Seed Cell Wall Storage Polysaccharides: Models to Understand Cell Wall Biosynthesis and Degradation[1]

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Cell wall storage polysaccharides (CWSPs) are found as the principal storage compounds in seeds of many taxonomically important groups of plants. These groups developed extremely efficient biochemical mechanisms to disassemble cell walls and use the products of hydrolysis for growth. To accumulate these storage polymers, developing seeds also contain relatively high activities of noncellulosic polysaccharide synthases and thus are interesting models to seek the discovery of genes and enzymes related to polysaccharide biosynthesis. CWSP systems offer opportunities to understand phenomena ranging from polysaccharide deposition during seed maturation to the control of source-sink relationship in developing seedlings. By studying polysaccharide biosynthesis and degradation and the consequences for cell and physiological behavior, we can use these models to develop future biotechnological applications.

CWSPs IN ENDOSPERMS

Galactomannans and the Mannan Family

Several species from various families are known to have seeds that store mannan, glucomannan, or galactomannan (Meier and Reid, 1982; Buckeridge et al., 2000b).

The mannan family comprises pure mannans, glucomannans, and galactomannans. The former is artificially defined as containing more than 90% of Man in the polysaccharide. Mannans are formed by mannosyl residues linked to each other by β-1,4-glycosidic linkages. When the main backbone chain also contains Glc, the polymer is called glucomannan. Both mannans and glucomannans can be substituted with single units of Gal linked to the main chain by α-1,6-linkages. In some species (Orchidaceae and Araceae, e.g. *Cattleya* and *Phylodendron*, respectively), mannan and glucomannan are acetylated and may not contain Gal (M.A.S. Tiné and M.S. Buckeridge, unpublished data). There is at least one report of the presence of pure mannan in pseudobulbs of an orchid, *Oncidium* (Wang et al., 2006). However, it is likely that the polymer is acetylated, as it is soluble upon extraction with hot water. Substitution with galactosyl residues to form a galactomannan or a galactoglucomannan and/or acetylation change hydrodynamic properties of the polysaccharides, impacting their solubility in water (McCleary et al., 1981).

This range of chemical structures gives rise to polymers with different biological functions. Mannans are present, for example, in palm (*Phoenix dactylifera*) and coffee (*Coffea arabica*) seeds, and because they are insoluble in water and display strong intermolecular interaction, their biological function is usually associated with conferring hardness to plant tissues. Several publications have led to the conclusion that the mannan present in tomato (*Solanum lycopersicum*) seed endosperm has as its primary function, to confer hardness and control radicle protrusion, rather than acting as a storage polysaccharide (Toorop et al., 1998). In tomato and lettuce (*Lactuca sativa*), the endosperm functions as controller of radicle protrusion affected by gibberellin and abscisic acid (ABA; e.g. Groot and Karssen, 1987, 1992; Dutta et al., 1997).

Endo-β-mannanase is the principal enzyme involved in mannan hydrolysis in tomato and several genes have been cloned with seed-specific expression (Bewley et al., 1997; Gong and Bewley, 2007). Nonogaki et al. (2000) localized transcripts of the genes *LeMAN1* and *LeMAN2* in the endosperm of tomato seeds. *LeMAN1* was associated with mobilization of mannan whereas *LeMAN2* was detected strictly in the endosperm cap and functions in radicle protrusion.

An exciting new discovery is the fact that endo-β-mannanase can perform transglycosylation (Schröder et al., 2004). Schröder et al. (2009) proposed the abbreviation MTH (for mannan tranglycosylase hydrolase) for this enzyme family in analogy with activities and structures of the xyloglucan transglycosylase hydrolase (XTH) family. Transglycosylation by MTH of CWSP (mannans, galactomannans, and glucoman-

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nans) would bring an interesting novel dimension to our understanding of how the mobilization system works: Galactomannan degradation in legumes, for instance, is thought to occur in nonstop mode, in contrast with storage xyloglucan disassembly, which can be halted by transglycosylation depending on growth rate (see below).

In palm and coffee endosperms, mannans are also storage compounds. In palms, they are slowly degraded and the Man is used for embryo development (De Mason et al., 1983). While the mannan is a reserve for the developing embryo, it also provides mechanical resistance such that the embryo is protected from damage during the long germination of palm seeds.

In legumes, the main function of the endosperm cell walls appears to be storage, with the yield of galactomannan reaching more than 30% of the seed dry weight in many species. These walls are thickened with galactomannan and in certain cases (e.g. fenugreek [*Trigonella foenum-graecum*] and *Schyzolobium parayba*) the cytoplasm disappears entirely. In these cases, the endosperm is nonliving and degradation is performed by enzymes (α-galactosidase, endo-β-mannanase, and exo-β-mannosidase) made in the aleurone layer. However, in many cases, although the walls are quite thick, they surround a cytoplasm in which protein bodies and other compounds such as raffinose and Suc are stored (Reid, 1971; Buckeridge and Dietrich, 1996; Buckeridge et al., 2000a).

The Leguminosae is one of the main plant families on Earth, with more than 18,000 species. Although the sample of species of this family that have been analyzed to date represents just a small fraction of the family members, about half of the species studied have an endosperm containing galactomannan. Together with their occurrence in many other important plant families, such as Palmae, Solanaceae, Convolvulaceae, Iridaceae, Araceae, and Orchidaceae, this makes the mannan family of polysaccharides one of the most widespread CWSP groups in plants.

The ratio of Man to Gal in legumes (the M:G ratio; Buckeridge et al., 2000a) varies from fully substituted polymers (e.g. fenugreek) to an average of ratio of 3:1 or 4:1 (carob [*Ceratonia siliqua*]). A typical distribution of M:G ratios in legumes gives a bimodal shaped curve (Buckeridge et al., 1995, 2000b), associated with the subfamily to which the species belong. Members of the subfamily Caesalpinioideae have poorly substituted galactomannans and it has been demonstrated that the polysaccharide is edited during deposition in the wall, presumably by a debranching α-galactosidase (Edwards et al., 1992). This renders a polymer that is relatively less water soluble. The subfamily Mimosoideae contains galactomannans that are partially branched with an average of two Mans per Gal, whereas Faboideae (or Papilionoideae) tends to have highly substituted galactomannans (M:G near 1:1) but usually with less polysaccharide per dry weight.

Because of their viscosity and solubility in water, it has been proposed that galactomannans can also play a role as imbibing substances (Reid and Bewley, 1979; Potomati and Buckeridge, 2002).The protection of the embryo against pathogens due to the physical barrier (highly viscous) that surrounds the embryo during early stages of development is another possible function. Although such roles can be played by galactomannan, the endosperms end up being degraded and the products of hydrolysis (monosaccharides) serve as source of carbon and energy to the growing embryo.

Lisboa et al. (2006) purified the endo-β-mannanase from *Sesbania virgata*, a fast-growing legume tree from the subfamily Faboideae. These authors found relatively high activity of endo-β-mannanase in the tip of the radicle. This activity decays as endo-β-mannanase increases in the endosperm. Thus, legumes probably have a similar mobilization system as tomato in their seeds.

During the 80s and the 90s, studies of galactomannan mobilization focused on the regulation of enzyme production by hormones and environmental factors and elucidating mechanisms used for seeds to control the entrance and loss of water in the endosperm (Reid and Bewley, 1979), the effect of water stress in slowing down galactomannan mobilization (Spyropoulos, 1982; Spyropoulos and Reid, 1988), and the effects of plant hormones on mobilization, especially ABA (Malek and Bewley, 1991). Dirk et al. (1999) found a correlation between the production of Gal and Man from mobilization and an increase in ADP-Glc pyrophosphorylase in starch-accumulating tissues of the seed.

ABA has been shown to interfere with the production of the galactomannan hydrolases (Reid and Meier, 1972; Seiler, 1977). ABA or endosperm leachate affected the production of enzymes in protoplasts of endosperm of carob seeds and also in isolated endosperms of fenugreek (Kontos and Spyropoulos, 1995; Kontos et al., 1996). Tonini et al. (2006) measured the endogenous levels of ABA in different tissues of seeds of *S. virgata* and found that the testa of these seeds have a relatively high concentration of ABA at the 1st d (16 nmol/g of fresh mass) and that it quickly decreases as germination proceeds. In the endosperm and embryo, ABA increases as germination proceeds. The testa has cells that are metabolically active, with activities of α-galatosidase and endo-β-mannanase, and so it is possible that the high concentration of ABA negatively regulates storage mobilization before the embryo requires the sugars for growth (Tonini et al., 2007).

Experiments performed with the addition of actinomycin-D (an inhibitor of transcription) and cycloheximide (an inhibitor of translation) to seeds of fenugreek, *Trifoliumin carnatum*, and *Medicago sativa* (Reid and Meier, 1972) and also with carob (Seiler, 1977), suggested that hydrolases are synthesized de novo. However, when seeds of *S. virgata* were incubated with α-amanitin, actinomycin-D, and cyclohehimide, different results were obtained (Tonini et al., 2010a), suggesting that the enzymes necessary for galactomannan degradation are already present, possibly in protein bodies. As storage protein degra-

dation occurs, the hydrolases are released to the storage cell wall. The existence of a connection between storage protein and cell wall polysaccharide mobilization is an attractive idea, since this would synchronize nitrogen and carbon delivery to the growing embryo. Furthermore, storage mobilization would be energetically more efficient, since the investment to produce the enzyme would come from the mother plant and the seed would not have to spend photoassimilates to supply energy for the respiration costs of enzyme production.

Experiments performed with addition and endogenous measurements of sugars, ABA, and ethylene indicated that galactomannan degradation in endosperms of *S. virgata* is controlled by a complex cross-talk mechanism involving several biochemical pathways (Tonini et al., 2010b). In these experiments, ethylene was shown to induce galactomannan mobilization.

Several species of angiosperms use galactomannans as storage compounds to feed the developing seedling until it reaches autotrophy. The use of galactomannan, as opposed to starch, offers advantages of other functions (e.g. regulation of water entrance and loss, protection against herbivory).

Remaining questions include how the program of gene expression is controlled such that enzymes are sent to the wall in the right proportions and act synergistically to completely degrade the storage wall at a rate that the metabolism of the seedling uses the products. How is the network of gene expression, enzyme production, and polysaccharide modification coordinated? What is the impact of this network organization on the physiological performance of the seedling? These questions highlight the importance that a systems biology approach will have in the interpretation of the biological function of the CWSP in plants.

Mannan and Galactomannan Biosynthesis

Grant Reid's group showed that galactomannans are synthesized in developing endosperms by a GDP-Man-dependent mannosyltransferase and an UDP-Gal-dependent galactosyltransferase (GalT). In certain species with high degree of Gal substitution, i.e. with lower M:G ratios such as fenugreek (M:G = 1.1) and guar (*Cyamopsis tetragonolobus*; M:G = 1.6), the M:G ratio is determined at synthesis, whereas in seeds of *Senna occidentalis* (M:G = 2.3–3.2) the synthesized galactomannan is edited in the endosperm by a specific α-galactosidase.

The genes that encode GalT from fenugreek (Edwards et al., 1999) and mannosyltransferase from guar (Dhugga et al., 2004) have been cloned and characterized. Two enzymes are active in a soluble form (Sandhu et al., 2009), making them very attractive for use in biotechnological applications since galactomannans with different degrees of branching can be used for different purposes in several industrial processes such as thickening in food formulations.

CWSPs IN COTYLEDONS

In contrast to the endosperm, arabinogalactan and xyloglucans are used as reserves in cotyledons. The cotyledon is an adapted leaf and integration of the metabolism of these polysaccharides involves different signaling mechanisms and other functions besides storage. As CWSP is degraded, cotyledons develop vascular bundles to transport the products to the growing plant parts.

Arabinogalactan

Pectin polymers are also found as CWSPs, notably arabinogalactan (Crawshaw and Reid, 1984; Parker, 1984; Buckeridge et al., 2000b). In cotyledons of lupin (*Lupinus angustifolius*), the chemical structure is a β-(1,4)-linked D-galactan with branches of α-(1,5)-Ara (Hirst et al., 1947). An exo-β-(1,4)-galactanase was purified from cotyledons of lupin that acts specifically on the galactan (Buckeridge and Reid, 1994). The gene that encodes lupin exogalactanase has been cloned (accession no. AJ011047).

The exogalactanase was incubated in vitro with soluble lupin seed galactan and also with cell walls isolated from cotyledons of lupin (Buckeridge et al., 2005). The purified enzyme was shown to release 82% of the galactosyl residues present in the isolated polysaccharide and 63% of the galactosyl residues present in cell wall ghosts as free Gal. Using an enzyme-gold conjugate of the exogalactanase, Buckeridge et al. (2005) observed that the degradation pockets previously described by Parker (1984) do not contain galactan. The remaining material has a composition of Rha (11%), Ara (16%), Gal (30%), Glc (20%), Xyl (20%), plus 35% of the wall as uronic acids, and is not attacked by enzymes during galactan degradation.

Biosynthesis of Storage Arabinogalactans

Little is known of the biosynthesis of arabinogalactans in storage tissues. The 1,4-β-linked galactan from lupin seeds is synthesized in microsomal fractions, has an optimal pH at 6.5, and is dependent on Mg^{2+}. The substrate of the GalT is UDP-Gal (Brickell and Reid, 1996). β-1,4-linked galactan, as well as the 1,3 to 1,6-β-linked galactan biosyntheses have also been studied in nonstorage tissues of flax (*Linum usitatissimum*; Goubet and Morvan, 1993) and pea (*Pisum sativum*; Abdel-Massih et al., 2003), but no genes of galactan biosynthesis have been characterized yet.

Xyloglucan

The Importance of Fine Structure to Physiology

The basic structure of storage xyloglucans is similar to the primary wall xyloglucans. They have a backbone composed of β-(1,4)-linked glucan with regular branching with α-(1,6)-linked xylosyl residues that can be

branched further with β-(1,2)-linked galactosyl residues. However, storage xyloglucan is not fucosylated.

Storage xyloglucans are based on blocks composed of Glc_4Xyl_3 and in the proposed nomenclature (Fry et al., 1993) are composed of XXXG, XLXG, XXLG, and XLLG in different proportions (Buckeridge et al., 1992). The only different structure found so far is the one from seeds of *Hymenaea courbaril* that is based on XXXXG and XXXXXG (along with XXXG) and several galactosylated versions of these oligosaccharides (Buckeridge et al., 1997; Tiné et al., 2006).

A comparison of the limit digest oligosaccharides obtained from *Copaifera*, tamarind (*Tamarindus indica*), and *Hymenaea* xyloglucans by action of *Trichoderma* endoglucanse at low concentrations, demonstrated that, under these conditions, the enzyme produces fragments with much higher M_r (e.g. up to 35 XXXG blocks; Tiné et al., 2003). It became clear that Gal distribution directly interfered with hydrolysis by endoglucanase, as *H. courbaril* xyloglucan was hydrolyzed faster by endoglucanase than the XXXG-based *Copaifera* xyloglucan.

Several of the oligosaccharides and polymer fragments of *H. courbaril* were purified and analyzed by mass spectrometry. The fine structure of the other members of the XXXXG family was reported by Tiné et al. (2006), allowing definition of the combination of XXXG and XXXXG oligomers in the same polymer, the proportions of which in *H. courbaril* is 2:1, respectively. To ease interpretation of these structures, Tiné et al. (2006) named XXXG as T and XXXXG as P. The combinatorial calculations on the basis of the data obtained by mass spectrometry suggested that xyloglucan from *H. courbaril* is composed of motifs of TPPT with additional T units at the sides. These blocks are not arranged randomly in the polysaccharide with implications then for the mechanism of biosynthesis and perhaps also as a defense strategy from consumption by herbivores.

Hymenaea xyloglucan is hydrolyzed faster than *Copaifera* (Tiné et al., 2003), suggesting that the fine structure makes its backbone more available to the enzymes. The *Hymenaea* xyloglucan binds more strongly to cellulose (Lima and Buckeridge, 2001) and to itself (Lima et al., 2004). *Hymenaea* xyloglucan precipitates from solution much more quickly than tamarind or *Copaifera* xyloglucan. The biological consequence of the polymer structure is that proportionally more carbon could be accumulated in the seed that contained a polymer like the one present in *H. courbaril*, because of its ability to adopt higher-order interactions.

Xyloglucan Degradation

Reis et al. (1987) found that in tamarind, the storage polymer is deposited between two primary walls that they termed inner and outer walls. During xyloglucan mobilization, only the storage wall disappears, leaving the inner and outer walls intact. This has also been observed in *H. courbaril* using CCRC-M1 antibody that binds to fucosylated xyloglucans (M.A.S. Tiné, M.R. Braga, G. Freshour, M. Hahn, and M.S. Buckeridge, unpublished data). Thus, enzymes have to be delivered to the storage wall by a process that includes passing through a fucosylated xyloglucan-containing primary wall without hydrolyzing it. The location of storage xyloglucans may possibly explain the lack of action of the exohydrolases on the polymers but only on oligosaccharides (see below).

Tiné et al. (2000b) found that the storage cell walls of *H. courbaril* forms protuberances that make bridges between cotyledon cells that store xyloglucan. These bridges seem to hold cells together along with a middle lamella, but at the same time with intercellular spaces that are thought to be filled with water during imbibition.

Four enzyme activities responsible for xyloglucan degradation were detected, purified, and characterized (α-xylosidase, β-galactosidase, β-glucosidase, and xyloglucan-endo-β-glucanase, later renamed xyloglucan endotransglycosylase; Edwards et al., 1985). Based on the purification and mode of action of nasturtium (*Tropaeolum majus*) enzymes, a disassembly model was proposed in which, after xyloglucan endotransglycosylase and β-galactosidase produced oligosaccharides, these were attacked first by β-glucosidase and then by α-xylosidase, culminating in the release of free monosaccharides (Fanutti et al., 1993).

In cotyledons of *Copaifera langsdorffii* and *H. courbaril* the β-galactosidases isolated (Alcântara et al., 1999 and 2006, respectively) are not able to release galactose from the polymer (unless it is in the terminal residue), but only from oligosaccharides with an XL motif on the nonreducing end. Furthermore, C.O. da Silva and M.S. Buckeridge (unpublished data) found that the enzyme from *Copaifera* will only release galactose from the XL position of a xyloglucan fragment with less than four blocks (TTTT). This extremely high specificity toward low M_r fragments, together with the fact that the optimum pH for both enzymes is at around 3.2 strongly suggest that the release of Gal is a rate-limiting step in xyloglucan disassembly. Another difference of the *Hymenaea* system in relation to nasturtium is that the XTH is fully dependent on the presence of oligosaccharides (Tiné et al. 2000a), having little or no xyloglucan endoglucanase activity. Tiné et al. (2000a) proposed a model in which the steps of xyloglucan disassembly (Alcântara et al., 1999; de Alcântara et al., 2006) are (1) transglycosylation, followed by (2) degalactosylation, and (3) XXXG/XXXXG disassembly by the α-xylosidase and β-glucosidase.

The consequence of such a finely controlled disassembly process suggests that the storage mobilization system of xyloglucans may have the capability of regulating the rate of mobilization by transglycosylation. Thus, storage polysaccharide mobilization may be coupled to the growth rate.

The Physiology of Xyloglucan Mobilization

Santos and Buckeridge (2004) found that approximately 60% of the carbon present in the cotyledons ends up in the aerial part of the seedling, whereas 30% stays in the cotyledon and only 10% goes to the roots. It has been observed that the pace of storage mobilization is directly related to the establishment of photosynthesis in the eophylls (first leaves of the seedling), and that the rate of xyloglucan mobilization is dependent on the growth rate, which indicates that xyloglucan catabolism is controlled by the source-sink relationship during early seedling growth.

Auxin is the principal hormone that controls xyloglucan storage mobilization (Hensel et al., 1991; dos Santos et al., 2004; Brandão et al., 2009). In *H. courbaril*, when seedlings were subjected to treatment with napthylphthalamic acid (an inhibitor of auxin transport), xyloglucan disassembly was arrested (dos Santos et al., 2004). The authors proposed that light signals (through source-sink relationship and photosynthesis or directly on cotyledons) together with auxin, tightly regulate xyloglucan mobilization. When aerial parts of the plant were excised, cotyledons halted disassembly and synthesized starch. This occurred until the lateral buds started to regenerate new branches, when xyloglucan mobilization restarted. These experiments clearly demonstrate a strong dependency of mobilization with growth, showing that the mobilization process is completely integrated with the physiology of the seedling.

More recently, Brandão et al. (2009) partially cloned the XTH and β-galactosidase as well as two genes of Suc metabolism (Suc Synthase and Alkaline Invertase). They found that the enzymes are synthesized de novo in the cotyledons and that this occurs under control of auxin. Both the expression of these genes (Brandão et al., 2009), as well as the activities of the xyloglucan hydrolases, seem to be controlled by the circadian rhythm (L.V. Amaral, H.P. Santos, and M.S. Buckeridge, unpublished data).

The discoveries made for *H. courbaril* suggests that we should adopt a system approach in the sense of Hammer et al. (2004) to further investigate it as a system that interacts through communication systems (auxin, photosynthesis, sugar sensing) with environmental factors such as temperature and light.

Xyloglucan Biosynthesis

Xyloglucan biosynthesis involves at least four biosynthetic enzymes, namely α-fucosyltransferase, β-GalT, α-xylosyltransferase, and β-(1,4)-glucan synthase (Faik et al., 2002).

Although Fuc is missing in storage xyloglucans, Faik et al. (2000) found that tamarind and nasturtium storage xyloglucans, as well as oligosaccharides obtained from tamarind, are among the best acceptors (6- to 7-fold higher than pea xyloglucan) for the α-fucosyltransferase that these authors isolated from pea and characterized biochemically.

According to the model proposed by Faik et al. (1997) the basic system for galactosylation of xyloglucan is that the motif $XXXX_3$ is the substrate for GalT, which galactosylate the middle XXXG forming XXXGXXLGXXXG.

The discovery of the genes related to xyloglucan biosynthesis (e.g. Cavalier et al., 2008) and the mechanisms by which the proteins interact in the cell, can be thought of as valuable tools to manipulate plant structure. Research in storage xyloglucan biosynthesis has participated decisively in the chain of discoveries that are leading us to understand these mechanisms. We then need to understand how the structural diversity of xyloglucan is produced in nature. It can be hypothesized that, as observed for galactomannans, xyloglucan structural diversity is partly a result of a tailoring process that is responsive to environmental conditions such as variations in temperature, light, and water availability (Buckeridge et al., 1992). In this case, XTH, expansins, and even exoenzymes such as β-galactosidase and α-fucosidase are candidates to be the editing elements.

CONCLUSION AND PERSPECTIVES

CWSP systems are very efficient in disassembly of cell wall polysaccharides and metabolism of the products. By understanding some key biochemical pathways such as the mechanism by which xyloglucan-containing cotyledons metabolize Xyl, it may be possible to use techniques of synthetic biology to reconstruct these pathways inside microorganisms to develop more efficient strategies for biomass fermentation to biofuels. Indeed, the way yeast (*Saccharomyces cerevisiae*) deals with pentoses is one of the main bottlenecks in lignocellulosic route of bioethanol production. The discovery that xyloglucans have a coding in their fine structure that has to be interpreted by hydrolytic enzymes, highlights the fact that we should not be looking only to enzymes, but also to polysaccharide fine structures to understand hydrolytic mechanisms.

The CWSP are synthesized by precise mechanisms and subsequently edited by hydrolases (exoenzymes) and transglycosylases (possibly by XTHs and MTHs too). This process is directly related to cell differentiation and because storage tissues contain relatively large proportions of polysaccharides and as a consequence a higher level of the proteins related to these phenomena, the storage wall model systems could be of great help to understand some aspects of cell differentiation.

One of the most interesting opportunities offered by studying CWSP systems is to understand how cells control the production of each class of cell wall polysaccharides. This is so because the storage walls accumulate proportionally large amounts of one type of polysaccharide. In most cases, cellulose synthesis is absent or very low in storage walls. Thus, the storage

cells of endosperms and cotyledons display altered genetic programs during seed development that produce walls with a single polymer. This poses the challenge of finding what are the conditions that lead to the assembly of such unique walls. This would help understanding of important aspects of cell differentiation, carbon partitioning, and may lead to important biotechnological applications.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AF017144 (*LeMAN1*), EU370969 (*LeMAN2*), AF184238 (*HcBGAL1*), EU370971 (*HcXTH1*), EU370968 (*HcAlkIN1*), and EU370970 (*HcSUS1*).

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