (Doblin *et al.,* 2003; Scheible and Pauly, 2004). Since we are interested in (hemi)cellulose backbone biosynthesis, next we will focus on the current knowledge about the enzymes involved in this process.

Genes involved in (hemi)cellulose backbone biosynthesis

The first plant *CesA* genes were identified by sequencing and analysis of random clones from a cotton-fibre cDNA library (Pear *et al*., 1996). Protein sequences derived from two cDNA clones from this library (*GhCesA1* and *GhCesA2*) contained stretches of amino acids that were highly similar to those of the *Acetobacter xylinum CesA* (Saxena *et al.*, 1990). Predominant among the conserved regions were four sub-domains U1 to U4 that are common to processive Family 2 GTs (Saxena *et al.*, 1995). These regions contain the conserved D, DxD, D residues and the QxxRW motif (where x stands for any amino acid), which were suggested to be involved in substrate binding and/or catalysis (Figure 3) (Charnock and Davies, 1999; Charnock *et al*., 2001; Saxena *et al*., 2001). In addition to the conserved motifs U1 to U4, a number of other conserved regions can be indicated in the plant CesA proteins. These regions include the N-terminal ‘zinc-finger’ domain, which is thought to be involved in interactions with other protein partners, two ‘hyper’variable regions and the transmembrane domains (indicated in Figure 3). From hydropathy plots, all CesA-encoded proteins were predicted to have two transmembrane domains at the N-terminus and five or six transmembrane domains at the C-terminus (Delmer, 1999). The

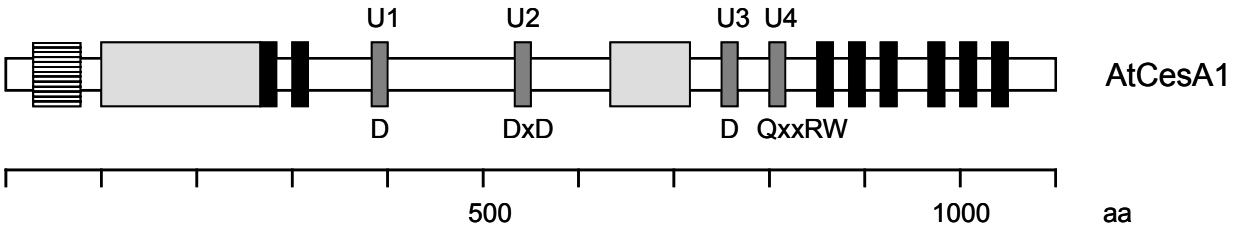


Figure 3. Schematic representation of the plant *CesA* genes using the *A. thaliana* CesA1 protein as a paradigm. The striped box at the amino terminus represents the zinc finger domain containing the CxxC motif. The variable regions are shown as two grey boxes. The black boxes indicate the eight putative transmembrane regions. Boxes U1 to U4 represent the four conserved domains containing the D, DxD, D, QxxRW motif. The conserved amino acids in domain U1 and U2 are thought to bind the UDP-Glc substrate, whereas the D, QxxRW residues in U3 and U4 are suggested to be involved in processivity.

large globular region containing the conserved GT residues is located on the cytoplasmic side of the plasma membrane (Delmer, 1999; Doblin *et al.*, 2003).

Direct evidence about the involvement of plant *CesA* genes in cellulose biosynthesis came from studies on two cellulose-deficient *Arabidopsis thaliana* mutants, *rsw1* (Arioli *et al*., 1998) and *irx3* (Turner and Somerville, 1997; Taylor *et al*., 1999). Genes with sequence similarity to cotton *CesA* have been isolated from many plant species. The genome sequences of *A. thaliana* and rice (*Oryza sativa*) have now made it possible to obtain information on the complete sets of cellulose synthases in these plants. In *A. thaliana*, at least ten distinct *CesA* genes have been identified, whereas the rice genome contains at least twelve different *CesA* members (Holland *et al.,* 2000; Richmond and Somerville, 2000).

In addition to the 'true' *CesA* genes, plants contain a large number of structurally related genes, called *cellulose synthase-like* (*Csl*). Although the CesA and Csl proteins vary in their degree of sequence similarity, they share several features. Like the *CesA* genes, all *Csl* gene products are integral membrane proteins and contain the D, DxD, D, QxxRW motif, that seems to be characteristic for the Family 2 processive GTs (Saxena *et al.*, 1995). On these grounds, it has been proposed that the *Csl* genes encode the catalytic subunits of the enzymes that synthesise the backbone of the non-cellulosic β-linked cell wall polysaccharides like xylan, xyloglucan, mannan and galactan (Richmond and Somerville, 2000, 2001). If the various *Csl* genes, like other Family 2 GTs, strictly make β-linkages, then a role in synthesis of α-linked polysaccharides, such as the pectic components HG, RG-I, is not very likely. It is however possible that linkage specificity is determined by subtle features in the active site of the protein (Stasinopolous *et al.*, 1999), and that the *Csl*s are able to make polysaccharides with both β- and α-linkages. However, since the nucleotide sugar donors are in the β-position, and Family 2 GTs makes glycosidic linkages with retention of anomeric configuration, it is unlikely that the members

of the Csl families play a role in the biosynthesis of α-linked polysaccharides (Carpita *et al.*, 1996). Except the CslD family, the Csl proteins lack the N-terminal zinc-finger domain.

By cluster analysis the Csl proteins have been subdivided into eight families, CslA through CslH (Figure 4). The *A. thaliana* genome contains six families of *Csl* genes (*CslA, B, C, D, E* and *G*) (Richmond and Somerville, 2000, 2001), which contain respectively 9, 6, 5, 6, 1, and 3 family members. Rice lacks the *CslB* and *CslG* genes and contains two other families, *CslF* and *CslH* (Hazen *et al.*, 2002). Rice has 10, 9, 4, 5, 7 and 2 members respectively of the *CslA*, *CslC*, *CslD*, *CslE*, *CslF*, and *CslH* families (Hazen *et al.*, 2002). The *CslF* and *CslH* genes may be responsible for the synthesis of polysaccharides found predominantly or only within the walls of grasses, such as mixed-linkage glucans (Richmond and Somerville, 2001; Hazen *et al.*, 2001). Conversely, the absence of *CslB* or *CslG* genes from the rice genome could implicate the involvement of these genes in the synthesis of polysaccharides predominantly found in dicots, such as xyloglucans (Richmond and Somerville, 2001).

Since the Csl proteins are suggested to participate in the biosynthesis of the non-cellulosic polysaccharides, they are expected to be located in the Golgi apparatus or endoplasmatic reticulum (ER) (Dhugga *et al.*, 2004; Scheible and Pauly, 2004). Immunolocalization and GFP-fusion experiments have revealed that the *A. thaliana* CslA, CslB, CslE, and CslG proteins are indeed localised to the Golgi (Richmond and

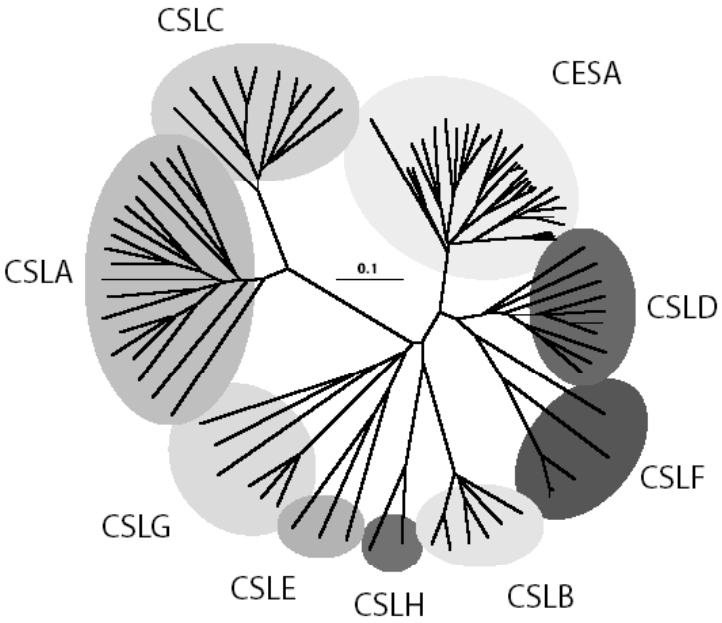


Figure 4. Unrooted, bootstrapped tree of the CesA superfamily. Clustal X (version 1.8) was used to create an alignment of available CesA/Csl protein sequences that was than bootstrapped (n= 5000 trials) to create the final tree. Subfamilies are encircled. Picture is taken from http://cellwall.stanford.edu.

Table 2. Overview of *cellulose synthase-like* (*Csl*) mutants and their major phenotypes.

Species and gene	Mutant	Mutant wall	Phenotype	Reference
AtCslA7	-	n.d.	embryo lethality, pollen tube growth affected	Goubet *et al.,* 2003
AtCslA9	rat4	↑ Gal in older tissue	resistant to bacterial attachment	Nam *et al.,* 1999; Zhu *et al.,* 2003
AtCslB6	-	Altered FTIR spectrum (cellulose region)	n.d.	Bonetta *et al.,* 2002
AtCslD3	kojak	n.d.	defective root hair growth	Favery *et al.,* 2001; Wang *et al.,* 2001

Abbreviations: *At, Arabidopsis thaliana; rat4,* resistant to *Agrobacterium* transformation*;* n.d., not determined; Gal, galactose.

Somerville, 2000). The CslD protein was also shown to be located in the endomembrane system, however in the ER instead of the Golgi (Favery *et al.*, 2001). Additionally, upon expression of a guar seed (*Cyamopsis tetragonoloba*) *CslA* cDNA in soybean somatic embryos, a high level of mannan synthase activity was localized to the Golgi (Dhugga *et al.*, 2004). All Csl proteins are integral membrane proteins with one or two transmembrane domains in the N-terminus and three to six transmembrane domains in the C-terminal region. It is generally believed that the proposed catalytic site of Csls with an even number of N-terminal transmembrane domains might face the cytosol, whereas the catalytic site of Csls with an odd number of N-terminal transmembrane domains might face the lumen of the Golgi apparatus (Doblin *et al*., 2003).

The function of most of the *Csl* genes remains unknown. Loss-of-functions mutations in several of the *Csl* genes resulted in a developmental phenotype (Table 2) however in neither case it is clear which type of cell wall polymer is affected. Recently, Dhugga *et al.* (2004) were the first to assign a *Csl* gene to the biosynthesis of a specific wall polysaccharide; a guar seed *CslA* gene was demonstrated to be involved in the formation of the β-1,4-mannan backbone of galactomannan, a hemicellulosic storage polysaccharide in endosperm walls. Furthermore, Liepman *et al.* (2005) revealed by heterologous expression that recombinant *A. thaliana* CslA proteins produce β-linked mannan and glucomannan polymers when supplied with GDP-mannose and UDP-glucose. Further investigation of the *Csl* gene families is required to determine what roles these genes play in hemicellulose biosynthesis.

XYLAN DEGRADATION

General aspects of cell wall disassembly

Following assembly of a primary cell wall, disassembly is necessary for processes in which transient, reversible wall loosening occurs, but where the overall structural integrity is maintained. Examples are cell expansion and, to a lesser extent, secondary wall formation. Cell wall disassembly is also characteristic of developmental events such as fruit ripening, seed germination, abscission and dehiscence. In these instances substantial and irreversible changes in wall architecture occur, resulting in a net wall depolymerization (Rose *et al*., 2003). The highly regulated process of cell wall disassembly requires the concerted action of a spectrum of cell wall modifying proteins including pectinases, glucanases, expansins, mannanases, xylanases and xyloglucan endotransglycosylase (XET) (Brummell and Labavitch, 1997; Brummell *et al*., 1997; Bewley *et al.*, 2000; Cosgrove, 2000; Thompson and Fry, 2001; Simpson *et al.*, 2003). For a coordinated wall disassembly, a synergistic enzyme action, where one class of enzymes might mediate the activity of other enzymes, is crucial. There are many ways in which this might occur. Degradation of a polymer backbone by endo-acting glycoside hydrolases generates small oligo- or polysaccharides that could subsequently be further degraded into mono- or disaccharides by exo-acting hydrolases. Alternatively, glycoside hydrolases that remove side chains from cross-linking polysaccharides might increase the ability of endo-acting glycoside hydrolases to act on the polymer backbone. The disruption of non-covalent polysaccharide interactions may also be an important component of synergistic cell wall disassembly, and in this regard, expansin may alter the accessibility of a range of substrates to their enzymes (Rose *et al.*, 2003).

Introduction to xylanases

Since we are interested in reducing the xylan content in the cell wall, the following sections focus on xylanases, the enzymes involved in the hydrolysis of xylan polymers. Due to the heterogeneity and complexity of the xylan polymers, complete hydrolysis of xylan requires the concerted action of several glycoside hydrolases. The most important are the endo-β-1,4-xylanases (xylanases, EC 3.2.1.8), which degrade the xylan backbone into xylo-oligosaccharides of varying lengths. For complete hydrolysis, exo-β-1,4-xylosidases (EC 3.2.1.37) cleave xylose monomers from the non-reducing end of xylo-oligosaccharides and xylobiose, while removal of the side chains is catalyzed by for instance α-glucuronidases (EC 3.2.1.139) and α-arabinofurosidases (EC 3.2.1.55) (Collins *et al.,* 2005).

At present, glycoside hydrolases (GHs) are classified into 99 families based on primary structure comparisons of the catalytic domains (see the carbohydrate active enzyme CAZY server at http://afmb.cnrs-mrs.fr/CAZY). Xylanases are primarily classified

into two families, GH10 and GH11, however enzymes with xylanase activity are also found in family 5, 7, 8 and 43 (Collins *et al.,* 2005). It is now widely established that xylanases, in common with the many plant cell wall hydrolases, often exhibit a modular structure comprised of catalytic domains linked to one or more non-catalytic carbohydrate binding modules (CBMs). CBMs attach the enzyme to the plant cell wall and increase the rate of hydrolysis by bringing the enzyme into intimate and prolonged contact with its substrate (Bolam *et al.*, 1998; Charnock *et al.*, 2000). Some CBMs have also been proposed to display additional functions, such as substrate disruption and feeding of single cellulose chains into the active site of the catalytic module (Tomme *et al.*, 1996). Similar to the catalytic modules, CBMs are divided into families based on amino acid sequence similarity. There are currently 43 families of CBMs (see http://afmb.cnrs-mrs.fr/CAZY/index.html) and these CBMs display substantial variation in ligand specificity. The majority of these modules bind to cellulose, xylan, mannan, chitin, or starch (reviewed by Boraston *et al.*, 2004). CBM families whose members bind xylan include CBM2b, CBM4, CBM6, CBM13, CBM22, CBM29, CBM35, CBM36, and CBM37 (Xie *et al.*, 2001; CAZY server).

Glycoside hydrolases often have complex structures consisting of multiple CBMs, and sometimes several catalytic modules, in addition to modules of unknown function. The CBMs in a single enzyme can be members of the same or different protein families (Tomme *et al.*, 1995). An enhanced polysaccharide binding has been observed for several modular glycoside hydrolases that contain multiple CBMs from the same family, such as CBM1 (Lindner *et al.*, 1996), CBM2 (Bolam *et al.*, 2001) and CBM29 (Freelove *et al.*, 2001).

Plant xylanases

More than 200 xylanases have been identified to date, but the great majority of these originate from bacteria and fungi. Microbial xylanases facilitate the degradation of plant cell walls and the products are subsequently used as an energy source for the microorganism. Current knowledge about plant xylanases is restricted to the few that have been purified from wheat (*Triticum aestivum*) (Cleemput *et al.*, 1997), barley (*Hordeum vulgare*) (Slade *et al.*, 1989; Banik *et al.*, 1996; Caspers *et al.*, 2001), maize (*Zea mays*) (Bih *et al.*, 1999; Wu *et al.*, 2002) and papaya (*Carica papaya*) (Chen and Paull, 2003). These xylanases are involved in different cellular processes. The barley X-1 protein has been shown to hydrolyze xylan polymers in germinating grains, thereby providing the embryo with carbohydrate nutrients (Caspers *et al.*, 2001). The maize ZmXyl is synthesized in the tapetum during anther development. This xylanase is suggested to hydrolyze the stigma wall so that the pollen tube can enter the transmitting track of the style (Wu *et al.*, 2003). The papaya xylanase CpaEXY1 may have a role in mesocarp softening during fruit

ripening (Chen and Paull, 2003). Irrespective of the function, the characterized plant xylanases are all proteins of about 35 kDa, which appear to be synthesized as much larger inactive precursors (>60 kDa). The inactive precursors consist of a GH10 domain preceded by a CBM22 module (Figure 5), whereas the active enzymes are composed of the catalytic GH10 domain alone (Chen and Paul, 2003; Simpson *et al.*, 2003). Analysis of the *A. thaliana* genome revealed a set of 12 putative *xylanase* (*Xyn*) genes (Henrissat *et al.*, 2001), of which at least three are coding for modular enzymes (Figure 5) (Suzuki *et al.*, 2002). The function of the *AtXyn* genes remains largely unknown. *AtXyn1* was shown to be expressed in vascular bundle cells and *AtXyn2* turned out to be expressed in the *A.*

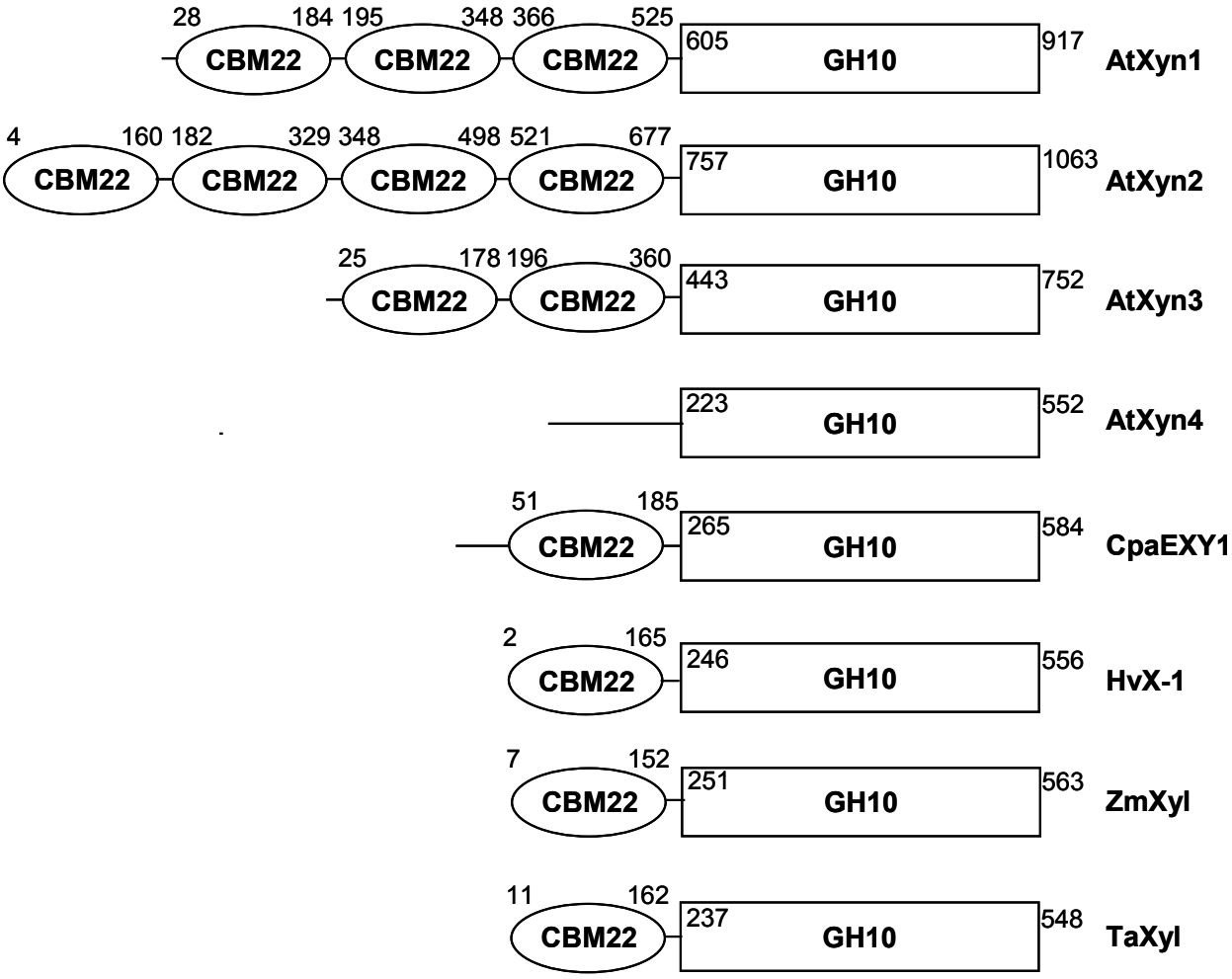


Figure 5. Schematic representation of plant xylanases. The putative CBM22 modules and GH10 catalytic domains are indicated. Numbers refer to the position of the first or last amino acid residue of each domain in the unprocessed protein sequence. Abbreviations: *At, Arabidopsis thaliana; Cpa, Carica papaya; Hv, Hordeum vulgare; Zm, Zea mays; Ta, Triticum aestivum.* The following protein sequences were used: BAA88262 (AtXyn1); AAC34334 (AtXyn2); AAD27896 (AtXyn3); AAC69373 (AtXyn4); AAN10199 (CpaEXY1); AAB51668 (HvX-1); AAF70549 (ZmXyl) and AAD41893 (TaXyl).

thaliana stems (Suzuki *et al.*, 2002). Both enzymes were therefore suggested to be involved in the rearrangement of xylan polymers in the cell wall.

Objectives and outline of the thesis

The research described in this thesis focuses on different approaches to modify the xylan content, and thereby also the attachment of lignin in the plant cell wall. *In planta* modification of xylan may improve the fibre characteristics resulting in an enhanced suitability for industrial applications. Instead of altering cell wall composition in flax, hemp or poplar, tobacco (*Nicotiana tabacum*) was selected as a model species, since it is widely used in fundamental cell wall research (Boudet, 1998, 2003; Boerjan *et al*., 2003). Additionally, tobacco has the advantage of being easily transformable and therefore amenable to genetic manipulation. Once interesting results are obtained in tobacco, technologies can be transferred into economically more important species.

Different approaches to generate transformants with altered xylan composition have been examined. (i) Decreasing the expression levels of putative xylan synthases, responsible for the polymerization of the xylan backbone structure. (ii) Specific degradation of the xylan polymer, which is already deposited in the cell wall. This can be achieved by the heterologous expression of xylan degrading enzymes. We selected AtXyn2, a modular Family 10 glycoside hydrolase from *A. thaliana*, for the heterologous expression in tobacco. AtXyn2 contains four consecutive CBM22 domains at the N-terminus, which can bring the enzyme into intimate and prolonged contact with its substrate. Therefore, AtXyn2 was expected to be a powerful tool for xylan degradation. Although the first approach seems more efficient, the applicability is limited due to the lack of information on genes involved in hemicellulose backbone biosynthesis.

Chapter 2 describes the generation of transgenic tobacco plants in which different *Csl* family members (*CslA, B, C, D, E* and *G*) were specifically down regulated. To achieve this, potato (*Solanum tuberosum*) *Csl* cDNA sequences were used to prepare inverted repeat (IR) constructs, which were directly transformed into tobacco. If one of the *Csl* families is coding for a xylan synthase, this strategy should consequently provide us with tobacco plants with altered xylan levels. To identify those transformants with an altered cell wall composition, monosaccharide compositional analysis of the wall polysaccharides was performed. Subsequently the effect of the *CslG* IR construct was studied on the RNA level by RT-PCR analysis and morphologically by microscopic analysis.

In **Chapter 3** we describe the isolation of a *NtCslE* and *NtCslG* cDNA clone from a xylogenic tobacco cDNA library. Sequence homologies, protein characteristics and relationships between *CslE* and *CslG* genes from different plant species are discussed. In addition, the size of the tobacco *CslE* and *CslG* gene family was investigated and the expression patterns of the isolated *Csl* genes were studied in wild type tobacco plants.

Chapter 4 describes the heterologous expression of AtXyn2, a modular Family 10 glycoside hydrolase from *A. thaliana* in tobacco. To investigate the function of *AtXyn2* and the role of the four CBM22 domains, the full length gene and a derivative, comprising the catalytic domain lacking the four CBM22 domains were introduced into tobacco. The activity and substrate specificity of the different proteins were studied and a model is proposed which might explain the regulation of AtXyn2 activity in the cell wall.

The thesis is concluded with a general discussion (**Chapter 5**) on the outcome of the different approaches to generate transformants with altered xylan composition. The limitations of the methodologies are described and future directions in research on the modification of the xylan content are suggested.

Chapter 2

Functional analysis of six *CslI* family members in *Nicotiana tabcum* by RNA inhibition

Monique Compier[1], Jean-Paul Joseleau[2], Katia Ruel[2], Richard G.F. Visser[1] and Jean-Paul Vincken[1]

[1] Graduate School Experimental Plant Sciences, Laboratory of Plant Breeding, Wageningen University, P.O. box 386, 6700 AJ Wageningen, The Netherlands.
[2] Centre de Recherches sur les Macromolécules Végétales (CERMAV), CNRS, BP 53, 38041 Grenoble Cedex 09, France

ABSTRACT

Plants contain at least eight families of *cellulose synthase-like* genes (*Csl*s) that have a strong structural similarity with the *cellulose synthases* (*CesA*s). Both the Csl and the CesA proteins are predicted to be integral membrane proteins and contain conserved motifs that are common to polymerizing β-glycosyltransferases. On these characteristics the *Csl* genes have been hypothesized to encode the enzymes that synthesize the non-cellulosic matrix polysaccharides. At present the biochemical function of most of the *Csl* genes has not been elucidated. This study reports the introduction of Inverted Repeat (IR) constructs, based on potato (*Solanum tuberosum*) *Csl* cDNA sequences, into tobacco (*Nicotiana tabacum*) in order to investigate the function of six different *Csl* families. Tobacco was chosen as a model species since it is widely used in fundamental cell wall research. Sugar compositional analysis of cell wall material isolated from *in vitro* grown *Csl* transformants revealed a reduction of xylose exclusively in the *CslG* transformants. Microscopic analysis of stem samples from mature *CslG* transformants revealed some very local effects like the multiplication of ray cells, enlargement of the size of vessels and the occurrence of abnormal fibres with unusually high starch content. Sugar compositional analyses of mature *CslG* transformants were ambiguous and a second series of *in vitro* grown *CslG* transformants did not confirm the decrease of xylose that was observed during the initial screening. Low xylose levels could not be linked to reduced *CslG* mRNA expression levels by RT-PCR analysis, and therefore it remains difficult to speculate on the biochemical function of *CslG* in tobacco. The possible involvement of factors like gene redundancy, low gene expression level and cell- or tissue-type specificity is discussed.

INTRODUCTION

A major breakthrough in plant polysaccharide biosynthesis research was the isolation of the first plant *cellulose synthase* genes (*CesA*s) from cotton (*Gossypium hirsutum*) by random cDNA sequencing. The deduced amino acid sequences of the cotton *CesA* gene products showed regions of homology with the proteins encoded by the bacterial *CesA* genes (Pear *et al.,* 1996). Evidence supporting the role of the plant *CesA* genes in cellulose biosynthesis came from studies on two *Arabidopsis thaliana* cellulose-deficient mutants, *rsw1* and *irx3* (Arioli *et al.,* 1998; Taylor *et al*., 1999). Comparisons of RSW1/CesA protein sequences with predicted protein sequences from the *A. thaliana* and rice (*Oryza sativa)* genomes, revealed the existence of a large superfamily of proteins that can be grouped into either cellulose synthase (CesA) or structurally related cellulose

synthase-like (Csl) sequences (Cutler and Somerville, 1997; Richmond and Somerville, 2000; Hazen *et al.*, 2002).

By cluster analysis the Csl proteins have been classified into eight families, CslA through CslH (Cutler and Somerville, 1997; Hazen *et al.,* 2002). Both the CesA and Csl classes of protein sequences possess the conserved motifs U1 to U4 that are common to polymerizing β-glycosyltransferases (Saxena *et al*., 1995). Besides these motifs, the *Csl* genes share only limited sequence identity with the *CesA* genes and they are therefore suggested to be involved in other processive glycosyltransferase reactions, such as the biosynthesis of non-cellulosic matrix polysaccharides like xylan, xyloglucan, mannan and galactan (Cutler and Somerville, 1997; Richmond and Somerville, 2000). Given the differences in cell wall composition between dicotyledons and graminaceous monocotyledons, it is expected that monocotyledons have another set of *Csl* genes than dicotyledons, such as those responsible for making mixed-linkage glucan. This is consistent with the fact that *A. thaliana* and rice share a number of *Csl* families, but also have a number of different classes (*A. thaliana CslA*, *CslB*, *CslC*, *CslD*, *CslE* and *CslG* and rice *CslA*, *CslC*, *CslD*, *CslE*, *CslF* and *CslH*) (Cutler and Somerville, 1997; Hazen *et al*., 2002).

Mutations in some of the *Csl* genes resulted in impaired development of root hairs (Favery *et al*., 2001; Wang *et al*., 2001), resistance to bacterial attachment (Zhu *et al*., 2003), or defective pollen tube growth and embryo lethality (Goubet *et al*., 2003), but in neither case it is clear which type of cell wall polymer is affected in these mutants. At present, only one of the *Csl* genes has been associated with the synthesis of a particular cell wall polysaccharide; Dhugga *et al*. (2004) provided the proof for the involvement of the guar seed (*Cyamopsis tetragonoloba*) *CslA* gene in the formation of the β-1,4-mannan backbone of galactoglucomannan in endosperm walls.

In order to reveal the function of the *Csl* genes, both forward and reverse genetics approaches can be applied. For the identification of natural *Csl* mutants, altered phenotypes have to be identified. Overall plant morphology and growth habit may be affected in *Csl* mutants; stunted plants may arise from the disruption of genes responsible for biosynthesis of the primary cell wall, while weaker plants may indicate defects in secondary cell wall biosynthesis (Turner and Somerville, 1997). It is however not very efficient to identify *Csl* mutants based on this criterion, since the phenotype of a *Csl* mutant is difficult to predict beforehand, and the growth habit and morphology of the plant can be affected by different mutations. Therefore, a reverse genetics approach seems more appropriate to reveal the function of the different *Csl* family members. Different tools for reverse genetics can be mentioned: transposon tagging (Parinov *et al*., 1999), large

transferred DNA (T-DNA) insertion mutations (Krysan *et al.*, 1999) and post-transcriptional gene silencing (PTGS) (Chuang and Meyerowitz, 2000).

PTGS is the sequence specific gene silencing induced by double-stranded RNA, mediated by 21-23 nucleotides small interfering RNAs (siRNAs) which are produced from long double-stranded RNAs by the RNAse II-like enzyme Dicer (Bernstein *et al*., 2001). The siRNAs are incorporated into an RNA-induced silencing complex (RISC) that targets and cleaves mRNA complementary to the siRNAs (Cerutti, 2003; Denli and Hannon, 2003). PTGS can be induced in plants by transforming them with either antisense (Rothstein *et al*., 1987) or co-suppression constructs (Napoli *et al.,* 1990), but this results only in a small proportion of silenced individuals. The application of Inverted Repeat (IR) constructs, containing two bi-directionally cloned transgene fragments separated by a spacer sequence, has been shown to result in an increase in both the number of silenced transformants and the degree of gene silencing (Smith *et al*., 2000; Wesley *et al*., 2001).

In this study we attempted to reveal the function of six different *Csl* families (*CslA*, *CslB*, *CslC*, *CslD*, *CslE* and *CslG*) in tobacco (*Nicotiana tabacum*) via an IR strategy. Potato (*Solanum tuberosum*) cDNA sequences coding for *Csl* family members were used to make IR constructs in order to specifically reduce the expression levels of the *Csl* genes in tobacco.

RESULTS AND DISCUSSION

Generation of *Csl* IR tobacco transformants

The function of six *Csl* families (*CslA*, *CslB*, *CslC*, *CslD*, *CslE* and *CslG*) was investigated in tobacco. Tobacco was chosen since it is widely used as a model species for studying the process of secondary cell wall formation (Boudet, 1998; Boerjan *et al*., 2003), although the sequence information on tobacco *Csl* genes is very limited. As potato cDNA clones (ESTs) coding for *Csl* genes were already available, it was first examined whether these sequences could be used to reveal the function of the six *Csl* families in tobacco by PTGS. Specific and effective gene silencing in heterologous systems via PTGS has been reported in different plant species (Salehuzzaman *et al.*, 1993; Burton *et al.,* 2000; Liu *et al*., 2002). Recently Benedito *et al.* (2004) showed that a 300 base pairs *phytoene desaturase* (*PDS*) fragment from lily elicited PTGS in *Nicotiana benthamiana*, in spite of the remote evolutionary relationship between the two species. The lily fragment showed 70% overall identity to the *PDS* from *N. benthamiana*. Although a *PDS* fragment from tomato (*Lycopersicon esculentum*) induced a stronger silencing effect in *N. benthamiana* than the lily fragment, the result clearly shows that heterologous gene silencing can be achieved,

Figure 1. Sequence alignment of potato cDNA clones (*StCsl)* with their tomato homologues (*LeCsl*). Nucleotides differing from the tomato sequences are indicated with solid black background. **A.** *StCslE* (cSTB47M10) and *LeCslE2* (cTOD2I22) share 93% sequence identity. The conserved region U1 is indicated above the sequence with an arrow. **B.** *StCslG1* (cSTB34J22) and *LeCslG5* (cTOF31A20) show 95% sequence identity. The conserved region U2 is indicated above the sequence with an arrow.

even with a monocotyledonous gene fragment in a dicotyledonous system (Benedito *et al.*, 2004).

BLAST analysis (Altschul *et al.*, 1997) of potato cDNAs against the NCBI EST database and alignment with available tomato *Csl s*equences (http://cellwall.stanford.edu) revealed a very high homology (up to 96% at the nucleotide level) between the *Csl* genes from potato and tomato, which are both solanaceous species. Figure 1 shows the alignment of the *StCslE* and *StCslG1* cDNA clones with their tomato homologues. The *CslE* and *CslG* cDNA clones share a sequence identity of respectively 93% and 95%. Because of the high homology between *Csl* genes from potato and tomato, it was assumed that the homology between *Csl* genes from potato and tobacco, also a solanaceous species, should be very high as well. Therefore, IR constructs were made based on potato cDNA sequences. In order to accomplish down regulation of a specific

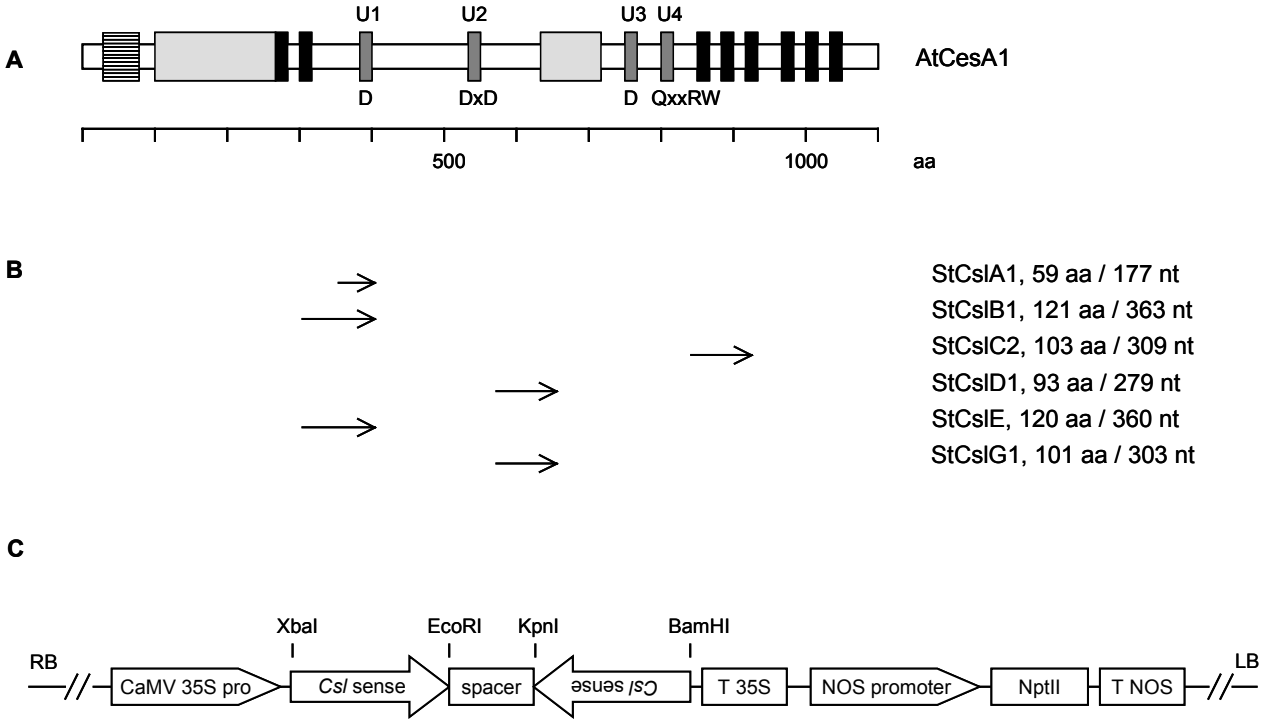


Figure 2. A. Schematic representation of the plant *CesA* genes using the *A. thaliana* CesA1 protein as a paradigm. The striped box at the amino terminus represents the zinc finger domain containing the CxxC motif. The variable regions are shown as two grey boxes. The black boxes indicate the eight putative transmembrane regions. Boxes U1 to U4 represent the four conserved domains containing the D, DxD, D, QxxRW motif. **B.** Size and position of the potato *Csl* cDNA fragments used for making the IR constructs, projected on the CesA protein. **C.** Schematic representation of the *Csl* IR T-DNA construct in the binary vector pGreen7K. The sense and antisense fragments are separated by a 158 base pairs spacer sequence which was obtained by PCR amplification from the pBIN19LUC-SBD vector (for details see Experimental Procedures section). Expression is driven by the CaMV35S promoter. *NptII*, neomycin phosphotransferase.

Csl family, potato cDNA clones were selected and PCR amplified such that the PCR products are located to a large extent outside the highly conserved regions U1 to U4 of the *Csl* genes (Figure 2A and 2B). The homology between the PCR products and their tomato homologues is very high: *StCslA1* 99%, *StCslB1* 96%, *StCslC2* 100% (a stretch of only 79 nucleotides), *StCslD1* 97% (a stretch of 126 nucleotides), *StCslE* 93% and *StCslG1* 95%. Due to the absence of sequence information on tobacco *Csl* genes, we could not predict beforehand whether this strategy would result in silencing of entire *Csl* families or specific family members. The IR constructs (Figure 2C), regulated by the constitutive CaMV35S promoter (Benfey and Chua, 1990), were introduced into tobacco via *Agrobacterium tumefaciens* mediated plant transformation. The transformants all developed normally and did not show any phenotypic differences with respect to their size, stem diameter and seed development in comparison with wild type tobacco and empty vector control transformants.

Screening of *in vitro* grown *CslI* transformants for alterations in hemicellulose composition

TFA hydrolysis of cell wall material, isolated from stems and leaves of about five weeks old *in vitro* grown tobacco plants, followed by HPLC analysis of monosaccharides, was performed to screen the *CslI* transformants for alterations in their hemicellulose composition. In Figure 3A a typical HPLC output is shown in which the peaks correspond to the different monosaccharides analysed. The monosaccharide composition of cell wall material from *in vitro* grown wild type tobacco plants is indicated in Figure 3B. Galactose and xylose and to a lesser extent also glucose and arabinose, are the most predominant sugars of the tobacco cell wall polysaccharides that are hydrolysed with 2 M TFA. Under mild hydrolysis conditions crystalline cellulose is not hydrolysed (Selvendran and Ryden, 1990) and therefore the glucose analysed is derived from xyloglucan, galactoglucomannan and callose (Gardner *et al.*, 2002). Hemicellulosic polysaccharides embedded in cellulose fibres are less accessible for TFA hydrolysis and are therefore underestimated in the

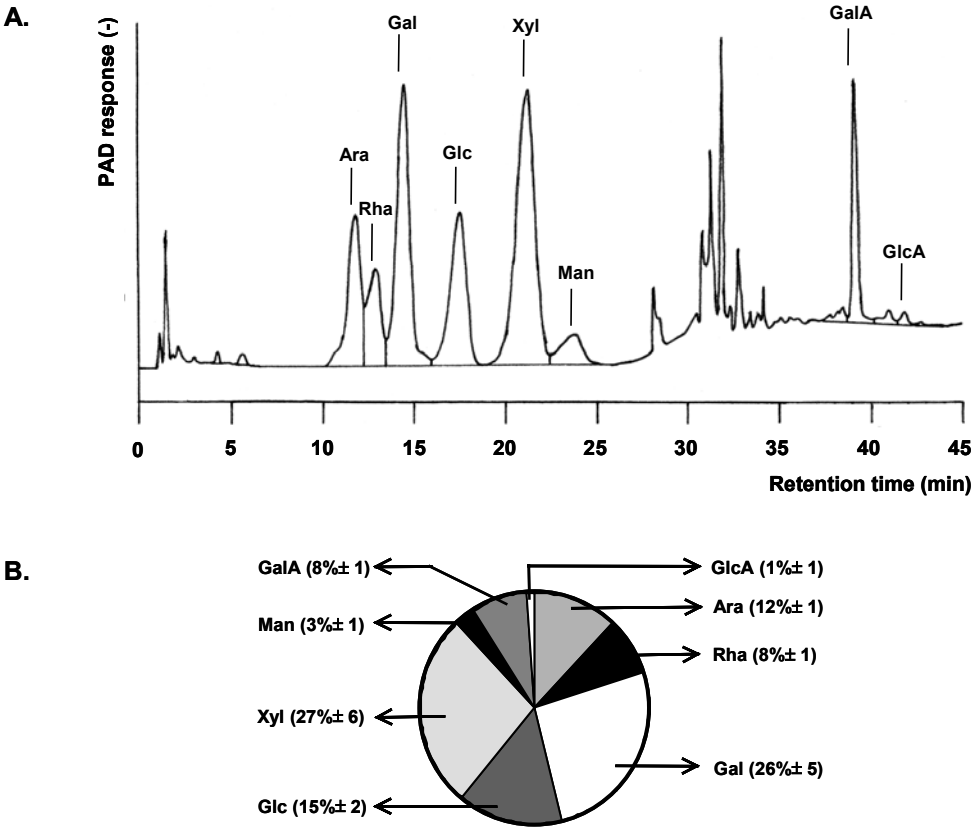
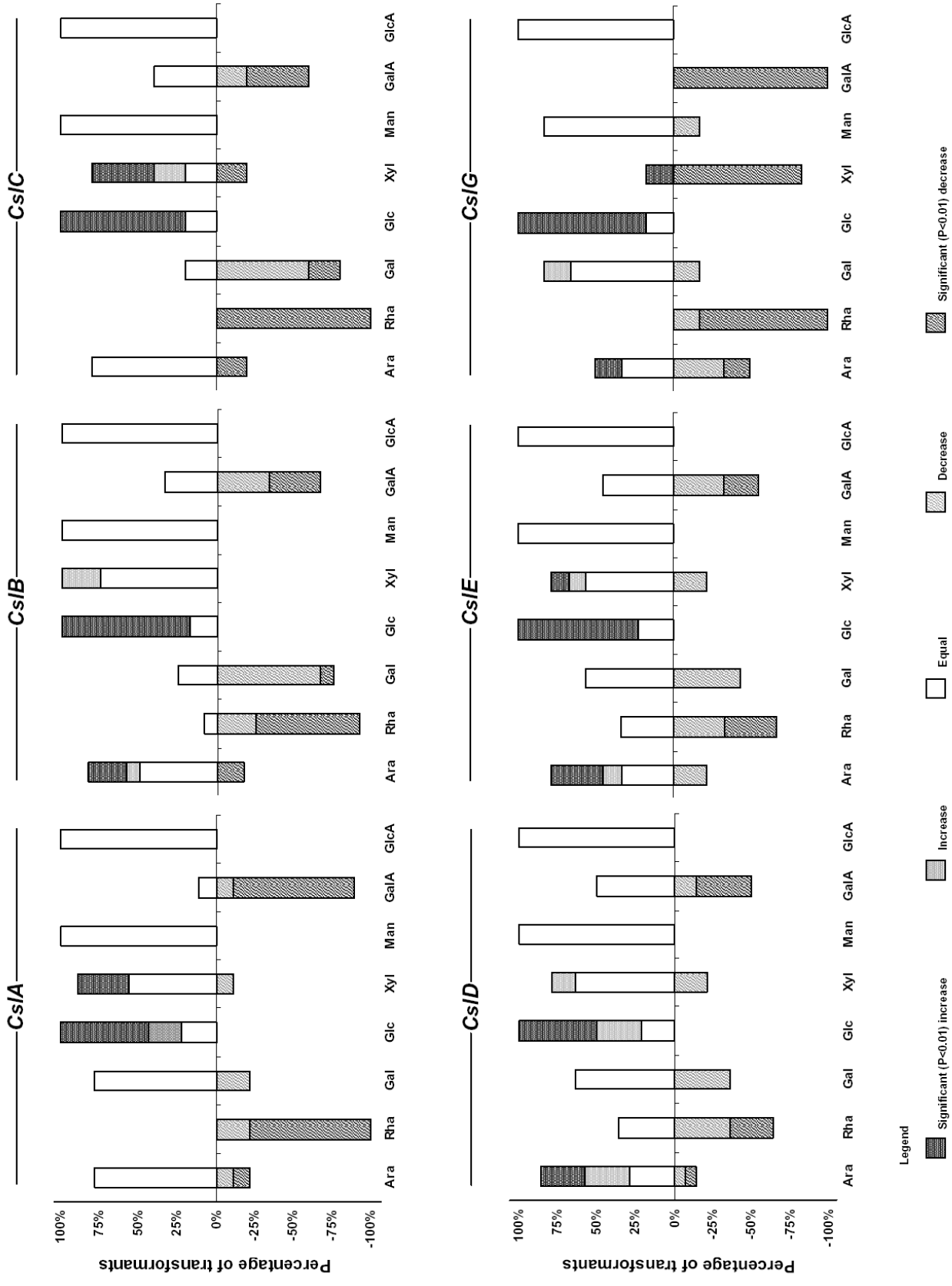


Figure 3. A. TFA hydrolysis products of the AIS of *in vitro* grown wild type tobacco plants. See experimental procedures for further details about hydrolysis conditions and HPLC analysis. **B.** Monosaccharide composition (mol %) of cell wall material isolated from *in vitro* grown wild type tobacco plants. The values represent the average of 14 individual plants and include the standard deviation.

analysis. Pauly *et al*. (1999) reported that about 18% of the xyloglucan in primary cell walls of pea (*Pisum sativum*) stems is trapped within or between cellulose microfibrils. This percentage did not correlate with a specific structural feature of the xyloglucan (Pauly *et al*., 1999) and therefore it might as well be applicable to xyloglucans from other dicotyledonous species, including tobacco.

Analysis of the *in vitro* grown *Csl* transformants (Figure 4) revealed a significant decrease of cell wall xylose in five out of six individual *CslG* transformants. In the young *in vitro* grown plants in which the cells have mainly primary walls, the xylose can be ascribed to both xylan and xyloglucan (Zablackis *et al*., 1995). The significant decrease of xylose in the *CslG* transformants suggests therefore the involvement of the *CslG* gene in either xylan or xyloglucan biosynthesis. Other changes in monosaccharides include a (significant) decrease of rhamnose and galacturonic acid in many transgenic lines. The galactose level was also slightly decreased in all transformants, except for the *CslG* plants. Since it is not likely that all *Csl* families are involved in the biosynthesis of rhamnogalacturonans, we have considered this decrease of rhamnose, galacturonic acid and galactose as a kind of transformation background. This is however rather speculative because empty vector control transformants were not included in the sugar compositional analysis. The glucose level was significantly increased in many transgenic lines. Different hypotheses can be put forward to explain the excess of glucose. One explanation is that alterations in the non-cellulosic polysaccharides (due to down regulation of the *Csl* genes) could result in modifications of cellulose crystallinity. In agreement with this, introduction of xyloglucan or (galacto)glucomannan in composite materials has been shown to result in a drastic reduction of the cellulose crystallinity (Whitney *et al*., 1995; Schröder *et al*., 2004). If cellulose becomes less crystalline, it could become more accessible for TFA hydrolysis. In this situation, the increase of glucose could be ascribed to cellulose. Another explanation may find its origin in the presence of starch in the AIS, which could be responsible for the excess of glucose. A third explanation is that down regulation of a specific hemicellulose could result in alterations in the abundance of the other non-cellulosic polysaccharides. In that case the glucose is likely to result from callose or xyloglucan. In all transgenic lines the amounts of mannose and glucuronic acid were comparable to wild type. The arabinose level was slightly increased in *CslD* and *CslE* transformants, as is the xylose level in *CslC* plants. These increases are however not significant and difficult to explain. Since the decrease of xylose in the *CslG* transformants seemed to be the most interesting result, it was decided to focus further analysis exclusively on these plants.

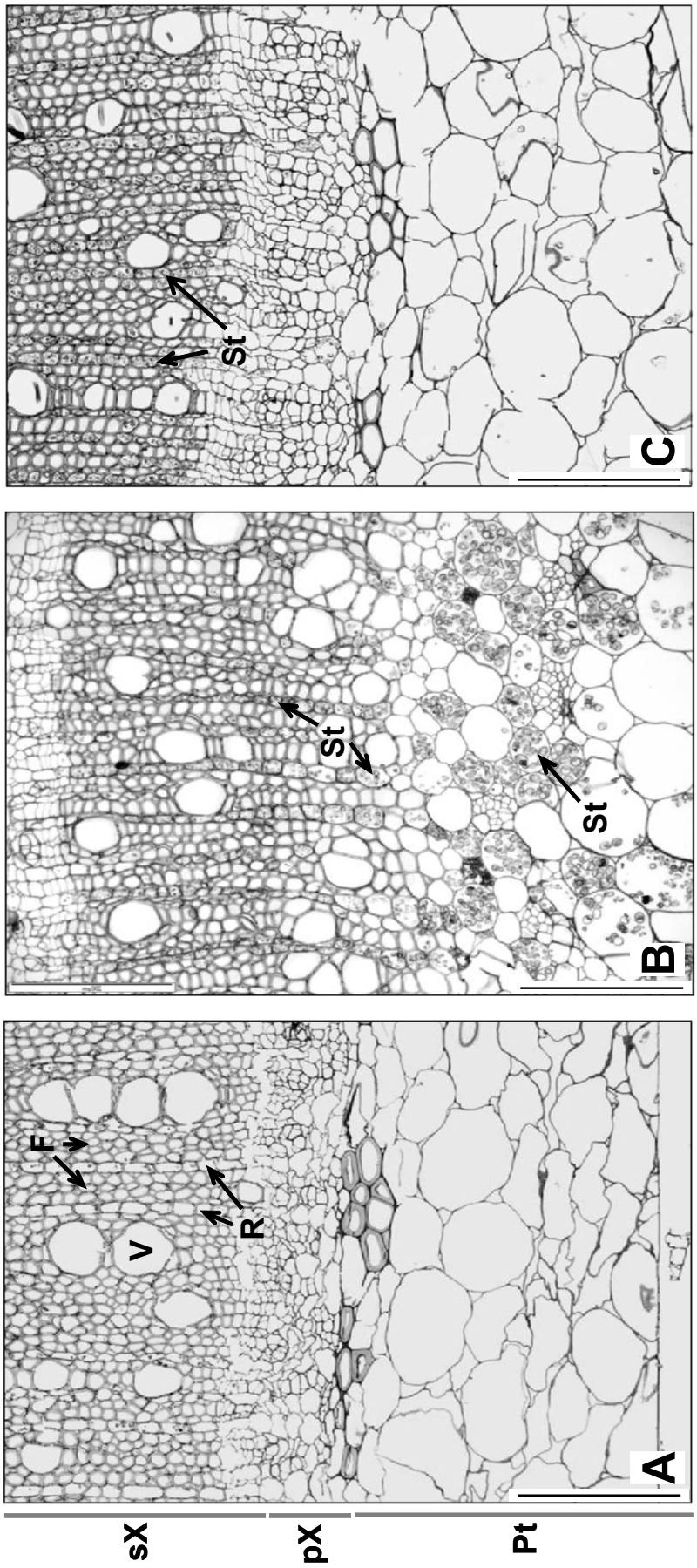

CslA
CslB
CslC
CslD
CslE
CslG
Ara
Rha
Gal
Glc
Xyl
Man
GalA
GlcA
100%
75%
50%
25%
0%
-25%
-50%
-75%
-100%
Percentage of transformants
Legend
Significant (P<0.01) increase
Increase
Equal
Decrease
Significant (P<0.01) decrease

Additional sugar compositional analyses of mature *CslG* transformants

Additional sugar compositional analyses were performed on stem material of mature *CslG* transformants in order to verify the results of the initial screening of young *in vitro* grown plant material. The additional analyses were carried out on xylem tissue isolated from internode 10 to 12 (the smallest number corresponding to the apex and the highest number corresponding to the older part of the stem) of five months old tobacco plants. Total acid hydrolysis by 72% sulphuric acid according to Saeman *et al*. (1954), followed by GC analysis of alditol acetates, revealed a change in several of the monosaccharides analysed (Table 1). In agreement with the initial screening results, all *CslG* transformants had lowered xylose contents in comparison with wild type tobacco. The slight reduction of arabinose in some of the transformants is also in agreement with earlier results. The decrease of mannose, glucose and to a lower extent also galactose, and the equal amounts of rhamnose are however contradictory. Most of the *CslG* plants showed a higher Glc/Xyl ratio. This ratio provides a global representation of the cellulose to hemicellulose ratio since in secondary wall tissues like the xylem, xyloglucans represent only a minor amount of the hemicelluloses, and therefore the amount of glucose may be regarded as representative of cellulose and the amount of xylose represents the xylans. The higher ratio appears to be the result of lower xylose content since xylose is generally more reduced than glucose. The decrease in xylose therefore suggests the involvement of *CslG* in xylan biosynthesis. The reduction in mannose could however also point towards a role of *CslG* in mannan biosynthesis. Therefore, the xylose and mannose levels were further investigated.

Selective hydrolysis of the hemicellulosic polysaccharides with 1M H_2SO_4, followed by GC analysis of alditol acetates, showed that the xylose and mannose levels in the selected *CslG* transformants were not significantly lower than in wild type samples. The results were confirmed by TFA hydrolysis (results not shown). Transformant *CslG-1* showed a very high level of glucose, which is in agreement with the initial screening results

Figure 4. Comparison of monosaccharide composition of cell wall material derived from various *in vitro* grown *Csl*-transformed tobacco plants. **Upper panel:** *CslA* transformants, 9 plants analysed; *CslB* transformants, 12 plants analysed; *CslC* transformants, 16 plants analysed. **Lower panel:** *CslD* transformants, 14 plants analysed; *CslE* transformants, 9 plants analysed; *CslG* transformants, 6 plants analysed. The X-axis shows the different hydrolysis products (neutral monosaccharides and uronic acids): arabinose, rhamnose, galactose, glucose, xylose, mannose, galacturonic acid and glucuronic acid. The Y-axis represents the percentage of transformants that showed a significant ($P<0.01$) increase (dotted black), increase (dotted gray), significant ($P<0.01$) decrease (striped black), decrease (striped gray) or no changes (white) in the amount of the specific hydrolysis products when compared to wild type tobacco. A non-significant increase or decrease of a specific monosaccharide means that the transformants showed values higher than the average plus standard deviation or values lower than the average minus standard deviation for that monosaccharide.


St
C
St
St
B
F
R
V
A
sX
pX
Pt

(Figure 4). This excess of glucose was suggested to result either from (hemi)cellulose or from residual starch in the samples that is not extracted by hot water extraction. In order to characterise the origin of this glucose an alkaline extraction (4.3 M NaOH) of the hemicelluloses was performed. Sugar compositional analysis of the alkaline extract confirmed the sugar analysis results of *CslG-1*. Since starch and hemicelluloses are extracted with 4.3 M NaOH, whereas cellulose is not, the excess of glucose is likely to result from either starch or hemicellullose. Further analysis demonstrated that the glucose was originating from starch. Treatment of the 4.3 M NaOH fraction with α-amylase released malto-dextrins and removed almost all glucose, as evidenced by the disappearance of the signals at δ 100.1, 73.7, 71.9, 71.5 and 60.8 ppm in the ^{13}C-NMR spectrum (results not shown).

Morphological characterization of mature *CslG-1* and *CslG-5* transformants

Light microscopic analysis of stem cross sections of the *CslG* transformants and wild type tobacco revealed the presence of starch in both the ray cells and the pith cells of the transformants (Figure 5). Additionally, a higher number of xylem rays and a higher amount of ray cells per ray could be observed. Figure 5B shows a less structured organization of the primary xylem and an enlarged size of the vessels of transformant *CslG-5*. This observation indicates a modification in cell differentiation and morphogenesis. Transmission electron microscopy (TEM) was used to investigate possible modifications at the ultrastructural level. Transformant *CslG-1* is distinguishable from the wild type because of the appearance of abnormal fibres with generally thinner walls and unusually high starch content (Figure 6). The detection of high amounts of starch in immature fibres and in ray and pith cells, brought additional evidence that the increase of glucose, observed during the sugar compositional analysis, most likely resulted from starch and not from hemicelluloses. Such an unusual accumulation of starch denotes a perturbed primary metabolism in the transformants, such that starch is only slowly utilized. The effects seemed to be very local and could therefore be due to low expression levels of the *Csl* genes or due to cell- or tissue-type specificity. Hamann *et al*. (2004) showed that there is a broad range of expression of the *CesA* and the various *Csl* genes. They analysed the *A. thaliana CesA* and *Csl* ESTs available in the TAIR database (http://www.arabidopsis.org) at July 1, 2003 and calculated the proportion of the *CesA* and *Csl* families. About 57% of the ESTs belonged to the *CesA* gene family whereas the *Csl* families represent the

Figure 5. Light micrographs of stem cross sections of three months old tobacco lines. **A.** Wild type (control). **B.** Transformant *CslG*-5. **C.** Transformant *CslG*-1. Bar represents 200 µm. The pith (Pt), primary and secondary xylem (pX and sX) are indicated. V: vessel; F: fibre cells; R: ray (cells). The relatively high amount of xylem rays and the high number of ray cells per ray are characteristic for the *CslG* transformants. The presence of starch in the ray cells and pith is clearly visible (indicated with St).

remaining 43% of the EST sequences (of which 12% *CslA,* 2% *CslB*, 15% *CslC*, 9% *CslD*, 2% *CslE* and 3% *CslG*) (Hamann *et al*., 2004). This suggests that at least some of the Csl proteins may catalyse the synthesis of only minor cell wall components or that they might exhibit cell- or tissue-type specificity. In both cases it is very difficult to reveal by sugar compositional analysis which cell wall polymer is affected in the *Csl* IR transformants. In this context, alterations in the content of a particular sugar residue are not easy to detect, since they are likely to be masked by the high amount of residues derived from major cell wall components, and also because of the fact that the number of normal cells within the analysed samples largely exceeds that of the abnormal cells.

RT-PCR analysis of *in vitro* grown *CslG* transformants

RT-PCR analysis was performed in order to reveal whether *CslG* mRNA expression was indeed down regulated in the *in vitro* grown *CslG* transformants. Because no sequence information on tobacco *CslG* genes was available, primers that were used to perform PCR analysis on tobacco cDNA were based on potato cDNA sequences. Different primer combinations and stringency conditions were used to enhance the likelihood that at least one primer pair had enough sequence homology to result in a PCR product. Amplification of the tobacco *Rubisco* gene was performed as a positive control due to its constitutive expression. *Rubisco* was shown to be expressed in both wild type tobacco and *CslG* transformants. Down regulation of *CslG* in the transformants and expression in wild type tobacco could however not be confirmed (results not shown). Low xylose levels could therefore not be linked to reduced *CslG* expression levels.

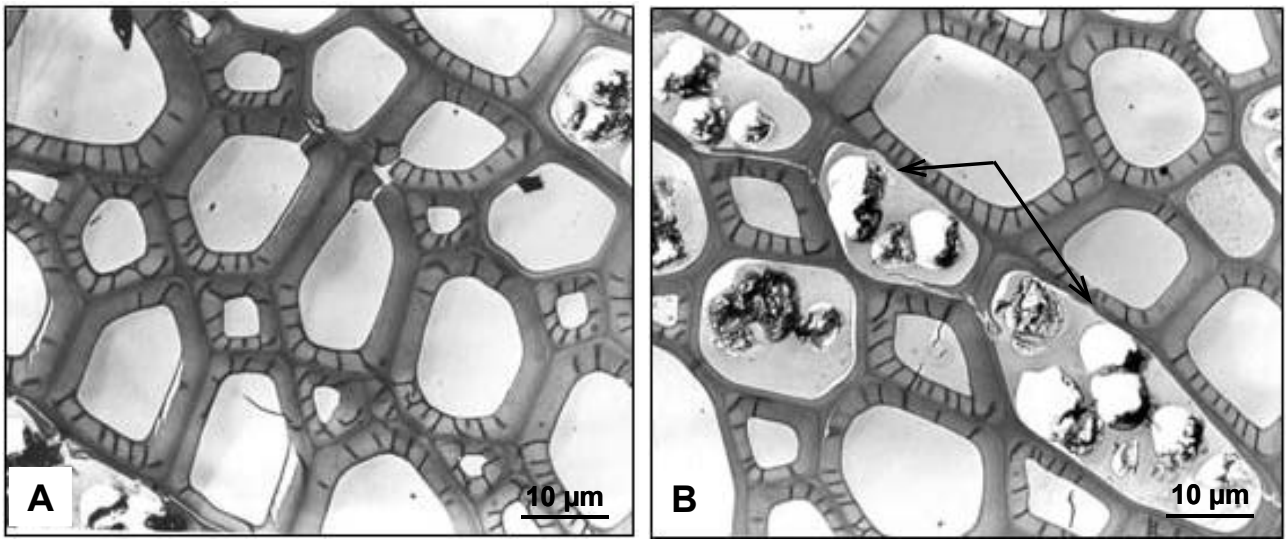


Figure 6. Cell wall ultrastructure in stem sections of three months old wild type and antisense *CslG* tobacco plants. A. Wild type tobacco (control). B. Antisense *CslG-1* transformant. Arrows indicate abnormal fibre cells with thin walls. Starch granules are clearly visible.

Generation and analysis of more *CslG* transformants

Tobacco transformation was repeated in order to obtain a higher number of *CslG* transformants. Seventeen individual *CslG* transformants were generated and again no phenotypic differences were found between wild type tobacco and the *CslG* plants. Sugar compositional analysis (TFA hydrolysis followed by HPLC analysis of monosaccharides) of the second series of *in vitro* grown *CslG* plants did not confirm the decrease of xylose that was observed in the initial screening. This inconsistency is difficult to explain since the analysed tobacco plants were in the same developmental stage as those of the initial screening. Based on the various results obtained after sugar compositional analysis of the *CslG* transformants, it remains very difficult to draw conclusions on the functional activity of the *CslG* family. Because no sequence information was available on tobacco *CslG* genes and because the low xylose content in the first series of transformants could not be correlated with reduced *CslG* expression levels, the analysis of the *CslG* plants was not continued. Instead, we started searching for tobacco *CslG* genes (Chapter 3 of this thesis). Once more sequence information on tobacco *CslG* sequences is available, the analysis of the *CslG* transformants might become easier.

Final remarks

In line with our results, insertional inactivation experiments of single genes from the *CslB* (*CslB5*), *ClsC* (*CslC4*) or *CslG* (*CslG1*) families in *A. thaliana* did not reveal any visible phenotype or a detectable change in cell wall polysaccharide composition (Richmond and Somerville, 2001). An explanation for the lack of phenotypes and the inconsistent sugar compositional analysis results could be that multiple *Csl* genes supply redundant function. In *A. thaliana*, at least four members of the *CslB* family have been shown to be co-expressed in the same cells and may therefore be functionally redundant (Richmond and Somerville, 2001). In that study, also *CslG1* and *CslG2* were shown to be co-expressed in *A. thaliana* and therefore these genes may be functionally redundant as well. It seems clear from these results that loss-of-function in several genes from the same family may be required to see any phenotypic effects and changes in cellular composition. In order to accomplish down regulation of entire *Csl* families in *A. thaliana*, Richmond and Somerville (2001) created antisense lines for several of the *Csl* families. Although it is unclear whether the antisense constructs resulted in down regulation of entire *Csl* families, this approach has also not yielded consistent results (Richmond and Somerville, 2001).

Since the outstanding challenge of determining *Csl* functions still remains, the potato IR constructs could be transformed into potato or new IR constructs could be prepared based on tobacco *CslG* sequences to generate transgenic tobacco lines. In principle it should be possible to use a reverse genetics approach to test the involvement of candidate genes in cell wall biosynthesis. However, taking into account the possible

redundancy, potential lethality, low level expression and cell or tissue-type specificity of at least some of the *Csl* family members, it has to be realised that the analysis of the transformants could remain very difficult. To avoid this problem a heterologous expression strategy can be used to determine the enzymatic activity of *Csl* gene products. Recently, Liepman *et al.* (2005) demonstrated by heterologous expression of four different *A. thaliana CslA* genes in *Drosophila* Schneider 2 cells that at least three of these genes can produce β-mannan polymers when GDP-mannose is supplied. It has to be noted that heterologous expression systems also have their limitations. The absence of glycosyltransferase activity can for example be a result of the fact that the *Csl* gene product does not use the tested nucleotide sugar as a donor substrate. It could also be caused by other factors like the absence of other members of a protein complex, or the absence of a suitable acceptor molecule to which sugar residues can be transferred. As the heterologous expression strategy and the reverse genetics approach both seem to have their advantages and limitations it remains very difficult to choose beforehand the best method for future studies on the enzymatic function and the biological role of the Csl proteins.

EXPERIMENTAL PROCEDURES

Preparation of Inverted Repeat (IR) constructs for plant transformation

Potato cDNA sequences (ESTs) coding for different *Csl* family members were used for the preparation of IR constructs (shown in Figure 2C). Based on availability and position in the *Csl* genes, the following cDNA clones were selected (name, accession number): *StCslA*1 (cSTB48I22, BG097871), *StCslB*1 (cSTB14M21, BE921502), *StCslC*2 (cSTB18G1, BE922122), *StCslD*1 (cSTA32C5, BE472127), *StCslE* (cSTB47M10, BG097543) and *StCslG*1 (cSTB34J22, BE924131). Numbering of the *Csl* family members is arbitrary and according to http://cellwall.stanford.edu. Primers were designed in order to amplify sense and antisense fragments from these sequences that are located outside the highly conserved regions U1 to U4 (shown in Figure 2B). Primers included 5' *Xba*I and 3' *Eco*RI sites (for sense fragment) and 5' *Bam*HI and 3' *Kpn*I sites (for antisense fragment). A 158 base pairs spacer fragment with 5' *Eco*RI and 3' *Kpn*I restriction sites was obtained by PCR amplification from the pBIN19LUC-SBD vector (Ji *et al.*, 2003). The sequences of the oligonucleotides used are listed in Table 2.

The amplified sense, spacer and antisense fragments were digested with respectively *Xba*I/*Eco*RI, *Eco*RI/*Kpn*I, *Kpn*I/*Bam*HI and ligated consecutively into a linearized pGEM-T cloning vector (Promega) digested with the same restriction endonucleases. Finally, the IR cassettes were excised from pGEM-T with *Xba*I/*Bam*HI and ligated in the corresponding sites of the binary vector pGreen7K (a pGreen derivative (Hellens *et al.*, 2000)). pGreen7K contains the Kanamycin resistance gene (*NptII*) and a *Hind*III/*Eco*RI inserted multiple cloning site (*Hind*III-*Xba*I-*Bam*HI-*Sma*I-*Eco*RI), flanked by the CaMV35S promoter and terminator (provided by B. Brandwagt, Phytopathology, Wageningen University).

Table 2. Oligonucleotide sequences used for PCR-amplification of potato *Csl* cDNA clones and spacer fragment. Introduced restriction sites *XbaI* (tctaga), *EcoRI* (gaattc), *BamHI* (ggatcc) and *KpnI* (ggtacc) are underlined.

PCR product	Forward primer (5'-3')	Reverse primer (5'-3')
StCslA1-sense	tctagaggataccaatggctgatgatga	gaattcggatcagtagaatcatcaagaactt
StCslA1-antisense	ggatccggataccaatggctgatgatga	ggtaccggatcagtagaatcatcaagaactt
StCslB1-sense	tctagaggctcccaaaacaccttctt	gaattcccaccgctaacaacgataaca
StCslB1-antisense	ggatccggctcccaaaacaccttctt	ggtaccccaccgctaacaacgataaca
StCslC2-sense	tctagaagctcattcttcccttctattc	gaattcccaccgctaacaacgataaca
StCslC2-antisense	ggatccggctcccaaaacaccttctt	ggtaccccaccgctaacaacgataaca
StCslD1-sense	tctagacaggctatcagggaaggtatgt	gaattcgcgaccatagcagcagataca
StCslD1-antisense	ggatcccaggctatcagggaaggtatgt	ggtaccgcgaccatagcagcagataca
StCslE-sense	tctagattgcatcttccattatttgag	gaattccatgactgataatattgtgttgat
StCslE-antisense	ggatccttgcatcttccattatttgag	ggtacccatgactgataatattgtgttgat
StCslG1-sense	tctagaatatcagctaggaaggcaatgtgt	gaattcctctagtgaagcaaggaactttt
StCslG1-antisense	ggatccatatcagctaggaaggcaatgtgt	ggtaccctctagtgaagcaaggaactttt
spacer	gaattcaaagttgcgcggaggagttgtgtt	ggtaccgtcggggtcggcgtggtg

All binary constructs were co-transformed with the helper plasmid pSoup (Hellens *et al.*, 2000) into *Agrobacterium tumefaciens* strain LBA4404 by electroporation. The integrity of the binary plasmids was tested by restriction analysis of plasmids isolated from *A. tumefaciens* cultures used for plant transformation.

Plant transformation

Nicotiana tabacum cv. Samsun NN plants were grown under sterile conditions on MS30-gelrite medium (Murashige and Skoog (1962) medium (4.4 g/l), Gamborg B5 vitamins, gelrite 3 g/l and sucrose 30 g/l, pH 5.7-5.9). *A. tumefaciens* containing the recombinant T-DNA constructs were cultured and used to infect tobacco leaf disks (upside-down in the dark for 2 days at 25°C). Leaf disks were washed in MS30 medium containing 250 mg/l carbenicillin (Carb) and transferred upside-up to solid callus inducing medium (MS30-gelrite plates containing 0.1 mg/l alpha-Naphtalene acetic acid (NAA), 1 mg/l 6-benzylaminopurine (BAP), 200 mg/l kanamycin (Kan) and 250 mg/l Carb). After a regeneration period of 2-4 weeks the formed calli were transferred to shoot inducing medium (MS20-gelrite plates containing 2 mg/l BAP, 0.2 mg/l NAA, 250 mg/l Carb and 100 mg/l Kan). After 4-5 weeks the regenerated shoots were rooted on 1/2MS15-gelrite medium containing 100 mg/l Kan, 200 mg/l claforan and 100 mg/l vancomycin. The formed plants were planted in soil and transferred to the greenhouse to generate mature plants.

TFA hydrolysis of cell wall polysaccharides followed by HPLC analysis

Five weeks old *in vitro* grown tobacco plants were harvested and after removal of the roots, the remaining stem and leaf materials were ground to a fine powder in liquid nitrogen. 0.5 g of ground material was extracted 3-4 times with 70% ethanol (v/v) and one time with aceton to remove low-M_r sugars and other metabolites, while retaining macromolecules (proteins and starch as well as wall polymers). Pellets were dried overnight at room temperature. The remaining alcohol insoluble solids (AIS) were subjected to acid

hydrolysis. Two mg of AIS were hydrolyzed with 2 M TFA for 2 h at 121°C. The samples were cooled down to room temperature and TFA was evaporated under a stream of air at 40°C. Pellets were dried and dissolved in 200 µl sterile water. The monosaccharides were analysed with a Dionex HPLC equipped with a Carbopac PA10 column (Dionex, Sunnyvale, California, USA). Ten µl sample was injected at 15 min with a Dionex AS3500 automated sampler. The eluent flow-rate was 1.5 ml/min, 0.3 M NaOH was added post-column and sugars were quantified with a pulsed amperometric detector (with gold electrode) (Dionex). Two eluents were used, eluent A (H_2O) and eluent B (0.5 M NaOH) for mixing the following gradient: 0 $\rightarrow$ 40.5 min, 100% eluent A; 40.5 $\rightarrow$ 50 min, linear gradient from 0-100% eluent B; 50 $\rightarrow$ 60 min, 100% eluent B.

Total acid hydrolysis or selective (TFA / H_2SO_4) hydrolysis of cell wall polysaccharides followed by gas chromatography (GC) analysis

Stems of mature tobacco plants were freeze-dried. After removal of the green bark and the pith, the remaining xylem tissue was ground (100 mesh) and extracted successively with a mixture of toluene and ethanol (2:1 (v/v)), twice with hot water and then freeze-dried. This was considered as crude cell wall material (CWM).

For total acid hydrolysis, 10 mg of CWM was pre-treated with 72% (w/w) H_2SO_4 (1.5 h, 30°C) followed by hydrolysis with 0.5 M H_2SO_4 for 4 h at 100°C, with the addition of *m*-inositol as an internal standard. After neutralization with $BaCO_3$, the constituent sugars were converted to their alditol acetate derivatives as in Mollard *et al.* (1997) and analyzed by GC according to Englyst and Cummings (1984). GC analysis was performed on a Hewlett-Packard 6850 equipped with a macrobore column (30 x 0.53 mm) of 3% SP 2380.

For selective hydrolysis, 10 mg of crude cell wall material was hydrolyzed with 2 M TFA for 1 h at 121°C or with 1M H_2SO_4 for 2 times 1 h at 121°C (after 1 h fresh 1M H_2SO_4 was added to the residue, followed by another 1 h incubation at 121°C). *m*-Inositol was added as an internal standard. Neutralisation, conversion to the alditol acetate derivatives and monosaccharide analysis were performed as described above.

Extraction of hemicelluloses

The crude CWM from wild type tobacco and transformant *CslG-1* were delignified with a mild chlorite treatment adapted from Ahlgren and Goring (1971). An aqueous suspension of CWM (100 mg) was treated with sodium chlorite (25 mg) buffered with acetic acid, for 1 h at 50°C. After washing with water, the delignified CWM was extracted with 4.3 M NaOH containing 1% $NaBH_4$ for 12 h at room temperature. The soluble material was neutralized with diluted acetic acid, dialyzed and freeze-dried. The recovered hemicelluloses (35 mg) were hydrolyzed with 2M TFA and analyzed by GC as described above.

Digestion of starch was carried out with α-amylase (SIGMA), and characterization of the released oligosaccharides was done by thin layer chromatography. The removal of starch was checked by 13C-NMR analysis at 75.46 MHz on a Bruker AM spectrometer.

Transmission electron microscopy (TEM)

Small pieces of stem (5-7 mm in length, 2 mm^2 in section) obtained by free hand sectioning with a razor blade were fixed in a freshly prepared mixture of 0.2% glutaraldehyde (v/v/), 2% *para*formaldehyde (w/v) in 0.05 M phosphate buffer (pH 7-7.2). After rinsing in phosphate buffer, they were dehydrated through a graded ethanol series up to 80% (v/v), then infiltrated and embedded in LR White resin (hard mixture, TAAB) and polymerized for 24 h at 50°C as described earlier (Ruel *et al*., 2002). General staining of polysaccharides on ultrathin sections was performed by the PATAg method according to Ruel *et al*. (1981).

RNA isolation and RT-PCR analysis

Approximately 1.5 g of ground plant material was transferred to microfuge tubes containing 3 ml RNA extraction buffer (50 mM Tris pH 9.0, 10 mM EDTA and 2% SDS (w/v)) and 3 ml phenol. Samples were homogenised and centrifuged for 10 min at 3000 rpm (4°C). The supernatants were subjected to phenol:chloroform extractions until no interface remained. Total RNA was obtained after successive isopropanol and LiCl precipitations. RNA was DNAseI treated and purified using the Gene-elute™ mammalian total RNA purification kit (Sigma).

Ten µg of total RNA was used for first-strand cDNA synthesis in a 50 µl reaction containing 100 ng primer polydT (5'-$[t]_{24}$-3'), dATP, dCTP, dGTP and dTTP, each at 0.5 mM final concentration, 4 mM DTT, 1* First Strand Buffer and 200 units Superscript™ II Reverse Transcriptase (Life Technologies), made to a total volume of 50 µl with sterile water. cDNA synthesis was performed at 42°C for 1 h. Five µl of cDNA was used in a standard PCR reaction with the following primer/Tm/cycle-number combinations: *StCslG*1sense FW and REV (see Table 4), Tm=50-60°C, 18-35 cycles. *StCslG*1-sense FW (see Table 4) and *StCslG*1-U4 REV (5'-ataagtgatgggattgagatgtga-3'), Tm=50-60°C, 35 cycles. *NtRubisco* (accession number Z14980) FW (5'-cagaaatcatcaggaaaggaaaca-3') and REV (5'-tctcttcacgttttcttgctcttg-3'), Tm=59°C, 30 cycles.

ACKNOWLEDGEMENTS

The work was funded by a grant within the EC Framework V (COPOL, QLK5-2000-01493) and is gratefully acknowledged.

Chapter 3

Molecular cloning and computational analysis of *Nicotiana tabacum* *CslE* and *CslG*

Monique Compier[1], Laurence Bindschedler[2,3], G. Paul Bolwell[2], Richard G.F. Visser[1] and Jean-Paul Vincken[1]

[1] Graduate School Experimental Plant Sciences, Laboratory of Plant Breeding, Wageningen University, P.O. box 386, 6700 AJ Wageningen, The Netherlands.
[2] Division of Biochemistry, School of Biological Sciences, Royal Holloway and Bedford New College, University of London, Egham, Surrey, TW20 0EX, United Kingdom.
[3] Present address: The BioCentre, University of Reading, Whiteknights, Reading, RG6 6AS, United Kingdom.

Submitted to *Phytochemistry* (with minor modifications).

ABSTRACT

Cellulose synthase-like (*Csl*) genes are hypothesised to encode the enzymes that polymerize the backbones of non-cellulosic polysaccharides of the plant cell wall. While *CslA* genes are beginning to be linked to mannan biosynthesis, no function has been assigned to any of the other *Csl* families. In previous experiments, Inverted Repeat (IR) constructs of potato (*Solanum tuberosum*) *CslG* were used to down regulate the expression level of tobacco (*Nicotiana tabacum*) *CslG* genes. Since xylose levels were lowered in the transgenics, we speculate about the involvement of *CslG* in xylan biosynthesis. However, it was not possible to analyse the *CslG* expression levels in the tobacco transformants due to the absence of sequence information on tobacco *CslG* genes. Therefore it required searching for tobacco *CslG* genes. The present work reports the isolation of the first full length tobacco *CslE* and *CslG* cDNA sequence from a cDNA library, constructed from mRNAs isolated from a xylogenic suspension cell culture of tobacco, known to be enriched in cells undergoing secondary wall biosynthesis. The protein sequences encoded by the *NtCslE* and *NtCslG* cDNA clones, contain eight putative transmembrane domains, alternating conserved and variable domains, and the processive glycosyltransferase signature, the D, DxD, D, QxxRW motif. Southern blot analysis revealed that the tobacco *CslE* and *CslG* gene families consist of two to four and at least three genes, respectively. Gene expression studies showed that C*slE* expression was highest in tissues associated with secondary wall biosynthesis, whereas *CslG* mRNA levels were highest in tissues undergoing primary wall formation.

INTRODUCTION

In addition to cellulose, plant cell walls contain pectin and hemicellulosic polysaccharides. Hemicelluloses comprise mainly xyloglucans, xylans and mannans. Xyloglucan is the main hemicellulose of the dicot primary cell wall, while xylans contribute approximately 5% of the primary cell wall in dicots, 20% of the primary cell walls of grasses and 20% of the secondary walls of both dicots and grasses (McNeil *et al*., 1984; Fry, 1988). Secondary wall xylans can establish tight interactions with cellulose (Awano *et al*., 2001) and covalent bonds with lignin (Hatfield *et al*., 1999), and may thereby be involved in regulating expansion and strengthening of the cell wall (Carpita, 1996). Xylans consist of a backbone of 1,4-linked β-xylosyl (Xyl) residues, some of which carry single arabinosyl (Ara) and / or 4-O-methyl-glucuronyl or glucuronyl (GlcA) residues, usually through α-1,2- or α-1,3-linkages. Many of the Xyl residues may be acetylated at C-2 and / or C-3. The Xyl residues may also be substituted with short side chains containing Ara, galactosyl (Gal) and Xyl

(Fry, 1988). The nature and extent of xylan substitution differs between plants, with monocots containing high levels of arabinosyl substituents, whereas dicots contain mainly glucuronyl groups (Coughlan and Hazlewood, 1993).

The understanding of the biosynthesis of the xylan heteropolysaccharide is still very limited. The xylan backbone is synthesized from UDP-xylose (UDP-Xyl) residues and its availability is thought to be controlled, at least partially, by the induction of the UDP-sugar converting enzymes UDP-glucose dehydrogenase (UGD) and UDP-glucuronate decarboxylase (UGAD) (Bolwell, 1993; Robertson *et al.*, 1995; Robertson *et al.*, 1996). The induction of UGD and UGAD results in a channeling of UDP-glucose (UDP-Glc) via UDP-glucuronic acid (UDP-GlcA) into the pool of UDP-Xyl, which can be utilized in the xylan biosynthesis (Figure 1). The xylan backbone is synthesized by a β-1,4-xylosyltransferase (xylan synthase (XS)). Xylosyltransferase activity has been found in enzyme preparations from different plant species, such as maize (*Zea mays*), bean (*Phaseolus vulgaris*), pea (*Pisum sativum*) and wheat (*Triticum aestivum*) (Bailey and Hassid, 1966; Odzuck and Kauss, 1972; Bolwell and Northcote, 1981; Baydoun *et al*., 1989; Gibeaut and Carpita, 1990; Porchia and Scheller, 2000, Kuroyama and Tsumuraya, 2001), but no known cognate cDNA sequence could be associated with the enzyme activity so far. The addition of side chains to xylans has been less investigated and only little is known about the way in which different glycosyltransferases interact to form the heteropolysaccharide. Porchia *et al*. (2002) were the first to report the presence of an arabinoxylan arabinosyltransferase (AX-AraT) in Golgi vesicles isolated from wheat seedlings. Although there is evidence that proteins in microsomal and Golgi membrane fractions are involved in the biosynthesis of the xylan heteropolysaccharide, to date none of these proteins has been completely purified or characterized and no sequence information is available on the proteins or the corresponding genes.

A number of model systems have been developed to assist in understanding secondary wall formation. Suspension-cultured cell systems have thin primary cell walls that are similar to those found in the meristematic cells of the plant. The cell culture systems can be induced to synthesize a secondary cell wall and are therefore useful for studying extensive morphological changes that occur during the secondary wall formation. An example is the *Zinnia* mesophyll cell system, which allowed the identification and characterization of candidate genes involved in secondary wall biosynthesis (Milioni *et al*., 2001). Blee *et al*. (2001) characterized a tobacco (*Nicotiana tabacum*) cell culture system transformed with the *Agrobacterium ipt* gene, that encodes an isopentenyl transferase (Memelink *et al.*, 1987). The *ipt* gene product is involved in cytokinin biosynthesis and over-expression of the gene leads to high levels of endogenous cytokinin. With optimized concentrations of sucrose and auxin, the cell line shows increased cell aggregation,

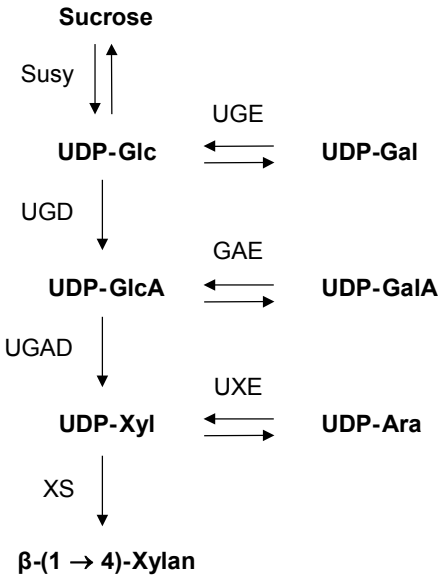


Figure 1. Schematic representation of UDP-sugar conversion and xylan biosynthetic pathway. Abbreviations: Susy: sucrose synthase; UGE: UDP-D-glucose 4-epimerase; UGD: UDP-D-glucose dehydrogenase; GAE: UDP-D-glucuronate 4-epimerase; UGAD: UDP-D-glucuronate decarboxylase; UXE: UDP-D-xylose 4-epimerase; XS: xylan synthase.

elongated cells and a five-fold increase in cell wall thickness. Unlike the *Zinnia* cell culture system, the xylogenic tobacco cell culture contains cells that remain meristematic, and therefore this system is convenient for sub-culturing. Cell cultures were found to have increased amounts of xylan, cellulose and phenolic components when compared to wild type tobacco cultures (Blee *et al*., 2001). The xylogenic tobacco cell culture is therefore a suitable model system for studying secondary cell wall formation, including xylan biosynthesis.

Changes in the activities of the enzymes involved in xylan biosynthesis were monitored after sub-culturing of the transformed tobacco cell culture. Maximum activities of UGD and UGAD occurred at three days after sub-culturing, whereas XS activity was detected after three days and showed a maximum at six days (Blee *et al.*, 2001). A cDNA library, constructed from mRNAs isolated from the xylogenic tobacco cell culture, three days after sub-culturing, was used for the isolation of cDNAs coding for UGD and UGAD (Bindschedler *et al*., 2005). Since XS activity could be detected after three days, this library might also be suitable for the isolation of XS cDNA clones.

In addition to the *cellulose synthase* (*CesA*) genes, plants contain at least eight families of *cellulose synthase-like* genes (*Csl*s) that have a strong structural similarity to the *CesA* genes. The *Csl* genes are suggested to be involved in the biosynthesis of non-cellulosic polysaccharides (Cutler and Somerville, 1997; Richmond and Somerville, 2001; Dhugga *et al*., 2004). Recently, functional assignment of the *Csl* gene family has begun with the identification of a mannan synthase (Dhugga *et al.*, 2004; Liepman *et al.*, 2005). *CslA* genes have been directly identified as mannan synthase in guar (*Cyamopsis tetragonoloba*) and *A. thaliana* (Dhugga *et al.*, 2004; Liepman *et al.*, 2005). Members of

the other *Csl* families are not functionally assigned yet. As part of our studies to understand the functional identity of polysaccharide synthases, we have carried out antisense expression of different *CesA* genes in potato (*Solanum tuberosum*) (Oomen *et al.,* 2004). Similarly, we expressed Inverted Repeat (IR) constructs, based on members of the *Csl* family (*CslA*, *B*, C, *D*, *E* and *G*) from potato into tobacco, and found only a *CslG* IR construct to result in a significant decrease of xylose in both young *in vitro* grown, and mature tobacco transformants (Chapter 2 of this thesis). This led us to speculate about the involvement of *CslG* in xylan biosynthesis. The observations on the *CslG* transformants were however ambiguous. It was not possible to analyse the *CslG* expression level in these plants due to the lack of sequence information on tobacco *CslG* genes or any indication of the number of tobacco *CslG*s and the percent similarity between those and the potato *StCslG1* cDNA sequence (cSTB34J22) of the IR construct. Therefore, no correlation could be drawn between the level of *CslG* mRNA expression and the amount of xylose.

In order to gain more knowledge on tobacco *Csl* genes and their possible involvement in xylan biosynthesis, we used a tobacco xylogenic suspension cell culture cDNA library, enriched in cells undergoing secondary wall biosynthesis, to clone the first full length tobacco *NtCslE* and *NtCslG* cDNAs. The size of the tobacco *CslE* and *CslG* gene families was investigated and additionally, differential expression of the *NtCslE* and *NtCslG* genes was determined in wild type tobacco tissues and xylogenic cell cultures. Differential expression of the *NtCslG* gene did not correlate with xylan synthesis. However, *NtCslE* gene expression was associated with xylogenesis, but not exclusively so.

RESULTS

Isolation and sequence analysis of *N. tabacum CslG* cDNA clones

We screened a cDNA library of mRNAs expressed in xylogenic tobacco cell cultures to obtain *Csl* family members associated with secondary wall synthesis (Blee *et al.*, 2001). This cDNA library has been used previously to obtain cDNAs coding for UGD and UGAD expressed during vascular differentiation (Bindschedler *et al.*, 2005). A 303 base pairs potato *CslG* cDNA fragment was used to screen the cDNA library. However, the screening did not result in the isolation of any positive cDNA clones, although it was performed under low stringent conditions. This result could either be explained by low expression of *CslG* mRNAs or, more likely, by low hybridization efficiency because of screening with a very short heterologous probe. The cDNA library was therefore screened with another probe, which is the full length *CslG2* cDNA clone from *Arabidopsis thaliana* (U11884). This screening resulted in the isolation of 13 full length cDNA clones. Sequencing of the clones,

followed by homology searches revealed that these clones encode a *NtCslE* and a *NtCslG* gene (seven and six identical clones, respectively), which show a clear similarity to other plant *Csl* genes. The open reading frames (ORFs) of the *NtCslE* and *NtCslG* cDNAs are respectively 2223 and 2235 base pairs long. Comparison of the tobacco *Csl* sequences with the *A. thaliana CslG2* cDNA clone that was used in the library screening, revealed that *NtCslE s*hares a sequence identity of 58% with *AtCslG2,* whereas *NtCslG* shares 61% identity with *AtCslG2*. The sequence identity between the *StCslG1* probe and *AtCslG2* is only 48%.

The size of the predicted protein sequences of *NtCslE* and *NtCslG* is respectively 740 and 744 amino acids. The molecular mass of both proteins was calculated to be 84 kDa, while the pI was predicted to be 7.0. Analysis of the peptide structure and hydrophobicity predicted that NtCslE and NtCslG have eight transmembrane domains with N- and C-termini in the cytosol. Two transmembrane domains are located at the N-terminus and the other six are detected in the C-terminal region as indicated in Figure 2. The NtCsl proteins have no obvious N-terminal signal sequence. These data suggest that the NtCsls are membrane proteins anchored to the plasma membrane, as predicted for the

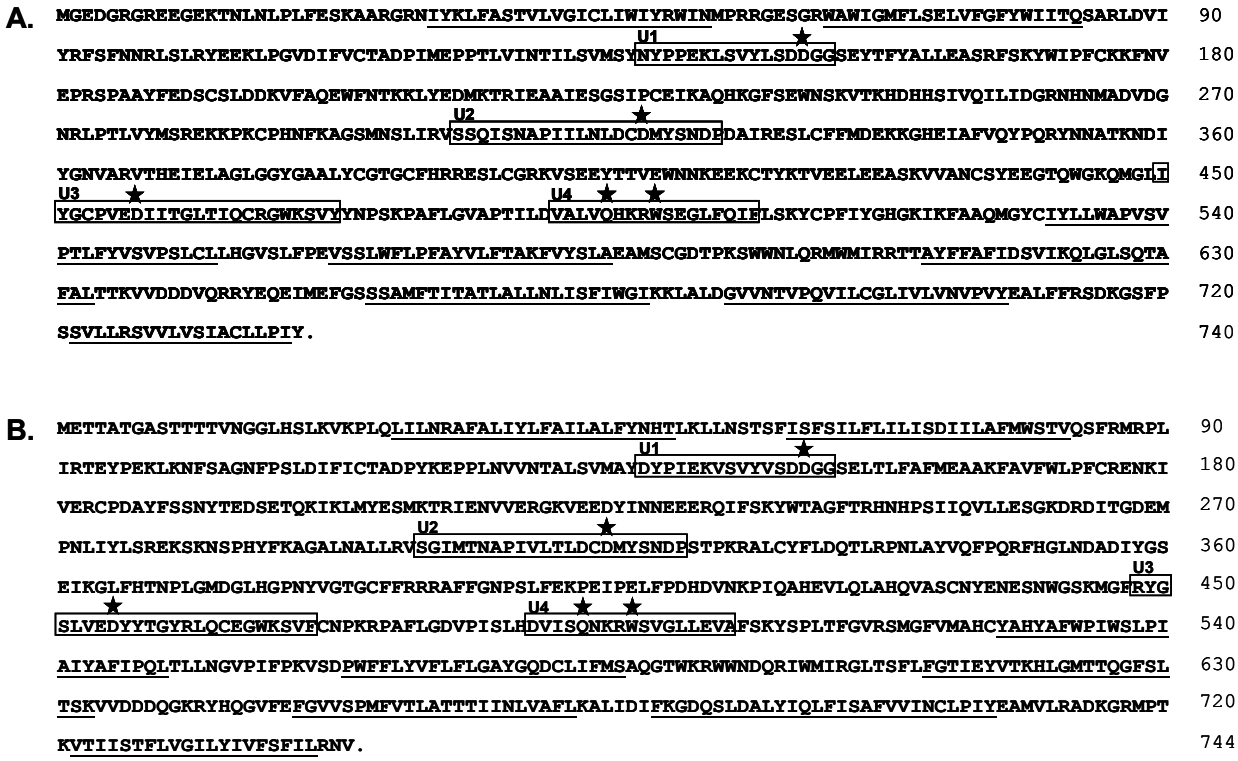


Figure 2. Amino acid sequence of the NtCslE (**A**) and NtCslG (**B**) proteins. Underlined amino acids correspond to the putative transmembrane domains of the proteins. The conserved subdomains U1 to U4 are marked with boxes. The positions of the three conserved Asp (D) residues and the QxxRW motifs characteristic for β-glycosyltransferases are indicated by asterisks.

CesA catalytic sub-unit (Delmer, 1999), or to a membrane in the endomembrane system. The latter option is however more likely, since Csl proteins are suggested to be involved in the biosynthesis of hemicelluloses, which is thought to occur in the ER and the Golgi apparatus (Dhugga *et al*., 2004; Scheible and Pauly, 2004).

Alignment with available *A. thaliana* and rice (*Oryza sativa*) CesA and Csl proteins (http://www.cellwall.stanford.edu) revealed multiple conserved residues in the NtCslE and NtCslG proteins (see Figure 2). Both proteins contain the four highly conserved sub-domains U1 to U4 that characterize the processive β-glycosyltransferases in plants and bacteria (Saxena *et al.*, 1995; Pear *et al.*, 1996). These regions contain three conserved aspartate (D) residues and the QxxRW motif, which have been proposed to be involved in substrate binding and catalysis. Surrounding these motifs, NtCslE and NtCslG share two variable regions with plant CesAs and other Csl proteins (Delmer, 1999; Favery *et al.*, 2001; Wang *et al.*, 2001; Goubet *et al*., 2003; Samuga and Joshi, 2004).

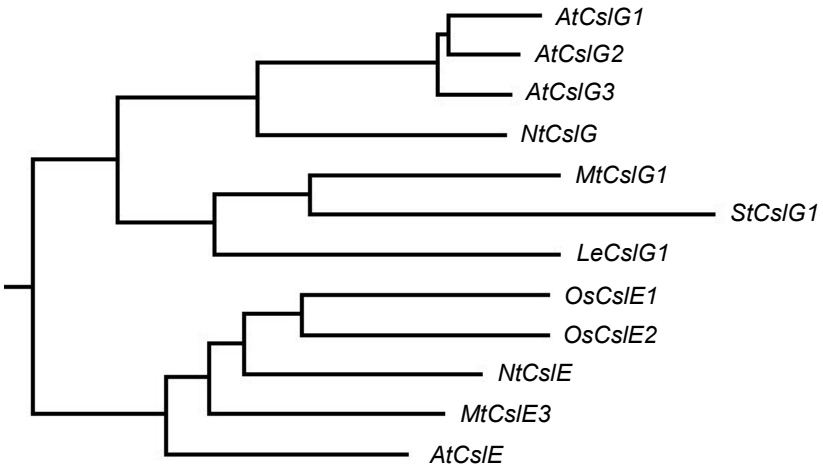


Figure 3. Phylogenetic tree showing the sequence divergence between several plant CslE and CslG proteins. Nomenclature of Csl proteins used is the same as described at http://cellwall.stanford.edu (*At*, *Arabidopsis thaliana*; *Nt*, *Nicotiana tabacum*; *Mt*, *Medicago truncatula*; *St*, *Solanum tuberosum*; *Le*, *Lycopersicon esculentum*; *Os*, *Oryza sativa*). The following full length protein sequences were used: *At*: AAL36396.1 (*CslE*), AAB63622.1 (*CslG1*), AAB63623.1 (*CslG2*), AAB63624.1 (*CslG3*); *Nt:* DQ127171 (*CslE*), DQ152918 (*CslG*); *Os*: AAL25129.1 (*CslE1*), AAL25130.1 (*CslE2*). The following EST sequences were assembled into contigs using the SeqMan program from the DNASTAR package: *Mt*: BF643693, BF647119, BE123967, AW686230, BF643348 (*CslE3*), AW560132, BI310491, BQ164832, AW125968, BG588396 (*CslG1*); *St*: BM408374, BE922091, BI920823, BG593274, BG595418 (*CslG1*); *Le*: AW738536, AW648880, BE436355, AW223674, AW223994 (*CslG1*). The MegAlign program from the DNASTAR package was used to align the (deduced) protein sequences according to the Clustal method (Higgins and Sharp, 1989) using a PAM250 weight table and gap penalties and gap-length penalties of 10.

Relation of *NtCslE* and *NtCslG* to other plant *Csl*s

The dendogram in Figure 3 represents the amino acid sequence homology between NtCslE and NtCslG and other plant CslE and CslG proteins. All Csl protein sequences used in this study were downloaded from the Stanford site (http://www.cellwall.stanford.edu). The dendogram clearly shows a separation of the CslE and CslG proteins in different clusters. The average sequence identity within the CslE and CslG clusters is 47% and 41%, respectively, while the identity between the clusters is about 30%. The CslG family is additionally divided into two different sub-groups. Interestingly, our NtCslG seems to be more closely related to AtCslG proteins than to the tomato, potato and *Medicago trunculata* CslG1 (Figure 3). BLAST analysis (Altschul *et al.*, 1997) of the *NtCslG* cDNA clone revealed a very high sequence homology to *LeCslG4* and *StCslG3* partial cDNA sequences. These results indicate that different plant species might have a varying number of CslG proteins. The fact that the *NtCslG*, *StCslG3* and *LeCslG4* (*CslG*s from different species) are more similar to each other than e.g. *StCslG1* and *3*, *LeCslG1* and *4* (*CslG*s from the same species), is a similar situation as for *CesA* and *CslD* genes (Holland *et al.*, 2000; Samuga and Joshi, 2004). It suggests that *CesA, CslD* and as well *CslG* gene structural divergence preceded the species divergence. This structural divergence could possibly be assigned to functionality divergence within the same *Csl* family.

Gene copy number determination by Southern blot analysis

To estimate the size of the *CslE* and *CslG* gene families in the *N. tabacum* genome, Southern blot analysis was performed on genomic DNA of *N. tabacum* using the cDNAs of *NtCslE* and *NtCslG* as a probe. Since *N. tabacum* is an amphidiploid, it contains the two complete genomes of its ancestors *Nicotiana sylvestris* and *Nicotiana tomentosiformis* (Kitamura *et al*., 2001). These two species were therefore included in the analysis. Hybridization with the *NtCslE* probe yielded two to four hybridizing bands in the digested *N. tabacum* DNA. Three bands are originating from *N. tomentosiformis,* whereas one band seems to be derived from *N. sylvestris* (Figure 4A). Three bands could be detected in digested *N. tabacum* DNA after hybridization with the *NtCslG* probe. Two bands seem to originate from *N. tomentosiformis,* while the other one is derived from *N. sylvestris* (Figure 4B). For the *CslE* probe, the number of hybridizing bands varies among the different restriction enzymes. None of the restriction enzymes used is likely to cut in the *NtCslE* and *NtCslG* cDNAs. If the gene structure is conserved between *A. thaliana* and *Nicotiana Csl* genes, introns are expected to be present in the *Nicotiana CslE* and *CslG* genes. Since the introns can harbour recognition sites for the restriction enzymes, it is possible that different *CslE* bands are originating from the same gene. The Southern blot results suggest that the *CslE* gene family in *N. tabacum* consists of about two to four genes, while

the *CslG* family consists of at least three genes. As cDNA sequences for both families can be quite divergent, it might be possible that either family possess more family members. However, we deliberately did not want to lower the stringency of the wash of the Southern blots, as we did not want cross-hybridization between *Csl* gene families.

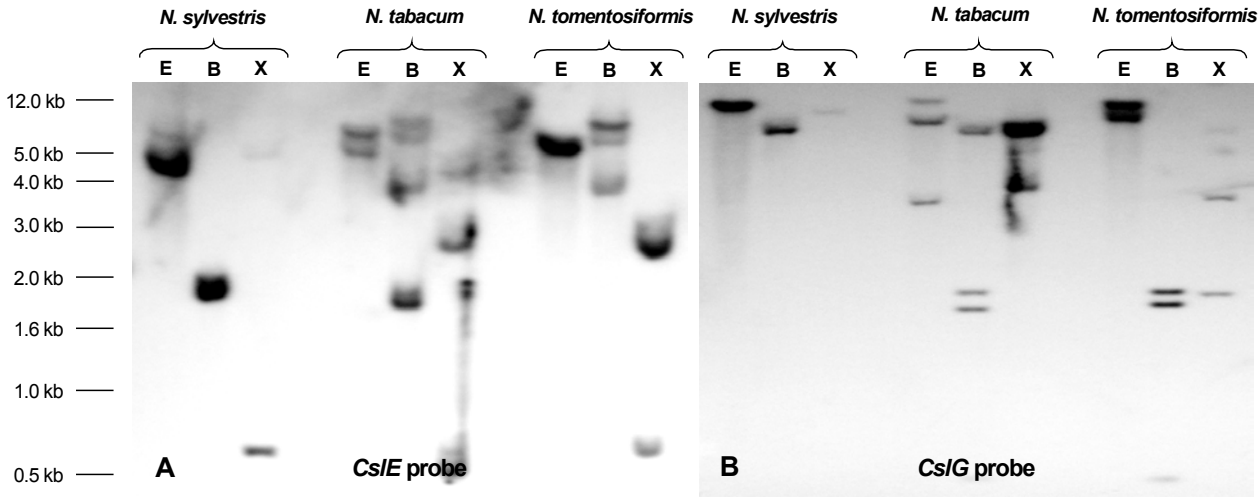


Figure 4. DNA gel blot analysis of *N. tabacum* and its ancestors *N. sylvestris* and *N. tomentosiformis* to detect *CslE* and *CslG* genes. Genomic DNA was digested with three different enzymes: *Eco*RI (E), *Bam*HI (B) and *Xba*I (X). 7.5 µg of restricted DNA was applied in each lane. Hybridization was performed with a 1.1 kb probe covering the 3' end of the *NtCslE* **(A)** or *NtCslG* **(B)** cDNAs. Molecular weight markers are indicated on the left.

Expression of *NtCslE* and *NtCslG* in xylogenic tobacco cell cultures and in tobacco plants

Expression of *NtCslE* and *NtCslG* was analysed by Northern blottings. *NtCslE* and *NtCslG* transcripts were detected in 1 to 10 days old xylogenic tobacco cell cultures. *NtCslE* and *NtCslG* were constitutively expressed, with the *CslE* expression level being higher than the *CslG* expression level (Figure 5A). Additionally, the *CslE* expression level was generally higher in the xylogenic cultures than in control cells (results not shown).

The expression pattern of *NtCslE* and *NtCslG* mRNAs was also investigated in wild type tobacco plants. Mature 2.5 months old plants carrying flower buds were used for the expression study. *NtCslE* was shown to be constitutively expressed *in planta*. The strongest signals were observed in roots and to a lower extent also in flower buds and leaves. In stems, the expression was low in the younger parts corresponding to internodes 1 to 3; the expression was higher in internodes 4, 7 to 10, increasing from 7 to 10. The expression dropped in internodes 11 and 12 (Figure 5B). Since secondary growth and

secondary cell wall formation should occur predominantly in secondary xylem and phloem, the stem from internodes 7 to 12 of mature tobacco plants was lyophilised and dissected into cortex, xylem and pith. The *CslE* mRNA that was detected in the older parts of the stem was mostly located in the pith and to a lower extent also in cortex and xylem (Figure 5B). *NtCslG* mRNAs were weakly detected in young internodes. Expression was highest in internode 1, while no detection was observed in the older internodes. The *CslG* mRNA transcript levels in roots and buds were comparable to internode 1, however expression turned out to be much higher in (developing) leaves (Figure 5B).

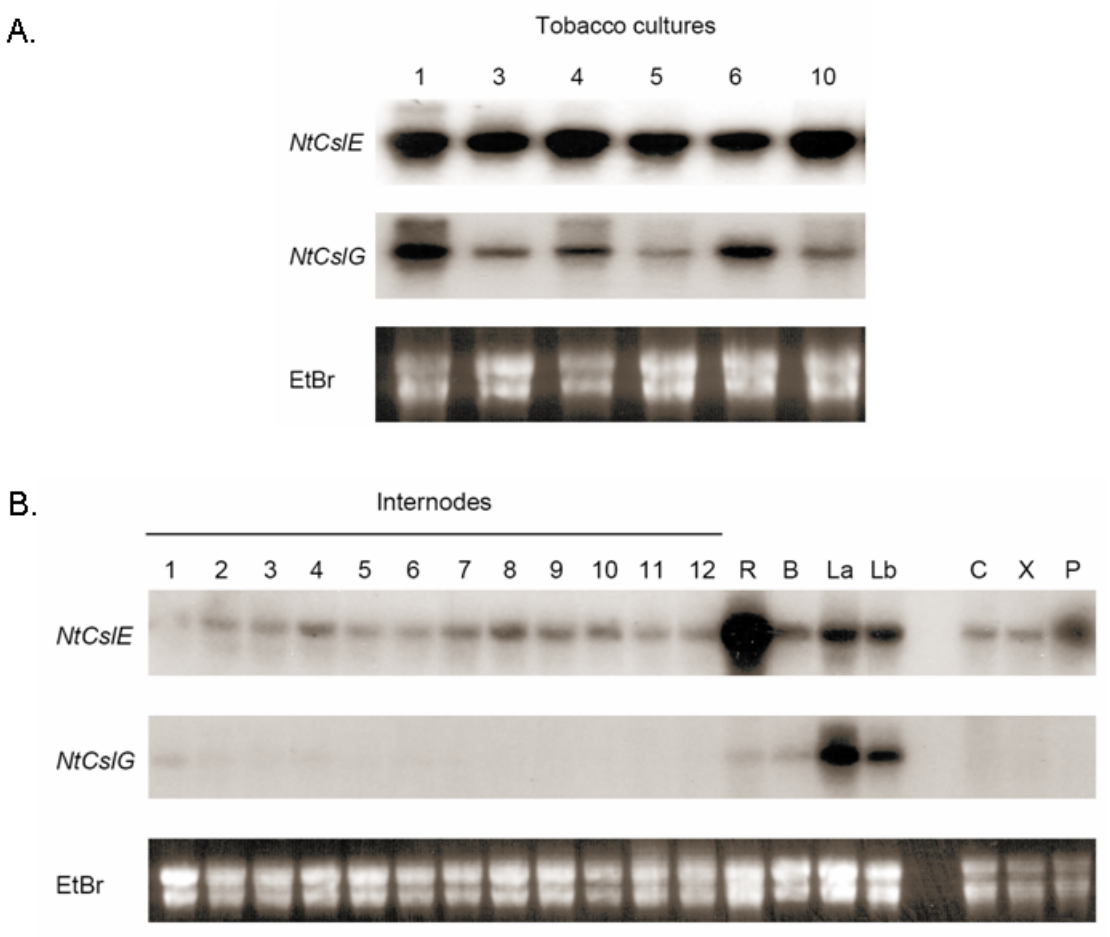


Figure 5. Expression study of *NtCslE* and *NtCslG* in xylogenic tobacco cell cultures and wildtype tobacco plants by Northern blotting. Ten micrograms of RNA were loaded in each lane. **A.** Expression in xylogenic tobacco cell cultures of different ages (indicated in days). **B.** Expression in tobacco stem internode 1-12 (the smallest number corresponding to the apex and the highest number corresponding to the older part of the stem), roots (R), flower buds (B), young developing leaves of 0-5 cm long (La), older leaves of more than 10 cm long (Lb) from 2.5 months old tobacco plants. RNA isolated from lyophilized cortex (C), xylem (X) and pith (P) of internode 7 to 12 of 2.5 months old tobacco plants was also included in the expression study. Loading of samples is shown as EtBr staining.

Sequence comparison of tobacco and potato *CslE* and *CslG* cDNA clones and interpretation of the consequences for the IR tobacco transformants

Down regulation of *CslG* in the IR tobacco transformants and expression of *CslG* in wild type tobacco could not be confirmed by an RT-PCR approach in which primers based on potato cDNA sequences were used to perform PCR on tobacco cDNA. In order to explain this result the *NtCslE* and *NtCslG* cDNA clones were aligned with the *StCslE* and *StCslG1* cDNA sequences used for the preparation of the IR constructs. Figure 6A shows that the sequence identity between *NtCslE* and the *StCslE* is around 84%. The sequence identity between *NtCslG* and the *StCslG1* seems to be much lower, only 52%, as indicated in Figure 6B. The absence of high sequence identity in the 5' end and 3' end region of the *StCslG1* cDNA sequence, the regions in which the RT-PCR primers were located, explains the difficulties in demonstrating the expression of this specific *CslG* gene in wild type tobacco.

DISCUSSION

Screening of the cDNA library made from three days old xylogenic tobacco cell cultures, with a full length *A. thaliana CslG2* cDNA clone as probe, has resulted in the cloning of *NtCslE* and *NtCslG* cDNAs. Both *NtCsl* clones share high sequence similarity with other plant *Csl* sequences. Since the library screening resulted in the isolation of multiple identical *NtCslE* and *NtCslG* cDNA clones, it can be concluded that *NtCslE* and *NtCslG* are the mainly expressed members of their respective families in three days old xylogenic cell cultures of tobacco.

Since the Csl proteins are thought to participate in the biosynthesis of non-cellulosic polysaccharides, they are expected to be located in the secretory pathway of the cell, that is, the ER and the Golgi apparatus. Immunolocalization and GFP studies with the *A. thaliana* Csl proteins have revealed that the Csl proteins are indeed localized to the Golgi apparatus (CslA, CslB, CslE and CslG) or ER (CslD) (Richmond and Somerville, 2000; Favery *et al.*, 2001). Additionally, soybean somatic embryos expressing a guar seed *(Cyamopsis tetragonoloba*) *CslA* cDNA, were found to obtain high levels of mannan synthase activity that localized to the Golgi (Dhugga *et al.*, 2004). NtCslE and NtCslG both have two N-terminal and six C-terminal transmembrane domains with N- and C-termini in the cytosol. This implies that the proposed catalytic site of NtCslE and NtCslG might face the cytosol.

Alignment of the *NtCslE* and *NtCslG* cDNA clones with the potato cDNA sequences that were used for the preparation of the IR constructs, revealed a very high sequence

Figure 6. Sequence alignment of *NtCslE* and *NtCslG* cDNA sequences with the potato cDNA clones used to prepare antisense *Csl* IR constructs (Chapter 2). Nucleotides differing from the tobacco sequences are indicated with solid background. **A.** *NtCslE* and *StCslE* (cSTB47M10) share 84% sequence identity. **B.** *NtCslG* and *StCslG1* (cSTB34J22) share 52% sequence identity.

homology (84%) between *NtCslE* and *StCslE*. A stretch of more than 23 nucleotides of complete identity, the lower size limit required for post-translational gene silencing using RNAi techniques (Thomas *et al.*, 2001), can be indicated. Therefore *NtCslE* might be silenced in the tobacco transformants. The sequence homology between *NtCslG* and the *StCslG1* cDNA clone turned out to be much lower (only 52%). Stretches of 23 nucleotides of complete identity cannot be found and therefore silencing of our *NtCslG* is not likely to occur in the IR tobacco transformants. The sequence divergence also becomes clear from the phylogenetic tree (Figure 3), which clearly shows that NtCslG and StCslG1 cluster in different sub-groups. The Southern blot results suggest the presence of at least two more *CslG* family members in tobacco, and it cannot be excluded that one of these genes shows a homology to *StCslG1* that is sufficient to accomplish gene silencing in tobacco.

The low sequence homology between the *NtCslG* and the *StCslG1* cDNA clone also explains the low hybridization efficiency during the library screen with the *StCslG1*

probe and possibly also the negative results of the RT-PCR analysis. The difficulties in demonstrating the expression of *CslG* in wild type tobacco by RT-PCR could however also be caused by a lack of expression of the specific gene in the young *in vitro* grown plants.

Although potato and tobacco are both solanaceous species, they seem to have a varying number of *Csl* genes. The Southern blot results suggest for example the presence of at least three *CslG* genes in tobacco, whereas more than four different *CslG* family members might be present in potato, according to the classification on the Stanford site. Tomato (*Lycopersicon esculentum*), another solanaceous species, seems to have more than seven different *CslG* genes (see http://www.cellwall.stanford.edu). Since the number and sequence similarity of *CslG* family members seems to differ a lot between solanaceous species, it is very risky to use *Csl* sequences from one solanaceous species to achieve gene silencing in another solanaceous species.

The tobacco xylogenic cell culture system developed by Blee *et al*. (2001) has been shown to be a valid model system for studying secondary cell wall formation. In this system, UGD and UGAD activity preceded XS activity, which could be detected from three days after sub-culturing. Bindschedler *et al.* (2005) revealed that *UGD* and *UGAD* mRNAs were present in 3 to 10 days old cell cultures, although the transcript levels were highest in 3 to 5 days old cultures. We have shown that *NtCslE* and *NtCslG* are both constitutively expressed throughout the culturing period, therefore it is not likely that these *CslE* and *CslG* genes are encoding for a XS involved in secondary wall synthesis. *NtCslG* could possibly be linked with primary wall biosynthesis, as it was shown to be expressed in young stems, roots, buds and (developing) leaves but not in older stems. Nevertheless, it cannot be excluded that another *NtCslG* with low similarity to the cloned cDNA might be dominantly expressed in tobacco stems and has a completely different expression pattern and function. *NtCslE* was more abundantly expressed in the xylogenic cell culture than in control cells. Additionally, expression was higher in older stem segments than in younger internodes. The *CslE* gene could therefore possibly be involved in secondary cell wall biosynthesis. This hypothesis needs however further investigation, since *NtCslE* was not exclusively expressed in the xylem tissue, as was observed for the UGAD proteins (Bindschedler *et al*., 2005). The fact that the expression profiles of *NtCslE* are not identical in stems and in the xylogenic cell cultures could imply that another *NtCslE* family member with low similarity to the cloned *NtCslE* is more abundant in tobacco stems or other parts of the plant than in cell cultures in which the cloned *NtCslE* strongly dominates. This is reinforced by the fact that the cloned *NtCslE* and *NtCslG* showed globally a much stronger signals on Northern blots for cell cultures than for the tobacco plants. In summary, an

involvement for *CslG* in xylan biosynthesis in tobacco, as indicated by heterologous gene silencing using potato cDNA sequences, is not supported by gene expression analysis.

EXPERIMENTAL PROCEDURES

Screening of cDNA library

A λZAPII cDNA library (Stratagene) that was made from poly-A^+ RNA isolated from a three days old cell suspension culture of a *Nicotiana tabacum* c.v. Petit Havana cell line, derived from a cultivar transformed with the *ipt* gene from *Agrobacterium* (Blee *et al*., 2001; Bindschedler *et al*., 2005). The titer of the cDNA library was estimated to be $7*10^9$ pfu/ml. One million phage plaques were screened with a 303 bp *StCslG1* fragment (cSTB34J22), [α-^{32}P]dCTP labelled with the rediprime II random prime labelling kit (Amersham Pharmacia Biotech), as a probe. The 303 bp *StCslG1* fragment was amplified by PCR with the primer pair 5'-atatcagctaggaaggcaatgtgt-3' / 5'-cctctagtgaagcaaggaactttt-3'. Membranes were pre-hybridized at 65°C for 3 h in modified Church buffer (Church and Gilbert, 1984). Hybridization was performed in modified Church buffer for 16 h at 60°C (low stringency because of screening with heterologous probe). Blots were washed at 60°C, two times in 2 x SSC, 0.1% SDS and two times in 1 x SSC, 0.1% SDS, for 15 min each time.

The *CslG2* cDNA clone (U11884) from *Arabidopsis thaliana* was obtained from the Arabidopsis Biological Resource Center (Columbus, Ohio, USA). The complete *CslG2* fragment was amplified by PCR with the primers 5'-cctgccggcgaaccatt-3' and 5'-ctgacccggtaagaacaaaactga-3' and used to screen the *N. tabacum* cell suspension culture cDNA library as described above with minor modifications. Hybridization was performed at 55°C (because of screening with highly heterologous probe) and blots were washed three times for 30 min at 60°C with 2 x SSC, 0.1% SDS. After two screening rounds 17 positive clones were obtained, of which the phagemids were excised using the ExAssist Interference-Resistant Helper Phage (Stratagene), resulting in a pBluescript vector containing the cDNA.

Sequencing and sequence analysis

The cDNA clones were sequenced by BaseClear (Leiden, The Netherlands) using the T3 (5'-aattaaccctcactaaaggg-3') and T7 (5'-gtaatacgactcactatagggc-3') primer and cDNA sequence-specific primers. Evaluation of sequencing data and assembly of contigs was performed with Lasergene (DNASTAR Inc., Madison, WI, USA) software packages. Nucleic acid and translated sequences were compared to GenBank non-redundant and EST databases using the BLASTN, BLASTX and TBLASTN sequence alignment programs (Altschul *et al*., 1997). Protein structure predictions were performed using publicly available programs (http://www.expasy.ch/tools). Clustal analysis and One pair alignment (DNASTAR Inc., Madison, WI, USA) software packages were used for further analysis and comparison of sequences. The *N. tabacum CslE* and *CslG* sequences have been deposited in the GenBank database (accession no. DQ127171 and DQ152918, respectively).

DNA isolation and Southern Blot analysis

Genomic DNA was isolated from *Nicotiana sylvestris*, *Nicotiana tomentosiformis* and *N. tabacum* cv Samsun NN. Approximately 1.5 g of ground plant material was transferred to centrifuge tubes containing 5 ml DNA extraction buffer (62% urea (w/v), 5 M NaCl, 1 M Tris-HCl pH 8.0, 0.5 M EDTA pH 8.0 and 20% sarcosyl (w/v)) and 5 ml 1:1 phenol:chloroform. Samples were homogenized, incubated at room temperature for 15 min and centrifuged for 10 min at 3000 rpm (4°C). The supernatants were subjected to phenol:chloroform

extractions until no interface remained. Genomic DNA was obtained after successive 4.4 M ammonium acetate pH 5.2, isopropanol and ethanol precipitations.

For Southern blot analysis, genomic DNA was RNAse treated and digested with *Eco*RI, *Bgl*II or Xba*I*. The products were resolved by electrophoresis through a 0.8% agarose gel (7.5 µg per lane) and transferred overnight onto a Hybond-N+ membrane (Amersham) by capillary transfer in 0.4 M NaOH. The DNA gel blots were pre-hybridized for 3 h at 65°C in modified Church buffer (Church and Gilbert, 1984). A 1163 bp *NtClsE* fragment and a 1143 bp *NtCslG* fragment were amplified by PCR with the primer pairs 5'-aacgatccagatgcaataagagaa-3' / 5'-tgcttcgtacactgggacattc-3' and 5'-atttagcctacgtccagtttcctc-3' / 5'-gtaggcatccttcctttatcagc-3' respectively. Hybridization was carried out for 16 h at 65°C in modified Church buffer with the amplified *NtCslE* or *NtCslG* fragments labeled with [α-^{32}P]dCTP by random primed labeling (Rediprime II random prime labeling kit, Amersham Pharmacia Biotech). The blots were washed at 65°C, two times in 2 x SSC, 0.1% SDS and two times in 1 x SSC, 0.1% SDS, for 15 min each time. The radioactively labeled blots were exposed to X-OMAT S and AR films (Kodak) at –80°C with intensifying screens.

RNA extraction and Northern Blot analysis

RNA was extracted from *N. tabacum* plants cv K326 or from tobacco cell cultures with guanidium thiocyanate-phenol according to Chomczynsky and Sacchi (1987) with slight modifications. Insoluble material was removed from the homogenization buffer by centrifugation at 1000 g for 15 min prior to addition of chloroform. After the first precipitation with isopropanol, the RNA pellets were washed with 75% ethanol and directly dissolved in 0.5% SDS. Solubilisation was facilitated by incubation at 55 °C, followed by centrifugation in a microfuge for 10 min at 10 000 rpm to remove insoluble particles.

Ten microgram of total RNA were loaded on 1.5% agarose gels for Northern blotting. A 1163 bp *NtClsE* fragment and a 1143 bp *NtCslG* fragment were amplified by PCR as described above. The PCR product was labeled with the High Prime DNA labeling kit according to the manufacturer (Roche) and used as a probe for Northern blot analysis. Hybridization conditions were as described before with the last wash at 0.5 * SSC, 0.1% SDS at 65 °C.

ACKNOWLEDGEMENTS

We thank the Arabidopsis Biological Resource Center (ABRC) at Ohio State University (USA) for providing the *A. thaliana* cDNA clone U11884. We are grateful to Jutta Tuerck (Advanced Technologies Cambridge (ATC), Cambridge, UK) for providing the seeds of *N. sylvestris* and *N. tomentosiformis*. The work was funded by a grant within the EC Framework V (COPOL, QLK5-2000-01493) and is gratefully acknowledged.

Chapter 4

Xyn2, a modular Family 10 glycoside hydrolase from *Arabidopsis thaliana*, displays a dual activity against β-1,4-linked xylans and glucans

Monique Compier, Richard G.F. Visser and Jean-Paul Vincken

Graduate School Experimental Plant Sciences, Laboratory of Plant Breeding, Wageningen University, P.O. box 386, 6700 AJ Wageningen, The Netherlands.

Submitted to *Plant Science*.

ABSTRACT

The putative *Arabidopsis thaliana Xyn2* gene is suggested to play a role in the remodeling of xylan in the cell wall. *AtXyn2* is coding for a modular enzyme, consisting of four contiguous CBM22 modules at the N-terminus, joined to a glycoside hydrolase Family 10 (GH10) catalytic domain. To reveal the function of AtXyn2 and the role of the four Family 22 CBMs, the full length AtXyn2 and a derivative, comprising the catalytic GH10 domain lacking the four CBMs, were ectopically expressed in tobacco (*Nicotiana tabacum*). A signal peptide and an RGS·$(HIS)_6$ -tag were fused to the N-terminus of the proteins to allow translocation into the cell wall and detection of the expressed proteins via the RGSHHHH epitope. The generated transformants were screened for the presence of the ectopic transcripts. Both transgenes were shown to be expressed in tobacco, however, presence of the corresponding proteins could not be demonstrated by Western Blot analysis, although different protein extraction procedures were used. We speculate that this could be caused by removal of the RGS·$(HIS)_6$ -tag due to either post-translational processing of AtXyn2 in tobacco or due to cleavage of the epitope-tag by a tobacco protease. Tobacco transformants expressing the complete AtXyn2 protein were found to possess a significantly higher wheat xylan and HE-cellulose degrading activity than wild type tobacco, whereas transgenic plants expressing the truncated protein did not. This result indicates that AtXyn2 displays a dual hydrolytic activity towards specific β-1,4-linked xylans and glucans. Additionally, the presence of the CBM22 domains adjacent to the catalytic module seems to play an essential role in the activity of the enzyme. The catalytic activity was found to be inversely proportional to the *AtXyn2* transcript level and therefore we speculate that high amounts of AtXyn2, or released oligosaccharides, could result in a negative feedback mechanism and subsequent inactivation or degradation of the enzyme. Based on our results and additional findings described in literature, a model is proposed which might explain the regulation of AtXyn2 activity in the plant cell wall.

INTRODUCTION

Plant cells show two types of cell wall deposition: the primary cell wall is generally synthesized during cell expansion in the first stages of development and the secondary wall is deposited in fully expanded and specialized cells. The secondary cell wall provides rigidity to cells that have a strengthening function and to those that are involved in the conduction of water. It is mostly composed of cellulose, hemicellulose and the aromatic compound lignin (Raven *et al*., 1992). Xylan, the most abundant hemicellulosic

component, plays a major role in the organization of secondary cell walls, as it can interact with lignin (Hatfield *et al*., 1999) and cellulose (Awano *et al*., 2001). The xylan layer may be important in maintaining the integrity of the cellulose and in helping protect the fibers against degradation by cellulases (Uffen, 1997). Xylan is a relatively complex polysaccharide comprising a backbone of xylosyl residues linked by β-1,4-glycosidic bonds. The main chain residues can be substituted to varying degrees with 4-O-methyl-glucuronyl, glucuronyl, acetyl and arabinofuranosyl groups (Fry, 1988; Gilbert and Hazlewood, 1993). The nature and the extent of xylan substitution vary between plants, with softwood and cereal xylans containing high levels of arabinosyl substituents, and hardwood xylan containing mainly glucuronyl groups (Coughlan and Hazlewood, 1993).

Controlled hydrolysis of xylan polymers and modification of the interaction with cellulose microfibrils is a prerequisite for the remodeling of plant cell walls during cell expansion and secondary cell wall formation (Goujon *et al*., 2003; Simpson *et al*., 2003). Xylan degradation also plays a role in other physiological processes such as fruit ripening (Chen and Paull, 2003), hydrolysis of the tapetum cell wall, pollen tube penetration into the stigma (Bih *et al*., 1999), and endosperm mobilization in germinated seeds (Slade *et al*., 1989). Several glycoside hydrolases are involved in the hydrolysis of xylan polymers, of which the most important are the endo-β-1,4-xylanases (xylanases) (EC 3.2.1.8). These enzymes degrade the xylan backbone into xylo-oligosaccaharides of varying lengths. For complete hydrolysis to xylose, xylanases cooperate with exo-β-1,4-xylosidases (EC 3.2.1.37), which cleave xylose from the non-reducing end of xylo-oligosaccharides. Little information is available on the plant enzymes that are involved in the remodeling of xylan during cell expansion and secondary wall formation. A putative xylanase has been found in a cell wall fraction prepared from tobacco cells having an extensive secondary cell wall (Blee *et al*., 2001). Goujon *et al*. (2003) identified the *Arabidopsis thaliana BXL1* gene, a putative β-xylosidase gene, which is proposed to be involved in the loosening of xylan during secondary cell wall formation. A search of the *Arabidopsis thaliana* genome sequence revealed 12 putative endo-β-1,4-*xylanase* (*Xyn*) genes (Henrissat *et al*., 2001), of which at least three are coding for modular enzymes (Simpson *et al*., 2003). The *A. thaliana Xyn1* gene (*AtXyn1*) is suggested to encode a modular xylanase, that plays a role in regulating secondary cell wall metabolism of vascular bundle cells (Suzuki *et al*., 2002). The function of the other *AtXyn* genes is unknown; however, at least some of them could be involved in the rearrangement of xylan polymers in the cell wall (Simpson *et al*., 2003).

At the time of writing, glycoside hydrolases are classified into 99 families, according to amino acid sequence similarity (see the carbohydrate-active enzyme CAZY server at http://afmb.cnrs-mrs.fr/CAZY (Coutinho and Henrissat, 1999)). Within this classification system, the xylanases belong mainly to Family 10, which contains plant, fungal and

bacterial enzymes, or to Family 11, which includes fungal and bacterial enzymes (Henrissat and Bairoch, 1996; Henrissat, 1998). Although the majority of enzymes of Family 10 are xylanases, this family also contains endo-β-1,3-xylanases (EC 3.2.1.32) and cellobiohydrolases (EC 3.2.1.91). Family 11 is monospecific, which means that it consists solely of xylanases (Collins *et al*., 2005). Additionally, a small number of enzymes with xylanase activity has been identified in Families 5, 7, 8 and 43 (Collins *et al*., 2005).

In common with other plant cell wall hydrolases, xylanases are generally modular enzymes in which the catalytic modules are joined, via a linker region, to one or more non-catalytic carbohydrate binding modules (CBMs) (Hall and Gilbert, 1988). The number of CBMs and their localization at the N- or C-terminus of the catalytic domain differ greatly between enzymes (Henrissat and Davies, 2000). CBMs have been grouped into more than 40 families, based on amino acid sequence similarity (see http://afmb.cnrs-mrs.fr/CAZY). Many of the characterized CBMs interact with cellulose, although CBMs that bind other substrates such as xylan, mannan, mixed-linkage glucan, chitin and starch have also been described (reviewed by Boraston *et al.*, 2004). CBM families whose members bind xylan include CBM2b, CBM4, CBM6, CBM13 and CBM22. Irrespective of ligand specificity, it appears that most CBMs mediate increased catalytic efficiency by increasing the effective enzyme concentration on the surface of the substrate (Bolam *et al*., 1998; Charnock *et al*., 2000).

Xylanases are widely distributed among bacteria, fungi and plant species (reviewed by Collins *et al*., 2005). The characterization of microbial xylanases revealed a lot of information about the catalytic properties of the enzymes. Current knowledge about plant xylanases is limited to the few enzymes that have been purified from wheat (*Triticum aestivum*) (Cleemput *et al*., 1997), barley (*Hordeum vulgare*) (Slade *et al*., 1989; Banik *et al.*, 1996; Caspers *et al.*, 2001), maize (*Zea mays*) (Bih *et al*., 1999; Wu *et al*., 2002) and papaya (*Carica papaya*) (Chen and Paull, 2003). The characterized plant xylanases are proteins of about 30 kDa, which appear to be synthesized as a much larger, inactive precursor polypeptide. The inactive precursors consist of a glycoside hydrolase Family 10 (GH10) catalytic domain preceded by a CBM22 module, whereas the active enzymes are composed of the catalytic GH10 domain alone (Chen and Paull, 2003; Simpson *et al*., 2003). Family 22 CBMs, formerly designated as X6, were originally defined as thermo-stabilizing modules, since their removal from thermophilic enzymes resulted in a decrease of the enzyme stability at elevated temperatures (Fontes *et al*., 1995; Hayashi *et al*., 1997). Later, the function of the X6 modules has been revised and they have been re-assigned as CBM22 based on its ability to bind four consecutive xylosyl residues (Charnock *et al.*, 2000).

The putative *Xyn2* gene from *A. thaliana* (*AtXyn2*) is coding for a modular enzyme, consisting of four Family 22 CBMs, joined to a GH10 catalytic module (Henrissat and Davies, 2000; Henrissat *et al*., 2001). This putative endo-β-1,4-*xylanase* gene has been shown to be expressed in the *A. thaliana* stem (Suzuki *et al*., 2002) and could possibly be involved in the rearrangement of xylan polymers in the cell wall. In the present study we introduced two different forms of AtXyn2, the full length protein and a derivative, comprising the catalytic module lacking the four CBMs, into tobacco (*Nicotiana tabacum*) to investigate the biological function of AtXyn2 and the role of the four Family 22 CBMs.

RESULTS

Generation of transgenic tobacco plants expressing *AtXyn2*

To investigate the biological function of AtXyn2 and the role of the Family 22 CBMs, a 'gain of function' approach was undertaken by which the full length *AtXyn2* and a derivative (GH10 catalytic module lacking the four CBMs), were ectopically expressed in tobacco after *Agrobacterium tumefaciens* mediated transformation. The full length gene will be further referred to as *AtXyn2*, whereas the truncated gene will be indicated as *AtXyn2ΔCBM*. The constructs used to generate transgenic plants are shown in Figure 1. The *AtXyn2* and *AtXyn2ΔCBM* sequences were preceded by the *Ntp303* signal sequence and a *RGS·(HIS)$_6$* -tag. The pollen-specific NTP303 glycoprotein of tobacco has been shown to be present in the pollen tube wall (Wittink *et al*., 2000) and therefore the corresponding signal peptide was used to target the xylanase proteins to the secretory pathway. The *RGS·(HIS)$_6$* -tag allows detection of the fusion proteins via the RGSHHHH epitope that is recognized by a highly specific monoclonal antibody. This enables us to discriminate between the expression of endogenous tobacco xylanases and the introduced AtXyn2 and AtXyn2ΔCBM. The transgenic tobacco plants are referred to as AtXyn2 #xx and AtXyn2ΔCBM #xx (#xx refers to the clone in that particular series of transformants).

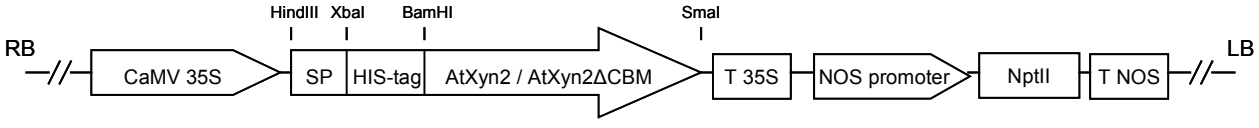


Figure 1. Schematic representation of the T-DNA constructs in the binary vector pGreen7K, used for the ectopic expression of *AtXyn2* and *AtXyn2ΔCBM* in tobacco. Both genes were under control of the CaMV35S promoter. The tobacco *Ntp303* signal peptide (SP) and RGS·$(HIS)_6$-tag (HIS-tag) were added to mediate respectively translocation into the cell wall and detection of the fusion proteins with antibodies directed against the RGSHHHH epitope. *NPTII*, neomycin phosphotransferase.

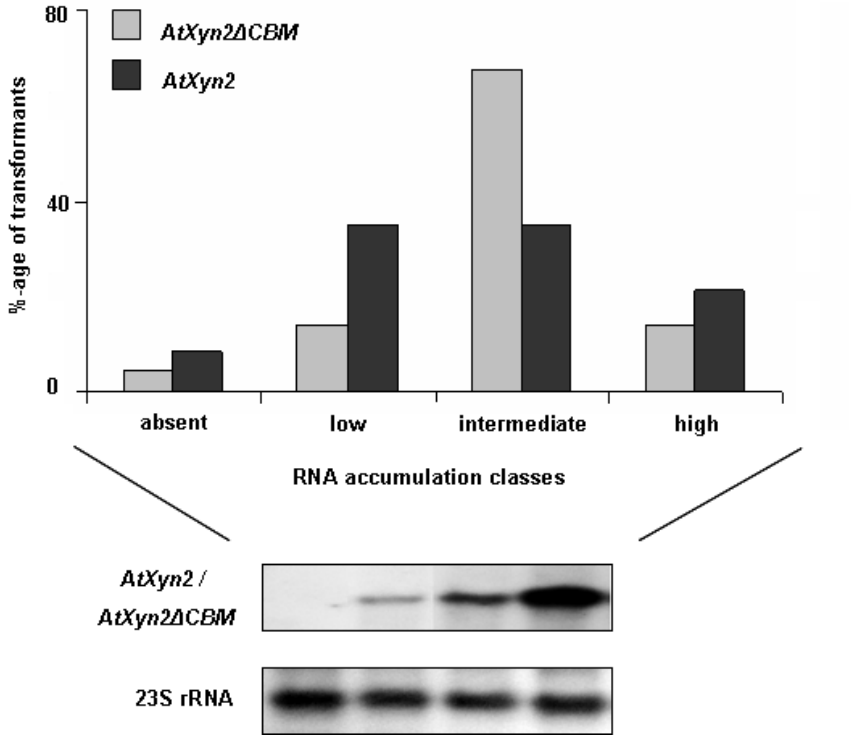


Figure 2. Expression levels of *AtXyn2* and *AtXyn2ΔCBM* in transgenic tobacco plants. The graph presents the distribution of the individual transformants over different classes of transgenic mRNA expression. The '*AtXyn2 / AtXyn2ΔCBM'* panel defines the four classes of mRNA accumulation in transgenic tobacco plants: absent, low, intermediate or high levels of the specific mRNAs. This classification is based on the results of a Northern blot analysis in which more than twenty transgenic plants of each series of transformants were screened for the presence of the ectopic transcript. For all transformants, 15 µg of total RNA was separated by gel electrophoresis, transferred onto a nylon membrane and hybridised with a ^{32}P-labelled 1.1 kb *AtXyn2ΔCBM* fragment. Hybridisation with a ribosomal RNA probe was performed as a loading control.

The transgenic plants were grown in the greenhouse. During growth, they all appeared phenotypically normal with respect to plant size, flower and seed development, when compared to wild type tobacco and empty vector control transformants.

Selection of transformants

More than twenty transgenic tobacco plants of each series of transformants were screened for the presence of the transcripts of the transgenes. Ninety one percent of the plants transformed with the *AtXyn2* construct were found to express the specific mRNA, whereas 95% of the *AtXyn2ΔCBM* transformants showed the presence of the ectopic transcript. Based on the intensity of the hybridization signals the transformants were divided into four different classes: no, low, intermediate and high levels of specific mRNA expression (Figure 2). Of each series of transformants, eight transgenic plants (showing low, intermediate or high expression levels) were randomly selected for further analysis.

DNA blot hybridization experiments were performed to analyze the copy numbers of the inserts. The copy number of the *AtXyn2* transformants correlated positively with the RNA levels of the transformants (one copy in the low expressers and two copies in the intermediate and high expressers). This does however not apply to the *AtXyn2ΔCBM* transformants (copy number varied from one to three) (results included in Figure 5). There is no clear explanation for this result.

No detection of AtXyn2 proteins

Since *AtXyn2* and *AtXyn2ΔCBM* mRNA is present in transgenic tobacco plants, we studied expression levels of the epitope-tagged xylanase proteins by Western blot analysis. A monoclonal antibody raised against the RGSHHHH epitope was used to detect the proteins. A standard protein extraction procedure, as described for NTP303 (Wittink *et al*., 2000), was not suitable to confirm the presence of the epitope-tagged xylanase proteins. The CBM22 domains could be very intimately associated with the xylan polymers in the cell wall (Linder and Teeri, 1997), and therefore the AtXyn2 proteins might not be easily extractable. To circumvent poor extraction of AtXyn2 due to anchoring in the cell wall, different protein extraction buffers were used. The buffers contained either a high salt concentration (extraction of ionically-bound proteins), 100 mM NaOH (alkaline solution which might saponify putative ester linkages), 8 M urea (chaotropic agent, helps simultaneously to swell cellulose fibres and to unfold proteins) or PVPP (binds polyphenols which influence the solubility of proteins). Both AtXyn2 and AtXyn2ΔCBM were not detectable on a Western blot, even when highly concentrated protein samples (up to 400 µg of total proteins per lane) were loaded on SDS-polyacrylamide gels (data not shown). The xylanase proteins could also not be visualized on Coomassie or silver stained gels. This result is very difficult to explain, since at least AtXyn2ΔCBM should be easily extractable from the transgenic tobacco samples, because of the lack of CBM22 domains. Therefore, it was investigated whether the difficulties in demonstrating the expression of the epitope-tagged xylanase proteins by Western Blot analysis, could be caused by post-translational processing in the tobacco plant.

Removal of the epitope-tag of AtXyn2 in tobacco?

Post-translational processing of endoxylanases has been reported in barley and papaya. The barley aleurone endoxylanase (XYN-1) precursor is processed by cysteine endoproteases to yield a mature active enzyme. During processing, N- and C-terminal residues are removed, leading to a reduction in molecular weight from 61.5 kDa to 34 kDa (Caspers *et al*., 2001). The papaya endoxylanase EXY1 is also suggested to be processed at the N- and C-terminus, reducing the size of the active enzyme from 65 kDa to 32.5 kDa (Chen and Paull, 2003). Processing sites and conserved amino acid sequences are

indicated in Figure 3A. Alignment of AtXyn2 with barley XYN-1 and papaya EXY1 revealed the presence of conserved amino acids and putative N- and C-terminal cleavage sites in AtXyn2 (Figure 3A and B). If cysteine endoproteases are also involved in post-translational processing of AtXyn2, the molecular weight of the mature AtXyn2 and AtXyn2ΔCBM fusion proteins will be reduced to 34.0 kDa and consequently the RGSHHHH epitopes will be removed (Figure 3B). This implies that the fusion proteins can no longer be detected on a Western Blot using RGS·HIS antibodies. Since the epitope-tagged CBM22 domains could also not be detected, the cleaved N- and C-terminal residues are likely to be degraded immediately after processing.

Another explanation for the fact that the epitope-tagged proteins could not be detected on a Western Blot, could find its origin in the three dimensional structure of the fusion proteins. If the N-terminal epitope-tag is protruding from the folded protein, the tag might be a target for cleavage by tobacco proteases. In order to investigate the option of cleavage of the RGS·$(HIS)_6$ -tag by tobacco proteases, a protein ladder, consisting of five different proteins of known size, each containing an N-terminal RGS·$(HIS)_6$ -tag, was

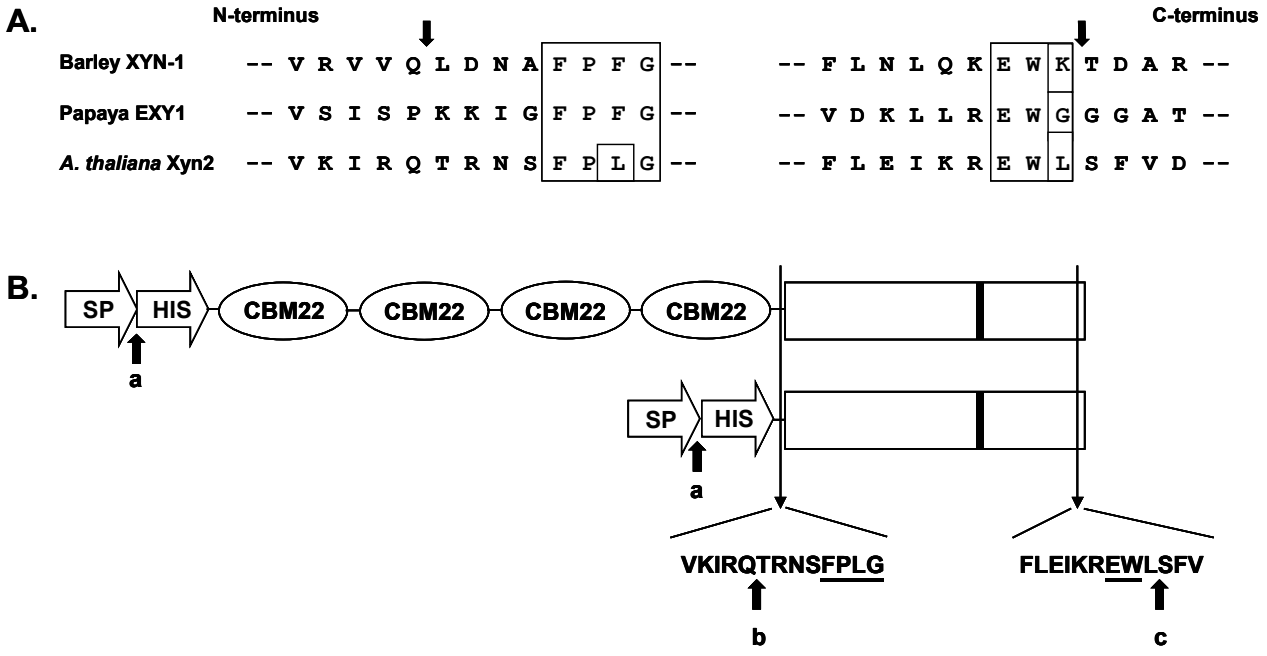


Figure 3. Post-translational processing of plant xylanases. **A.** Comparison of the N- and C-terminal sequences of barley XYN-1, papaya EXY1 and AtXyn2. The conserved amino acid motifs FP(F/L)G and EW are indicated with boxes. Arrows indicate the potential proteolysis sites, based upon barley XYN-1 post-translational processing. **B.** Post-translational processing of AtXyn2 and AtXyn2ΔCBM. Arrow **a** denotes the predicted signal peptide cleavage site. Arrow **b** and **c** indicate the potential post-translational cleavage sites shown in part A.

incubated in protein extracts of wild type tobacco. After incubation in tobacco extract without EDTA and protease inhibitor, the tagged proteins of the size marker were no longer detectable on a Western Blot, whereas the marker incubated in tobacco extract supplemented with EDTA and protease inhibitor could still be detected (results not shown). This result revealed that tobacco contains a protease that could be responsible for removal of the epitope-tag.

Grinding of the plant material and protein extraction occurred at 4°C, and the protein extraction buffer contained EDTA and protease inhibitor. Therefore, removal of the RGS·$(HIS)_6$ -tag from the AtXyn2 and AtXyn2ΔCBM fusion proteins is likely to occur already *in planta*. An important question is whether processing occurs intra- or extracellularly. Due to the presence of the NTP303 signal peptide, the AtXyn2 and AtXyn2ΔCBM proteins are supposed to be secreted into the apoplast. Therefore, cleavage of the RGS·$(HIS)_6$ -tag is expected to occur *in planta* after translocation into the extracellular space.

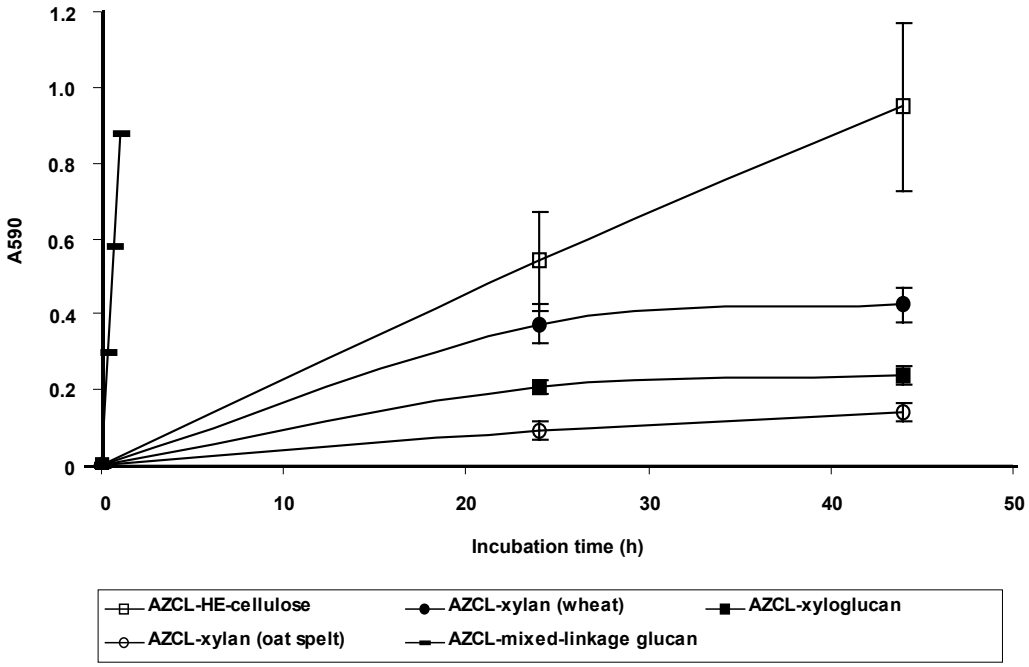


Figure 4. Release of soluble dyed fragments from AZCL-substrates during incubation with protein extract prepared from *in vitro* grown wildtype tobacco plants. Protein concentrations in the reaction mixtures were 0.5 mg/ml (AZCL-mixed-linkage glucan), 1.0 mg/ml (AZCL-HE-cellulose and AZCL-xyloglucan) or 2.0 mg/ml (AZCL-xylan, wheat and oat spelt). Error bars indicate the standard deviation (SD). SDs for AZCL-beta-1,3-1,4-glucan were not included for clarity of the figure. The SD values were 0.09, 0.255 and 0.290 for respectively 20, 40 and 60 minutes incubation.

Enzyme assays

In order to be able to discriminate between the hydrolytic activities in the *AtXyn2* and *AtXyn2ΔCBM* transformants and wild type tobacco, the endogenous hydrolytic activity was first determined in wild type tobacco extract. The substrate specificity of AtXyn2 has not been studied before. The major enzymes of the Family 10 glycoside hydrolases are xylanases, however, substrate specificity studies have revealed that not all enzymes within this family are entirely specific for xylan, and that some of them may also be active on cellulosic substrates (Claeyssens and Henrissat, 1992; Biely *et al*., 1997). Therefore, hydrolytic activities of the wild type tobacco extract were determined towards several polymeric substrates. Figure 4 shows that endo-glucanase as well as endo-xylanase activity could be measured in the tobacco extracts. This implies that different types of hydrolytic enzymes are likely to be active in the wild type situation. The hydrolytic activity was highest on mixed-linkage glucan and relatively low on HE-cellulose, wheat and oat spelt xylan and xyloglucan (Figure 4). When using an AZCL-substrate concentration of 1%, the A590 values were relatively low (A590 values were up to 4.0 when e.g. pure xylanase was tested (results not shown)) and therefore it can be concluded that the substrate concentration is non-limiting. The hydrolytic activity was proportional to the amount of protein in the reaction mixtures (results not shown) and increased, in case of HE-cellulose and mixed-linkage glucan, linearly with the length of incubation. The fact that the hydrolytic activities towards wheat xylan, oat spelt xylan and xyloglucan did not increase linearly with the length of incubation, could possibly be explained by a loss of the stability of the specific hydrolases during incubation.

Protein extracts from selected AtXyn2 and AtXyn2ΔCBM transformants (selection occurred on the basis of RNA expression levels) did not show any increase or decrease of the hydrolytic activity towards oat spelt xylan and xyloglucan, when compared to wild type tobacco (results not shown). The mixed-linkage glucan degrading activity was also not significantly changed, however, because of the relatively high activity in wild type tobacco extracts, a slight increase of this hydrolytic activity can not be excluded for the AtXyn2 transformants (results not shown). Figure 5 shows that the HE-cellulose and wheat xylan degrading activities clearly differ between the series of transformants. The AtXyn2 transformants #1, #7 and #13 showed a significant (P=0.05) increase of the hydrolytic activity against HE-cellulose and wheat xylan, whereas the other selected transformants did not. The hydrolytic activity of AtXyn2 transformants #1, #7 and #13 was not increased towards oat spelt xylan. This result was unexpected, since wheat xylan is more branched that oat spelt xylan, and consequently the wheat xylan backbone is less accessible to the enzyme. In agreement with our results, XYN10B from the ruminal protozoan *Polyplastron*

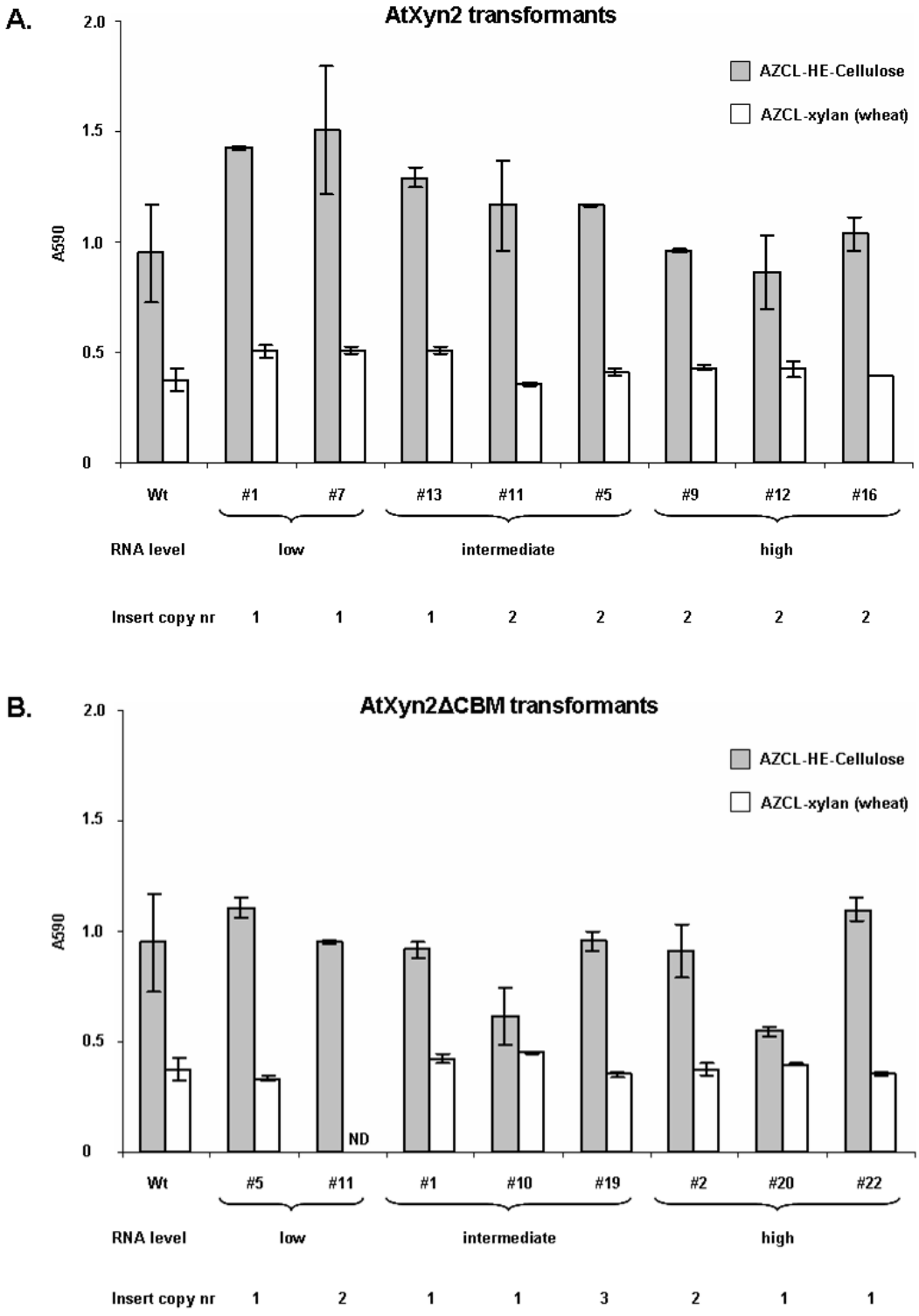


Figure 5. Comparison between AZCL-HE-cellulose and AZCL-xylan (wheat) degrading activity in *in vitro* grown wild type (Wt) tobacco and transgenic tobacco plants expressing *AtXyn2* or *AtXyn2ΔCBM*. Incubation with AZCL-HE-cellulose for 44h, incubation with wheat AZCL-xylan for 24 h. Error bars indicate the standard deviation. ND: not determined. The numbers refer to the independent transgenic plant lines. Levels of transgene mRNA expression of the specific transformants and transgene copy numbers are indicated below the graphs.

multivesiculatum also appeared to display a much higher activity towards the highly branched wheat xylan than to the much less substituted oat spelt xylan (Devillard *et al*., 2003). An explanation for this could find its origin in the presence of different xylanases in plants and protozoan, acting towards either wheat xylan or oat spelt xylan.

The amount of hydrolytic activity was inversely proportional to the *AtXyn2* transcript level of the transformants, i.e. AtXyn2 plants that showed the highest HE-cellulose and wheat xylan degrading activity contained the lowest levels of transgene mRNA. This correlation did not exist for the AtXyn2ΔCBM plants; the selected transformants showed hydrolytic activities comparable to wild type against HE-cellulose and wheat xylan. An exception is AtXyn2ΔCBM transformant #20, a high expresser, which showed a significant (P=0.05) reduction of the HE-cellulose degrading activity (Figure 5B). There is no clear explanation for this result.

DISCUSSION

This study reports the introduction of the RGS·$(HIS)_6$ -tagged AtXyn2 and a derivative, comprising the catalytic GH10 domain lacking the four CBMs, into tobacco. Although expression of the transgenes was established, presence of the corresponding epitope-tagged proteins could not be demonstrated. We speculate that this could be caused by removal of the RGS·$(HIS)_6$ - tag either as a result of post-translational processing of the AtXyn2 and AtXyn2ΔCBM proteins in tobacco, or due to N-terminal cleavage of the epitope-tag by a tobacco protease. In line with our results, the RGS·$(HIS)_6$ -tagged fungal elicitor protein ECP2 could also not be detected by RGS·HIS antibodies on a Western Blot after infiltration of the epitope-tagged proteins in the apoplast of tomato leaves. Removal of the epitope-tag occurred irrespective of the kind of tag used (Peter van Esse, Phytopathology, Wageningen University, pers. communication), and therefore it seems to be likely that the epitope-tags are protruding from the folded proteins, and become a target for proteolytic cleavage. In contrast to the results in tobacco and tomato, intracellular expression of the RGS·$(HIS)_6$ -tagged IRX3 protein in *A. thaliana* could be confirmed by Western Blot analysis using the RGS·HIS antibody (Taylor *et al*., 2000). Also the epitope-tagged ECP2 could be detected on a Western Blot after infiltration in the apoplast of *A. thaliana* leaves (Peter van Esse, Phytopathology, Wageningen University, pers. communication). A possible explanation for these apparently conflicting observations could be that the protease involved in cleavage of the epitope-tag is present in the extracellular space in solanaceous species, but not in *A. thaliana.*

Substrate specificity

AtXyn2 was shown to display a dual hydrolytic activity towards both wheat xylan and HE-cellulose. Since the artificial HE-cellulose substrate most closely resembles amorphous cellulose, our result implies that AtXyn2 is likely to degrade β-1,4-glucans, like cellulose and arabinoxylan in the plant cell wall.

The dual hydrolytic activity might find its origin in the structural similarity of the xylan and cellulose polymers. Both polymers consist of β-1,4-linked D-glycosyl residues; the only difference between a glucosyl and xylosyl residue is the CH_2OH group at the C-5 atom, which is present in glucose and absent in xylose (Fry, 1988). In line with this, cellulases showing both endo-β-1,4-glucanase and endo-β-1,4-xylanase activity have been reported. An example is the endo-β-1,4-glucanase from *Thermomonospora* sp., which contains a single active site for the hydrolysis of both xylan and carboxymethyl cellulose (Jagtap and Rao, 2005). Secondly, the dual activity of AtXyn2 could be related to the binding specificity of the CBM22 domains, which could influence the mode of action of the cognate enzyme (Boraston *et al*., 2004). The CBM22 module has been shown to bind to various xylans, mixed-linkage glucan and HE-cellulose (Charnock *et al.*, 2000). The Family 22 CBMs of *Clostridium stercorarium* Xyn10B were shown to bind more strongly to mixed-linkage glucan than to xylan, and to change the specificity of the glycoside hydrolase Family 10 xylanase such that it displayed primarily β-1,3-1,4-glucanase activity. Removal of the CBM22 domains from the catalytic module drastically reduced the activity towards mixed-linkage glucan, however the xylan degrading activity was unaffected by truncation (Araki *et al*., 2004).

Suzuki *et al.* (2002) reported that the modular AtXyn1 protein possibly functions as a xylanase in plants, since over-expression of *AtXyn1* in *A. thaliana* caused an increase in xylan degrading activity in the transgenic plants. However, compared to our results, in this study a very low amount of activity was measured, both in the wild type plants and in the *AtXyn1* transformants. It is uncertain whether AtXyn1 also displays activity against other polymeric substrates than xylan, such as HE-cellulose, xyloglucan or mixed-linkage glucan. It remains to be established whether AtXyn1 also contains a dual hydrolytic activity.

Xylanase inhibitors

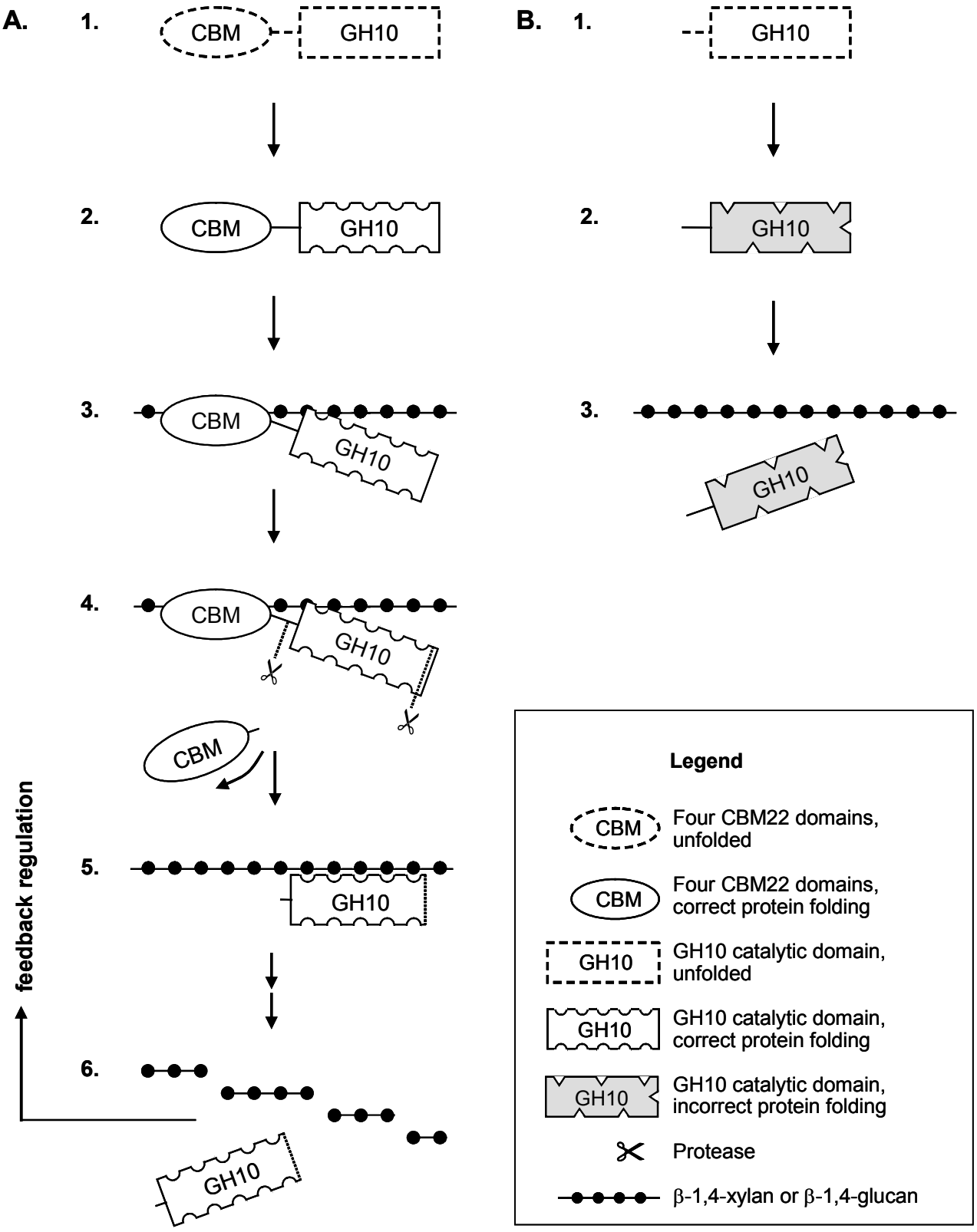
In order to obtain a complete understanding of the functional role of endoxylanases in plants, the possible natural inhibitors of these enzymes should also be taken into account. The overall inhibition of endoxylanase activity has been extensively studied in cereals (reviewed by Bellincampi *et al*., 2004; Goesaert *et al.*, 2004; Igawa *et al*., 2004). To date, two distinct classes of xylanase inhibitors, with different structures and specificities have been described in cereals, the XIP-type and the TAXI-type inhibitors (Gebruers *et al*.,

2004; Juge *et al*., 2004). The XIP-type inhibitors identified so far are specific for fungal endoxylanases Family 10 and 11 and ineffective against other xylanases of bacterial or plant origin (Flatman *et al*., 2002; Juge *et al*., 2004). The TAXI-type xylanase inhibitors inhibit Family 11 xylanases of bacterial and fungal origin, but have no activity towards Family 10 xylanases (Bellincampi *et al.*, 2004). Because all cereal xylanases identified to date belong to glycoside hydrolase Family 10, TAXI-type xylanase inhibitors probably do not play a role as such in plant growth and development (Bellincampi *et al.*, 2004). The observation that XIP- and TAXI-type inhibitors do not show any activity against Family 10 plant xylanases, lead us to conclude that the activity of plant xylanases is likely to be regulated in a different way than through the action of inhibitors.

Model for the regulation of AtXyn2 activity

Based on our results and findings described in literature, we propose a model for the regulation of AtXyn2 activity in the cell wall (Figure 6). After correct folding of the protein, the CBM22 domains of AtXyn2 are thought to target the enzyme to specific regions of the cell wall and to concentrate the enzyme on to the polysaccharide substrates (Figure 6A 1-2-3) (Boraston *et al*., 2004). Since we have shown that AtXyn2 displays a dual hydrolase activity against both wheat xylan and HE-cellulose, the polymeric substrate can either be arabinoxylan or amorphous cellulose. The AtXyn2 protein is latently present and remains inactive until it is processed by an endoprotease (Figure 6A 4), as is the case for barley XYN-1 and papaya EXY1 (Caspers *et al*., 2001; Chen and Paull, 2003). It might be suggested that this protease is only able to reach AtXyn2 when cell wall remodeling has started by e.g. endo-glucanases, and the cellulose-hemicellulose network is loosening. This needs however further investigation. If similar proteases are involved as in barley and papaya, the N- and C-terminal 717 and 52 amino acid residues respectively, are removed during processing of AtXyn2 (see Figure 3), reducing the molecular weight from 121.9 kDa to 34.0 kDa, by which an active enzyme is formed (Figure 6A 4-5-6). It remains unclear whether binding of the catalytic domain to the polymeric substrate occurs before or after post-translational processing.

Interestingly, the AtXyn2 transformants that showed the highest wheat xylan and HE-cellulose degrading activity contained the lowest levels of transgene mRNA. This observation could imply that AtXyn2 activity is regulated on the protein level, as well on the mRNA level. A high concentration of active AtXyn2 protein might lead to lethality. Therefore, either high amounts of AtXyn2, or its products after processing of the intact enzyme, or released oligosaccharides could result in a negative feedback mechanism, and consequently lead to degradation or inactivation of the enzyme. This remains however rather speculative and needs further investigation.


A.
1.
CBM
GH10
2.
CBM
GH10
3.
CBM
GH10
4.
CBM
GH10
CBM
5.
GH10
6.
GH10
feedback regulation
B.
1.
GH10
2.
GH10
3.
GH10
Legend
CBM
Four CBM22 domains, unfolded
CBM
Four CBM22 domains, correct protein folding
GH10
GH10 catalytic domain, unfolded
GH10
GH10 catalytic domain, correct protein folding
GH10
GH10 catalytic domain, incorrect protein folding
Protease
β-1,4-xylan or β-1,4-glucan

Tobacco transformants expressing the *AtXyn2ΔCBM* gene did not show any increased catalytic activity against wheat xylan and HE-cellulose, irrespective of the transgene mRNA level. This result could imply that removal of the CBM22 domains results in incorrect protein folding and thereby to a non-functional enzyme (Figure 6B). This is in agreement with results obtained from studies on barley XYN-1. Heterologous expression of a truncated barley XYN-1, which lacks the first 129 amino acids and therefore most of its CBM22 module, resulted in a largely inactive enzyme. Since even minor truncations of the N- or C-terminal domains severely reduced the activity and the solubility of the enzyme, it was concluded that the complete N- and C-terminal domains of the barley xylanase are required for the correct folding of the protein (Caspers *et al*., 2001). In contrast to plant xylanases, microbial and protozoan xylanases have been shown to retain catalytic activity after removal of N-terminal sequences. Physico-chemical properties and substrate specificity are e.g. identical for *Polyplastron multivesiculatum* XYN10B with or without the CBM22 domain (Devillard *et al*., 2003). Another example is the truncated XynZ endoxylanase from *Clostridium thermocellum* that retains its activity when it is synthesized in transgenic tobacco plants (Herbers *et al*., 1995). The differences in catalytic activity of truncated plant-, microbial- and protozoan xylanases imply that functional differences exist between xylanases from different types of organisms.

In this study we have shown that removal of the CBM22 domains from AtXyn2 results in a loss of enzyme activity. Therefore, the CBM22 domains of AtXyn2 seem to play an important role in the folding and stability of the enzyme and probably a minor role in the substrate specificity. The CBM22 domains are suggested to immobilize the enzyme in proximity of the substrate and mediate the correct folding and the stability of the protein. The activity of the protein is probably regulated in its turn by post-translational cleavage by an endoprotease.

Figure 6. Model for the regulation of AtXyn2 activity. **A**. After correct folding of the AtXyn2 protein (1-2), the CBM22 domains mediate binding of the enzyme to the polymeric substrate, thereby increasing the effective enzyme concentration on the surface of the substrate (3). The polymeric substrate can be either β-1,4-xylan (arabinoxylan) or β-1,4-glucan (amorphous cellulose). The AtXyn2 enzyme remains inactive until it is N- and C-terminally processed by an endoprotease (4). After post-translational processing, the active enzyme hydrolyses the xylan or glucan polymers during several steps into small oligosaccharides (5-6). The fate of the CBM22 domains remains unknown. In this model, the catalytic GH10 domain is suggested to bind the polymeric substrate after post-translational processing. This is however rather speculative; it is also possible that binding proceeds processing of the enzyme. High amounts of released oligosaccharides could trigger a negative feedback mechanism, resulting in degradation or inactivation of the AtXyn2 enzyme. The feedback mechanism could as well be triggered by high amounts of AtXyn2, or by its products released after processing of the intact enzyme. For clarity reasons, this is not included in the figure. **B.** The AtXyn2ΔCBM protein lacks the four N-terminal CBM22 domains. Removal of the N-terminal CBM22s results in incorrect protein folding (1-2) and therefore in a less stable and inactive enzyme (3).

Concluding remarks

The results presented in this paper suggest a dual activity of AtXyn2 against arabinoxylan and amorphous cellulose. The cellulose degrading activity appeared to be even higher than the xylan degrading activity. This raises the question whether AtXyn2 should be considered as a cellulase instead of a xylanase. The answer remains unclear, since, based on the *in vitro* experiments, we can not conclude whether the active AtXyn2 enzyme is also involved in the hydrolysis of both polymers *in planta*, or that it degrades either xylan or cellulose in an *in vivo* situation.

AtXyn2 is suggested to play a role in the remodeling of xylan polymers in the cell wall. The involvement of AtXyn2 in cell wall metabolism has not been demonstrated in this study. Further analysis of the transgenic plants, such as sugar compositional analysis of the cell walls or microscopic analysis, may be useful for clarifying the function of AtXyn2 *in planta*. Additionally, *AtXyn2* knock out mutants or silencing transformants, may be required to determine the relationship between the activity of AtXyn2 and plant morphology. Functional analysis of *AtXyn2* could also be performed in other plant species, like tobacco, using their *AtXyn2* homologues genes for down regulation.

EXPERIMENTAL PROCEDURES

Preparation of constructs for plant transformation

BAC clone T27I1 (*Arabidopsis thaliana* chromosome I) was obtained from the Arabidopsis Biological Resource Center (Columbus, Ohio, USA). This BAC clone contains amongst others the genomic DNA sequence of the putative endo-β-1,4-*xylanase AtXyn2* (T27I1.7), that belongs to the glycoside hydrolase Family 10 (GH10) and contains four N-terminal carbohydrate binding modules of Family 22 (CBM22) (http://afmb.cnrs-mrs.fr/CAZY/). The full length *AtXyn2* and the Family 10 catalytic module devoid of the four CBM22s (*AtXyn2ΔCBM*), both with 5' *Bam*HI and 3' *Sma*I restriction sites, were obtained by PCR amplification from BAC clone T27I1. The sequences of the oligonucleotides used are listed in Table 1.

Standard cloning procedures were applied to assemble the *AtXyn2* and *AtXyn2ΔCBM* constructs (Figure 1) from four DNA fragments: (1) a *Ntp303* signal peptide for translocation into the cell wall (*Hind*III/*Xba*I) (accession number X61146; Weterings *et al*., 1992), (2) a RGS·$(HIS)_6$ -tag for antibody detection (*Xba*I/*Bam*HI), (3) a full length or truncated *AtXyn2* fragment (*Bam*HI/*Sma*I), and (4) a pGreen7K plasmid (a pGreen derivative (Hellens *et al*., 2000)). The pGreen7K plasmid, provided by B. Brandwagt (Phytopathology, Wageningen University), already contained the Kanamycin resistance gene (*NptII*) and a *Hind*III/*Eco*RI inserted multiple cloning site (*Hind*III-*Xba*I-*Bam*HI-*Sma*I-*Eco*RI) flanked by the Cauliflower Mosaic virus 35S promoter (Benfey and Chua, 1990) and the NOS terminator sequence. The oligonucleotide sequences used for the construction of the signal peptide and the RGS·HIS-tag are listed in Table 1. The constructs were sequenced by BaseClear (Leiden, The Netherlands) using T3 (5'-aattaaccctcactaaaggg-3') and T7 (5'-gtaatacgactcactatagggc-3') primers and sequence-specific primers in order to verify their correctness. The AtXyn2 and AtXyn2ΔCBM fusion proteins have a predicted molecular mass of respectively

Table 1. Oligonucleotide sequences used for construction of signal peptide and RGS·HIS fragment and for PCR-amplification of the *A. thaliana endoxylanase* fragments. Introduced restriction sites *Hind*III (aagctt), *Xba*I (tctaga), *Bam*HI (ggatcc) and *Sma*I (cccggg) are underlined.

PCR product	Forward primer (5'-3')	Reverse primer (5'-3')
Signal peptide	agcttatgggaagtggtaaagtaacatttgtggctt-tgctactttgcctctccgtaggggtgatagctt	ctagaagctatcacccctacggagaggcaaagt-agcaaagccacaaatgttactttaccacttcccata
RGS·HIS tag	ctagaagaggatcgcatcaccatcaccatcacg	gatccgtgatggtgatggtgatgcgatcctctt
$(CBM22)_4$-GH10	ggatcccttaacattgtaatgaacggtgac	cccgggttaaagatctataatcacatcaactgga
GH10	ggatccgttcgtaaacgcaatgtttgcctcaa	cccgggttaaagatctataatcacatcaactgga

121.9 and 46.4 kDa. After cleavage of the signal peptide the predicted molecular masses are 119.6 and 44.1 kDa, respectively.

The binary constructs were co-transformed with the helper plasmid pSoup (Hellens *et al*., 2000) into *Agrobacterium tumefaciens* strain LBA4404 by electroporation. The integrity of the binary plasmids was tested by restriction analysis of plasmids isolated from *A. tumefaciens* cultures used for plant transformation.

Plant transformation

Nicotiana tabacum cv. Samsun NN plants were grown under sterile conditions on MS30-gelrite medium (Murashige and Skoog (1962) medium (4.4 g/l), Gamborg B5 vitamins, gelrite 3 g/l and sucrose 30 g/l, pH 5.7-5.9). *A. tumefaciens* containing the recombinant T-DNA constructs were cultured and used to infect tobacco leaf disks (upside-down in the dark for two days at 25 °C). Leaf disks were washed in MS30 medium containing 250 mg/l carbenicillin (Carb) and transferred upside-up to solid callus inducing medium (MS30-gelrite plates containing 0.1 mg/l alpha-Naphtalene acetic acid (NAA), 1 mg/l 6-benzylaminopurine (BAP), 200 mg/l kanamycin (Kan) and 250 mg/l Carb). After a period of 2-4 weeks calli were transferred to shoot inducing medium (MS20-gelrite plates containing 2 mg/l BAP, 0.2 mg/l NAA, 250 mg/l Carb and 100 mg/l Kan). After 4-5 weeks the regenerated shoots were rooted on 1/2MS15-gelrite medium containing 100 mg/l Kan, 200 mg/l claforan and 100 mg/l vancomycin. The formed plants were planted in soil and transferred to the greenhouse to generate mature plants. The presence of the neomycin phosphotransferase (*NPTII*) gene was tested by Southern Blot analysis.

DNA isolation and Southern blot analysis

Genomic DNA was isolated from leaf material of *in vitro* grown *N. tabacum* (wild type and regenerated transformants) plants. Approximately 1.5 g of ground material was transferred to microfuge tubes containing 5 ml DNA extraction buffer (62% urea (w/v), 5 M NaCl, 1 M Tris-HCl pH 8.0, 0.5 M EDTA pH 8.0 and 20% sarcosyl (w/v)) and 5 ml phenol:chloroform. Samples were homogenised, incubated at room temperature for 15 min and centrifuged for 10 min at 2000 g (4°C). The supernatants were subjected to phenol:chloroform extractions until no interface remained. Genomic DNA was obtained after successive 4.4 M ammonium acetate pH 5.2, isopropanol and ethanol precipitations.

To determine the copy number of the *AtXyn2* and *AtXyn2ΔCBM* inserts, genomic DNA was RNAse treated and digested with *Eco*RI or *Hind*III. The products were resolved by electrophoresis through a 0.8% agarose gel (7.5 µg per lane) and transferred overnight onto a Hybond-N+ membrane (Amersham) by capillary transfer in 2 x SSC. The DNA gel blots were pre-hybridised for 3 h at 65°C in modified Church

buffer (Church and Gilbert, 1984). Hybridisation was carried out for 16 h at 65°C in modified Church buffer with a 722 bp *NPTII* fragment labelled with [α-^{32}P]dCTP by random primed labelling (Rediprime II random prime labelling kit, Amersham). The *NPTII* fragment was obtained by PCR amplification from the pGreen7K vector using oligonucleotides 5'-tcggctatgactgggcacaacaga-3' and 5'-aagaaggcgatagaaggcgatgcg-3'. The blots were washed at 65°C, two times in 2 x SSC, 0.1% SDS, two times in 1 x SSC, 0.1% SDS and 2 times in 0.5 x SSC, 0.1% SDS, for 15 min each time. The radioactively labelled blots were exposed to X-OMAT S and AR films (Kodak) at –80°C with intensifying screens.

RNA isolation and Northern blot analysis

To screen the regenerated transformants for the expression of the specific transgenes, total RNA was isolated from five weeks old *in vitro* grown tobacco plants. Approximately 0.2 g fresh plant material was used for RNA isolation with Trizol Reagent (Life Technologies), according to the manufacturer's instructions. Equal amounts of RNA (15 µg) were separated in denaturing formaldehyde 1.5% (w/v) agarose gels. After electrophoresis the RNA was transferred overnight onto a Hybond-N nylon membrane (Amersham) by capillary transfer in 10 x SSC. The RNA blots were pre-hybridised for 4 h at 65°C in modified Church buffer (Church and Gilbert, 1984). Hybridisation was carried out for 16 h at 65°C in modified Church buffer with a [α^{32}P]dCTP labelled 1.1 kb *AtXyn2ΔCBM* fragment, amplified from BAC clone T27I1, using oligonucleotides GH10 forward and reverse (Table 1) for PCR amplification. Labelling was performed with a Rediprime II random prime labelling kit (Amersham) according to supplier's protocol. The membranes were washed at 65°C, 2 times 15 min in 2 x SSC, 1 x SSC and 0.5 x SSC successively, each containing 0.1% SDS. The radioactively labelled blots were exposed to X-OMAT S and AR Scientific Imaging films (Kodak) at –80°C with intensifying screens. After stripping of the blots, hybridisation with a 2.0 kb [α^{32}P]dCTP labelled fragment of a tobacco 23S ribosomal RNA gene was performed as a control. The 2.0 kb ribosomal fragment was amplified from tobacco genomic DNA using oligonucleotides 5'-tcggggagttgaaaataagcata-3' and 5'-agcttcatagggtctttctgtcca-3' for PCR amplification.

Protein extraction

About five weeks old *in vitro* grown tobacco plants were harvested and after removal of the roots, the remaining stem and leaf material was ground in liquid nitrogen with a mortar and pestle. To obtain the optimal extraction buffer, aliquots of tobacco powder were added to several protein extraction buffers (0.5 g powder per ml buffer, 4°C). The following buffers were used: 50 mM Tris-HCl (pH 6.8), 10% (w/v) sucrose, 1% (v/v) β-mercaptoethanol, 1% (w/v) SDS (Wittink *et al*., 2000); 50 mM Tris-HCl (pH 6.8), 0.5 M NaCl, 0.1% (v/v) Tween 20, 5 mM EDTA, protease inhibitor (Complete, Roche) (adapted from Herbers *et al*., 1996); 8 M Urea in 50 mM HEPES (pH 7.5) (Fry, 1988); 100 mM NaOH; 250 mM sodiumphosphate buffer (pH 6.0), 0.5 M NaCl, 5 mM EDTA, 0.1% (v/v) Tween 20, protease inhibitor (Complete, Roche), 37.5 mg/ml PVPP ('native' protein extraction buffer; adapted from Vincken *et al*., 1998). The samples were vortexed and incubated for 2 h at 4°C with gentle shaking. After centrifugation (20 min, 4500 g, 4°C) the supernatant, containing the soluble protein fraction, was collected. The protein samples were concentrated with Centricon Plus-20 devices (Millipore) according to supplier's protocol. Protein concentrations were determined with an ESL protein assay (Roche) using BSA as standard. Equal amounts of protein were analysed by SDS-PAGE and immunoblots and used for measuring the glycoside hydrolase enzyme activities.

Incubation of RGS·$(HIS)_6$ -tagged size marker in protein extract of wild type tobacco

Protein extracts were prepared from wild type tobacco as described above, with minor modifications. Different 'native' protein extraction buffers were used, either lacking or supplemented with 5 mM EDTA and

protease inhibitor (Complete, Roche). Non-concentrated protein extracts were used for incubation with RGS·$(HIS)_6$ -tagged size marker. A batch of RGS·$(HIS)_6$ -tagged protein size marker (Qiagen) was dissolved in 100 µl 50 mM Tris, pH 7.5, so that the final concentration of the individual proteins varied from 50-75 ng/µl. One or 2 µl of epitope-tagged size marker was added to respectively 19 and 18 µl of the tobacco extracts. The mixtures were incubated at room temperature for about 15 h. As a control, RGS·$(HIS)_6$-tagged size marker was incubated in 50 mM Tris, pH 7.5. Protein separation, electro blotting and Western blot analysis, using RGS·HIS antibodies, were performed as described below.

Enzyme assays

Endo-β-1,4-xylanase and endo-β-1,4-glucanase activities were measured using AZCL-xylan (oat), AZCL-arabinoxylan (wheat), AZCL-β-1,3-1,4-glucan (barley), AZCL-HE-cellulose or AZCL-xyloglucan (Megazyme, Bray, Co. Wicklow, Ireland) as substrates. For each assay, 0.5, 1 or 2 mg 'native' protein extract (in 100 µl) was added to 900 µl assay buffer (50 mM sodium citrate, 10 mg/ml chloramphenicol, supplemented with 1% AZCL-substrate). The mixtures were incubated at 30°C for 40 min – 43h, with shaking at 1400 rpm. At different time points, including T=0, 100 µl samples were taken. The samples were placed on ice and insoluble material was removed by centrifugation at 14000 rpm for 2 min at 4°C. The supernatant, containing water-soluble blue products released from insoluble AZCL-substrates due to enzyme activity, was collected. The absorbency of the supernatant was measured at 590 nm with a spectrophotometer. Enzyme activities are expressed in absorbency units corrected for the T=0 values.

Electrophoretic separation of proteins

Protein samples were separated with SDS-PAGE according to the discontinuous buffer system of Laemmli (1970) using 12% SDS-polyacrylamide gels (Mini-Protean II apparatus, Bio-Rad). Proteins were visualised by either Coomassie Brilliant Blue R-250 or silver staining.

Western blot analysis

The proteins separated by SDS-PAGE were electroblotted onto nitrocellulose in 192 mM glycine, 25 mM Tris base, 0.1% (w/v) SDS, 20% (v/v) ethanol, pH 8.3 (Mini-gel transfer apparatus, Bio-Rad). The non-specific binding sites for immunoglobulins on the nitrocellulose membrane were blocked for 2 h with 3% (w/v) BSA (Sigma) in TBS (TBS is 10 mM Tris base, 150 mM NaCl, pH 7.5). After blocking, the membrane was incubated for 2 h with a 1:1000 dilution of the primary anti-RGS·HIS antibody (Qiagen) in a TBS solution containing 3% (w/v) BSA. The membrane was washed twice in TBS-TT buffer (TBS-TT buffer is 20 mM Tris base, 500 mM NaCl, pH 7.5, 0.05% (v/v) Tween 20, 0.2% (v/v) Triton X-100) and once in TBS buffer for 10 min each time. After this, the membrane was incubated for 2 h with a horseradish peroxidase (HRP) conjugated sheep anti-mouse antibody (Amersham), 1:2000 diluted in TBS buffer containing 10% (w/v) non-fat dried milk (ELK, Campina Melkunie BV). The membrane was washed with TBS-TT buffer four times for 10 min each time and subsequently immersed in Supersignal®ULTRA substrate working solution (Pierce), a mixture of equal parts of ULTRA luminol/enhancer solution and ULTRA stable peroxide solution. Finally the membrane was exposed for 1 min - 20 h to X-OMAT S or AR Scientific Imaging films (Kodak) at room temperature.

ACKNOWLEDGEMENTS

We thank the Arabidopsis Biological Resource Center (ABRC) at Ohio State University (USA) for providing the BAC clone T27I1 (*A. thaliana* chromosome I). The work was funded by a grant within the EC Framework V (COPOL, QLK5-2000-01493) and is gratefully acknowledged.

Chapter 5

General Discussion

GENERAL DISCUSSION

The experiments described in this thesis have been performed to explore the possibilities of altering the xylan content, and thereby also the attachment point of lignin, in the plant cell wall. Modification of the cell wall characteristics of plant fibres could result in an increased fibre quality and therefore in an enhanced suitability for industrial applications. More specifically we aimed at: (i) Reduction of xylan content by decreasing the expression level of xylan backbone synthesizing enzymes. (ii) Degradation of already deposited xylan polymers by the introduction of xylan degrading enzymes (xylanases). The results described in the previous chapters are discussed in this chapter, particularly with respect to the different approaches that were used.

Alteration of xylan content by down regulation of backbone synthesizing enzymes

Although in the past years considerable progress has been made in characterizing the structure of the hemicellulosic cell wall polysaccharides, little is known about the biosynthetic enzymes that produce them. At the start of the work described in this thesis (April 2001) the enzymes involved in xylan biosynthesis were not identified. Analysis of the *Arabidopsis thaliana* genome sequence revealed a set of *cellulose synthase-like* (*Csl*) genes, which were important candidate genes for hemicellulose biosynthesis (Cutler and Somerville, 1997; Richmond and Somerville, 2000). Although a *CslA* gene from guar (*Cyamopsis tetragonoloba*) was recently shown to catalyze the accumulation of a β-linked mannan when expressed in soybean cells (Dhugga *et al.*, 2004), the biochemical function of the other *Csl* families remains still unclear. To test the involvement of the candidate *Csl* genes in hemicellulose (in particular xylan) biosynthesis, a heterologous gene silencing approach was applied, in which Inverted Repeat (IR) constructs, based on potato (*Solanum tuberosum*) *Csl* cDNA sequences were used to down regulate the expression level of *Csl* genes in tobacco (*Nicotiana tabacum*) (Chapter 2). If one of the *Csl* family members is coding for a xylan synthase, this strategy consequently would provide us with transgenic tobacco plants with altered xylan levels.

Analysis of the IR tobacco transformants led us to speculate about the involvement of *CslG* in xylan biosynthesis. This speculation was however difficult to substantiate as reduced xylan levels could not be related to reduced *CslG* mRNA expression levels due to the lack of sequence information on tobacco *CslG* genes (Chapter 2). The isolation of a *NtCslE* and *NtCslG* cDNA clone from a tobacco cDNA library allowed us to compare the potato *Csl* sequences with tobacco *Csl* sequences. While the homology between *StCslE* and *NtCslE* turned out to be sufficient to, theoretically, achieve gene silencing in tobacco, the *StCslG1* and *NtCslG* sequences were too divergent to accomplish heterologous gene silencing (Chapter 3). However, since the tobacco *CslG* family seems to consist of at least

two more family members (Chapter 3), it cannot be excluded that one of the other genes shows sufficient homology to *StCslG1* to fulfill gene silencing in tobacco. Gene expression studies revealed that *NtCslG* is mainly expressed in tissues associated with primary wall biosynthesis (Chapter 3). This result argues against a role for *NtCslG* in xylan biosynthesis.

In principle, the heterologous gene silencing strategy should be useful to identify enzymes involved in cell wall biosynthesis. An advantage of this approach is that it directly results in a reduced amount or absence of a particular mRNA and the corresponding protein. Even when a 100% knock out is lethal, plants can be generated which show a range of reduced protein levels and consequently also a range of the phenotype of interest. Unfortunately, we were not successful to identify a xylan synthase by using the heterologous gene silencing approach and some drawbacks have to be mentioned. Analysis of the transformants became very difficult since no sequence information was available on the genes that were subjected to silencing. This favors the use of a homologous gene silencing strategy. However, taking into account factors like plasticity of cell wall architecture, genetic redundancy, low level of gene expression, and cell- or tissue type specificity of at least some of the *Csl* family members (Richmond and Somerville, 2001), the analysis of the transformants could still remain very difficult.

Alternative approaches to identify candidate genes involved in hemicellulose biosynthesis

Despite predictions that hundreds of enzymes may be required to produce the diverse network of glycosidic linkages within the hemicellulose matrix (Perrin *et al.*, 2001), very few genes encoding enzymes involved in the biosynthesis of hemicelluloses have been described. To date, a number of glycosyltransferases (GTs) involved in side chain addition have been identified. The enzymes that are necessary to assemble the hemicellulose backbones remain largely unidentified. In the next section we will summarize the state of the art knowledge on hemicellulose biosynthetic enzymes and the methodologies that have been used to identify and characterize the genes (summarized in Table 1).

The first approach to identify candidate genes involved in (hemi)cellulose biosynthesis, is the use of transcriptional profiling. Genes that change in expression levels in concert with changes in wall biosynthesis can be considered as candidate genes. Transcriptional profiling of developing cotton (*Gossypium hirsutum*) fibre cells undergoing rapid cellulose deposition resulted in the identification of the first plant *cellulose synthase* (*CesA*) gene (Pear *et al*., 1996). A similar approach was used to identify a candidate β-mannan synthase (*ManS*) gene from developing guar (*Cyamopsis tetragonoloba*) seeds undergoing deposition of galactomannan, a seed storage polysaccharide (Dhugga *et al*., 2004). The transcriptional profiling approach is most effective when applied to relatively

Table 1. Glycosyltransferases involved in hemicellulose biosynthesis. Only genes that have been biochemically characterized or for which mutants have been identified are included.

Species and Gene	Activity	Methodology	Reference
PsFTase	xyloglucan α-1,2 fucosylT	biochemical purification	Perrin *et al.,* 1999
AtFUT1	xyloglucan α-1,2 fucosylT	heterologous expression in mammalian COS cells	Perrin *et al.,* 1999
		mur2 mutant (chemically mutagenized)	Vanzin *et al.,* 2002
TfGMGT	galactomannan α-1,6-galactosylT	biochemical purification, heterologuos expression in *Pichia pastoris*	Edwards *et al.,* 1999
		heterologous expression in tobacco seeds	Reid *et al.,* 2003
AtMUR3	xyloglucan β-1,2 galactosylT	*mur3* mutant (chemically mutagenized), heterologous expression in *Pichia pastoris*	Madson *et al.,* 2003
AtXT1	xyloglucan α-1,2 xylosylT	heterologous expression in *Pichia pastoris*	Faik *et al.,* 2002
CtManS	mannan synthase	transcriptional profiling, heterologous expression in soybean somatic embryos	Dhugga *et al.,* 2004
AtCslA	mannan synthase	heterologous expression in *Drosophila* S2 cells	Liepman *et al.,* 2005

Abbreviations: Ps, Pisum sativum; At, Arabidopsis thaliana; Tf, Trigonella foenum-graecum; Ct, Cyamopsis tetragonoloba; FTase, fucosyltransferase; FUT1, fucosyltransferase 1; GMGT, galactomannan galactosyltransferase; XT1, xylosyltransferase 1; ManS, mannan synthase; CslA, cellulose synthase-like Family A; T, transferase.

homogenous tissues that devote most cellular resources to the process of interest. The identification of plant tissues and an experimental design that satisfies these criteria is however very difficult. It should be emphasized that this strategy is valuable for the identification of candidate genes and development of hypotheses regarding their function. However, it cannot be used to prove the actual role of the candidate gene in cell wall biosynthesis.

The second strategy, and frequently the most laborious, is biochemical purification of the enzymes involved in polysaccharide biosynthesis. Peptide sequence information

derived from the purified protein can subsequently be used to identify cDNA clones or genes that encode the protein. The availability of genome sequences is advantageous for the identification of corresponding genes or cDNA sequences, it is however not a prerequisite. The identity of the isolated enzymes has to be confirmed by activity assays. At present the biochemical purification strategy has been successful only for a pea (*Pisum sativum*) xyloglucan fucosyltransferase (*FTase*) (Perrin *et al*., 1999) and a galactomannan galactosyltransferase from fenugreek (*Trigonella foenum-graecum*) (*TfGMGT*) (Edwards *et al*., 1999). No backbone GTs have yet been identified in this manner, perhaps because these proteins are not highly abundant, unstable during purification procedures, or only function in multi-protein complexes. Additionally, the activity assays can be very difficult, especially since acceptor substrates are rarely available, and usually need to be prepared.

Mutant screens have also led to the identification of several hemicellulose biosynthetic enzymes. Screening of chemically mutagenized *A. thaliana* plants for abnormal cell wall monosaccharide composition yielded two mutant lines (*mur2* and *mur3*) that had a severe reduction in cell wall fucose content (Reiter *et al.*, 1997). The *mur2* plants were shown to contain a mutation in the fucosyltransferase *AtFUT1* resulting in a loss of enzyme function and absence of xyloglucan fucosylation (Vanzin *et al.*, 2002). In the *mur3* plants, a mutation in a xyloglucan galactosyltransferase gene was shown to result in an altered xyloglucan structure. The fucogalactosyl side-chains, which are important in binding xyloglucan to cellulose, were completely absent (Madson *et al.*, 2003). The major limitation of this approach is that it is indirect and laborious. The phenotype is difficult to predict beforehand and can be caused by multiple mutations. Additional factors like genetic redundancy and cell- or tissue type specificity of certain genes undoubtedly can make the analysis very complicated.

During the last years, progress has been made in identifying and characterizing some of the enzymes involved in hemicellulose biosynthesis, in particular in side chain addition. These enzymes can be used in yeast-two hybrid or affinity-based purification experiments (immuno-precipitation or tag-based purification) to demonstrate whether they function in a multi-protein complex. Characterization of the interacting proteins may reveal information about additional enzymes involved in the biosynthesis of the specific polysaccharide. This approach allows the usage of GTs involved in side chain addition to obtain more knowledge about the backbone synthesizing enzymes. Immuno-precipitation experiments already showed that *A. thaliana* CesA4, 7 and 8 are all components of the same protein complex (Taylor *et al*., 2000, 2003).

Finally, a proteomics approach could be applied to provide a set of candidate cell wall biosynthetic enzymes. This innovative approach, which has been rapidly developing during the last years, involves the isolation and subsequent characterization of complex protein mixtures from distinct subcellular compartments or the apoplast / cell wall. The

characterization involves protein gel electrophoresis followed by proteolytic digestion and sequencing of derived peptides. The sequence information can subsequently be used to assign the protein to a specific protein class, based on sequence homology with known genes and proteins. The public availability of entire genome sequences for *A. thaliana* and rice (*Oryza sativa*) may favor the use of proteomics approaches for these species. However, the availability of entire genome sequences is not a prerequisite. For example, the proteomics strategy has been successfully used to identify a set of proteins related to secondary wall formation, including a peroxidase, polyphenol oxidase/laccase and extensin, from a xylogenic tobacco cell culture (Blee *et al.*, 2001).

Once candidate genes for hemicellulose biosynthesis have been identified, the enzymatic activities of these genes have to be determined. Commonly used strategies to reveal the function of a candidate gene include gene silencing and heterologous expression. Since the gene silencing strategy has already been discussed in the previous section, here we focus on heterologous expression. The heterologous expression strategy requires functional enzyme assays along with expression systems capable of producing enzymatically active proteins. A number of hemicellulose biosynthetic enzymes have been shown to exhibit activity when expressed in heterologous systems (Edwards *et al.*, 1999; Perrin *et al.*, 1999; Faik *et al.*, 2002; Madson *et al.*, 2003; Reid *et al.*, 2003; Dhugga *et al.*, 2004; Liepman *et al.*, 2005). Regarding the relatively high number of enzymatic activities that have been determined by this approach, the interspecies gene assays seem to be very promising. However, a lack of GT activity can be due to the absence of several factors crucial for enzyme activity, such as additional members of a protein complex, a suitable donor substrate or a suitable acceptor molecule to which sugar residues can be transferred. For example, the enzyme that produces the xyloglucan backbone is thought to lose activity in the absence of an associated xylosyltransferase (Hayashi, 1989; White *et al.*, 1993), and multiple isoforms of CesA proteins are required for cellulose biosynthesis (Saxena and Brown, 2005).

It might have become clear that the different approaches for the identification and functional analysis of genes involved in hemicellulose biosynthesis all have their advantages and limitations. It therefore remains difficult to predict beforehand the best strategy for studying the process of hemicellulose biosynthesis. Regarding the goal of our research, it has to be noted that if one of these approaches leads to the identification of an enzyme that synthesizes the xylan backbone, the corresponding gene still has to be silenced *in planta* in order to achieve an alteration of the xylan content and lignin attachment in the cell wall. An exception is the mutant approach, which directly provides knock out plants.

Degradation of xylan polymers by the introduction of a xylan degrading enzyme

Another approach to generate transformants with altered xylan levels is the specific degradation of the xylan polymers, which are already deposited in the cell wall. In order to achieve this, AtXyn2, a modular Family 10 glycoside hydrolase (GH10) from *A. thaliana* was heterologously expressed in tobacco (Chapter 4). AtXyn2 contains four consecutive CBM22 domains at the N-terminus, which can bring the enzyme into intimate and prolonged contact with its substrate. Therefore, AtXyn2 was expected to be a powerful tool for xylan degradation. This putative endo-β-1,4-*xylanase* was later reported to be expressed in the *A. thaliana* stem (Suzuki *et al.,* 2002) and suggested to be involved in the remodeling of xylan polymers in the cell wall. Our study revealed some very intriguing aspects about AtXyn2. First, we have shown that AtXyn2 displays a dual activity against β-1,4-linked xylans and glucans. The degrading activity against glucan appeared to be even higher than that towards xylan. This raises the question whether AtXyn2 should be considered as a cellulase instead of a xylanase. Second, the catalytic activity of AtXyn2 was shown to be inversely proportional to the *AtXyn2* transcript level. This result led us to speculate that high amounts of AtXyn2, or its hydrolysis products, could result in a negative feedback mechanism and subsequent inactivation or degradation of the enzyme.

AtXyn2 appeared to degrade the highly branched wheat arabinoxylan better than the much less substituted oat spelt xylan. It has been reported that the stalks of *N. tabacum* contain mainly linear unsubstituted xylan (Eda *et al.,* 1976). Therefore, we expected that *AtXyn2* expression did not have a large impact on the xylan levels of the respective tobacco transformants. In addition, the AtXyn2 activity seemed to be extensively controlled via the negative feedback mechanism. As a result of the low intrinsic activity of AtXyn2 we expected only subtle differences in the xylan level between wild type plants and transformants. Therefore, we did not pursue a more detailed characterization of the cell walls of the transformants, but rather focused on clarifying the function of this gene *in planta*. As tobacco has a higher fibre content compared to *A. thaliana*, tobacco is more favorable to study cell wall processes like xylogenesis, in which AtXyn2 might catalyze the rearrangement of xylan polymers. Therefore, we continued with the isolation of the tobacco homologues of *AtXyn2* (see next section). Once these genes are available, they can be used in a homologous gene silencing strategy to reveal their function *in planta*.

Molecular cloning and sequence analysis of tobacco GH10 enzymes

The 1122 base pairs *GH10* fragment of *AtXyn2* was used to screen the cDNA library constructed from mRNAs isolated from three days old xylogenic tobacco cell cultures[1].

[1] For cDNA library details and screening of the cDNA library, we refer to the Experimental Procedures section in Chapter 3.

The screening resulted in the isolation of eight full length cDNA clones. Sequencing of these clones followed by homology searches revealed that they encode two different putative endo-β-1,4-*xylanase* genes, *NtXyn1* and *NtXyn2*. The coding regions of the *NtXyn1* and *NtXyn2* cDNAs are both 2757 base pairs long and share 96% sequence identity. The size of the predicted protein sequences is 918 amino acids; the amino acid sequences share 96% identity. The *N. tabacum Xyn1* and *Xyn2* sequences have been deposited in the GenBank database (accession no. DQ152919 and DQ152920, respectively).

The putative *NtXyn* genes are coding for modular enzymes that consist of three consecutive CBM22 domains joined to a GH10 catalytic module (Figure 1A). The majority of enzymes of the GH10 family are endo-β-1,4-xylanases (xylanases), however substrate specificity studies have shown that this family also contains endo-β-1,3-xylanases or cellobiohydrolases (see the carbohydrate-active enzyme CAZY server at http://afmb.cnrs-mrs.fr/CAZY). All GH10 proteins have the conserved active site motif [G/T/A]-X-X-[L/I/V/N]-X-[I/V/M/F]-[S/T]-E-[L/I/Y]-[D/N]-[L/I/V/M/F] (X represents any amino acid residue) (Henrissat, 1997, prosite 00591). Comparison of the amino acid sequences of the putative catalytic GH10 domains of NtXyn1 and NtXyn2 (amino acid 571 until 919) with this conserved motif revealed that the tobacco enzymes nearly match the consensus sequence (Figure 1B). A substitution of V_{797} for L/I/Y was found in the NtXyn proteins. This substitution results only in a minor change in the nature of the amino acid. The amino acids E_{695} and E_{796} correspond to the conserved amino acids that have been shown to play a crucial role in hydrolysis, see Figure 1B (Tull *et al.,* 1991; MacLeod *et al*., 1994).

The surface of the ligand binding cleft of CBM22 domains contains a tryptophan (W) and two tyrosine (Y) residues. These amino acid residues are highly conserved among CBM22 members and have been shown to be involved in xylose binding in one of the CBM22 domains of *Clostridium thermocellum* Xyn10B (Xie *et al.,* 2001). The W and the first Y residue are likely to form hydrophobic stacking interactions with the xylan substrate, whereas the second Y residue is suggested to interact with the ligand via the hydroxyl group (Xie *et al.,* 2001). The first CBM22 domain of NtXyn has these conserved amino acids, whereas in the second and third CBM22 module, one Y residue is replaced with either W or phenylalanine (F) (Figure 1B). In the substitution of Y with W, two aromatic residues are exchanged. This minor difference is not likely to affect the binding affinity with the xylan substrate. Additonally, Xie *et al.* (2001) revealed that a *Clostridium thermocellum* CBM22 mutant, in which the second Y residue was changed into F, shows only a modest reduction in affinity for xylan. It can therefore be suggested that the three CBM22 domains of NtXyn might be able to bind xylan. However, Family 22 CBMs have not only been shown to bind to various xylans, but also to mixed-linkage glucan and HE-cellulose (Charnock *et al.,* 2000). In Chapter 4 we have shown that AtXyn2 is very likely to display a

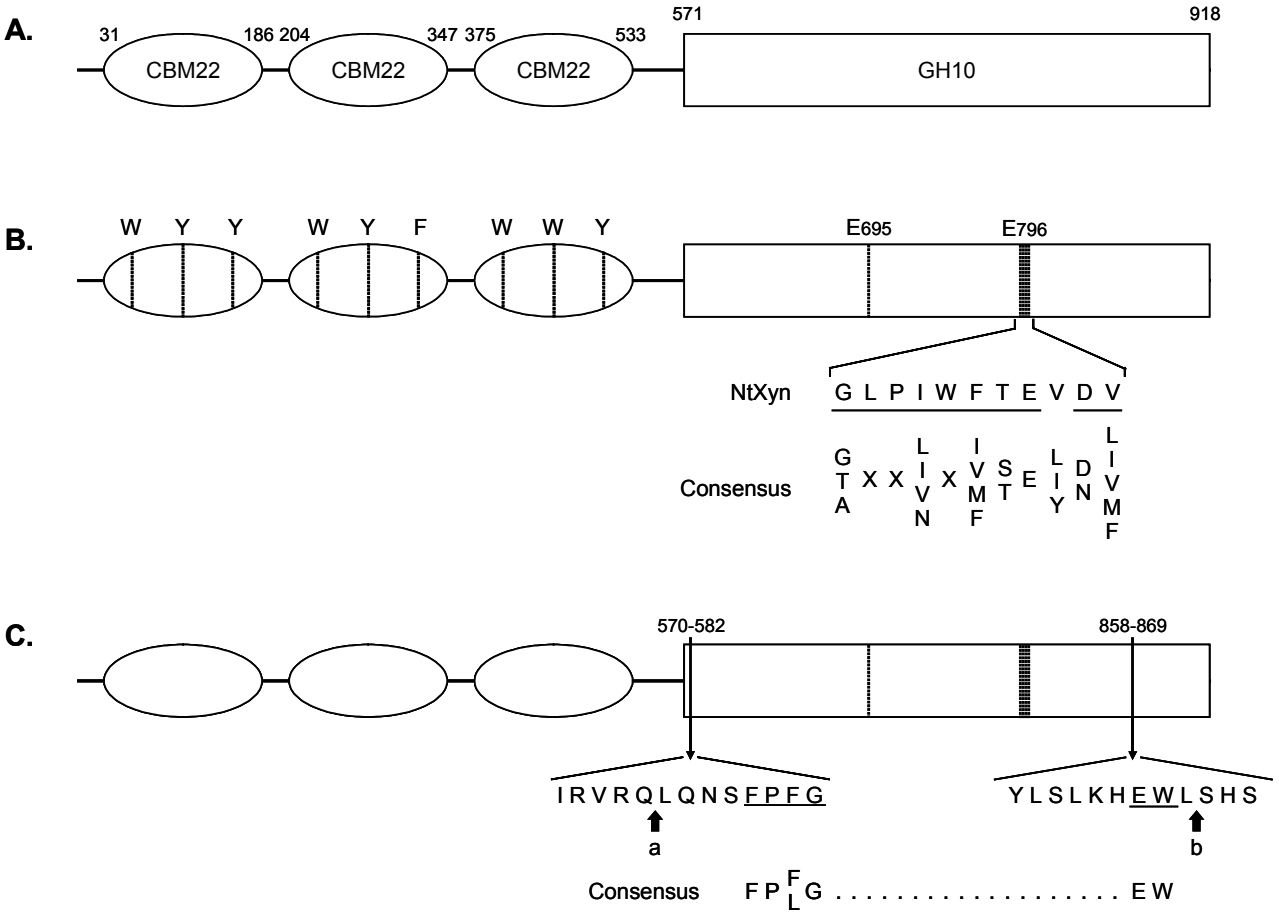


Figure 1. Schematic representation of predicted NtXyn proteins. **A.** Three consecutive CBM22 modules and a GH10 catalytic domain are indicated. The numbers refer to the first or last amino acid residue of each domain. **B.** Conserved amino acid residues involved in interaction with xylan are indicated above the CBM22 domains. E_{695} and E_{796} refer to the conserved catalytic residues important for hydrolysis. A comparison of the amino acid sequences of residues 789-799 of NtXyn and the consensus sequence of the glycoside hydrolase Family 10 active site is shown. Amino acid residues that match the consensus sequence are underlined. X can be any amino acid residue. **C.** Post-translational processing of NtXyn. Numbers refer to amino acid positions. The consensus sequence, determined in Chapter 4, is indicated. Amino acids that match the consensus are underlined. Arrow **a** and **b** indicate the putative post-translational cleavage sites, based upon barley XYN-1 post-translational processing (described in Chapter 4).

dual activity against arabinoxylan and amorphous cellulose. Therefore, (heterologous) expression of NtXyn in for instance *Escherichia coli*, yeast or plants, followed by analysis of the hydrolytic activity against several polymeric substrates, is necessary to reveal the substrate specificity of the enzymes. If post-translational processing is essential to activate the NtXyn enzymes (discussed in next section), (heterologous) expression *in planta* would be preferable.

BLAST analysis (Altschul *et al.*, 1997) of the deduced NtXyn protein sequences revealed a very high sequence homology to a putative hybrid aspen (*Populus tremula x*

tremuloides) xylanase (PttXyn10) and Xyn1 from *A. thaliana* (74% identity to PttXyn10 and 66% identity to AtXyn1). Like NtXyn, the PttXyn10 and AtXyn1 proteins also contain three CBM22 modules at the N-terminal side of the GH10 domain. *AtXyn1* is predominantly expressed in vascular bundles (Suzuki *et al.,* 2002) and *PttXyn10* has been shown to be highly up-regulated in wood tissue undergoing secondary cell wall formation (Aspeborg *et al.,* 2005). Based on these results AtXyn1 and PttXyn10 are suggested to be involved in xylan hydrolysis or remodeling during xylogenesis (Suzuki *et al.,* 2002; Aspeborg *et al.,* 2005). This putative function can therefore also be suggested for our NtXyn proteins. In order to reveal the biological function of *NtXyn*, knock-out mutants or transgenic tobacco plants in which the *NtXyn* levels are drastically reduced, are required.

Post-translational processing of NtXyn

In Chapter 4 we proposed a model, which might explain the regulation of AtXyn2 activity in the cell wall (Chapter 4, Figure 6). In this model, AtXyn2 is suggested to remain inactive until it is processed by endoproteases, as is also described for barley (*Hordeum vulgare*) XYN-1 and papaya (*Carica papaya*) EXY1 (Caspers *et al.,* 2001; Chen and Paull, 2003). The conserved amino acid motifs, the N-terminal FP(F/L)G sequence, the C-terminal EW motif, and their protease cleavage sites are shown in Figure 3 (Chapter 4). Sequence comparison of NtXyn with AtXyn2, Xyn-1 and EXY1 sequences revealed the presence of these conserved amino acids and the putative N- and C-terminal cleavage sites also in NtXyn (Figure 1C). This result implies that NtXyn is also subjected to post-translational processing. During processing of NtXyn, the N- and C-terminal 574 and 53 amino acid residues, respectively, will probably be cleaved off, thereby reducing the molecular weight and activating the enzyme. In conclusion, our model proposed for the regulation of AtXyn2 activity in the cell wall seems also valid for the NtXyn proteins. Interestingly, the FPLG motif is also present at amino acid position 391-394, in the third CBM22 domain. However, the biological relevance of this additional protease recognition site is presently unknown.

Concluding remarks

In this chapter we described different approaches to alter the xylan content in the cell wall. The identification of candidate genes involved in xylan biosynthesis and subsequent down-regulation remains a useful strategy, although several limitations and difficulties of this approach have to be kept in mind. Another approach is the degradation of xylan polymers by the introduction of xylan degrading enzymes. Further analysis of the *NtXyn* genes described in this chapter could reveal whether these genes are coding for functional endo-β-1,4-xylanases. If so, the *NtXyn* genes can be used to generate transgenic tobacco plants with increased or decreased *NtXyn* expression levels in order to alter the xylan level and thereby also attachment of lignin in the cell wall. If NtXyn, like AtXyn2, displays a dual

hydrolytic activity, the heterologous expression of characterized bacterial or fungal xylanases, would be a better alternative to degrade xylan polymers *in planta*. Extensively investigated microbial xylanases originate for example from *Bacillus*, *Aspergillus* and *Trichoderma* species as reviewed by Collins *et al.* (2005).

REFERENCES

Ahlgren, P.A. and Goring, D.A.I. (1971) Removal of wood components during chlorite delignification of black spruce. *Can. J. Chem*. **49**, 1271-1275.

Akin, D.E., Morrison III, W.H., Rigsby, L.L. and Dodd, R.B. (2001) Plant factors influencing enzyme retting of fiber and seed flax. *J. Agric. Food Chem.* **49**, 5778-5784.

Altschul, S.M., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman. D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucl. Acids Res*. **25**, 3389-3402.

Anterola, A.M. and Lewis, N.G. (2002) Trends in lignin modification: a comprehensive analysis of the effects of genetic manipulations/mutations on lignification and vascular integrity. *Phytochem.* **61**, 221-294.

Araki, R., Ali, M.K., Sakka, M., Kimura, T., Sakka, K. and Ohmiya, K. (2004) Essential role of the family-22 carbohydrate-binding modules for β-1,3-1,4-glucanase activity of *Clostridium stercorarium* Xyn10B. *FEBS letters* **561**, 155-158.

Arioli, T., Peng, L., Betzner, A.S., Burn, J., Wittke, W., Herth, W., Camilleri, C., Höfte, H., Plazinski, J., Birch, R., Cork, A., Glover, J., Redmond, J. and Williamson, R.E. (1998) Molecular analysis of cellulose biosynthesis in *Arabidopsis*. *Science* **279**, 717-720.

Aspeborg, H., Schrader, J., Coutinho, P.M., Stam, M., Kallas, A., Djerbi, S., Nilsson, P., Denman, S., Amini, B., Sterky, F., Master, E., Sandberg, G., Mellerowicz, E., Sundberg, B., Henrissat, B. and Teeri, T.T. (2005) Carbohydrate-active enzymes involved in the secondary wall biogenesis in hybrid aspen. *Plant Physiol.* **137**, 983-997.

Awano, T., Takabe, K. and Fujita, M. (2001) Xylan and lignin deposition on the secondary wall of *Fagus crenata* fibers. In *Molecular Breeding of Woody Plants* (Morohoshi, N. and Komamine, A., eds). The Netherlands: Elsevier Science B.V., pp. 137-142.

Bacic, A., Harris, P.J. and Stone, B.A. (1988) Structure and function of plant cell walls. In *The Biochemistry of Plants* (ed. J. Priess), Academic Press, New York, pp. 297-371.

Bailey, R.W. and Hassid, W.Z. (1966) Xylan synthesis from uridine-diphospate-D-xylose by particulate preparations from immature corn-cobs. *Proc. Natl. Acad. Sci. USA* **56**, 574-580.

Banik, M., Garrett, T.P. and Fincher, G.B. (1996) Molecular cloning of cDNAs encoding (1→4)-beta-xylan endohydrolases from the aleurone layer of germinated barley (*Hordeum vulgare*) *Plant Mol. Biol.* **31**, 1163-1172.

Baydoun, E.A.H., Waldron, K.W. and Brett, C.T. (1989) The interaction of xylosyltransferase and glucuronyltransferase involved in glucuronoxylan synthesis in pea (*Pisum sativum*) epicotyls. *Biochem. J.* **257**, 853-858.

Bellincampi, D., Camerdella, L., Delcour, J.A., Desseaux, V., D'Ovidio, R., Durand,A., Elliott, G., Gebruers, K., Giovane, A., Juge, N., Sorensen, J.F., Svensson, B. and Vairo, D. (2004) Potential physiological role of plant glycosidase inhibitors. *Biochim. Biophys. Acta* **1696**, 265-274.

Benedito, V.A., Visser, P.B., Angenent, G.C. and Krens, F.A. (2004) The potential of virus-induced gene silencing for speeding up functional characterization of plant genes. *Genet. Mol. Res.* **3**, 323-341.

Benfey, P.N. and Chua, N.H. (1990) The Cauliflower Mosaic Virus 35S promoter: Combinatorial regulation of transcription in plants. *Science* **250**, 959-966.

Bernstein, E., Caudy, A.A., Hammond, S.M. and Hannon, C.J. (2001) Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* **409**, 363-366.

Bewley, J.D., Banik, M., Bourgault, R., Feurtado, J.A., Toorop, P. and Hilhorst, H.W.M. (2000) Endo-β-mannanase activity increases in the skin and outer pericarp of tomato fruits during ripening. *J. Exp. Bot.* **51**, 529-538.

Biely, P., Vrsanska, M., Tenkanen, M. and Kluepfel, D. (1997) Endo-β-1,4-xylanase families: differences in catalytic properties. *J. Biotech*. **57**, 151-166.

Bih, F.Y., Wu, S.S., Ratnayake, C., Walling, L.L., Northnagel, E.A. and Huang, A.H. (1999) The predominant protien on the surface of maize pollen is an endoxylanase synthesized by a tapetum mRNA with a long 5' leader. *J. Biol. Chemistry* **274**, 22884-22894.

Bindschedler, L.V., Wheatley, E., Gay, E., Cole, J., Cottage, A. and Bolwell, G.P. (2005) Characterization and expression of the pathway from UDP-glucose to UDP-xylose in differentiating tobacco tissue. *Plant Mol. Biol.* **57**, 285-301.

Blee, K.A., Wheatley, E.R., Bonham, V.A., Mitchell, G.P., Robertson, D., Slabas, A.R., Burrell, M.M., Wojtaszek, P. and Bolwell, G.P. (2001) Proteomic analysis reveals a novel set of cell wall proteins in a transformed tobacco cell culture that synthesizes secondary walls as determined by biochemical and morphological parameters. *Planta* **212**, 404-415.

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

Bolam, D.N., Ciruela, A., McQueen-Mason, S., Simpson, P., Williamson, M.P., Rixon, J.E., Boraston, A., Hazlewood, G.P. and Gilbert, H.J. (1998) Pseudomonas cellulose-binding domains mediate their effects by increasing enzyme substrate proximity. *Biochem. J.* **331**, 775-781.

Bolam, D.N., Xie, H.F., White, P., Simpson, P.J., Hancock, S.M., Williamson, M.P. and Gilbert, H.J. (2001) Evidence for synergy between family 2b carbohydrate binding modules in *Cellulomonas fimi* Xylanase 11A. *Biochem.* **40**, 2468-2477.

Bolwell, G.P. (1993) Dynamic aspects of the plant extracellular matrix. *Int. Rev. Cytol.* **146**, 261-324.

Bolwell, G.P. and Northcote, D.H. (1981) Control of hemicellulose and pectin synthesis during differentiation of vascular tissue in bean (*Phaseolus vulgaris*) callus and in bean hypocotyl. *Planta* **152**, 225-233.

Bonetta, D.T., Facette, M., Raab, T.K. and Somerville, C.R. (2002) Genetic dissection of plant cell wall biosynthesis. *Biochem. Soc. Trans.* **30**, 298-301.

Boraston, A.B., Bolam, D.N., Gilbert, H.J. and Davies, G.J. (2004) Carbohydrate-binding modules: fine-tuning polysaccharide recognition. *Biochem. J.* **382**, 769-781.

Boudet, A.M. (1998) A new view of lignification. *Trends Plant Sci.* **3**, 67-71.

Boudet, A.M. (2003) The supramolecular organization of the lignified wall. In *The plant cell wall* (ed. J.K.C. Rose). Blackwell Publishing, Oxford, UK.

Boudet, A.M., Chabannes, M., Goffner, D. *et al.* (1998) Controlled down-regulation of genes involved in the last steps of lignin synthesis may significantly change the lignin profiles of plants. Proceedings of the 'Molecular breeding of woody species', Tokyo, Japan, 26-27 August 1988, pp. 1-21.

Boudet, A.M., Kajita, S., Grima-Pettenati, J. and Goffner, D. (2003) Lignins and lignocellulosics: a better control of synthesis for new and improved uses. *Trends Plant Sci.* **8**, 576-581.

Brady, J.D., Sadler, I.H. and Fry, S.C. (1996) Di-isodityrosine, a novel tetrameric derivative of tyrosine in plant cell wall proteins: a new potential cross-link. *Biochem. J.* **315**, 323-327.

Brown, R.M. jr. (1996) The biosynthesis of cellulose. *J. Macromol. Sci. Pure App. Chem.* **VA33**, 1345-1373.

Brummell, D.A. and Labavitch, J.M. (1997) Effect of antisense suppression of endopolygalacturonase activity on polyurodine molecular weight in ripening tomato fruit and in fruit homogenates. *Plant Physiol.* **115**, 717-725.

Brummell, D.A., bird, C.R., Schuch, W. and Bennett, A.B. (1997) An endo-1,4-β-glucanase expressed at high levels in rapidly expanding tissues. *Plant Mol. Biol.* **33**, 87-95.

Burton, R.A., Gibeaut, D.M., Bacic, A., Findlay, K., Roberts, K., Hamilton, A., Baulcombe, D.C. and Fincher, G.J. (2000) Virus-induced silencing of a plant cellulose synthase gene. *Plant Cell* **12**, 691-705.

Carpita, N., Tierney, M. and Campbell, M. (2001) Molecular biology of the plant cell wall: searching for the genes that define structure, architecture and dynamics. *Plant Mol. Biol.* **47**, 1-5.

Carpita, N.C. (1996) Structure and biogenesis of the cell walls of grasses. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **47**, 445-476.

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

Carpita, N.C. and McCann, M. (2000) The cell wall. In *Biochemistry and Molecular Biology of Plants* (eds B. Buchanan, W. Gruissem and R. Jones). American Society of Plant Physiologists, Rockeville, USA, pp. 52-108.

Carpita, N.C., McCann, M. and Griffing, L.R. (1996) The plant extracellular matrix: news from the cell's frontier. *Plant Cell* **8**, 1451-1463.

Caspers, M.P.M., Lok, F., Sinjorgo, K.M.C., van Zeijl, M.J., Nielsen, K.A. and Cameron-Mills, V. (2001) Synthesis, processing and export of cytoplasmic endo-β-1,4-xylanase from barley aleurone during germination. *Plant J.* **26**, 191-204.

Cerutti, H. (2003) RNA interference: travelling in the cell and gaining functions? *Trends Genet.* **19**, 39-46.

Charnock, S.J. and Davies, G.J. (1999) Structure of the nucleotide-diphospho-sugar transferase, SpsA from *Bacillus subtilis*, in native and nucleotide-complexed forms. *Biochem.* **38**, 6380-6385.

Charnock, S.J., Bolam, D.N., Turkenburg, J.P., Gilbert, H.J., Ferreira, L.M., Davies, G.J. and Fontes, C.M. (2000) The X6 'thermostabilizing' domains of xylanases are carbohydrate-binding modules: structure and biochemistry of the *Clostridium thermocellum* X6b domain. *Biochem.* **39**, 5013-5021.

Charnock, S.J., Henrissat, B. and Davies, G.J. (2001) Three-dimensional structures of UDP-sugar glycosyltransferases illuminate the biosynthesis of plant polysaccharides. *Plant Physiol.* **125**, 527-531.

Chen, N.J. and Paull, R.E. (2003) Endoxylanase expressed during papaya fruit ripening: purification, cloning and characterization. *Func. Plant Biol.* **30**, 433-441.

Chomczynsky, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156-159.

Chuang, C.F. and Meyerowitz, E.M. (2000) Specific and heritable genetic interference by double-stranded RNA in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. USA* **97**, 4985-4990.

Church, M.G. and Gilbert, W. (1984) Genomic sequencing. *Proc. Natl. Acad. Sci. USA* **81**, 1991-1995.

Claeyssens, M. and Henrissat, B. (1992) Specificity mapping of cellulolytic enzymes: classification into families of structurally related proteins confirmed by biochemical analysis. *Protein Science* **1**, 1293-1297.

Clarke, J.H., Rixon, J.E., Ciruela, A., Gilbert, H.J. and Hazlewood, G.P. (1997) Family-10 and Family-11 xylanases differ in their capacity to enhance the bleach ability of hardwood and softwood paper pulps. *Appl. Microbiol. Biotechnol.* **48**, 177-183.

Cleemput, G., Hessing, M., van Oort, M., Deconynck, M. and Delcour, J.A. (1997) Purification and characterization of β-D-xylosidase and an endo-xylanase from wheat flour. *Plant Physiol.* **113**, 377-386.

Collins, T., Gerday, C. and Feller, G. (2005) Xylanases, xylanase families and extremophilic xylanases. *FEMS Micobiol. Rev.* **29**, 3-23.

Cosgrove, D.J. (1999) Enzymes and other agents that enhance cell wall extensibility. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **50**, 391-417.

Cosgrove, D.J. (2000) Loosening of plant cell walls by expansins. *Nature* **407**, 321-326.

Coughlan, M.P. and Hazlewood, G.P. (1993) Beta-1,4-D-xylan-degrading enzyme systems: biochemistry, molecular biology and applications. *Biotechnol. Appl. Biochem.* **17**, 259-289.

Coutinho, P.M. and Henrissat, B. (1999) Carbohydrate-active enzyme server (CAZY) at URL: http://afmb.cnrs-mrs.fr/~cazy/CAZY/.

Coutinho, P.M., Stam, M., Blanc, E. and Henrissat, B. (2003) Why are there so many carbohydrate-active enzyme-related genes in plants? *Trends Plant Sci.* **8**, 563-565.

Cutler, S. and Somerville, C. (1997) Cellulose synthesis: cloning in silico. *Curr. Biol.* **7**, R108-R111.

De Vries, J.A., Voragen, A.G.J., Rombouts, F.M. and Pilnik, W. (1981) Extraction and purification of pectins from alcohol insoluble solids from ripe and unripe apples. *Carbohydr. Polym.* **1**, 117-127.

Delmer, D. D. and Amor, Y. (1995) Cellulose biosynthesis. *Plant Cell* **7**, 987-1000.

Delmer, D.P. (1999) Cellulose biosynthesis: Exciting times for a difficult field of study. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **50**, 245-276.

Denli, A.M. and Hannon, G.J. (2003) RNAi: an ever-growing puzzle. *Trends Biochem. Sci.* **28**, 196-201.

Devillard, E., Bera-Maillet, C., Flint, H.J., Scott, K.P., Newbold, C.J., Wallace, R.J., Jouany, J-P. and Forano, E. (2003) Characterization of XYN10B, a modular xylanase from the ruminal protozoan *Polyplastron multivesiculatum*, with a family 22 carbohydrate-binding module that binds to cellulose. *Biochem. J.* **373**, 495-503.

Dhugga, K.S. (2001) Building the wall: genes and enzyme complexes for polysaccharide synthases. *Curr. Op. Plant Biol.* **4**, 488-493.

Dhugga, K.S., Barreiro, R., Whitten, B., Stecca, K., Hazebroek, J., Randhawa, G.S., Dolan, M., Kinney, A.J., Tomes, D., Nichols, S. and Anderson, P. (2004) Guar seed β-mannan synthase is a member of the cellulose synthase super gene family. *Science* **303**, 363-366.

Doblin, M.S., Vergara, C.E., Read, S., Newbigin, E. and Bacic, A. (2003) Plant cell wall biosynthesis: making the bricks. In *The plant cell wall* (ed. J.K.C. Rose). Blackwell Publishing, Oxford, UK.

Ebringerova, A. and Heinze, T. (2000) Xylan and xylan derivatives – biopolymers with valuable properties. Naturally occurring xylan structures, isolation procedures and properties. *Macromol. Rapid Commun.* **21**, 542-556.

Ebskamp, M.J.M. (2002) Engineering flax and hemp for an alternative to cotton. *Trends Biotech.* **20**, 229-230.

Eda, S., Ohnishi, A. and Kato, K. (1976) Xylan isolated from the stalk of *Nicotiana tabacum*. *Agric. Biol. Chem.* **40**, 359-364.

Edwards, M.E., Dickson, C.A., Chengappa, S., Sidebottom, C., Gidley, M.J. and Grant Reid, J.S. (1999) Molecular characterisation of a membrane-bound galactosyltransferase of plant cell wall matrix polysaccharide biosynthesis. *Plant J.* **19**, 691-697.

Elmayan, T. and Vaucheret, H. (1996) Expression of single copies of a strongly expressed *35S* transgene can be silenced post-transcriptionally. *Plant J.* **9**, 787-797.

Emons, A.M.C. and Mulder, M. (2000) How the deposition of cellulose microfibrils builds cell wall architecture. *Trends Plant Sci.* **5**, 35-40.

Emons, A.M.C., Schel, J.H.N. and Mulder, M. (2002) The geometrical model for microfibril deposition and the influence of the cell wall matrix. *Plant Biol.* **4**, 22-26.

Englyst, H.N. and Cummings, J.H. (1984) Simplified method for the measurement of total non-starch polysaccharides by gas-liquid chromatography of constituent sugars as alditol acetates. *Analyst* **109**, 937-942.

Faik, A., Price, N.J., Raikhel, N.V. and Keegstra, K. (2002) An *Arabidopsis* gene encoding an α-xylosyltransferase involved in xyloglucan biosynthesis. *Proc. Natl. Acad. Sci. USA* **99**, 7797-7802.

Favery, B., Ryan, E., Foreman, J., Linstead, P., Boudonck, K., Steer, M., Shaw, P. and Dolan, L. (2001) *KOJAK* encodes a cellulose synthase-like protein required for root hair cell morphogenesis in *Arabidopsis*. *Genes Dev*. **15**, 79-89.

Flatman, R., McLauchlan, W.R., Juge, N., Furniss, C., Berrin, J.G., Hughes, R.K., Manzanares, P., Ladbury, J.E., O'Brien, R. and Williamson, G. (2002) Interactions defining the specificity between fungal xylanases and the xylanase-inhibiting protein XIP-1 from wheat. *Biochem. J.* **365**, 773-781.

Fontaine, A.S., Bout, S., Barrière, Y. and Vermerris, W. (2003) Variation in cell wall composition among forage maize (*Zea mays* L.) inbred lines and its impact on digestibility: analysis of neutral detergent fiber composition by pyrolysis-gas chromatography-mass spectrometry. *J. Agric. Food Chem.* **51**, 8080-8087.

Fontes, C.M.G.A., Hazlewood, G.P., Morag, E., Hall, J., Hirst, B.H. and Gilbert, H.J. (1995) Evidence for a general role for non-catalytic thermostabilizing domains in xylanases from thermophilic bacteria. *Biochem. J.* **307**, 151-158.

Freelove, A.C.J., Bolam, D.N., White, P., Hazlewood, G.P and Gilbert, H.J. (2001) A novel carbohydrate-binding protein is a component of the plant cell wall-degrading complex of *Piromyces equi. J. Biol. Chem.* **276**, 43010-43017.

Fry, S.C. (1988) The growing plant cell wall: chemical and metabolic analysis. Longman Scientific & Technical, Essex, U.K.

Fry, S.C., York, W.S., Albersheim, P., Darvill, A., Hayashi, T., Joseleau, J.-P., Kato, Y., Lorences, E.P., Maclachlan, G.A., McNeil, M., Mort, A.J., Reid, J.S.G., Seitz, H.U., Selvendran, R.R., Voragen, A.G.J. and White, A.R. (1993) An unambiguous nomenclature for xyloglucan-derived oligosaccharides. *Physiol. Plant* **89**, 1-3.

Gardner, S.L., Burrell, M.M. and Fry, S.C. (2002) Screening of *Arabidopsis thaliana* stems for variation in cell wall polysaccharides. *Phytochem*. **60**, 241-254.

Gaspar, Y., Johnson, K.L., McKenna, J.A., Bacic, A. and Schultz, C.J. (2001) The complex structure of arabinogalactan-proteins and the journey towards understanding function. *Plant Mol. Biol.* **47**, 161-176.

Gebruers, K., Brijs, K., Courtin, C.M., Fierens, K., Goesaert, H., Rabijns, A., Raedschelders, G., Robben, J., Sansen, S., Sorensen, J.F., Van Campenhout, S. and Delcour, J.A. (2004) Properties of TAXI-type endoxylanase inhibitors. *Biochim. Biophys. Acta* **1696**, 213-221.

Gibeaut, D.M. (2000) Nucleotide sugars and glycosyltransferases for synthesis of cell wall matrix polysaccharides. *Plant Physiol. Biochem.* **38**, 69-80.

Gibeaut, D.M. and Carpita, N.C. (1990) Separation of membranes by flotation centrifugation for in vitro synthesis of plant cell wall polysaccharides. *Protoplasma* **156**, 82-93.

Gibeaut, D.M. and Carpita, N.C. (1994) Biosynthesis of plant cell wall polysaccharides. *FASEB J.* **8**, 904-915.

Gilbert, H.J. and Hazlewood, G.P. (1993) Bacterial cellulases and xylanases. *J. Gen. Microbiol.* **139**, 187-194.

Goesaert, H., Elliott, G., Kroon, P.A., Gebruers, K., Courtin, C.M., Robben, J., Delcour, J.A. and Juge, N. (2004) Occurrence of proteinaceous endoxylanase inhibitors in cereals. *Biochim. Biophys. Acta* **1696**, 193-202.

Goubet, F., Misrahi, A., Park, S.K., Zhang, Z., Twell, D. and Dupree, P. (2003) *AtCslA7*, a cellulose synthase-like putative glycosyltransferase, is important for pollen tube growth and embryogenesis in *Arabidopsis*. *Plant Physiol*. **131**, 547-557.

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

Grima-Pettenati, J. and Goffner, D. (1999) Lignin genetic engineering revisited. *Plant Science* **145**, 51-65.

Gruppen, H., Hoffman, R.A., Kormelink, F.J.M., Voragen, A.G.J., Kamerling, J.P. and Vliegenthart, J.F.G. (1992) Characterization by 1H NMR spectroscopy of enzymatically derived oligosaccharides from alkali-extractable wheat-flour arabinoxylan. *Carbohydr. Res.* **233**, 45-64.

Hall, J. and Gilbert, H.J. (1988) The nucleotide sequence of a carboxymethylcellulase from *Pseudomonas fluorescens* subsp. *cellulosa*. *Mol. Gen. Genet.* **213**, 112-117.

Halpin, C., Knight, M.E., Foxon, G.A., Campbell, M.M., Boudet, A.-M., Boon, J.J., Chabbert, B., Tollier, M.T. and Schuch, W. (1994) Manipulation of lignin quality by down-regulation of cinnamyl alcohol dehydrogenase. *Plant J.* **6**, 339-350.

Hamann, T., Osborne, E., Youngs, H.L., Misson, J., Nussaume, L. and Somerville, C. (2004) Global expression analysis of *CESA* and *CSL* genes in Arabidopsis. *Cellulose* **11**, 279-286.

Hatfield, R.D., Ralph, J. and Grabber, J.H. (1999) Cell wall cross-linking by ferulates and diferulates in grasses. *J. Sci. Food Agric.* **79**, 403-407.

Hayashi, H., Takagi, K., Fukumura, M., Kimura, T., Karita, S., Sakka, K. and Ohmiya, K. (1997) Sequence of *xynC* and properties of XynC, a major component of the *Clostridium thermocellum* cellulosome. *J. Bacteriol.* **179**, 4246-4253.

Hayashi, T. (1989) Xyloglucans in the primary cell wall. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **40**, 139-168.

Hazen, S.P., Scott-Craig, J.S. and Walton, J.D. (2002) Cellulose synthase-like genes of rice. *Plant Physiol.* **128**, 336-340.

Hellens, R.P., Edwards, E.A., Leyland, N.R., Bean, S and Mullineaux, P.M. (2000) pGreen: a versatile and flexible binary Ti vector for *Agrobacterium*-mediated plant transformation. *Plant Mol. Biol*. **42**, 819-832.

Henrissat, B. (1997) Supplementary data are available at http://www.expasy.org/cgi-bin/nicesite.pl?PS00591.

Henrissat, B. (1998) Glycosidase families. *Biochem. Soc. Trans.* **26**, 153-156.

Henrissat, B. and Bairoch, A. (1996) Updating the sequence-based classification of glycosyl hydrolases. *Biochem. J.* **316**, 695-696.

Henrissat, B. and Davies, G. (2000) Glycoside hydrolases and glycosyltransferases: families, modules and implications for genomics. *Plant Physiol.* **124**, 1515-1520.

Henrissat, B., Coutinho, P.M. and Davies, G.J. (2001) A census of carbohydrate-active enzymes in the genome of *Arabidopsis thaliana*. *Plant Mol. Biol.* **47**, 55-72.

Herbers, K., Flint, H.J. and Sonnewald, U. (1996) Apoplastic expression of the xylanase and β(1-3,1-4) glucanase domains of the xyn D gene from *Ruminococcus flavefaciens* leads to functional polypeptides in transgenic tobacco plants. *Mol. Breeding* **2**, 81-87.

Herbers, K., Wilke, I. and Sonnewald, U. (1995) A thermostable xylanase from *Clostridium thermocellum* expressed at high levels in the apoplast of transgenic tobacco lines has no detrimental effects and is easily purified. *Bio/Technology* **13**, 63-66.

Higgins, D.G. and Sharp, P.M. (1989) Fast and sensitive multiple sequence alignments on a microcomputer. *Comput. Appl. Biosci.* **2**, 151-153.

Holland, N., Holland, D., Helentjaris, T., Dhugga, K., Xoconostle-Cazares, B. and Delmer, D.P. (2000) A comparative analysis of the cellulose synthase (*CesA*) gene family in plants. *Plant Physiol.* **123**, 1313-1323.

IENICA (2000) Summary report for European Union Fibre Crops (www.ienica.net/reports/FRANCE).

Igawa, T., Ochiai-Fukuda, T., Takahashi-Ando, N., Ohsato, S., Shibata, T., Yamaguchi, I. and Kimura, M. (2004) New TAXI-type xylanase inhibitor genes are inducible by pathogens and wounding in hexaploid wheat. *Plant Cell Physiol.* **45**, 1347-1360.

Jagtap, S. and Rao, M. (2005) Purification and properties of a low molecular weight 1,4-β-D-glucan glucohydrolase having one active site for carboxymethyl cellulose and xylan from an alkalothermophilic *Thermomonospora* sp. *Biochem. Biophys. Res. Comm.* **329**, 111-116.

Ji, Q., Vincken, J.-P., Suurs, L.C.J.M. and Visser, R.G.F. (2003) Microbial starch-binding domains as a tool for targeting proteins to granules during starch biosynthesis. *Plant Mol. Biol*. **51**, 789-801.

Juge, N., Payan, F. and Williamson, G. (2004) XIP-1, a xylanase inhibitor protein from wheat: a novel protein function. *Biochim. Biophys. Acta* **1696**, 203-211.

Jung, H.J.G. and Buxton, D.R. (1994) Forage quality variation among maize inbreds: Relationships of cell-wall composition and in vitro degradability for stem internodes. *J. Sci. Food Agric.* **66**, 313-322.

Keegstra, K. and Raikhel, N. (2001) Plant glycosyltransferases. *Curr. Op. Plant Biol.* **4**, 219-224.

Kimura, S., Laosinchai, W., Itoh, T., Cui, X., Linder, C.R. and Brown, R.M. jr (1999) Immunogold labelling of rosette terminal cellulose-synthesizing complexes in the vascular plant *Vigna angularis. Plant Cell* **11**, 2075-2085.

Kitamura, S., Inoue, M., Shikazono, N. and Tanaka, A. (2001) Relationships among *Nicotiana* species revealed by the 5S rDNA spacer sequence and fluorescence in situ hybridization. *Theor. Appl. Genet.* **103**, 678-686.

Krysan, P.J., Young, J.C. and Sussman, M.R. (1999) T-DNA as an insertional mutagen in Arabidopsis. *Plant Cell* **11**, 2283-2290.

Kuroyama, H. and Tsumuraya, Y. (2001) A xylosyltransferase that synthesizes β-(1→4)-xylans in wheat (*Triticum aestivum* L.) seedlings. *Planta* **213**, 231-240.

Laemmli, U.K. (1970) Cleavage of structural proteins during assembly of the heads of bacteriophage T4. *Nature* **227**, 680-685.

Lapierre, C., Pollet, B., Petit-Conil, M., Toval, G., Romero, J., Pilate, G., Leple, J.C., Boerjan, W., Ferret, V.V., De Nadai, V. and Jouanin, L. (1999) Structural alterations of lignins in transgenic poplars with depressed cinnamyl alcohol dehydrogenase or caffeic acid O-methyltransferase activity have an opposite impact on the efficiency of industrial kraft pulping. *Plant Physiol.* **119**, 153-164.

Liepman, A.H., Wilkerson, C.G. and Keegstra, K. (2005) Expression of cellulose synthase-like (*Csl*) genes in insect cells reveals that *CslA* family members encode mannan synthases. *Proc. Natl. Acad. Sci. USA* **102**, 2221-2226.

Lindner, M. and Teeri, T.T. (1997) The roles and function of cellulose-binding domains. *J. Biotech.* **57**, 15-28.

Lindner, M., Salovuori, I., Ruohonen, L. and Teeri, T.T. (1996) Characterization of a double cellulose-binding domain. *J. Biol. Chem.* **35**, 21268-21272.

Liu, Y., Schiff, M., Marathe, R. and Dinesh-Kumar, S.P. (2002) Tobacco *Rar1*, *EDS1* and *NPR1/NIM1* like genes are required for N-mediated resistance to tobacco mosaic virus. *Plant J.* **30**, 415-429.

MacDougall, A.J., Brett, G.M., Morris, V.J., Rigby, N.M., Ridout, M.J. and Ring, S.G. (2001) The effect of peptide-pectin interactions on the gelation behaviour of a plant cell wall pectin. *Carbohydr. Res.* **335**, 115-126.

MacLeod, A.M., Lindhorst, T., Withers, S.G. and Warren, R.A. (1994) The acid/base catalyst in the exoglucanase / xylanase from *Cellulomonas fimi* is glutamic acid 127: evidence from detailed kinetic studies of mutants. *Biochem.* **33**, 6371-6376.

Madson, M., Dunand, C., Li, X., Verma, R., Vanzin, G.F., Caplan, J., Shoue, D.A., Carpita, N.C. and Reiter, W.-D. (2003) The *MUR3* gene of Arabidopsis encodes a xyloglucan galactosyltransferase that is evolutionary related to animal exostosins. *Plant Cell* **15**, 1662-1670.

McDougall, G.J., Morrison, I.M., Stewart, D., Weyers, J.D.B. and Hillman, J.R. (1993) Plant fibres: Botany, chemistry and processing for industrial use. *J. Sci. Food Agric.* **62**, 1-20.

McNeil, M., Darvill, A.G., Fry, S.C. and Albersheim, P. (1984) Structure and function of the primary cell walls of plants. *Ann. Rev. Biochem.* **53**, 625-663.

Memelink, J., Hoge, J.H.C. and Schilperoort, R.A. (1987) Cytokinin stress changes the developmental regulation of several defence-related genes in tobacco. *EMBO J.* **6**, 3579-3583.

Milioni, D., Sado, P-E., Stacey, N.J., Domingo, C., Roberts, K. and McCann, M. (2001) Differential expression of cell-wall-related genes during the formation of tracheary elements in the *Zinnia* mesophyll cell system. *Plant Mol. Biol.* **47**, 221-238.

Mollard, A., Domon, J.M., David, H. and Joseleau, J-P. (1997) Xylose-rich polysaccharides from the primary walls of embryogenic cell line of *Pinus caribaea*. *Int. J. Biol. Macromol*. **21**, 189-194.

Moore, P.J., Darvill, A.G., Albersheim, P. and Staehelin, A.L. (1986) Immunogold localozation of xyloglucan and rhamnogalacturonan I in the cell walls of suspension-cultured sycamore cells. *Plant Physiol.* **82**, 787-794.

Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bioassay with tobacco tissue culture. *Physiol. Plant* **15**, 473-497.

Nam, J., Mysore, K.S., Zheng, C., Knue, M.K., Matthysse, A.G. and Gelvin, S.B. (1999) Identification of T-DNA tagged *Arabidopsis* mutants that are resistant to transformation by *Agrobacterium. Mol. Gen. Genet*. **261**, 429-438.

Napoli, C., Lemieux, C. and Jorgensen, R. (1990) Introduction of a chimeric chalcone synthase gene into *Petunia* results in reversible co-suppression of homologous genes in trans. *Plant Cell* **2**, 279-289.

O'Neill, M.A. and York, W.S. (2003) The composition and structure of plant primary cell walls. In *The Plant Cell Wall.* (ed. J.K.C. Rose), Blackwell Publishing, Oxford, U.K.

O'Neill, M.A., Albersheim, P. and Darvill, A. (1990) The pectic polysaccharides of primary cell walls. In *Methods in Plant Biochemistry*, Vol. 2 (ed. P.M. Dey), Academic Press, London, pp. 415-441.

Odzuck, W. and Kauss, H. (1972) Biosynthesis of pure araban and xylan. *Phytochem.* **11**, 2489-2494.

Onysko, K.A. (1993) Biological bleaching of chemical pulps – a review. *Biotechnol. Advances* **11**, 179-198.

Oomen, R.J.F.J., Doeswijk-Voragen, C.H.L., Bush, M.S., Vincken, J.-P., Borkhardt, B., Van den Broek, L.A.M., Corsar, J., Ulvskov, P., Voragen, A.G.J., McCann, M.C. and Visser, R.G.F. (2002) *In muro* fragmentation of the rhamnogalacturonan I backbone in potato (*Solanum tuberosum* L.) results in a reduction and altered location of the galactan and arabinan side-chains and abnormal periderm development. *Plant J.* **30**, 403-413.

Oomen, R.J.F.J., Tzitzikas, E.N., Bakx, E.J., Straatman-Engelen, I., Bush, M.S., McCann, M.C., Schols, H.A., Visser, R.G.F. and Vinkcen, J.P. (2004) Modulation of the cellulose content of tuber cell walls by antisense expression of different potato (*Solanum tuberosum* L.) *CesA* clones. *Phytochem*. **65**, 535-546.

Pallesen, B.E. (1996) The quality of combine-harvested fibre flax for industrial puposes depends on the degree of retting. *Ind. Crops Prod.* **5**, 65-78.

Parinov, S., Sevugan, M., Ye, D., Yang, W.C., Kumaran, M. and Sundaresan, V. (1999) Analysis of flanking sequences from dissociation insertion lines: a database for reverse genetics in *Arabidopsis*. *Plant Cell* **11**, 2263-2270.

Pauly, M., Albersheim, P., Darvill, A. and York, W.S. (1999) Molecular domains of the cellulose/xyloglucan network in the cell walls of higher plants. *Plant J.* **20**, 629-639.

Pear, J.R., Kawagoe, Y., Schreckenogst, W.E., Delmer, D.P. and Stalker, D.M. (1996) Higher plants contain homologs of the bacterial *celA* genes encoding the catalytic subunit of cellulose synthase. *Proc. Natl. Acad. Sci. USA* **93**, 12637-12642.

Perrin, R., Wilkerson, C. and Keegstra, K. (2001) Golgi enzymes that synthesize plant cell wall polysaccharides: finding and evaluating candidates in the genomic area. *Plant Mol. Biol.* **47**, 115-130.

Perrin, R.M. (2001) How many cellulose synthases to make a plant? *Curr. Biol.* **11**, R213-R216.

Perrin, R.M., DeRocher, A.E., Bar-Peled, M., Zeng, W., Norambuena, L., Orellana, A., Raikhel, N.V. and Keegsta, K. (1999) Xyloglucan fucosyltransferase, an enzyme involved in plant cell wall biosynthesis. *Science* **284**, 1976-1979.

Porchia, A.C. and Scheller, H.V. (2000) Arabinoxylan biosynthesis: Identification and partial characterization of β-1,4-xylosyltransferase from wheat. *Physiol. Plant.* **110**, 350-356.

Porchia, A.C., Sorensen, S.O. and Scheller, H.V. (2002) Arabinoxylan biosynthesis in wheat. Characterisation of arabinosyltransferase activity in golgi membranes. *Plant Physiol.* **130**, 432-441.

Raven, P.H., Evert, R.F. and Eichhorn, S.E. (1992) Biology of plants. Fifth edition. Worth Publishers New York, USA.

Reid, J.S.G., Edwards, M.E., Dickson, C.A., Scott, C. and Gidley, M.J. (2003) Tobacco transgenic lines that express fenugreek galactomannan galactosyltransferase constitutively have structurally altered galactomannans in their seed endosperm cell walls. *Plant Physiol.* **131**, 1487-1495.

Reiter, W.-D. and Vanzin, G.F. (2001) Molecular genetics of nucleotide sugar interconversion pathways in plants. *Plant Mol. Biol.* **47**, 95-113.

Reiter, W.-D., Chapple, C. and Somerville, C.R. (1997) Mutants of *Arabidopsis thaliana* with altered cell wall polysaccharide composition. *Plant J.* **12**, 335-345.

Richmond, T.A. and Somerville, C.R. (2000) The cellulose synthase superfamily. *Plant Physiol.* **124**, 495-498.

Richmond, T.A. and Somerville, C.R. (2001) Integrative approaches in determining Csl function. *Plant Mol. Biol*. **47**, 131-143.

Robertson, D., Beech, I. and Bolwell, G.P. (1995) Regulation of the enzymes of UDP-sugar metabolism during differentiation of French bean. *Phytochem*. **39**, 21-28.

Robertson, D., Smith, C. and Bolwell, G.P. (1996) Inducible UDP-glucose dehydrogenase from French bean (*Phaseolus vulgaris* L.) locates to vascular tissue and has alcohol dehydrogenase activity. *Biochem. J.* **313**, 311-317.

Rose, J.K.C., Catalá, C., Gonzalez-Carranza, Z.H. and Roberts, J.A. (2003) Cell wall disassembly. In *The plant cell wall* (ed. J.K.C. Rose). Blackwell Publishing, Oxford, UK.

Rothstein, S.J., Dimaio, J., Strand, M. and Rice, D. (1987) Stable and heritable inhibition of the expression of nopaline synthase in tobacco expressing antisense RNA. *Proc. Natl. Acad. Sci. USA* **84**, 8439-8443.

Ruel, K., Barnoud, F. and Eriksson K-E. (1981) Micromorphological and ultrastructural aspects of spruce wood degradation by wild-type *Sporotrichum pulverulentum* and its cellulase-less mutant Cel 44. *Holzforschung* **35**, 151-171.

Ruel, K., **Montiel M-D., Goujon, T., Jouanin, L., Burlat, V. and Joseleau, J-P.** (2002) Interrelation between lignin deposition and polysaccharide matrices during the assembly of plant cell walls. *Plant Biol*. **4**, 2-8.

Saeman, J.F., Moore, W.E., Mitchell, R.L. and Millett, M.A. (1954) Techniques for the determination of pulp constituents by quantitative paper chromatography. *Tappi* **37**, 336-343.

Salehuzzaman, S.N., Jacobsen, E. and Visser, R.G. (1993) Isolation and characterization of a cDNA encoding granule-bound starch synthase in cassava (*Manihot esculenta Crantz*) and its antisense expression in potato. *Plant Mol. Biol.* **23**, 947-962.

Samuga, A. and Joshi, C.P. (2004) Cloning and characterisation of cellulose synthase-like gene, *PtrCSLD2* from developing xylem of aspen trees. *Physiol. Plant.* **120**, 631-641.

Sarkanen, S. (1998) Template polymerization in lignin biosynthesis. In *Lignin and Lignan Biosynthesis* (eds N.G. Lewis and S. Sarkanen), American Chemical Society Symposium, Washington, DC, pp. 194-208.

Saxena, I.M. and Brown, R.M. jr. (2005) Cellulose biosynthesis: current views and evolving concepts. *Annals Botany* **96**, 9-21.

Saxena, I.M., Brown, R.M. jr. and Dandekar, T. (2001) Structure-function characterization of cellulose synthase relationship to other glycosyltransferases. *Phytochem.* **57**, 1135-1148.

Saxena, I.M., Brown, R.M. jr., Fevre, M., Geremia, R.A. and Henrissat, B. (1995) Multidomain architecture of β-glycosyltransferases: implications for mechanism of action. *J. Bact.* **177**, 1419-1424.

Saxena, I.M., Lin, F.C. and Brown, R.M. jr. (1990) Cloning and sequencing of the cellulose synthase catalytic subunit gene of *Acetobacter xylinum*. *Plant Mol. Biol.* **15**, 673-683.

Scheible, W-R. and Pauly, M. (2004) Glycosyltransferases and cell wall biosynthesis: novel players and insights. *Curr. Op. Plant Biol.* **7**, 1-11.

Schols, H.A. and Voragen, A.G.J. (1994) Occurrence of pectic hairy regions in various plant cell wall materials and their degradability by rhamnogalacturonase. *Carbohydr. Res.* **256**, 83-95.

Schröder, R., Wegrzyn, T.F., Bolitho, K.M. and Redgwell, R.J. (2004) Mannan transglycosylase: a novel enzyme activity in cell walls of higher plants. *Planta* **219**, 590-600.

Seifert, G.J. (2004) Nucleotide sugar interconversions and cell wall biosynthesis: how to bring the inside to the outside. *Curr. Opin. Plant Biol.* **7**, 277-284.

Selvendran, R.R. and Ryden, R. (1990) Isolation and analysis of plant cell walls. In: Dey, P.M. (Ed.), Carbohydrates, Chapter 16. Academic Press, London, pp. 549-579.

Sharma, H.S.S., Faughey, G. and Lyons, G. (1999) Comparison of physical, chemical, and thermal characteristics of water-, dew, and enzyme-retted flax fiber. *J. Appl. Polym. Sci.* **74**, 139-143.

Simpson, D.J., Fincher, G.B., Huang, A.H.C. and Cameron-Mills, V. (2003) Structure and function of cereal and related higher plant (1→4)-β-xylan endohydrolases. *J. Cereal Sci.* **37**, 111-127.

Slade, A.M., Hoj, P.B., Morrice, N.A. and Fincher, G.B. (1989) Purification and characterization of three (1→4)-β-D-xylan endohydrolases from germinated barley. *Eur. J. Biochem.* **185**, 533-539.

Smith, N.A., Singh, S.P., Wang, M.B., Stoutjesdijk, P.A., Green, A.G. and Waterhouse, P.M. (2000) Total silencing by intron-spliced hairpin RNAs. *Nature* **407**, 319-320.

Sørensen, S.O., Pauly, M., Bush, M.S., Skjot, M., McCann, M.C., Borkhardt, B. and Ulvskov, P. (2000) Pectin engineering: Modification of potato pectin by *in vivo* expression of an endo-1,4-β-D-galactanase. *Proc. Natl. Acad. Sci. USA* **97**, 7639-7644.

Stasinopoulos, S.J., Fisher, P.R., Stone, B.A. and Stanisich, V.A. (1999) Detection of two loci involved in (1,3)-β-glucan (curdlan) biosynthesis by *Agrobacterium* sp. ATCC31749, and comparative sequence analysis of the putative curdlan synthase gene. *Glycobiol.* **9**, 31-41.

Stephen, A.M. (1982) Other plant polysaccharides. In *The polysaccharides*, Vol 2 (ed. G.O. Aspinall), Academic Press, New York, pp. 97-113.

Stone, B.A. and Clarke, A.E. (1992) *Chemistry and biology of (1,3)-β-glucans,* La Trobe University Press, Melbourne.

Suzuki, M., Kato, A., Nagata, N. and Komeda, Y. (2002) A xylanase, AtXyn1, is predominantly expressed in vascular budles, and four putative xylanase genes were identified in the *Arabidopsis thaliana* genome. *Plant Cell Physiol.* **43**, 759-767.

Taylor, N.G., Howells, R.M., Huttly, A.K., Vickers, K. and Turner, S.R. (2003) Interactions among three distinct CesA proteins essential for cellulose synthesis. *Proc. Natl. Acad. Sci. USA* **100**, 1450-1455.

Taylor, N.G., Laurie, S. and Turner, S.R. (2000) Multiple cellulose synthase catalytic subunits are required for cellulose synthesis in Arabidopsis. *Plant Cell* **12**, 2529-2539.

Taylor, N.G., Scheible, W.R., Cutler, S., Somerville, C.R. and Tuner, S.R. (1999) The *irregular xylem3* locus of *Arabidopsis* encodes a cellulose synthase required for secondary wall synthesis. *Plant Cell* **11**, 769-779.

Terashima, N., Fukushima, K., He, L.F. and Takabe, K. (1993) In *Forage Cell Wall Structure and Digestibility* (eds. H.G. Jung, D.R. Buxton, R.D. Hatfield and J. Ralph), ASA-CSSA-SSSA, Madison, WI, pp.247-270.

Thomas, C.L., Jones, L., Baulcombe, D.C. and Maule, A.J. (2001) Size constraints for targeting post-transcriptional gene silencing and for RNA-directed methylation in *Nicotiana benthamiana* using potato virus X vector. *Plant J.* **25**, 417-425.

Thompson, J.E. and Fry, S.C. (2001) Restructuring of wall-bound xyloglucan by transglycosylation in living plant cells. *Plant J.* **26**, 23-34.

Tomme, P., Warren, R.A.J. and Gilkes, N.R. (1995) Cellulose hydrolysis by bacteria and fungi. *Adv. Microb. Physiol.* **37**, 1-80.

Tull, D., Withers, S.G., Gilkes, N.R., Kilburn, D.G., Warren, R.A. and Aebersold, R. (1991) Glutamic acid 274 is the nucleophile in the active site of a 'retaining' exoglucanase from *Cellulomonas fimi. J. Biol. Chem.* **266**, 15621-15625.

Turner, S.R. and Somerville, C.R. (1997) Collapsed xylem phenotype of *Arabidopsis* identifies mutants deficient in cellulose deposition in the secondary cell wall. *Plant Cell* **9**, 689-701.

Uffen, R.L. (1997) Xylan degradation: a glimpse at microbial diversity. *J. Ind. Microbiol. Biotechnol.* **19**, 1-6.

Van Roekel, G. (1994) Hemp pulp and paper production. *J. Int. Hemp Association* **1**, 12-14.

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

Vincken, J.-P., Zabotina, O.A., Beldman, G. and Voragen, A.G.J. (1998) Xyloglucan endotransglycosylase activities in apples ripening-related: implications for fruit juice processing. *J. Sci. Food and Agriculture* **78**, 46-52.

Wang, X., Cnops, G., Vanderhaeghen, R., De Block, S., Van Montagu, M. and Van Lijsebettens, M. (2001) *AtCslD3*, a cellulose synthase-like gene important for root hair growth in *Arabidopsis*. *Plant Physiol.* **126**, 575-586.

Wesley, S.V., Helliwell, C.A., Smith, N.A., Wang, M.B., Rouse, D.T., Liu, Q., Gooding, P.S., Singh, S.P., Abbott, D., Stoutjesdijk, P.A., Robinson, S.P., Gleave, A.P., Green, A.G. and Waterhouse, P.M. (2001) Construct design for efficient, effective and high-throughput gene silencing in plants. *Plant J.* **27**, 581-590.

Weterings, K., Reijnen, W., van Aarssen, R., Kortstee, A., Spijkers, J., van Herpen, M., Schrauwen, J. and Wullems, G. (1992) Characterization of a pollen-specific cDNA clone from *Nicotiana tabacum* expressed during microgametogenesis and germination. *Plant Mol. Biol.* **18**, 1101-1111.

Whetten, R.W., MacKay, J.J. and Sederoff, R.R. (1998) Recent advances in understanding lignin biosynthesis. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **49**, 585-609.

White, A.R., Xin, Y. and Pezeshk, V. (1993) Xyloglucan glucosyltransferase in Golgi membranes from *Pisum sativum* (pea). *Biochem. J.* **294**, 231–238.

Whitney, S.E.C., Brigham, J.E., Darke, A.H., Grand-Reid, J.S. and Gidley, M.J. (1995) *In vitro* assembly of cellulose/xyloglucan networks: ultrastructural and molecular aspects. *Plant J.* **8**, 491-504.

Whitney, S.E.C., Gothard, M.G.E., Mitchell, J.T. and Gidley, M.J. (1999) Roles of cellulose and xyloglucan in determining the mechanical properties of primary plant cell walls. *Plant Physiol.* **121**, 657-663.

Wittink, F.R.A., Knuiman, B., Derksen, J., Capkova, V., Twell, D., Schrauwen, J.A.M. and Wullems, G.J. (2000) The pollen-specific gene Ntp303 encodes a 69-kDa glycoprotein associated with the vegetative membranes and the cell wall. *Sex Plant Reprod.* **12**, 276-284.

Wu, S.S.H., Suen, D.F., Chang, H.C. and Huang, A.H.C. (2002) Maize tapetum xylanase is synthesized as a precursor, processed and activated by a serine protease and deposited on the pollen. *J. Biol. Chem.* **277**, 49055-49064.

Xie, H., Gilbert, H.J., Charnock, S.J., Davies, G.J., Williamson, M.P., Simpson, P.J., Raghothama, S., Fontes, C.M.G.A., Dias, F.M.V., Ferreira, L.M.A. and Bolam, D.N. (2001) *Clostridium thermocellum* Xyn10B carbohydrate-binding module 22-2: the role of conserved amino acids in ligand binding. *Biochem.* **40**, 9167-9176.

Zablackis, E., Huang, J., Muller, B., Darvill, A.G. and Albersheim, P. (1995) Structure of plant cell walls. Characterization of the cell wall polysaccharides of *Arabidopsis thaliana* leaves. *Plant Physiol.* **107**, 1129-1138.

Zhu, Y., Nam, J., Carpita, N.C., Matthysse, A.G. and Gelvin, S.B. (2003) Agrobacterium-mediated root transformation is inhibited by mutation of an Arabidopsis cellulose synthase-like gene. *Plant Physiol.* **133**, 1000-1010.

SUMMARY

Natural fibres have a wide range of technological applications, such as in paper and textile industries. The basic properties and the quality of plant fibres are determined by the composition of the plant cell wall. Characteristic for fibres are thick secondary cell walls, which consist of cellulose microfibrils in a matrix of lignin and hemicelluloses. The major hemicellulose component of the dicot secondary wall is xylan. This component can establish tight interactions with cellulose and covalent bonds with lignin and is thereby involved in strengthening of the wall. Since high amounts of hemicelluloses as well as lignins have a negative effect on the industrial processing of fibres, the development of plants with altered cell wall composition is of great value. This thesis focuses on modification of the hemicellulose (in particular xylan) content and thereby also the attachment of lignin in the cell wall. Different approaches to generate transgenically modified plants with altered xylan composition have been examined. (i) Decreasing the expression levels of putative xylan synthases, responsible for the polymerisation of the xylan backbone. (ii) Degradation of already deposited xylan polymers by the introduction of xylan degrading enzymes (xylanases). In our research, tobacco (*Nicotiana tabacum*) is used as a model species, since it is widely used in fundamental cell wall research. Once interesting results are obtained in tobacco, technologies can be transferred into economically more important species, such as flax (*Linum usitatissimum*) and poplar (*Populus* spp).

At the start of the work described in this thesis, genes involved in hemicellulose backbone biosynthesis were not identified yet. This favoured the use of a set of candidate genes, the *cellulose synthase-like* genes (*Csl*s), which are generally suggested to be involved in the biosynthesis of the non-cellulosic cell wall polysaccharides. Plants contain at least eight families of *Csl*s (*CslA* though *H*), which all show the conserved motifs common to polymerizing β-glycosyltransferases. In order to investigate the function of six *Csl* families (*CslA*, *B*, *C*, *D*, *E* and *G*), we introduced Inverted Repeat (IR) constructs, based on potato (*Solanum tuberosum*) *Csl* cDNA sequences into tobacco (**Chapter 2**). If one of the *Csl* family members is coding for a xylan synthase, this strategy consequently provides us with transgenic tobacco plants with altered xylan levels. Sugar compositional analysis of cell wall material isolated from *in vitro* grown IR transformants revealed a reduction of xylose exclusively in the *CslG* transformants. These data led us to speculate about the involvement of *CslG* in xylan biosynthesis. Microscopic analysis of stem samples from mature *CslG* transformants revealed some very local effects like the multiplication of ray cells, enlargement of the size of vessels and the occurrence of abnormal fibres with thin walls and unusually high starch content. This result could indicate the tissue- or cell-type specificity of the *CslG* gene. Sugar compositional analyses of mature *CslG* plants

were rather ambiguous and a second series of *in vitro* grown *CslG* transformants did not confirm the decrease of xylose that was observed during the initial screening. Additionally, the absence of tobacco *CslG* sequence information did not allow us to link the reduced xylose levels with altered *CslG* mRNA expression levels. Therefore, it required searching for tobacco *CslG* genes. **Chapter 3** reports the isolation of full length tobacco *CslE* and *CslG* cDNA sequences from a cDNA library, constructed from mRNAs isolated from a xylogenic suspension cell culture of tobacco. The proteins encoded by the *NtCslE* and *NtCslG* cDNA clones, contain eight putative transmembrane domains, alternating conserved and variable domains, and the processive glycosyltransferase signature. Southern blot analysis revealed that the tobacco *CslE* and *CslG* gene families consist of two to four and at least three genes, respectively. Gene expression studies in wild type tobacco tissues showed that *CslE* expression was highest in tissues associated with secondary wall biosynthesis, whereas *CslG* mRNA levels were highest in tissues undergoing primary wall formation. An involvement for *CslG* in xylan biosynthesis in tobacco, as indicated by the heterologous IR approach (**Chapter 2**), is not supported by the gene expression analysis.

Apart from modifying the xylan content by down regulation of putative xylan backbone synthesizing enzymes, a second strategy to generate transformants with altered xylan composition was applied. This strategy involved the specific degradation of the xylan polymers, which are already deposited in the cell wall, by the introduction of a xylanase. **Chapter 4** describes the heterologous expression of AtXyn2, a modular enzyme from *Arabidopsis thaliana*, consisting of four contiguous CBM22 modules at the N-terminus, joined to a glycoside hydrolase Family 10 (GH10) catalytic domain, into tobacco. The full length *AtXyn2* as well as a derivative, comprising the catalytic GH10 domain lacking the four CBMs, was ectopically expressed in tobacco. An N-terminal signal peptide and RGS·$(HIS)_6$ -tag mediated translocation into the cell wall and detection of the protein. Although both transgenes were successfully expressed in tobacco, presence of the corresponding proteins could not be demonstrated. We speculate that this could be caused by removal of the RGS·$(HIS)_6$ -tag due to either post-translational processing of AtXyn2 in tobacco or due to cleavage of the epitope-tag by a tobacco protease. Our data showed that tobacco transformants, expressing the complete AtXyn2 protein, possessed a significantly higher wheat xylan and HE-cellulose degrading activity than wild type tobacco. This result indicated that AtXyn2 displays a dual hydrolytic activity towards specific β-1,4-linked xylans and glucans. Since the catalytic activity was inversely proportional to the *AtXyn2* transcript level, we speculate that high amounts of AtXyn2, or released oligosaccharides, could result in a negative feedback mechanism and subsequent inactivation or degradation of the enzyme. The cellulose degrading activity of AtXyn2 appeared to be even higher than the xylan degrading activity. This raises the question

whether AtXyn2 should be considered as a cellulase instead of a xylanase. Transgenic tobacco plants expressing the truncated AtXyn2 protein did not show an altered hydrolytic activity against any of the substrates tested. It can therefore be concluded that the presence of the CBM22 domains adjacent to the catalytic module seems to play an essential role in the enzyme activity. Based on our results and additional information found in literature, we propose a model, which might explain the regulation of AtXyn2 activity in the plant cell wall. This model suggests that the CBM22 domains are responsible for immobilization of the enzyme in proximity of the substrate and for correct folding and stability of the protein. The enzyme activity is in turn suggested to be regulated by post-translational cleavage by an endoprotease.

The thesis is concluded with a general discussion (**Chapter 5**) on the outcome of the different approaches to generate tobacco transformants with reduced xylan content. Although both approaches initially seemed to be very promising, we were not successful in altering the xylan level in the cell wall. The drawbacks related to the down regulation of *Csl* candidate genes are discussed. Alternative approaches are therefore essential to identify xylan synthases and to modulate the xylan content in the cell wall. Various strategies to identify and characterise genes involved in hemicellulose biosynthesis are discussed. The heterologous expression of AtXyn2 was expected to result in a reduction of the xylan level in the cell wall. However, the dual substrate activity and the regulation of AtXyn2 activity, make this enzyme less suitable for reduction of the xylan content. Heterologous expression of well-studied microbial xylanases could be a suitable alternative to degrade xylan polymers *in planta*.

SAMENVATTING

Natuurlijke vezels hebben een brede technologische toepasbaarheid, waaronder in de papier- en textielindustrie. De fysische eigenschappen en kwaliteit van plantenvezels worden met name bepaald door de samenstelling van de celwand. Typerend voor vezelcellen zijn de dikke secundaire celwanden die voornamelijk bestaan uit cellulose microfibrillen, ingebed in een matrix van lignine en hemicelluloses. De belangrijkste hemicellulose component in de secundaire celwand van dicotyle planten is xylaan. Dit polymeer kan een niet-covalente interactie aangaan met cellulose en covalent binden aan lignine. Als gevolg hiervan levert xylaan een belangrijke bijdrage aan de stevigheid van de celwand. De aanwezigheid van grote hoeveelheden hemicellulose en lignine heeft een negatief effect op de industriële verwerking van vezels. Het is daarom van groot belang om planten te ontwikkelen met een veranderde celwand samenstelling. Dit proefschrift richt zich op het veranderen van de hoeveelheid hemicellulose (met name xylaan) in de celwand, wat tegelijkertijd effect kan hebben op de verankering van lignine. Verschillende strategieën zijn toegepast om transgene planten met een veranderde xylaan hoeveelheid te genereren. (i) Reductie van het expressieniveau van kandidaat xylaan synthases, die verantwoordelijk zijn voor de synthese van de xylaan hoofdketen. (ii) Afbraak van aanwezige xylaan polymeren door het introduceren van xylaan afbrekende enzymen (xylanases). Binnen dit onderzoek is tabak (*Nicotiana tabacum*) als modelsysteem gebruikt omdat deze soort algemeen gebruikt wordt voor fundamenteel onderzoek aan celwanden. Wanneer interessante resultaten zijn verkregen in tabak, kunnen de geschikte technologieën toegepast worden in de voor de vezelindustrie economisch belangrijke gewassen, zoals vlas (*Linum usitatissimum*) en populier (*Populus* spp.).

Op het moment dat het onderzoek beschreven in dit proefschrift van start ging, waren er nog geen genen bekend, die betrokken zijn bij de biosynthese van hemicellulose hoofdketens. Daarom is gebruik gemaakt van een set kandidaatgenen, de *cellulose synthase-achtige* genen (*Csl*s), waarvan gesuggereerd wordt dat ze betrokken zijn bij de biosynthese van celwand polysacchariden anders dan cellulose. In planten zijn tenminste acht *Csl* families aanwezig (*CslA* tot en met *H*), die allen de geconserveerde aminozuren specifiek voor polymeriserende β-glycosyltransferases bezitten. Om de functie van zes *Csl* families op te helderen (*CslA*, *B*, *C*, *D*, *E* en *G*) zijn Inverted Repeat (IR) constructen gebaseerd op aardappel (*Solanum tuberosum*) *Csl* cDNA sequenties geïntroduceerd in tabak (**Hoofdstuk 2**). Wanneer één van de *Csl* familieleden codeert voor een xylaan synthase, zal deze strategie resulteren in transgene tabaksplanten met een veranderde hoeveelheid xylaan. Analyse van de suikersamenstelling van celwand materiaal, geïsoleerd uit *in vitro* opgegroeide IR transformanten, liet uitsluitend in de *CslG* transformanten een reductie van xylose zien. Op grond van deze data zou *CslG* mogelijk

betrokken kunnen zijn bij xylaan biosynthese. Microscopische analyse van stengelmateriaal van volgroeide *CslG* transformanten liet een aantal zeer locale effecten zien, zoals de vermeerdering van het aantal mergstraalcellen en een toename in de vezelgrootte. Tevens werden afwijkende vezelcellen met dunne celwanden en een verhoogd zetmeelgehalte waargenomen. Dit resultaat kan duiden op weefsel- of celtypespecificiteit van het *CslG* gen. De resultaten van de suikerbepaling van volgroeide *CslG* planten waren niet eenduidig; de analyse van een tweede serie *in vitro* opgegroeide *CslG* transformanten bevestigde niet de afname van xylose zoals die was waargenomen in de eerste serie. Vanwege het gebrek aan sequentie-informatie van tabak *CslG* genen was het niet mogelijk om de afname in xylose te koppelen aan veranderde *CslG* mRNA expressieniveaus. Daarom was het van belang om *CslG* genen van tabak te isoleren. **Hoofdstuk 3** beschrijft de screening van een cDNA bank, gemaakt van de mRNAs geïsoleerd uit een xylogene celsuspensie van tabak. De screening resulteerde in de isolatie van de volledige cDNA sequentie van tabak *CslE* en *CslG*. De corresponderende eiwitten (respectievelijk NtCslE en NtCslG) bevatten acht mogelijke transmembraan domeinen, afwisselend geconserveerde en variabele domeinen, en een processief glycosyltransferase motief. DNA analyse liet zien dat het tabaksgenoom twee tot vier *CslE* genen bevat en tenminste drie *CslG* genen. Genexpressie studies in wild type tabaksweefsels toonden aan dat de expressie van *CslE* het hoogst was in weefsels waarin een hoge mate van secundaire celwandvorming plaats vindt. De *CslG* mRNA niveaus waren daarentegen het hoogst in weefsels waarin voornamelijk primaire celwandvorming plaatsvindt. De betrokkenheid van *CslG* bij de xylaan biosynthese, zoals gesuggereerd werd door de heterologe IR benadering (**Hoofdstuk 2**), wordt niet ondersteund door deze genexpressie resultaten.

Naast het veranderen van het xylaangehalte door middel van het uitschakelen van mogelijke enzymen betrokken bij de biosynthese van de xylaan hoofdketen, werd een tweede strategie toegepast waarin een xylanase in tabak werd geïntroduceerd om de reeds aanwezige xylaan polymeren af te breken. In **Hoofdstuk 4** wordt de heterologe expressie van *AtXyn2* in tabak beschreven. AtXyn2 is een modulair enzym afkomstig van *Arabidopsis thaliana*, dat vier opeenvolgende Familie 22 koolhydraat bindingsmodules (CBM22) aan de N-terminus bevat, die gekoppeld zijn aan een glycoside hydrolase Familie 10 (GH10) katalytisch domein. Zowel het volledige *AtXyn2* gen als een gedeelte ervan, bestaande uit enkel het katalytisch domein, zijn ectopisch in tabak tot expressie gebracht. Een additioneel N-terminaal signaalpeptide en een RGS·$(HIS)_6$-label zorgden voor respectievelijk translocatie naar de celwand en de mogelijkheid om de eiwitten te detecteren. Alhoewel beide transgenen in tabak tot expressie kwamen, kon de aanwezigheid van de corresponderende eiwitten niet aangetoond worden. We speculeren dat dit mogelijk veroorzaakt kan zijn door verwijdering van het RGS·$(HIS)_6$-label als gevolg

van post-translationele modificatie van AtXyn2 in tabak of vanwege de verwijdering van het label door een tabaksprotease. Resultaten lieten zien dat extracten van tabakstransformanten waarin het volledige AtXyn2 eiwit tot expressie komt, een significant hogere hydrolyse activiteit hebben ten aanzien van tarwe xylaan en HE-cellulose dan die van wild type tabaksplanten. Hieruit kan geconcludeerd worden dat AtXyn2 een tweeledige hydrolytische activiteit heeft voor β-1,4-xylanen en -glucanen. De katalytische activiteit was omgekeerd evenredig met het expressieniveau van *AtXyn2*. Daarom speculeren we dat grote hoeveelheden AtXyn2, of vrijgekomen oligosacchariden, kunnen resulteren in inactivering of afbraak van het enzym. De cellulose afbrekende activiteit van AtXyn2 was groter dan de xylaan afbrekende activiteit. Dit werpt de vraag op of AtXyn2 beschouwd moet worden als een cellulase in plaats van een xylanase. Transgene tabaksplanten waarin alleen het katalytische domein van AtXyn2 tot expressie kwam, lieten geen veranderde hydrolytische activiteit zien ten aanzien van de geteste substraten. Hieruit kan geconcludeerd worden dat de aanwezigheid van de CBM22 domeinen voorafgaand aan de katalytische module essentieel is voor de activiteit van het enzym. Op basis van onze resultaten, aangevuld met kennis uit de literatuur, is een model opgesteld dat de regulatie van de enzymactiviteit van AtXyn2 beschrijft. Dit model suggereert dat de CBM22 domeinen het enzym in aanwezigheid van het substraat immobiliseren. Tevens zijn de CBM22 domeinen verantwoordelijk voor correcte vouwing en stabiliteit van het eiwit. De enzymactiviteit wordt op zijn beurt gereguleerd via post-translationele modificatie door een endoprotease.

Het proefschrift wordt afgesloten met een algemene discussie (**Hoofdstuk 5**). In dit hoofdstuk worden de twee strategieën vergeleken die gebruikt zijn om transgene tabaksplanten met verlaagde xylaan hoeveelheden te verkrijgen. Alhoewel beide benaderingen theoretisch gezien veelbelovend waren, zijn we er niet in geslaagd om het xylaan niveau in de celwand te veranderen. De nadelen met betrekking tot het uitschakelen van *Csl* kandidaatgenen worden besproken. Alternatieve methodes zijn noodzakelijk om xylaan synthases te identificeren en de xylaan hoeveelheid in de celwand te reguleren. Hiertoe worden verschillende alternatieven voorgesteld om genen te identificeren die betrokken zijn bij hemicellulose biosynthese. Er werd verwacht dat heterologe expressie van AtXyn2 zou resulteren in een afname van de hoeveelheid xylaan in de celwand. Echter, de tweeledige substraatactiviteit AtXyn2 en de complexe regulatie van de enzymactiviteit, maken dit enzym niet ideaal om de xylaan hoeveelheid te verlagen. Heterologe expressie van goed gekarakteriseerde microbiële xylanases kan een goed alternatief bieden om *in planta* xylaan polymeren af te breken.

NAWOORD

Nu het proefschrift klaar is rest mij alleen nog het schrijven van het nawoord. Met veel plezier kijk ik terug op de afgelopen vier jaar bij de vakgroep Plantenveredeling. In dit nawoord wil ik iedereen bedanken die hier direct of indirect een bijdrage aan heeft geleverd.

Allereerst wil ik mijn promotor Richard en mijn co-promotor Jean-Paul bedanken voor de goede samenwerking. Richard, bedankt voor de mogelijkheid om dit onderzoek als promotie-onderzoek uit te mogen voeren bij de vakgroep Plantenveredeling. Ik waardeer het vertrouwen dat je in me hebt gehad en de ruimte die ik kreeg om samen met Jean-Paul een eigen invulling aan het project te kunnen geven. J-P, jouw bijdrage aan het onderzoek is voor mij zeer belangrijk geweest. Jouw enthousiasme en optimisme hebben mij erg geholpen wanneer de resultaten weer eens niet zo waren als verwacht. Zonder jouw suggesties en kritische blik had dit proefschrift er heel anders uitgezien. Daarnaast wil ik jou en Marian hartelijk danken voor de gastvrijheid en jullie persoonlijke interesse.

I would like to thank all COPOL partners for the pleasant meetings and fruitful discussions. Additionally, I would like to express special gratitude to Paul, Laurence, Jean-Paul and Katia for their interest in my project and the input in the manuscripts.

Alle collega's en oud-collega's van de vakgroep Plantenveredeling wil ik bedanken voor de gezellige sfeer zowel tijdens het werk als in de koffiepauzes. In het bijzonder wil ik de analisten van het moleculair, weefselkweek en biochemisch lab bedanken voor al het werk dat direct of indirect voor mij gedaan is. Marian, Irma, Elly, Fien, Petra, Marjan, Isolde, Dirkjan en Luc, ik heb de samenwerking met jullie allen als erg plezierig ervaren! Annie, Letty, Theo en Hans, bedankt voor alle administratieve en technische werkzaamheden, maar zeker ook het gezellige 'geklets' over serieuze en minder serieuze zaken. Vele analyses in het lab waren niet mogelijk zonder een goede verzorging van de planten bij Unifarm. Mart, Henk en Bert, dank jullie wel! I could share a lot with my roommates and cell wall colleagues Jaap and Wole. Good luck with finishing your own research and I wish you all the best!

Met veel plezier kijk ik terug op alle etentjes en uitstapjes met vrienden en collega's. Clementine & Ben, Ronald & Ruurd, Marjolein & Hans, Francis & Francel (en Janne natuurlijk!), Luisa & Rob, Berlinda & Erik, Marian & Bart, Géraldine, Corine, Geertjan & Miriam, Bart & Debby, Ruth & Ronny, Jeannette & Ben, Tristan & Enif, jullie allen bedankt voor het leuke contact en de gezelligheid.

Clementine en Ronald, jullie zijn de afgelopen jaren erg betrokken geweest bij mij en mijn onderzoek. We hebben samen veel successen en tegenslagen gedeeld, zowel op wetenschappelijk als op persoonlijk gebied. Ik hecht veel waarde aan onze vriendschap en ben blij dat jullie mijn paranimfen willen zijn!

Bijzondere dank ook aan mijn lieve ouders voor alle steun en medeleven tijdens de afgelopen jaren. Pap en Mam, dank jullie wel dat ik altijd bij jullie terecht kan voor een luisterend oor en advies. Jullie positieve en relativerende instelling is voor mij erg belangrijk geweest!

Lieve Maarten, jij hebt een heel belangrijke bijdrage geleverd aan het tot stand komen van dit proefschrift. Ik ben jou veel dank verschuldigd voor je steun, suggesties en geduld tijdens de afgelopen jaren maar zeker ook voor al je hulp bij de lay-out en bij het maken van figuren. Nu beide proefschriften klaar zijn sluiten we een mooie en bijzondere periode af. *Nuestro viaje todavía continúa…*

Monique

CURRICULUM VITAE

Monique Godefrida Maria Compier werd op 30 november 1977 geboren in Schijndel. In juni 1996 behaalde zij het V.W.O. diploma aan het R.K. Gymnasium Beekvliet te St. Michielsgestel. In datzelfde jaar begon zij haar studie Biologie aan de Katholieke Universiteit Nijmegen. Haar stages heeft zij verricht bij de vakgroep Celbiologie van de Plant van de Katholieke Universiteit Nijmegen en bij Mars Incorporated (Masterfoods), Nottingham, United Kingdom. In februari 2001 behaalde zij *cum laude* haar doctoraal diploma. In april van datzelfde jaar begon zij als toegevoegd onderzoeker bij het Laboratorium voor Plantenveredeling van de Wageningen Universiteit. Het onderzoek vond plaats binnen het door de EU gefinancierde project 'Integrated control of polysaccharide and lignin biosynthesis to improve cellulose content and availability and fibre quality' en heeft geleid tot dit proefschrift. Sinds 1 september 2005 heeft zij een aanstelling als adviseur WBSO met specialisatie Veredeling bij SenterNovem in Zwolle.

Education Statement of the Graduate School

Experimental Plant Sciences

Issued to: Monique G.M. Compier
Date: 7 November 2005
Group: Laboratory of Plant Breeding, Wageningen University

1) Start-up phase	*date*
► **First presentation of your project**	
Integrated control of polysaccharide and lignin biosynthesis to improve cellulose content and availability and fibre quality	November 28, 2001
► **Writing a project proposal**	
► **Writing an introduction chapter for thesis**	
Introduction to hemicellulose biosynthesis and degradation in tobacco cell walls	2001-2005
► **MSc courses**	
► **Laboratory use of isotopes**	
Course 'Radiation Hygiene, level 5B'	October 30-November 2, 2001
Subtotal Start-up Phase	*6.0 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
PhD students day 2002, Wageningen	January 24, 2002
PhD students day 2003, Utrecht	March 27, 2003
► **EPS theme symposia**	
Theme symposium IV, 2001, Nijmegen	December 13, 2001
Theme symposium III, 2001, Utrecht	December 20, 2001
Theme symposium III, 2002, Utrecht	October 11, 2002
Theme symposium III, 2003, Amsterdam	December 11, 2003
► **Research discussion**	
Weekly meetings at Plant Breeding	2001-2005
► **National meetings**	
ALW meeting Lunteren 2003, Plant Sciences	April 7-8, 2003
ALW meeting Lunteren 2004, Plant Sciences	April 5-6, 2004
ALW meeting Lunteren 2005, Plant Sciences	April 4-5, 2005
► **Seminars (series), workshops and symposia**	
Plant Breeding Seminar Series	2001 - 2005
Frontiers in Plant Developments Seminar Series	2002-2004
► **International symposia and conferences**	
COPOL meeting, London, United Kingdom	May 2-4, 2001
COPOL meeting, Grenoble, France	January 25-27, 2002
COPOL meeting, Barcelona, Spain	October 4-6, 2002
COPOL meeting, Copenhagen, Denmark	March 6-8, 2003
COPOL meeting, Gent, Belgium	December 10-12, 2003
COPOL meeting, Wageningen, The Netherlands	July 1-3, 2004
► **Presentations**	
Oral presentation at COPOL meeting, Grenoble, France	January 26, 2002
Oral presentation at COPOL meeting, Barcelona, Spain	October 5, 2002
Oral presentation at COPOL meeting, Copenhagen, Denmark	March 7, 2003
Oral presentation at COPOL meeting, Gent, Belgium	December 11, 2003
Oral presentation at COPOL meeting, Wageningen, The Netherlands	July 2, 2004
Oral presentation at Meeting Plant Breeding, Genetics & Breeding and PPO	May 17, 2002
► **Excursion**	
Subtotal Scientific Exposure	*18.4 credits*

3) In-Depth Studies	*date*
► **EPS courses or other PhD course**	
Protein engineering Agro Food Biotech	November 17-19, 2001
Summer course "Glycoscience"	June 23-27, 2002
► **Journal club**	
► **Individual research training**	
Subtotal In-Depth Studies	*1.6 credits**

4) Personal development	*date*
► **Skill training courses**	
► **Organisation of PhD students day, course or conference**	
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*0 credits**

TOTAL NUMBER OF CREDIT POINTS*	**26.0 credits***

** A credit represents a normative study load of 40 hours of study*

The work described in this thesis was carried out in the Graduate School Experimental Plant Sciences at the Laboratory of Plant Breeding, Wageningen University. Main financial support was provided by the EU project 'Integrated control of polysaccharide and lignin biosynthesis to improve cellulose content and availability and fibre quality' (COPOL, QLK5-2000-01493). The J.E. Jurriaanse Stichting financially supported the reproduction of this thesis.

Cover: Motif of an Indian textile handcraft (Otovalo, Ecuador).

Printed at Ponsen & Looijen B.V., Wageningen.