Plant Biotechnology Journal (2006) **4**, pp. 145–167 doi: 10.1111/j.1467-7652.2005.00169.x

Review

Plant cell wall biosynthesis: genetic, biochemical and functional genomics approaches to the identification of key genes

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Received 5 July 2005;
revised 21 September 2005;
accepted 24 September 2005.
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Keywords: cellulose synthase-like genes, functional genomics, genetic approaches, glycosyltransferases, polysaccharide biosynthesis, stress tolerance, wall proteins.

Summary

Cell walls are dynamic structures that represent key determinants of overall plant form, plant growth and development, and the responses of plants to environmental and pathogen-induced stresses. Walls play centrally important roles in the quality and processing of plant-based foods for both human and animal consumption, and in the production of fibres during pulp and paper manufacture. In the future, wall material that constitutes the major proportion of cereal straws and other crop residues will find increasing application as a source of renewable fuel and composite manufacture. Although the chemical structures of most wall constituents have been defined in detail, the enzymes involved in their synthesis and remodelling remain largely undefined, particularly those involved in polysaccharide biosynthesis. There have been real recent advances in our understanding of cellulose biosynthesis in plants, but, with few exceptions, the identities and modes of action of polysaccharide synthases and other glycosyltransferases that mediate the biosynthesis of the major non-cellulosic wall polysaccharides are not known. Nevertheless, emerging functional genomics and molecular genetics technologies are now allowing us to re-examine the central questions related to wall biosynthesis. The availability of the rice, *Populus trichocarpa* and *Arabidopsis* genome sequences, a variety of mutant populations, high-density genetic maps for cereals and other industrially important plants, high-throughput genome and transcript analysis systems, extensive publicly available genomics resources and an increasing armoury of analysis systems for the definition of candidate gene function will together allow us to take a systems approach to the description of wall biosynthesis in plants.

Introduction

The plant cell wall represents the single major determinant of plant structure and is of fundamental importance in plant growth and development, resistance to pathogen invasion, quality of plant-based foods and the properties of plant fibres and fuels. In contrast to animals, in which specialized skeletal systems provide structural integrity and physical support, the strength, flexibility, texture and overall shape of higher plants depend on the cumulative properties of walls which surround individual cells (Bacic *et al*., 1988; Carpita and Gibeaut, 1993). We now know that the wall is a complex, diverse and dynamic entity, changing throughout the processes of cell division, growth and differentiation. It is a metabolically active compartment responsible for cell adhesion, is involved in cell–cell communication and is selectively permeable. Different cell types, of which there are approximately 40 within a plant, are distinguished from each other by the chemistry and organization of their walls, and even adjacent cells of the same type differ in the fine structure of some components in a way that is related to their developmental fate or history of exposure to environmental conditions. Thus, the compositions of walls

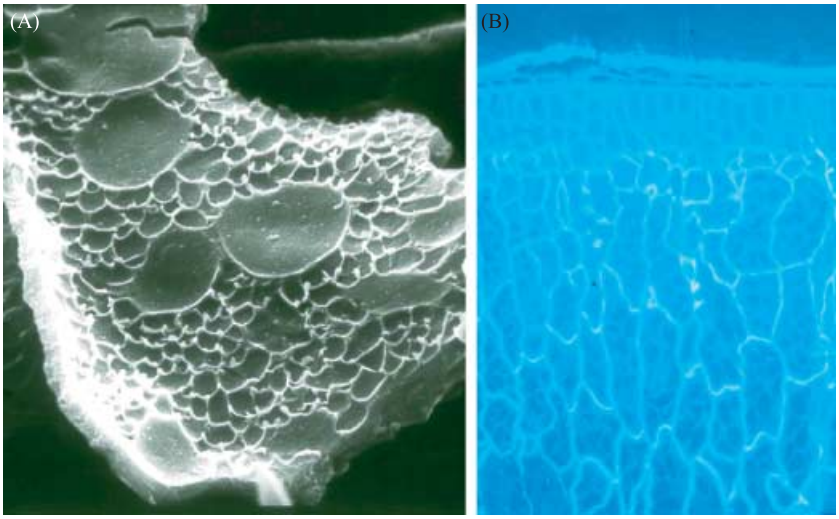

Figure 1 Starchy endosperm walls from barley grain and a model of the organization of wall components. (A) Scanning electron micrograph of a cell wall fragment isolated from the starchy endosperm of mature barley grain, showing impressions on the inner surface left by starch granules during grain development (from Fincher, 1975). (B) Section of the outer starchy endosperm, aleurone layer and seed coat of mature barley grain, stained with Calcofluor White. The outline of the walls is clearly visible (Hrmova and Fincher, 2001; photograph kindly provided with permission by Drs Leslie MacLeod and Meredith Wallwork).

vary depending upon the growth phase, cell type, cell position and local region within the wall (Carpita *et al.*, 2001; Trethewey and Harris, 2002). Walls are also involved in defence responses, when rapid formation of a cross-linked protein network and the deposition of callose and polyphenolics (e.g. lignin) create a physical barrier to invading microbial pathogens. Thus, the wall is essential for plant development and survival.

Primary walls of dicotyledonous plants consist of a cellulosic network embedded in a matrix of complex polysaccharides, of which xyloglucans and pectic polysaccharides are most abundant (Fincher and Stone, 1986; Bacic *et al.*, 1988; Carpita and Gibeaut, 1993; McCann and Roberts, 1994). Walls of the Poales and related commelinoid monocots, including wheat and barley, are organized in essentially the same way, although glucuronoarabinoxylans and (1,3;1,4)-β-D-glucans predominate in the matrix phase of these species, whereas levels of pectic polysaccharides, glucomannans and xyloglucans are relatively low (Bacic *et al.*, 1988; Carpita and Gibeaut, 1993; McCann and Roberts, 1994; Smith and Harris, 1999) (Figure 1). The (1,3)-β-D-glucan, callose, is found in certain specialized walls or at specific stages of wall development (Stone and Clarke, 1992). During secondary wall development, cellulose and matrix phase polysaccharides, such as heteroxylans and heteromannans, are deposited in a highly ordered pattern to form a rigid complex that can withstand enormous compressive forces. In some cell types, lignin can also be deposited throughout the wall during secondary development, but lignin biology will not be addressed here.

As our understanding of the importance and contributions of walls and their constituents to agro-industrial processes, such as paper and pulping, food quality and texture, malting and brewing, bioethanol production, dietary fibre and ruminant digestibility, grows, there are increasing attempts to apply genetic manipulation and conventional breeding techniques to modify the quality and quantity of individual components. A great deal of this effort has been directed towards lignin, for which genes encoding the enzymes in the biosynthetic pathway have been characterized (Boerjan *et al.*, 2003). In contrast, manipulation of the major wall components, the polysaccharides, has been hampered by the paucity of our knowledge of the mechanisms and control of the biosynthetic steps, coupled with a limited understanding of the physical and chemical interactions between wall components that influence characteristics such as porosity, elasticity and mechanical strength. A major contributing factor to our poor understanding of the enzymes involved in wall biosynthesis has been that many of the enzymes are large, membrane-bound proteins that have resisted extensive biochemical efforts for their purification in an active form. As a result, it has generally not been possible to obtain amino acid sequence information and the isolation of corresponding genes and complementary DNAs (cDNAs) has proved difficult. However, there has been significant, if rather limited, recent progress on describing the genetic and enzymatic processes that underlie the biosynthesis of wall polysaccharides, particularly in the area of cellulose biosynthesis (Doblin *et al.*, 2002; Lai-Kee-Him *et al.*, 2002; Tanaka *et al.*, 2003; Taylor *et al.*, 2003; Burton *et al.*, 2004), callose biosynthesis (Doblin *et al.*, 2001; Him *et al.*, 2001; Hong *et al.*, 2001a; Li *et al.*, 2003), xyloglucan biosynthesis (Perrin *et al.*, 1999; Faik *et al.*, 2000, 2002; Madson *et al.*, 2003; Li *et al.*, 2004) and, more recently, heteromannan biosynthesis (Dhugga *et al.*, 2004; Liepman *et al.*, 2005). Future advances are likely to be made through emerging molecular biological and functional genomics approaches, while the public availability of the *Arabidopsis*, rice and *Populus* genome sequences, coupled with extensive,

associated genomics resources (*Arabidopsis* Genome Initiative, 2000; Ware *et al*., 2002; Brunner *et al*., 2004), will greatly facilitate the identification of the genes involved in wall biosynthesis and related cellular processes.

In this review, recent advances in our knowledge of the genes responsible for wall synthesis, assembly and remodelling are presented, and the role of some functional genomics technologies in allowing these advances is described. In addition, the procedures that are available to confirm the functions of candidate genes are examined, together with the potential for the exploitation of enhanced knowledge of plant cell wall biosynthesis in the generation of crops with enhanced stress tolerance and increased utility in industrial processes that rely on wall utilization.

Classification of major enzymes in wall polysaccharide biosynthesis

General classification of glycosyltransferases

Polysaccharide synthases and glycosyltransferases are classified within the overall 'glycosyltransferase' (GT) class of carbohydrate-modifying enzymes (Coutinho and Henrissat, 1999; http://afmb.cnrs-mrs.fr/CAZY/). GTs are amongst the largest groups of enzymes found, and can be divided into more than 70 families based on sequence similarities, the existence of certain motifs, hydrophobic cluster analysis (HCA) and their catalytic specificity (Campbell *et al*., 1997; Imberty *et al*., 1999; Ross *et al*., 2001; Rosen *et al*., 2004). In general, homology amongst and within GT families is low, which makes the definition of function based on sequence similarity very difficult (Breton and Imberty, 1999).

Catalytic specificity, which encompasses the type of activated sugar nucleotide, the type of glycosyl residue that the enzyme transfers and the type of linkage formed, is another useful means of classification of GTs. Because most GTs catalyse specific reactions, there are a large number of GTs in any organism. Despite this fact, the biochemical activity of only a small number of plant GTs has been demonstrated so far (Keegstra and Raikhel, 2001). The reason why the functions and identities of many GTs have remained elusive is because of difficulties in the heterologous expression of cDNAs, in identifying the correct substrates for the expressed enzyme and, in some instances, the lack of commercially available sugar nucleotide donors (Zhang *et al*., 2003). About 1.6% of the *Arabidopsis* genome is made up of genes that encode GTs (Egelund *et al*., 2004). This value accounts for more than 400 genes from 41 GT families, compared with approximately 200 genes in the human genome (Breton *et al*., 2001; Scheible and Pauly, 2004). One of the reasons why plants have so many GTs is believed to be for the synthesis of complex cell wall polysaccharides and glycoconjugates that display tissue- and cell-type specificity (Coutinho *et al*., 2003).

Type I polysaccharide synthases

Within the overall GT group, two quite distinctive classes of GTs can be identified. First, the type I polysaccharide synthase group includes enzymes that catalyse the iterative incorporation of glycosyl residues from sugar nucleotide donors into molecular backbones that constitute the main chains of wall polysaccharides (Perrin *et al*., 2001; Vergara and Carpita, 2001; Bonetta *et al*., 2002). These are integral membrane proteins with multiple transmembrane (TM) helices, and have been difficult, if not impossible, to purify by traditional biochemical methods (Delmer, 1999). The type I polysaccharide synthases are believed to remain in close association with the nascent polysaccharide during its elongation, and are involved in the biosynthesis of homopolysaccharides such as cellulose, the (1,3)-β-D-glucan callose and, possibly, (1,3;1,4)-β-D-glucan (Gibeaut, 2000; Saxena and Brown, 2000; Richmond and Somerville, 2001; Bonetta *et al*., 2002; Doblin *et al*., 2003). The other putative function for integral membrane proteins is the synthesis of the backbones of other matrix phase heteropolysaccharides, including xyloglucans, arabinoxylans and glucomannans (Perrin, 2001; Vergara and Carpita, 2001; Dhugga *et al*., 2004). For plant cell wall biosynthesis, the polysaccharide synthases are believed to be encoded by multigene families of the cellulose synthase (*CesA*) and cellulose synthase-like (*Csl*) groups (Figure 2), the glucan synthase-like (*GSL*) genes (Figure 3) and, possibly, by other genes (Richmond and Somerville, 2000; Bonetta *et al*., 2002). The CesA and Csl enzymes are large proteins, with about 850–1000 amino acid residues, and contain three to six TM helices towards the COOH terminus and one to two towards the NH_2 terminus. They share a common D_D_D_QxxRW motif, which is believed to be involved in sugar binding and catalytic activity of the enzyme (Richmond and Somerville, 2001). The GSL proteins, which have been implicated in callose biosynthesis, are also large integral membrane proteins; they have molecular masses of greater than 200 kDa, but lack the D_D_D_QxxRW motif (Cui *et al*., 2001; Doblin *et al*., 2001; Him *et al*., 2001; Hong *et al*., 2001a; Jacobs *et al*., 2003; Li *et al*., 2003).

Type II glycosyltransferases

The second distinct class of GTs within the overall GT group contains enzymes that are also referred to as glycosyltransferases,

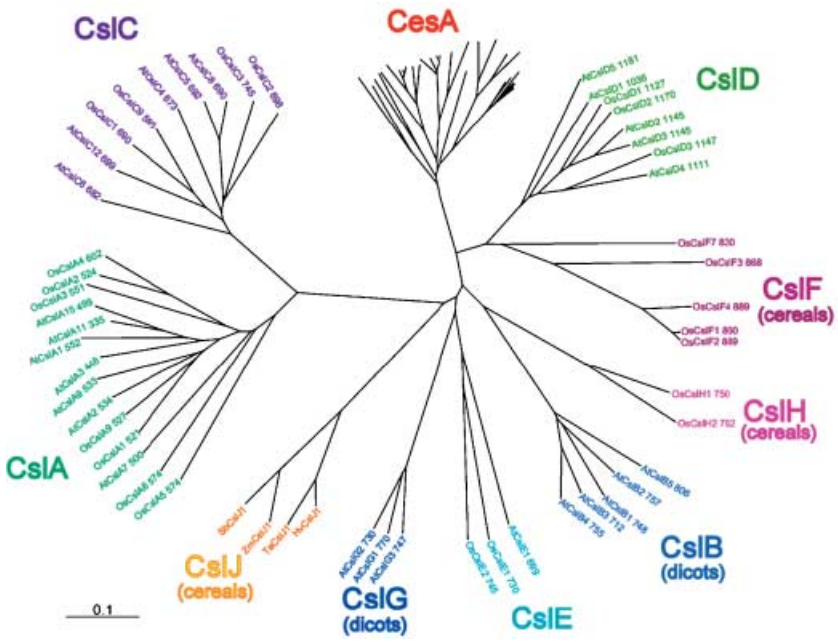

Figure 2 Cellulose synthase and cellulose synthase-like gene families in higher plants. The cellulose synthase (*CesA*) and cellulose synthase-like (*Csl*) gene families from higher plants contain 50 or more members and have been classified by Richmond and Somerville (2000) (http://cellwall.stanford.edu/). The *CslJ* group is found in certain cereals (A. J. Harvey, N. J. Shirley, R. A. Burton and G. B. Fincher, unpublished data 2005). While the functions of several *CesA* genes in cellulose biosynthesis have been confirmed, the functions of the *Csl* genes have been far more difficult to demonstrate.

but in a more restricted sense insofar as they transfer glycosyl residues from the donor to a polysaccharide backbone in a single event. Here, this second, type II class of GT enzymes is designated GlyT. They have a single TM helix that spans the membrane and functions as an anchor, together with a short cytosolic NH_2 terminus, an extended hydrophilic stem region and a globular catalytic domain within the lumen of the Golgi towards the COOH terminus of the protein (Kleene and Berger, 1993; Breton and Imberty, 1999; Gibeaut, 2000; Keegstra and Raikhel, 2001; Perrin *et al*., 2001; Sterling *et al*., 2001; Pagny *et al*., 2003). The relative ease with which this group of GTs can be dissociated from membranes with mild detergents in an active form has enabled some progress on their characterization through classical biochemical routes (Edwards *et al*., 1999). Examples of the GlyT class of enzyme are the α-L-arabinosyltransferases, assumed to add single α-L-arabinofuranosyl substituents to the (1,4)-β-D-xylan backbone of arabinoxylans, and the α-D-xylosyltransferases, which add single α-D-xylosyl residues to the (1,4)-β-D-glucan backbone of xyloglucans (Faik *et al*., 2002).

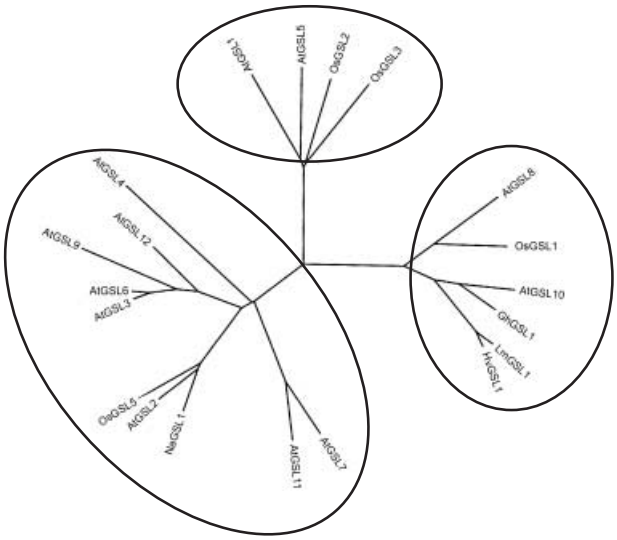

Figure 3 Phylogeny of glucan synthase-like (*GSL*) genes. Sequences of full-length GSL proteins from the http://cellwall.stanford.edu website were aligned, and an unrooted phylogenetic tree was created using programs accessed via the Australian National Genomic Information Service (ANGIS) website (http://www.angis.org.au). The GSL proteins are divided into three distinct clades. Species designations are as follows: At, *Arabidopsis thaliana*; Gh, *Gossypium hirsutum*; Hv, *Hordeum vulgare*; Lm, *Lolium multiflorum*; Na, *Nicotiana alata*; Os, *Oryza sativa*.

The cellulose synthase (*CesA*) gene family

Identification of *CesA* genes

Cellulose comprises unbranched chains of (1,4)-β-D-glucopyranosyl residues. Each chain has a variable degree of polymerization (DP) of up to 6000 and 14 000 in primary and secondary walls, respectively (Fincher and Stone, 2004). Three dozen or more chains of (1,4)-β-D-glucopyranose are arranged in highly ordered parallel arrays to make para-crystalline microfibrils of about 3 nm in diameter in primary walls and 5–10 nm in diameter in secondary walls (Brown, 1996; Jarvis, 2003; Fincher and Stone, 2004).

Isolation of active cellulose synthases has been difficult to achieve for a number of reasons, including the loss of activity during disruption of the plant tissue prior to membrane

enrichment. This loss of activity might be attributable, in part at least, to the instability of the rosette structure during cell rupture (Feingold *et al*., 1958; Doblin *et al*., 2002; Lai-Kee-Him *et al*., 2002). The only convincing example of significant cellulose synthase activity *in vitro* was achieved in microsomal fractions of blackberry cell suspension culture by Lai-Kee-Him *et al*. (2002).

Given these difficulties with the biochemical approach, the use of transcript analysis by Pear *et al*. (1996) to identify a gene encoding a CesA from cotton fibres was seen as a critical breakthrough in our ability to define and manipulate the molecular mechanisms and genetic basis for cellulose synthesis in higher plants. The identities of several other *CesA* genes were confirmed (Arioli *et al*., 1998; Burton *et al*., 2000), and it was soon realized that the *CesA* gene family had multiple members (Figure 2). Thus, there are 10 *CesA* genes in *Arabidopsis thaliana*, 12 in rice, about 12 in maize, 18 in *Populus trichocarpa* (Djerbi *et al*., 2005) and at least eight in barley (Richmond and Somerville, 2000; Doblin *et al*., 2002; Burton *et al*., 2004). It is likely that the individual *CesA* genes or groups of *CesA* genes are independently controlled in different tissues and during primary or secondary wall biosynthesis.

Cellulose-synthesizing rosette terminal complexes

Electron microscopy has shown that cellulose is synthesized in plants by large plasma membrane-associated protein particles that are known as rosette terminal complexes (Roelofsen, 1958; Brown *et al*., 1976; Mueller and Brown, 1980; Haigler and Blanton, 1996; Ha *et al*., 1998; Kimura *et al*., 1999; Saxena and Brown, 2000; Perrin *et al*., 2001; Doblin *et al*., 2002). Biochemical evidence and transcript analyses now corroborate previous suggestions that several distinct CesA proteins might be necessary for their correct assembly. Complementation of cellulose synthesis in *Arabidopsis* mutants indicates that a number of *CesAs* are required to synthesize cellulose, and that members of the gene family are not functionally redundant (Arioli *et al*., 1998; Taylor *et al*., 2000; Perrin, 2001). It now appears that groups of three *CesA* genes are co-ordinately transcribed in many plant species (Tanaka *et al*., 2003; Burton *et al*., 2004), which suggests that the three different cellulose synthase enzymes encoded by each group of *CesA* genes aggregate into active cellulose-synthesizing rosettes. It also seems likely that two distinct groups of three CesA enzymes are required for cellulose synthesis in primary and secondary walls (Doblin *et al*., 2002; Eckardt, 2003; Tanaka *et al*., 2003; Taylor *et al*., 2003; Burton *et al*., 2004). Doblin *et al*. (2002) calculated that the size estimated for rosette terminal complexes from numerous electron microscopy studies was consistent with the presence of a total of 36 CesA proteins arranged in six groups of six individual proteins.

A key objective of future work will be to define just where the groups of three co-ordinately expressed CesA proteins are placed within this complex (Doblin *et al*., 2002; Read and Bacic, 2002) and to identify additional ancillary proteins that might be required for catalytic activity (e.g. KORRIGAN; Szyjanowicz *et al*., 2004) and/or for establishing and maintaining the rosette structure.

The cellulose synthase-like (*Csl*) gene family

Subfamilies of *Csl* genes

The *Csl* genes can be divided into eight subfamilies, designated *CslA*, *CslB*, *CslC*, *CslD*, *CslE*, *CslF*, *CslG* and *CslH* (Figure 2), each of which contains multiple genes (Hazen *et al*., 2002; Goubet *et al*., 2003). In *Arabidopsis*, there are 30 known *Csl* genes and, in rice, about 37 (Hazen *et al*., 2002; Somerville *et al*., 2004).

Characterization of *Csl* genes

The *Csl* group is widely believed to encode enzymes that direct the synthesis of backbone chains of non-cellulosic wall polysaccharides, such as heteroxylans, xyloglucans and heteromannans, although it remains possible that some members might also be involved in cellulose biosynthesis (Richmond and Somerville, 2000; Doblin *et al*., 2001, 2003). It is also noteworthy that not all *Csl* subfamilies are represented in higher plant groups. Thus, the *CslB* and *CslG* subfamilies are found only in dicotyledons and gymnosperms, whereas the *CslF* and *CslH* groups are found only in monocotyledons (Figure 2). It is assumed that the different distribution of these subfamilies reflects differences in polysaccharide components in monocots and dicots (Carpita and Gibeaut, 1993; Smith and Harris, 1999). For example, the (1,3;1,4)-β-D-glucans are only found in the walls of the Poales and related commelinoids (Smith and Harris, 1999), and the *CslF* and *CslH* groups would therefore represent prime candidates for the (1,3;1,4)-β-D-glucan synthases.

Despite these clues on distribution, the availability of cell wall mutants in many plant species, extensive transcript analyses in which *Csl* transcriptional activity has been linked to wall composition (Richmond and Somerville, 2001), the high level of gene collinearity that has enabled comparative genomics to become a powerful tool in gene analysis (Sorrells

et al., 2003) and the potential use of targeted techniques (Burton *et al*., 2000) to down-regulate specific *Csl* genes, the enzyme activity of only one of the many members of the *Csl* gene family has been positively identified. Thus, it was shown recently that a *CslA* gene from guar (*Cyamopsis tetragonoloba*) encodes a (1,4)-β-mannan synthase (Dhugga *et al*., 2004). This result was confirmed by Liepman *et al*. (2005), who expressed *Arabidopsis CslA* genes heterologously in *Drosophila* S2 cells.

Transcript analyses of the *Csl* genes

Established transcript profiling procedures, including quantitative polymerase chain reaction (PCR) and microarray technologies, coupled with sophisticated statistical analysis and access to extensive expressed sequence tag (EST) databases, have provided clues as to the functions of specific groups of co-expressed genes, especially when it is possible to correlate transcriptional activity with cell wall composition and polysaccharide fine structure (Pear *et al*., 1996; Dhugga *et al*., 2004). Analysis of the *Arabidopsis* Affymetrix ATH-1 DNA chips by Hamann *et al*. (2004) has provided the definition of the transcript profiles of all but one (*CslB3*) of the *Csl* genes, together with the 10 *CesA* genes, in a range of tissues or organs at different developmental stages. This confirmed that the *Csl* genes are expressed at consistently lower levels than the *CesA*s, and indicated that a number of genes in this superfamily are regulated by circadian rhythm, ethylene and salt levels. However, there are few clear expression patterns in these data that might be linked to the progressive changes occurring in the cell wall during growth, or to the specialized nature of some walls in particular plant organs, which in turn might link a gene to a specific polysaccharide product. Until recently, the involvement of only a few of the Csl proteins in a number of growth processes had been documented, most notably when a mutation in *AtCslD3* resulted in a 'bald' root phenotype in which root hairs were absent (*kjk*; Favery *et al*., 2001), when *AtCslA7* was implicated in pollen tube growth and embryogenesis (Goubet *et al*., 2003) and when the insertion of a T-DNA in *AtCslA9* caused a decrease in the number of lateral roots with a concomitant increase in resistance to transformation by *Agrobacterium tumefaciens* (*rat*4; Zhu *et al*., 2003a,b). A *Nicotiana alata CslD* gene has also been implicated in cellulose biosynthesis in pollen tubes (Doblin *et al*., 2001).

Why are the functions of so few *Csl* genes known?

To date, only a few members of the *CslA* group have been assigned a well-defined biological function and, although the *CslA* gene subfamily contains a number of (1,4)-β-D-mannan synthase genes (Liepman *et al*., 2005), it is unclear as to whether all genes in the *CslA* subfamily encode (1,4)-β-D-mannan synthases. Progress in the search for genes that direct the biosynthesis of pectic polysaccharides has also been slow. Overall, the *Arabidopsis* genome is believed to contain more than 700 genes involved in cell wall metabolism (Henrissat *et al*., 2001), and the roles of only two of more than 170 genes that are predicted to be involved in pectin biosynthesis have been defined (Somerville *et al*., 2004). Why has progress in this area proved to be so difficult to realize, despite the increasingly sophisticated genomic and technical resources now available?

First, the abundance of messenger RNAs (mRNAs) corresponding to the key genes involved in wall biosynthesis is very often extremely low, and the actual levels of the biosynthetic enzymes themselves might not need to be especially high (Dhugga, 2005). Generally, this has made it difficult to correlate mRNA abundance of *Csl* genes with high levels of a particular polysaccharide in the walls of a specific tissue or cell type. Second, analyses of either mutants or transgenic plants in which selected *Csl* genes have been silenced or down-regulated have not been particularly helpful. It has become clear that silencing or down-regulating a specific member of a gene family can lead to the compensatory expression of other genes that might obscure the function of the silenced or down-regulated gene (Burton *et al*., 2000). It is also clear that another common problem associated with reverse genetic approaches is the lack of an obvious phenotype in the manipulated plants (Bouche and Bouchez, 2001). The full *Arabidopsis* genome sequence shows that there is a high level of gene redundancy, with only 35% of genes classified as unique and with 37% of genes belonging to families with five or more members (*Arabidopsis* Genome Initiative, 2000). Because all of the *CesA* and most of the *Csl* genes are members of such multigene families, it is not surprising that gene knockout approaches *per se* do not provide sufficient information on which to base the assessment of gene function, and therefore silencing of an entire family may be required before an effect is seen in the plant. Such strategies also need to be linked to more in-depth analyses of the phenotype, where detailed chemical changes in the affected walls would be most instructive.

Third, it is widely believed that the biosynthesis of non-cellulosic wall polysaccharides might require the concerted action of more than one enzyme to form large multimeric complexes (Buckeridge *et al*., 2004), as observed for cellulose biosynthesis (Doblin *et al*., 2002). A requirement for an additional member of the same or different Csl enzyme

subfamily for synthase activity, or a requirement for a completely unknown protein, could complicate functional assays to the extent that conclusions might not be confidently drawn. For example, if two individual *CslH* genes, or one *CslH* gene in combination with one *CslF* gene, were required for (1,3;1,4)-β-D-glucan synthesis in cereals, a much higher degree of complexity and hence uncertainty would be imposed on the functional analysis systems being employed. Finally, it is possible that we are not looking in the right place. So far, most work has been based on a candidate gene approach, in which the candidates are usually selected from the *Csl* gene family (Figure 2); however, the reasons for choosing candidate genes from this family are not always compelling. For example, members of the large *GSL* gene family, which are known to include callose synthase genes (Stone and Clarke, 1992; Hong *et al*., 2001a; Jacobs *et al*., 2003), might be involved in the synthesis of wall polysaccharides other than (1,3)-β-D-glucans.

Procedures for the functional analysis of *Csl* genes

Heterologous expression systems

There are three general approaches that might be taken for the functional analysis of candidate genes. First, the gene or its cDNA could be expressed heterologously *in vitro* and the activity proposed for the gene product could be measured directly. The second route involves various loss-of-function systems, such as gene silencing or mutant analysis, while the third approach could be via several gain-of-function systems.

Heterologous expression of candidate genes in a range of systems that do not normally synthesize the polysaccharide of interest, coupled with direct measurement of the enzyme activity of the expressed protein (Liepman *et al*., 2005), can prove particularly valuable. Examples of heterologous expression systems include *Escherichia coli*, yeast, *Pichia*, *Drosophila* embryo (Benting *et al*., 2000) and other insect cells, and Chinese hamster ovary (CHO) cells. It has been shown that, in some instances, different isoforms or homologues of a single enzyme isoform will be preferentially expressed in soluble, active form (Persans *et al*., 2001). Because many of the enzymes of interest are likely to be membrane bound, their functional analysis through heterologous expression will critically depend on the ability to produce correctly folded proteins in lipid phases or liposomes (Opekarová and Tanner, 2003).

One way to circumvent the need to provide an artificial membrane is to transiently express these enzymes in plant cells, in which all the components for correct processing, folding and targeting are likely to be present. A number of transient *in planta* expression systems have been described using either viruses (Voinnet *et al*., 2003) or *Agrobacterium* (Komarnytsky *et al*., 2004) as the infectious agent. Intelligent design of gene constructs may also be possible, in which proteins that have previously been shown to require partners for efficient function, such as the CesA proteins, are co-expressed at similar levels from a polycistronic mRNA driven by a single strong promoter (Bonnal *et al*., 2003; El Amrani *et al*., 2004; http://ifr31w3.toulouse.inserm.fr/IRESdatabase/).

Loss-of-function systems

Gene silencing induced at the post-transcriptional level, either through double-stranded RNA interference (dsRNAi) methods (Waterhouse and Helliwell, 2003) or by viral vectors (Baulcombe, 1999), is one of the most commonly used loss-of-function techniques. In particular, effective dsRNAi methods, both transient and in stably transformed plants, have been developed as a rapid loss-of-function approach to define gene function, through down-regulation of the specific gene of interest (Fire *et al*., 1998; Wesley *et al*., 2004). Although this approach has yielded valuable information, there are a number of problems with the technique. Silencing may not be complete and sufficient active protein might be synthesized from the reduced levels of mRNA to allow the plant to function normally, or functionally redundant gene homologues may mask the silencing (Burton *et al*., 2000). It is possible to down-regulate entire gene families using a highly conserved gene fragment that is found in all members of the multigene family or, as reported by Abbott *et al*. (2002), for the inactivation of three genes in the lignin biosynthesis pathway, multigene constructs may be used. However, the down-regulation of cell wall genes on a whole plant level may be undesirable or even lethal and, for this reason, the use of more targeted silencing regimes employing tissue-specific or inducible promoters will be a key consideration for the future success of gene silencing procedures in plants.

In addition to the post-transcriptional gene silencing methods, a number of powerful procedures involving gene silencing are now available for the definition of gene function, together with the requisite genetic resources. These include natural and chemically induced mutant populations, and T-DNA and transposon-tagged mutant lines in many plant species. There are a number of practical drawbacks in the generation and use of these mutant populations, as large numbers of lines are often required to achieve insertional saturation, and the mapping of each of the insertions can be time consuming and expensive. Two methods have been developed recently which do not require such large starting numbers and which rely on quicker and cheaper PCR-based methods for screening of pools of mutagenized

individuals. The first is the Delete-a-gene® approach (Li *et al*., 2002a), which can remove parts of genes, whole genes or entire tandem arrays of genes by fast neutron bombardment, and gives a much more efficient coverage of the genome than methods based on insertional mutagenesis. The second method is Targeted Induced Local Lesions IN Genomes, or TILLING, which employs ethyl methanesulphonate (EMS) mutagenesis to induce point mutations (McCallum *et al*., 2000; Colbert *et al*., 2001; Henikoff and Comai, 2003; Till *et al*., 2003a, 2003b; Caldwell *et al*., 2004; Slade *et al*., 2005). TILLING not only provides the opportunity to obtain an allelic series of mutations in the target gene, as has been successfully performed in hexaploid wheat to generate a near null allele of the waxy phenotype (Slade *et al*., 2005), but also enables the identification of natural allelic variants that may be useful in breeding programmes (Till *et al*., 2003a).

Gain-of-function systems

To resolve possible disadvantages of loss-of-function assays, a small number of gain-of-function systems have been developed for plant genes (Zhang, 2003; Tani *et al*., 2004). These systems are based on the over-expression of a gene in a place or time in which it is not normally expressed, and activation T-DNA tagging using four tandem repeats of a strong promoter's enhancer elements (Bassham and Raikhel, 2000; Zhang, 2003; Tani *et al*., 2004). Thus, in a gain-of-function approach, plant genes can be expressed in systems such as *Arabidopsis*, tobacco, yeast, *Drosophila*, *Physcomitrella* and a range of mammalian cell lines; again it is preferable to choose a system in which the polysaccharide of interest is not normally present.

The moss *Physcomitrella patens* is fast growing in popularity as a model functional genomics system as a result of a number of features that make it easy to handle, grow and transform (Frank *et al*., 2005). It has the great advantage over seed plants that targeted homologous recombination is an efficient, routine procedure (Schaefer and Zryd, 1997), such that modification of a gene should allow the generation of both a null allele and an allelic series. In terms of plant cell wall biology, *P. patens* has a number of other attractive features which could be exploited in functional analyses. Some *PpCesA* and *PpCsl* genes have already been cloned (Roberts *et al*., 2002; Roberts and Roberts, 2004), but, to date, of the four *PpCesA*s identified, none appears to be an orthologue of the secondary cell wall-specific genes found in seed plants (Taylor *et al*., 2003; Burton *et al*., 2004). This is not surprising as mosses lack specialized vascular tissues, but it provides scope for the heterologous expression of a set of plant *CesA* genes that might have a direct impact on the formation of the secondary wall terminal rosette complex, and hence on cellulose microfibril structure. Disruption of the CesAs in the *P. patens* terminal complex through homologous recombination targeted to areas of the genes thought to be involved in protein–protein interactions may therefore provide an avenue to explore how the catalytic subunits interact to form a complex, and how the integration of 'foreign' *CesA* genes may make it possible to manipulate the form of the cellulosic product. The expression of members of the grass-specific *Csl* families could equally well lead to the synthesis of some novel or hybrid polysaccharide products.

Other potentially valuable genetic resources for the functional analysis of genes that mediate wall biosynthesis, and in which gene expression in specific tissues and cells can be examined or manipulated, are the libraries of *GAL4* rice and *Arabidopsis* enhancer trap lines (Wu *et al*., 2003; Johnson *et al*., 2005). The tissue specificity of the endogenous enhancer or promoter element is reflected directly by the expression pattern of the *gfp* in each independent transformant, with some lines showing exquisite tissue- or cell-type specificity and others displaying more widespread expression. The potential use of such lines in the exploration of gene function lies in the proven phenomenon of GAL4 transactivation. If a second gene, also linked to the GAL4 upstream activating sequence (UAS), is co-transformed into a chosen enhancer trap line, this gene should be subject to transactivation by the pre-existing GAL4 transcription factor, producing exactly the same expression pattern as the *gfp*. Therefore, by careful selection of the parental transgenic line, expression or down-regulation, for example by means of a dsRNAi construct, of a second gene of interest will potentially be tightly controlled and the location of expression can be predicted. This is a powerful tool for those genes which may be deleterious when manipulated at a whole plant level, and also narrows the search for a phenotype to a clearly defined area. Expression of selected *Csl* genes under the GAL4 UAS in carefully chosen *Arabidopsis* enhancer trap lines should allow us to target gene expression to certain cell types and subsequently to test for the appearance of a novel polysaccharide in this location.

The glucan synthase-like (*GSL*) genes

The callose synthases of higher plants are most likely encoded by the *GSL* gene family. Callose is a plant (1,3)-β-D-glucan that is not found in all cell walls, but is deposited in a number of specialized walls or wall-associated structures during normal plant development and also in response to wounding or pathogen challenge (Stone and Clarke, 1992). Like cellulose

synthases, callose synthases are located at the plasma membrane and have proved to be difficult to identify using biochemical means (Li *et al*., 2003), despite being relatively stable *in vitro*. A major advance came from work on the genes responsible for the synthesis of a very similar (1,3)-β-D-glucan in fungi. In the yeast *Saccharomyces cerevisiae*, the *Fks* (*FK506 supersensitive*) genes encode (1,3)-β-D-glucan synthases, as shown by analyses of mutants (Douglas *et al*., 1994; Inoue *et al*., 1995; Mazur *et al*., 1995) and biochemical studies (Inoue *et al*., 1995). Searches of EST databases for plant genes with similarity to the *Fks* genes identified a family of candidate genes, called *Glucan Synthase-Like* (*GSL*) (Saxena and Brown, 2000; Cui *et al*., 2001).

GSL gene families

Plant *GSL* genes are usually members of multigene families, with 12 in *Arabidopsis* (Hong *et al*., 2001a), 13 in rice, more than nine in *Populus trichocarpa* (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html) and at least eight in barley (M. Schober, A. K. Jacobs, R. A. Burton and G. B. Fincher, unpublished data 2005). The GSL proteins can be divided into three distinct clades (Figure 3; Doblin *et al*., 2001). Proteins in the clade containing *At*GSL1 and *At*GSL5 are clearly distinct from the other GSL proteins, being 100–150 amino acids shorter at their NH_2 terminus, and are encoded by genes that contain less than three exons. In contrast, the other *Arabidopsis GSL* genes have approximately 40 exons (Richmond and Somerville, 2000; Doblin *et al*., 2001; Hong *et al*., 2001a; Verma and Hong, 2001). The *GSL* genes from barley and rice can also be similarly divided (Richmond and Somerville, 2000; Jacobs *et al*., 2003). It has been proposed that each of the 12 *Arabidopsis* genes is responsible for callose synthesis in a different location within the plant (Hong *et al*., 2001a), and the expression of particular *GSL* genes specifically in tissues that produce callose, such as the expression of *GhGSL1* in cotton fibres (Cui *et al*., 2001) and *NaGSL1* in pollen tubes (Doblin *et al*., 2001), supports this suggestion. It should be noted that, at this stage, the *GSL* gene numbering systems for the different species do not indicate orthology, although it has been proposed that orthologous *GSL* genes from different species may be functionally related and produce callose in the same tissue or at the same developmental stage (Doblin *et al*., 2001).

The GSL proteins

Predicted GSL proteins are large, with about 1800–1950 amino acid residues (Saxena and Brown, 2000; Hong *et al*., 2001a; Li *et al*., 2003), and are predicted to contain 13–16 TM helices (Cui *et al*., 2001; Doblin *et al*., 2001; Verma and Hong, 2001; Østergaard *et al*., 2002; Li *et al*., 2003). They contain an NH_2 terminal domain of about 350–500 amino acid residues that is predicted to be cytoplasmic, followed by a predicted membrane region with six TM helices. A large central domain of about 620–780 amino acid residues is predicted to be cytoplasmic and, at the COOH terminal end, there is another membrane region predicted to contain between eight and 10 TM helices. Differences in the number of TM helices alter the location of the COOH terminus, although most predictions place it in the cytoplasm.

A biochemical approach has now been successfully used to provide evidence that GSL proteins are involved in callose synthesis. Antibodies have provided an indirect link between *GhGSL1* (Cui *et al*., 2001), *HvGSL1* (Li *et al*., 2003) and *AtGSL6* (Hong *et al*., 2001a) and callose synthase activity through the detection of high-molecular-weight polypeptides in fractions enriched for callose synthase activity. A direct biochemical link was established by Li *et al*. (2003), who used peptide mass fingerprinting to identify *Hv*GSL1 as the major polypeptide in a callose-synthesizing region of a native gel.

Functional analyses have also been used to determine *GSL* function. Two groups have associated the absence of callose from cell wall appositions (papillae) that form beneath infection sites with null mutations in *AtGSL5* (Jacobs *et al*., 2003; Nishimura *et al*., 2003), strongly implicating *At*GSL5 in wound-activated callose synthesis. However, there are no published reports of loss-of-function mutants for other *GSL* genes, and gain-of-function experiments with *GSL* genes are yet to produce convincing data.

Although there is considerable evidence that the GSL proteins are involved in (1,3)-β-D-glucan synthesis, they do not contain a recognized uridine-5′-diphosphoglucose (UDP-Glc) binding motif. There is therefore some speculation as to whether these proteins constitute the catalytic subunit, or whether they are a pore-forming unit in the CalS (Callose synthase) complex, based on their low-level structural similarity to bacterial and eukaryotic transporters (Douglas *et al*., 1994; Cui *et al*., 2001; Hong *et al*., 2001a; Dijkgraaf *et al*., 2002). However, it is also possible that GSL proteins utilize a novel motif for UDP-Glc binding and are catalytically active.

Callose synthase complexes

In common with the cellulose synthases, callose synthases of plants may consist of complexes containing a number of proteins. The GSL proteins have been identified in high-molecular-weight complexes on gels (Li *et al*., 2003; Kjell

et al., 2004), but other polypeptides were also present in the region containing the GSL proteins. Similarly, several polypeptides are usually present in callose synthase-enriched fractions, but it remains unclear whether they are integral components of a callose synthase complex. Some potential complex components have been identified by their interaction with GSL proteins. The *Gh*GSL1 protein from cotton fibres binds calmodulin *in vitro* in the presence of Ca^{2+} (Cui *et al*., 2001) and yeast-two-hybrid analyses indicate that *At*GSL6, the putative cell-plate callose synthase, interacts with the cell-plate-associated dynamin-like protein, phragmoplastin, and a novel UDP-Glc transferase (UGT1) (Hong *et al*., 2001b). More than one active site, and therefore more than one GSL protein, may be present in each putative callose synthase complex. Polymers of (1,3)-β-D-glucan can form triple helices of parallel chains (Stone and Clarke, 1992; Pelosi *et al*., 2003), suggesting the activity of three closely associated catalytic subunits (Lai-Kee-Him *et al*., 2001).

Glycosyltransferase (*GlyT*) genes in wall biosynthesis

Although the functions of many mammalian and bacterial GlyTs have now been defined, a relatively limited number of the GlyTs that function in plant cell wall biosynthesis have been characterized and these are described below.

Galacturonosyltransferase (family GT8)

Two allelic mutants (*qua1-1* and *qua1-2*) of *Arabidopsis* showed a dwarfed and weak cell adhesion phenotype (Bouton *et al*., 2002). Analysis of wall composition showed a 25% decrease in galacturonic acid (GalA) content. The decrease in GalA, but not in neutral sugars, was similar to that in a related mutant (*Quasimodo1*) of pectin biosynthesis, and the decrease in homogalacturonan in mutants compared with the wild-type was further confirmed with the homogalacturonan-specific antibodies JIM5 and JIM7. The *QUA1* gene is a putative α-D-galacturonosyltransferase, but its protein product has not been characterized biochemically (Bouton *et al*., 2002).

Galactosyltransferase (family GT34)

Edwards *et al*. (1999) purified and partially sequenced a 51-kDa putative galactomannan (1,6)-α-D-galactosyltransferase protein from fenugreek (*Trigonella foenum-graecum*) that was used to isolate a cDNA clone from developing endosperm. A truncated cDNA clone lacking the TM helix was expressed in *Pichia pastoris* and the expressed protein showed galactomannan α-D-galactosyltransferase activity (Edwards *et al*., 1999). Over-expression of the gene in tobacco resulted in an increase in galactosylation of the mannan backbone by 25% from the T1 to the T2 generation (Reid *et al*., 2003). Following the isolation of the *GalT* gene from fenugreek, an orthologue of this gene was isolated from *Lotus japonicum* (Edwards *et al*., 2004). Post-transcriptional gene silencing of the *GalT* gene resulted in an increase in the mannose : galactose (Man : Gal) ratio in the galactomannan from 1.2 to 6, suggesting the down-regulation of α-D-galactosyltransferase activity in developing *L. japonicum* endosperm (Edwards *et al*., 2004).

Xylosyltransferase (family GT34)

Faik *et al*. (2002) used the fenugreek α-D-galactosyltransferase (GalT) peptide sequence to identify eight homologous genes in *Arabidopsis* (Keegstra and Raikhel, 2001). The cDNAs corresponding to these were isolated and expressed in *Pichia* for the analysis of associated biochemical activity (Faik *et al*., 2002). One of the putative genes showed α-D-xylosyltransferase (XylT) activity on cello-oligosaccharides. The truncated version of *At*XylT, unlike the fenugreek GalT, was not active following heterologous expression (Faik *et al*., 2002). In the same study, a pea homologue was isolated from detergent-washed microsomes and showed α-XylT activity. The PsXylT catalysed the transfer of xylose from UDP-xylose to cello-oligosaccharides bigger than DP5, unlike *At*XylT, which also used cellotetraose as an acceptor (Faik *et al*., 2002). It was concluded that these α-D-XylT genes were involved in the substitution of main chain (1,4)-β-D-glucosyl residues with α-D-xylosyl residues during xyloglucan biosynthesis.

Fucosyltransferase (family GT37)

A 63-kDa α-fucosyltransferase (FuT) enzyme purified from microsomal fractions of pea epicotyls was used to generate amino acid sequence data (Perrin *et al*., 1999; Faik *et al*., 2000). Biochemical characterization of the native protein showed that the active form is an oligomer with a higher molecular weight (approximately 250 kDa) than predicted from the corresponding cDNA. PsFuT1 catalyses the last step of pea cell wall xyloglucan synthesis by transferring fucose from GDP-fucose to oligo-xyloglucosides through a (1,2)-α linkage.

The peptide sequence of the pea enzyme was subsequently used to isolate a homologous gene from *Arabidopsis* (*AtFuT1*), which was later characterized by expression in mammalian COS cells (Perrin *et al*., 1999). *PsFuT1* cDNA has 62.3% amino acid sequence identity with *AtFuT1* and the enzymes

share common short sequence motifs (Perrin *et al*., 1999; Faik *et al*., 2000; Keegstra and Raikhel, 2001). It has also been shown that *mur2*, a loss-of-function mutant of *AtFuT1* on chromosome 2 of *Arabidopsis*, exhibits a 98% reduction in xyloglucan fucosylation (Reiter *et al*., 1997; Keegstra and Raikhel, 2001; Vanzin *et al*., 2002).

Glucuronosyltransferase (family GT47)

A *Nicotiana plumbaginifolia* T-DNA insertion mutant, *nolac-H18* (non-organogenic callus with loosely attached cells), was used by Iwai *et al*. (2002) to isolate a glucuronosyltransferase gene. The gene was named *NpGUT1* because of its sequence similarities with animal homologues (Iwai *et al*., 2002). Cell wall composition analysis of normal and *nolac-H18* mutant calli supported a β-D-glucuronosyltransferase function for *NpGUT1*, which is believed to participate in the transfer of glucuronic acid (GlcA) residues to type II rhamnogalacturonans in pectin. Iwai *et al*. (2002) also showed that the lack of GlcA in the mutants, and ultimately in the pectic rhamnogalacturonan II fraction, affects the formation of borate cross-links.

Galactosyltransferase (family GT47)

In a similar approach, the gene responsible for the *Arabidopsis mur3* mutant was positionally cloned and isolated (Madson *et al*., 2003). Cell wall analyses of *mur3* plants showed a 50% decrease in fucose content in xyloglucans (Reiter *et al*., 1997). Detailed studies of *mur3* cell walls revealed that a β-D-galactosyl residue was missing from a specific xylosyl residue of xyloglucan, which ultimately meant that there would be no fucose in this structure. Further to the loss of the galactosyl residue, the addition of galactosyl residues to another xylosyl residue was enhanced (Madson *et al*., 2003). The lack of the galactosyl residue on the xylosyl residue suggested that *mur3* encoded a xyloglucan β-D-galactosyltransferase. The *mur3* cDNA was expressed in *Pichia* to confirm β-D-galactosyltransferase (GalT) activity. Extracts from *Pichia* cells expressing the GalT enzyme showed an approximate 25-fold increase in the incorporation of radiolabelled galactosyl residues into ethanol-insoluble xyloglucan, compared with an empty vector control (Madson *et al*., 2003).

Proteins of the primary wall

Wall proteins, which generally comprise less than 10% of the dry weight of the primary wall, are now recognized as critical components in maintaining both the physical and biological functions of the plant extracellular matrix. They are not restricted solely to the wall itself, but also form structural and functional elements of the plasma membrane–cell wall continuum. This continuum is vital for the perception of signals from the external environment.

Proteins extracted from the wall were historically seen as structural elements. It has now become clear that many of these proteins fulfil a variety of roles in addition to that of structural components. Chemical analysis shows that the protein component is rich in the amino acids hydroxyproline/proline (Hyp/Pro), serine/threonine (Ser/Thr) and glycine (Gly), particularly in hydroxyproline-rich glycoproteins (HRGPs) and glycine-rich proteins (GRPs) (Showalter, 1993; Cassab, 1998; Johnson *et al*., 2003). The HRGPs consist of the extensins (Showalter, 1993; Kieliszewski and Lamport, 1994; Cassab, 1998), arabinogalactan-proteins (AGPs; Fincher *et al*., 1983; Nothnagel, 1997; Gaspar *et al*., 2001; Showalter, 2001) and proline-rich proteins (PRPs; Chen and Varner, 1985; Tierney *et al*., 1988; Fowler *et al*., 1999).

The specific interactions of HRGPs and GRPs with themselves and with other wall components are still largely unknown. They are proposed to interact with the major carbohydrate components of the wall to form a complex network (Bacic *et al*., 1988; Fincher, 1992; Carpita and Gibeaut, 1993). Changes during development and exposure to abiotic and biotic stresses influence the nature of these interactions and alter wall composition and structure (Cassab and Varner, 1988). The wall also contains other families of proteins vital to wall assembly and remodelling during growth, development and stress responses. These include enzymes such as hydrolases, proteases, glycosidases, peroxidases and esterases, expansins and wall-associated kinases (WAKs). Bioinformatic analysis of genomic sequences has revealed that many of these wall proteins are encoded by multigene families (Schultz *et al*., 2002; Cosgrove, 2003).

Extensins

Extensins are abundant glycoproteins of the wall and are the best characterized structural proteins. In general, extensin genes encode proteins with a signal peptide followed by a repetitive region rich in Pro/Hyp, with the main repeat motif being SP_4. They are rich in Hyp and Ser, and valine (Val), tyrosine (Tyr), lysine (Lys) and histidine (His) are moderately abundant. Extensins are basic proteins with isoelectric points of ~10 due to the Lys content. Carbohydrate comprises approximately 50% of their mass and consists of *O*-linked side-chains that include single galactopyranosyl (Galp) residues attached to Ser, as well as mono-, di- and tri-(1,2)-

β-linked arabinosides or a tetra-arabinoside (αAra*f*1-3βAra*f*1-2βAra*f*1-2βAra*f*1-) attached to Hyp. The addition of arabinosides to blocks of Hyp induces the extended polyproline-II conformation, a rod-like helical structure stabilized by glycan side-chains, that characterizes extensins (van Holst and Varner, 1984).

In *Arabidopsis*, there is a large family of at least 22 extensin genes (Merkouropoulos *et al*., 1999; Yoshiba *et al*., 2001; Schultz *et al*., 2002; Johnson *et al*., 2003). The *Arabidopsis* extensins can be divided into different classes depending on their repeat motifs and using features of extensins characterized from other dicot species (Johnson *et al*., 2003). Searches of the rice genome database (http://www.tigr.org/tigr-scripts/e2k1/irgsp.spl) have failed to find 'true' extensins, that is, proteins containing both $SP_{3–4}$ and some combination of the amino acids Val, Tyr, Lys and His, which are features of the *Arabidopsis* extensins.

Arabinogalactan-proteins

AGPs are highly glycosylated proteoglycans, usually containing 1–10% protein (Johnson *et al*., 2003). AGP protein backbones are typically rich in Hyp/Pro, alanine (Ala), Ser and Thr. The Hyp residues are usually substituted by type II arabino-3,6-galactans (AGs; 5–25 kDa), although short arabinosides are found (Nothnagel, 1997). Most AGPs are anchored, sometimes transiently, to the plasma membrane by glycosylphosphatidylinositol (GPI) anchors (Youl *et al*., 1998). AGPs are secreted from plant cells in which they are either anchored to the plasma membrane or are soluble in the extracellular matrix or cell wall.

The AGPs are proposed to have a specific function during root formation (van Hengel and Roberts, 2002; van Hengel *et al*., 2002), the promotion of somatic embryogenesis (e.g. van Hengel *et al*., 2004), pollen tube guidance (Roy *et al*., 1998), alternation between sporophytic and gametophytic transitions in the ovules (Acosta-Garcia and Vielle-Calzada, 2004), resistance to root-dependent transformation by *Agrobacterium* (Gaspar *et al*., 2004) and xylem differentiation in *Zinnia* and *Arabidopsis* (Motose *et al*., 2004).

In *Arabidopsis*, at least 47 genes are predicted to encode AGP protein backbones, and most of these are predicted to be GPI anchored (Johnson *et al*., 2003). These have been divided into several subclasses on the basis of their biased amino acid composition and their modular structure, and include 13 classical AGPs, 12 AG-peptides (mature protein backbones of 10–17 amino acids), three basic AGPs that include a short Lys-rich region and 21 FLAs (fasciclin-like AGPs) that have, in addition to predicted AGP-like glycosylated regions, putative cell adhesion domains known as fasciclin domains (Johnson *et al*., 2003; Schultz *et al*., 2004). The recent identification of a *salt overly sensitive* (*sos5*) mutant with a mis-sense mutation in the fasciclin domain of *AtFLA4* (Shi *et al*., 2003) indicates that these domains are important for FLA function.

Proline-rich proteins

PRPs and 'hybrid' proline-rich proteins (HyPRPs) represent another family of HRGPs that accumulate in the wall (Carpita and Gibeaut, 1993; Showalter, 1993; Cassab, 1998; Johnson *et al*., 2003). They are proteins with an NH_2 terminal secretion signal, followed by a coding sequence biased for Pro residues, often in blocks of KPPVY(K) that can be repeated over 40 times in the one polypeptide. Because PRPs generally have a K residue preceding PP, PRPs are thought to be either minimally glycosylated with arabino-oligosaccharides or not to be glycosylated at all (Kieliszewski *et al*., 1995). In *Arabidopsis*, four genes encoding chimeric *PRPs* (*AtPRP1*, *AtPRP2*, *AtPRP3* and *AtPRP4*) have been characterized (Fowler *et al*., 1999). At least 17 PRPs have been identified in the *Arabidopsis* annotated protein database and have been placed into three subclasses (Showalter, 1993; Fowler *et al*., 1999; Schultz *et al*., 2002; Johnson *et al*., 2003). PRPs accumulate in the wall in response to wounding or elicitor treatment and are rapidly insolubilized by oxidative cross-linking through similar mechanisms to those proposed for extensins (Johnson *et al*., 2003).

Glycine-rich proteins

Unlike most other plant structural proteins, GRPs generally do not contain Pro-rich sequences and are not known to be glycosylated (Keller, 1993; Showalter, 1993; Sachetto-Martins *et al*., 2000; Johnson *et al*., 2003). The Gly-rich domains of plant GRPs consist of sequence repeats that can be summarized by the formula $(Gly)_nX$, where X can be any amino acid and *n* is generally 1–5. A number of different types of Gly-rich repeats have been identified, including GGGX, GGXXXGG and GXGX. A large number (approximately 100) of Gly-rich proteins are present in the *Arabidopsis* genome (Schultz *et al*., 2002; Johnson *et al*., 2003). Sachetto-Martins *et al*. (2000) have provided an extensive review of the expression patterns of GRPs, showing that they are developmentally regulated and are induced by physical, chemical and biological factors. Up-regulation of GRP genes in response to water stress, wounding and pathogen attack suggests a protective role for GRPs.

Expansins

Expansins are particularly important wall-associated proteins (McQueen-Mason *et al*., 1992; Cosgrove *et al*., 2002; Cosgrove, 2003) and have been implicated in the acid growth phenomenon of walls in elongating cells. Two multigene families of expansins, namely α-expansins (*EXP*) and β-expansins (*EXPB*), have been recognized and are believed to participate in wall loosening during growth (Cosgrove *et al*., 2002; Cosgrove, 2003). A third group of related genes, termed expansin-like (*EXPL*), are found in both the *Arabidopsis* and rice genomes, but their role, if any, in wall loosening has not been established (Cosgrove, 2003).

In *Arabidopsis*, there are 26 members of the *EXP* gene family, five members of the *EXPB* gene family and three members of the *EXPL* gene family, whereas, in rice, there are 33, 18 and four members, respectively (www.bio.psu.edu/expansins/). The functional and evolutionary reasons for the large numbers of expansin genes are unclear. It has been suggested that the two major classes (α and β) function in essentially the same manner, but perhaps target different polysaccharides in the cell wall (Cosgrove *et al*., 2002). The expansin proteins have a modular two-domain structure: one domain comprises a series of conserved cysteine (Cys) residues with sequence similarity to family 45 endoglucanases, and the second, COOH terminal domain is thought to be involved in polysaccharide binding. The proteins have been purified and assayed for their ability to 'loosen' associations between cellulose molecules and other wall polysaccharides *in vitro*, but they have no known enzymatic activity (Cosgrove *et al*., 2002).

Remodelling of polysaccharides following deposition into the wall

Primary cell walls of plants are dynamic structures that change during normal growth and development, and in response to external stimuli. Walls respond to changing requirements for strength, flexibility and porosity, and to abiotic and biotic stresses (Bacic *et al*., 1988; Carpita *et al*., 2001; Doblin *et al*., 2003; Fincher and Stone, 2004). In this section, selected examples of polysaccharide remodelling and the enzymes that mediate the changing fine structures of wall polysaccharides are presented. Specifically, attention is focused on proposed mechanisms for the alteration in xyloglucan and heteroxylan fine structure and molecular size, both of which exert dramatic effects on physical structure and function of both the polysaccharides and wall properties, together with the potential roles of polysaccharide hydrolases in wall metabolism. It is quite possible that post-synthetic remodelling of wall polysaccharides, particularly the matrix phase constituents, could prove to be a general phenomenon in the normal growth and development of plant cell walls (Carpita *et al*., 2001; Doblin *et al*., 2003; Fincher and Stone, 2004).

Role of arabinoxylan arabinofuranohydrolase (AXAH) enzymes in remodelling

Heteroxylans are characteristic components of the primary walls of plants, where they consist of a (1,4)-linked β-D-xylan backbone, substituted with terminal α-L-arabinofuranosyl residues, mostly at the C(*O*)3 but also at the C(*O*)2 positions of the backbone; α-D-glucuronosyl substituents may also be present (Vietor *et al*., 1994). The content of arabinoxylans in the walls of maize and barley coleoptiles increases only slightly during growth, but there are significant changes in their fine structure, in particular, the ratio of substituted to unsubstituted xylosyl residues decreases with maturity (Gibeaut and Carpita, 1991; Inouhe *et al*., 1997; Gibeaut *et al*., 2005). The removal of the arabinosyl residues is believed to be mediated by AXAHs (Ferre *et al*., 2000; Lee *et al*., 2001). Gibeaut and Carpita (1991) suggested that a high degree of arabinosyl substitution of the xylan backbone in the newly synthesized polysaccharide would increase its solubility during passage through the endomembrane system and cause it to be loosely bound after deposition into the wall. As the wall matures, the decreasing degree of substitution would allow closer alignment with and increased hydrogen bonding to cellulose and other wall components, and would result in the arabinoxylan becoming more tightly bound to the wall (Carpita, 1984; Suzuki *et al*., 2000).

The potential role of AXAHs in remodelling wall arabinoxylans is implied rather than proven at this stage. A family 51 AXAH purified from extracts of 7-day-old barley (*Hordeum vulgare* L.) had an apparent M_r of about 65 kDa and released L-arabinose from cereal cell wall arabinoxylans (Ferre *et al*., 2000; Lee *et al*., 2001). Using cDNAs encoding barley AXAHs as probes, preliminary transcript analyses in tissues in which wall arabinoxylans undergo rapid modification, such as in growing coleoptiles and the early developing grain, have shown extremely high levels of mRNAs for the AXAH enzymes. The AXAHs are encoded by a gene family of about eight members in rice and may play key roles in wall metabolism in cereals and other members of the Poaceae, including the modification of arabinoxylan fine structure during wall deposition, maturation or expansion, or in wall turnover and the hydrolysis of arabinoxylans in germinated grain.

Role of xyloglucan transglycosylases/hydrolases (XTHs) in polysaccharide remodelling

Xyloglucans are major matrix phase components of walls in many plants, and consist of a backbone of (1,4)-β-D-glucan substituted with xylosyl, galactosyl and sometimes fucosyl residues (Vincken *et al*., 1997; Pauly *et al*., 2001). The xyloglucans are subject to modification following their initial deposition into the wall, both with respect to their ability to bind to cellulose and other wall polysaccharides, and with respect to their fine structural features and substitution patterns (Pauly *et al*., 2001; Gibeaut *et al*., 2005). Furthermore, the molecular sizes of xyloglucans can be altered after their deposition into the cell wall. The XTHs, which are abundant in the apoplastic space (Rose *et al*., 2002), can hydrolyse xyloglucan backbones and, in some cases, transfer one of the hydrolytic products directly on to the non-reducing terminus of another xyloglucan chain. Some XTHs only act as transglycosylases (Kallas *et al*., 2005). The transglycosylation activity results in a rapid increase in the molecular size of some xyloglucan molecules and might be necessary for 'grafting' newly formed xyloglucan chains on to existing wall polysaccharides (Farkas *et al*., 1992; Fry *et al*., 1992; Fry, 2004).

Sequences encoding XTHs are surprisingly abundant in barley EST databases, given the relatively low levels of xyloglucans in the walls of most barley tissues (Fincher, 1992; Gibeaut *et al*., 2005). There are at least 22 XTH genes in barley (Strohmeier *et al*., 2004) and about 30 in rice (Yokoyama and Nishitani, 2004). It is possible that some of these enzymes might also be active on the more abundant matrix phase polysaccharides, namely the arabinoxylans and the (1,3;1,4)-β-D-glucans (Strohmeier *et al*., 2004). Indeed, molecular modelling experiments based on the three-dimensional structure of an XTH from poplar (Johansson *et al*., 2004) predicted that the three-dimensional dispositions of amino acid residues in the substrate-binding and catalytic sites of XTHs and microbial (1,3;1,4)-β-D-glucan endohydrolases would be similar. Furthermore, they revealed similarities with the active sites of family GH11 (1,4)-β-D-xylan endohydrolases. Thus, the molecular modelling establishes a structural and evolutionary connection between XTHs, (1,3;1,4)-β-D-glucan endohydrolases and xylan endohydrolases. A role for XTHs in the modification of highly abundant (1,3;1,4)-β-D-glucans and arabinoxylans in walls of the commelinoid monocots would be consistent with the apparent over-representation of XTH sequences in cereal EST databases, and with the abrupt increase in the molecular size of heteroxylans that has been observed in suspension-cultured maize cells following the deposition of the polysaccharide into the walls (Kerr and Fry, 2003).

Role of hydrolytic enzymes in wall synthesis

There is mounting genetic evidence that hydrolytic enzymes participate in polysaccharide biosynthesis and wall assembly. Thus, a point mutation in the *Arabidopsis KOR* gene, which encodes a (1,4)-β-D-glucanase, is associated with a large decrease in wall cellulose. The precise mechanism of action of the enzyme in cellulose synthesis is unknown, but it might be involved in trimming or otherwise 'editing' nascent cellulose chains (Szyjanowicz *et al*., 2004), or possibly in releasing newly synthesized chains from the biosynthetic enzymes. Our transcript analyses of genes encoding enzymes that hydrolyse major wall polysaccharides, including (1,4)-β-D-xylan (1,3;1,4)-β-D-glucan and (1,4)-β-D-mannan, show that barley tissues in which net wall synthesis is occurring generally have significant levels of mRNAs for these endohydrolases (R. A. Burton, N. J. Shirley and G. B. Fincher, unpublished data 2005). Furthermore, a major quantitative trait locus (QTL) for barley grain (1,3;1,4)-β-D-glucan content co-segregates with a gene encoding a (1,3;1,4)-β-D-glucanase (Han *et al*., 1995).

Regulation of cell wall biosynthesis

There is little or no information available on the regulation of either polysaccharide synthases or GlyTs during the deposition and assembly of polysaccharides in walls or during wall remodelling. The regulation of synthases, particularly cellulose synthases, can now be examined at the transcriptional, translational, enzymatic and cellular levels. Increasingly reliable techniques, such as yeast one- and two-hybrid systems, can be used to identify proteins that bind to particular DNA promoter elements and to build models of protein complexes that are involved in the regulation of specific genes (Thaminy *et al*., 2003; Iyer *et al*., 2005). Reliable transcript analysis techniques, such as quantitative real-time PCR, could also be used for the analysis of mRNA transcript levels of genes in specific cell types under specific conditions, in attempts to correlate these data with wall polysaccharide composition. Hormone-responsive elements found in gene promoters would allow the development of testable hypotheses as to whether hormones regulate the synthesis of cellulose and non-cellulosic wall polysaccharides. Up- or down-regulation of key transcription factor genes could also be used to evaluate the overall importance of the transcription factors and their potential for manipulation.

A central, but as yet unanswered, biological question is whether the composition of sugar nucleotide pools acts as a determinant of wall composition, or whether these pools simply react passively to changing metabolite flux, which

might occur during the withdrawal of sugar nucleotides for the biosynthesis of a specific wall polysaccharide. The primary sugar nucleotide involved in the synthesis of wall polysaccharides is UDP-D-glucose, which represents the activated sugar donor for the biosynthesis of cellulose, xyloglucans and (1,3;1,4)-β-D-glucans (Feingold and Avigad, 1980; Reiter and Vanzin, 2001; Gu and Bar-Peled, 2004; Seifert, 2004; Zhang *et al*., 2005). Oxidation of the UDP-D-glucose by UDP-D-glucose dehydrogenase forms UDP-D-glucuronate, which can be decarboxylated by UDP-D-glucuronate decarboxylase (UXS) to form UDP-D-xylose. The UDP-D-xylose becomes the glycosyl donor for the biosynthesis of xyloglucans and arabinoxylans, and the UDP-L-arabinose that is formed by epimerization of the UDP-D-xylose is used for arabinoxylan and pectic polysaccharide biosynthesis. Zhang *et al*. (2005) showed, in preliminary experiments, that elevated levels of *HvUXS* mRNAs in selected barley tissues appeared to correspond to higher contents of wall arabinoxylan, but, at this stage, a number of technical and theoretical constraints preclude unequivocal conclusions that the activity of the *Hv*UXS enzymes has a direct effect on carbon flux through the sugar nucleotide interconversion pathways or on cell wall composition.

Future applications of wall biology in biotechnology

For millennia, cell walls, in the form of timber and plant fibres such as cotton and linen, have been an integral part of human societies. In more recent times, as our understanding grows of how different wall components contribute to food quality and texture, dietary fibre, paper and pulping, and ruminant digestibility, we are increasingly modifying the quality and quantity of wall components through genetic manipulation. So far, much of this effort has been directed towards lignin, because the relevant biosynthetic enzymes are characterized and genes corresponding to these enzymes have been cloned (Hu *et al*., 1999; Sederoff, 1999; Boudet, 2002; Humphreys and Chapple, 2002; and references cited therein). Not surprisingly, manipulation of lignin structure or quantity has often resulted in unexpected changes in the quantity and/or composition of the cellulosic and non-cellulosic polysaccharides of the wall, again reflecting the plasticity of cellular metabolism in compensating for changes in key processes. The recent advances in our understanding of the molecular aspects of cell wall polysaccharide synthases and GlyTs now offer opportunities to directly manipulate crops and other plants, and thereby to enhance the quality and processing efficiencies of plant-based products.

Cereal (1,3;1,4)-β-D-glucans and arabinoxylans can exert a disproportionate influence on many aspects of cereal processing, and this is often associated with the propensity of the polysaccharides to form aqueous solutions of high viscosity (Fincher and Stone, 2004). The important malting barley quality characteristic, hot water extract, reflects the extent of starchy endosperm modification during the malting process and depends, in part at least, on the extent of cell wall degradation in the germinated grain (Bamforth, 1993). Thus, extracts will depend not only on cell wall thickness and the levels of wall polysaccharides in ungerminated barley grain, but also on the amounts and speed of synthesis of hydrolytic enzymes that are generated upon germination. During wort separation and beer filtration, incompletely degraded (1,3;1,4)-β-D-glucans in particular, but also arabinoxylans, from barley malt and cereal adjuncts can cause inconvenient filtration difficulties, and can contribute to undesirable haze formation in the final beer (Bamforth, 1993).

In contrast with these undesirable effects, high-molecular-weight water-soluble arabinoxylans have positive attributes in bread-making, where they can improve dough water absorption, loaf volume and breadcrumb texture (Cawley, 1964). To release higher levels of the important high-molecular-weight water-soluble arabinoxylans from walls, microbial endoxylanases are often added as 'improvers' during bread-making. This treatment is particularly valuable in whole grain flours, although a range of endogenous xylanase inhibitors in wheat flour can counteract the positive effects of added xylanases (Sorensen *et al*., 2004; Trogh *et al*., 2004). Cereal (1,3;1,4)-β-D-glucans might also prove useful in bread-making, where they could be used as a flour supplement to increase water absorption of dough. In any case, manipulation of the expression of genes involved in (1,3;1,4)-β-D-glucan and arabinoxylan synthesis or modification could significantly enhance the bread-making properties and nutritional value of wheat.

From the nutritional viewpoint, high levels of soluble arabinoxylans and (1,3;1,4)-β-D-glucans can have negative effects in feed formulations for monogastric animals such as pigs and poultry (Choct *et al*., 1995). These 'anti-nutritive' effects are attributable to the increased viscosity of gut contents, which results not only in reduced rates of diffusion of digestive enzymes, but also in reduced absorption of digestion products, and hence in reduced growth rates. For poultry, this problem is compounded by the production of 'sticky' faeces, arising from poor digestion, which can cause handling and hygiene problems for producers (Bhatty, 1993; Fincher and Stone, 2004). Again, manipulation of (1,3;1,4)-β-D-glucan and arabinoxylan levels in cereal grains could greatly

enhance the value of specific grains for animal feed formulations (Von Wettstein *et al*., 2003).

In contrast, wall polysaccharides have a number of beneficial effects in human diets. Thus, cereal arabinoxylans and (1,3;1,4)-β-D-glucans make up a large part of the dietary fibre fraction that is so important for human bowel health (Topping and Wong, 1994). Microorganisms in the lower bowel can metabolize both wall-derived polysaccharides and resistant starch to produce volatile fatty acids and other compounds that appear to provide protection against some cancers (Yamada, 2000; Paulsen, 2002). High concentrations of (1,3;1,4)-β-D-glucans can also be useful for non-insulin-dependent diabetics, because they can lower the glycaemic index by modulating glucose and insulin responses following food intake (Hodge *et al*., 2004). In addition, high levels of (1,3;1,4)-β-D-glucan and other non-cellulosic wall polysaccharides in food have been linked to lower serum cholesterol concentrations (Anderson *et al*., 1991; Behall *et al*., 2004). Thus, cereal (1,3;1,4)-β-D-glucans have considerable potential as functional food ingredients in human diets (Brennan and Cleary, 2005). On the basis of these observations, there is potential to improve the nutritional value of bread and other cereal-based foods, either through supplementation with (1,3;1,4)-β-D-glucans from other cereals or through the manipulation of arabinoxylan and (1,3;1,4)-β-D-glucan levels in wheat grain itself.

At the fibre level, higher plant cell walls are major constituents of wood, wood-derived building products and paper. Wood, reeds, dried grass and the residues of crop plants, such as wheat stubble, are composed predominantly of cellulose and the non-cellulosic polysaccharides of cell walls that remain essentially intact following senescence of the plant tissue (Boudet *et al*., 2003). In most cases, these residues contain lignocellulosic complexes that are difficult to degrade enzymatically, but this very property, coupled with their structural strength, makes wall polysaccharides highly suitable for paper production and as building materials (Pilate *et al*., 2002). Similarly, the cell walls of plant residues have been used by humans as a fuel for heating and for conversion to other energy forms. Although direct combustion of wood and plant residues will probably decline in the future, wall polysaccharides will have a key role in the emerging bioethanol industry, where cellulosic plant crop residues are replacing starch as the primary source of fermentable sugar for ethanol production as recent advances in the catalytic efficiency of the relevant hydrolytic enzymes makes the process economically feasible (http://www.eere.energy.gov/RE/bio_fuels.html). Should this occur, new information on genes encoding wall polysaccharide synthases and GlyTs could be used to manipulate wall composition as a means of facilitating subsequent hydrolysis of crop residues and increasing the yields of ethanol during fermentation processes. For example, the ratios of pentose- and hexose-based wall polysaccharides in vegetative tissues could be manipulated, together with the fine structures that mediate polysaccharide–polysaccharide or polysaccharide–lignin interactions in walls, to increase the speed and extent of hydrolysis of cereal straw residues to their monosaccharide constituents, and thence the rate and extent of fermentation of released sugars to ethanol. Similar manipulations would also have considerable potential for increasing the digestibility and nutritional value of pasture and crop residues in animal production.

Pectins, a class of wall polysaccharides found in abundance in many fruits and vegetables, are now considered as one of the most versatile hydrocolloids used in industry as texture modifiers and stabilizers (e.g. gelling, thickening, emulsifying). The first genetically modified food on supermarket shelves was FlavrSavr™ tomatoes in which the endopolygalacturonase enzyme was down-regulated using anti-sense RNA technology in order to prolong the shelf life of tomatoes and enhance their processing characteristics (Tucker and Mitchell, 1993; and references cited therein). Pectins continue to be a major target for industrial manipulation (Willats *et al*., 2001; and references cited therein) by engineering plants with hydrolytic enzymes that modify pectin structure. This can be achieved either before the deposition of pectins into the wall, for example by over-expressing arabinanase in the Golgi apparatus (Skjøt *et al*., 2001), or after deposition of the polysaccharide into the wall, for example by over-expressing (1,4)-β-galactan endohydrolase (Sørensen *et al*., 2000; Ulvskov *et al*., 2005).

An emerging field of application for plant-based wall polysaccharides is as potentially valuable therapeutic agents, which arises from their ability to modulate the human immune systems (for reviews, see Yamada, 2000; Paulsen, 2002). Evidence is accumulating that some polysaccharides, particularly type II AGs/AGPs and pectins, can activate certain types of immune cells as well as the complement system. Because the fine structure of these wall-based polysaccharides is critical for their biological activity, the ability to define and manipulate the structure of these polysaccharides will be important in the production of therapeutic grade products for use in human health.

To date, our attention to wall polysaccharides in biotechnology has been focused on the enzymatic degradation of cellulose and non-cellulosic polysaccharides. In future biotechnological applications, our increased knowledge of biosynthetic mechanisms will also provide opportunities to manipulate the fine structures of the polysaccharides, their

interactions within the wall, their relative abundance in the wall during plant growth, and their functionality in nutrition and agro-industrial processes. The forthcoming challenge will be to balance commercial demands for novel plants with manipulated wall compositions against the structural and other functional requirements of walls that are essential for growth and development, and hence plant survival.

Concluding remarks

Our repertoire of functional genomics tools and candidate gene analysis systems is steadily increasing. However, rapid future progress in the definition of the functions of *Csl* genes and other genes that participate in wall synthesis and remodelling will probably be best tackled through a systems approach, in which biophysical, developmental, genetic and *in vitro* composition analyses are focused on the problem in an integrated way (Somerville *et al*., 2004). Thus, a combination of genome analyses and comparative genetics, high-density genetic and QTL mapping, map-based cloning, high-throughput, non-biased transcript analyses, proteomics and metabolomics will help us to understand the groups of genes and their products that control wall synthesis. Increasingly, we should be able to relate these analyses to wall polysaccharides in individual cell types in plant tissues and, using microanalyses of wall composition in conjunction with antibodies specific for particular wall polysaccharides, it will be possible to define gene expression at all stages from transcription to the deposition of known polysaccharides into the wall. These approaches must ultimately lead to the identification of candidate genes that mediate wall synthesis, for which novel, independent procedures will be available for the final assignment and testing of gene function. Understanding gene and enzyme function in wall biosynthesis will undoubtedly create new opportunities for the development of crops with enhanced productivity, nutritional value and biotechnological potential.

Acknowledgements

This work was supported by the Grains Research and Development Corporation and the Australian Research Council. We thank Ed Newbigin, Neil Shirley, Monika Doblin, Bruce Stone and Jose Varghese for invaluable assistance with aspects of the work.

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