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Review

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Review

The inside and outside: topological issues in plant cell wall biosynthesis and the roles of nucleotide sugar transporters

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Abstract

The cell wall is a complex extracellular matrix composed primarily of polysaccharides. Noncellulosic polysaccharides, glycoproteins and proteoglycans are synthesized in the Golgi apparatus by glycosyltransferases (GTs), which use nucleotide sugars as donors to glycosylate nascent glycan and glycoprotein acceptors that are subsequently exported to the extracellular space. Many nucleotide sugars are synthesized in the cytosol, leading to a topological issue because the active sites of most GTs are located in the Golgi lumen. Nucleotide sugar transporters (NSTs) overcome this problem by translocating nucleoside diphosphate sugars from the cytosol into the lumen of the organelle. The structures of the cell wall components synthesized in the Golgi are diverse and complex; therefore, transporter activities are necessary so that the nucleotide sugars can provide substrates for the GTs. In this review, we describe the topology of reactions involved in polysaccharide biosynthesis in the Golgi and focus on the roles of NSTs as well as their impacts on cell wall structure when they are altered.

Key words: cell wall biosynthesis, glycosyltransferases, Golgi, nucleotide sugar transporters, polysaccharides

The synthesis of plant cell wall polysaccharides

The plant cell wall represents the major component of the dry mass of plant tissues and is the major contributor to both the form and properties of plant structures. It is a very complex extracellular matrix composed primarily of polysaccharides, but it also contains structural proteins and lignin in certain tissues. The polysaccharides in this matrix are cellulose; and the noncellulosic polysaccharides, hemicelluloses and pectins. Cell wall polysaccharides are synthesized by glycosyltransferase (GT) enzymes that are able to take an activated sugar in the form of a nucleotide sugar and then transfer it onto a specific acceptor (Keegstra and Raikhel 2001; Scheible and Pauly 2004; Oikawa et al. 2013). Because of the large diversity and complexity of the polysaccharides forming the plant cell wall, a large number of GTs are encoded in plant genomes. For instance, in Arabidopsis, more than 550 putative GTs have been identified based on their gene sequences in the Carbohydrate Active Enzymes database (CAZy; http://www.cazy.org/). These enzymes are located throughout the secretory pathway including the endoplasmic reticulum (ER), where the *N*-glycosylation of glycoproteins begins; the Golgi apparatus, where most hemicellulose and pectin biosynthesis takes place and the plasma membrane, where cellulose and callose are synthesized.

Cellulose is the major polysaccharide found in plants, representing roughly one-third of the total mass in many cases. It consists of long parallel linear β-1,4-D-glucan chains assembled into microfibrils by hydrogen bonds (McFarlane et al. 2014). Its synthesis occurs in the plasma membrane, catalyzed by cellulose synthases (CESAs), a set of enzymes that belong to the Glycosyltransferase Family 2 (GT2). The current model for cellulose synthesis catalyzed by CESAs indicates that the glucose donor uridine diphosphate (UDP)-Glc is taken into the cytosol and transformed into an

elongating polymer that is deposited in the extracellular space (Sethaphong et al. 2013; McNamara et al. 2015). Recently, the crystal structure of the bacterial CESA complex BcsA-BcsB from *Rhodobacter sphaeroides* was determined, and data confirm the model proposed in which the CESA complex takes the substrate from the cytosol and the cellulose polymer is exported across the plasma membrane (Morgan et al. 2013). Thus, the mechanism of cellulose biosynthesis does not require the transport of UDP-glucose from the cytosol to the extracellular space. The completion of the Arabidopsis genome sequence revealed the presence of 10 CESA genes with an average of 64% sequence identity at the protein level (Holland et al. 2000; Richmond and Somerville 2000; McFarlane et al. 2014). Some are involved in cellulose synthesis for the primary cell wall, whereas others are involved in the synthesis of secondary cell wall cellulose. These proteins range between 985 and 1088 amino acids in length and have eight putative transmembrane segments (Somerville 2006).

Synthesis of the backbones of hemicelluloses and pectin

Unlike cellulose synthesis, biosynthesis of most of the hemicelluloses, and all pectic polysaccharides present in the wall, occurs in the Golgi apparatus (Keegstra and Raikhel 2001; Oikawa et al. 2013). A set of CESA-related sequences have been detected in the Arabidopsis genome, and they have been termed cellulose synthase-like genes (CSLs) (Richmond and Somerville 2000). It has been proposed that the backbone of the hemicelluloses mannan, xyloglucan and mixed-linkage glucan is synthesized by members of different families of CSL proteins. Liepman et al. (2005) showed, by heterologous expression of *CSLA* genes, that some members of this family have mannan synthesis activities. It has also been suggested that three CSLD genes participate in mannan synthesis. The overexpression of CSLD5 and both CSLD2 and CSLD3 in tobacco led to increased guanidine diphosphate-mannose (GDP-Man) transferase activity into endogenous acceptors (Verhertbruggen et al. 2011; Yin et al. 2011). Nevertheless, a different role for CSLD3 has been suggested during cell wall synthesis in tip-growing root-hair cells, where it is not located in the Golgi apparatus but in the plasma membrane. This signifies a role in β-1,4 glucan synthesis (Park et al. 2011). Therefore, more evidence is needed to get a better understanding of the roles that these proteins play in cell wall biosynthesis.

It has been demonstrated that a member of the cellulose synthase-like C family, CSLC4, encodes for a β-1,4-D-glucan synthase, located in the Golgi apparatus, during biosynthesis of the xyloglucan backbone (Cocuron et al. 2007; Davis et al. 2010). It is possible that other members belonging to this family are responsible for the xyloglucan backbone synthesis in plants. Finally, members of the cellulose synthase like F (CSLF) and cellulose synthase like H families have been shown to participate in the biosynthesis of β-(1→3,1→4)-mixed glucans (Burton et al. 2006; Doblin et al. 2009). Interestingly, Wilson et al. (2015) provided evidence that CSLF6 is located in the plasma membrane, suggesting that some steps of the biosynthesis of mixed-linked glucans might occur at the plasma membrane.

Cellulose synthase-like GTs may work via a mechanism similar to the one proposed for CESA, and the substrates might be taken from the cytosol while the nascent polymer is delivered into the Golgi lumen (Reyes and Orellana 2008). However there could be one exception for mannan biosynthesis. Since CSLA9 was reported to have the active site facing the lumen of the organelle, this suggests using a Golgi GDP-Man pool for its synthesis (Davis et al. 2010).

A different class of GTs participates in the synthesis of the xylan hemicellulose backbone and the pectin homogalacturonan (HG). A member of the glycosyltransferase family 47 (GT47) domain known as IRX10 has been shown to have xylan xyolsyltransferase activity (Jensen et al. 2014; Urbanowicz et al. 2014). In addition, two members of the glycosyltransferase family 43 (GT43) domain known as IRX9 and IRX14, along with their paralogs IRX9L and IRX14L, are also needed for xylan synthesis, but their actual activities remain to be determined. All of these enzymes are predicted to be type II membrane-bound proteins in which the N-terminus is cytosolic and the C-terminal portion, containing the catalytic domain, faces the lumen of the Golgi (Brown et al. 2007; Lee et al. 2007; Rennie and Scheller 2014). Interestingly, some recent data indicate that IRX9, IRX10 and IRX14 form a xylan synthase protein complex (Zeng et al. 2016). In HG synthesis, biochemical evidence obtained in the pea suggests that it occurs in the lumen of the Golgi apparatus (Sterling et al. 2001). A member of the GT8 family with UDP-galacturonic acid (GalA) transferase activity towards an HG acceptor was subsequently identified in Arabidopsis as a galacturonosyl transferase 1 (GAUT1) (Sterling et al. 2006), after which it was demonstrated that, along with GAUT7, it forms part of a protein complex able to synthesize HG (Atmodjo et al. 2011). These results indicate that the backbones of xylan and HG are made in the lumen of the Golgi and therefore, need a lumenal pool of nucleotide sugars. Interestingly, both xylan and HG have been shown to be components of an arabinogalactan protein (APAP1) suggesting that some interconnections between classes of polysaccharides might exist (Tan et al. 2013).

Synthesis of the polysaccharide branches

Many GTs involved in the synthesis of side chains present in xyloglucan have been identified. The xyloglucan fucosyl transferase gene (AtFUT1/MUR2) was cloned from Arabidopsis and shown to encode a type II membrane-bound protein with its catalytic domain located in the Golgi lumen (Perrin et al. 1999; Wulff et al. 2000). The *mur2* mutant displayed a lack of fucose (Fuc) in xyloglucan (Vanzin et al. 2002). The dwarf mutant *mur3* is affected in a gene encoding for a xyloglucan galactosyltransferase, another predicted type II membrane-bound protein (Madson et al. 2003), and the strong phenotype of the *mur3* mutant is associated with less-galactosylated xylose (Xyl) on xyloglucan side chains (Kong et al. 2015). Three xyloglucan xylosyltransferases are encoded by members of the GT34 family, the the XXT1, XXT2 and XXT5 enzymes, identified as capable of adding Xyl from UDP-Xyl to cellohexaose. These enzymes also belong to the type II membrane-bound proteins (Faik et al. 2002; Chevalier et al. 2010; Zabotina et al. 2008).

The glucuronic acid (GlcA) substitutions in xylan, which produce the glucuronoxylan (GX) highly abundant in the secondar cell walls of Arabidopsis, are added by GUX1 and GUX2 enzymes that are members of the GT8 family (Mortimer et al. 2010; Bromley et al. 2013). In contrast to dicots, xylans from grasses are extensively arabinosylated by members of the GT61 family (Anders et al. 2012).

Pectin side chains are also synthesized by type II membrane proteins. GALACTAN SYNTHASE 1, GALACTAN SYNTHASE 2 and GALACTAN SYNTHASE 3 (GALS1, GALS2 and GALS3) were described as members of the GT92 family located in the Golgi apparatus. The GALS1 protein catalyzes β-1,4-galactan synthesis in vitro using β-1,4-galactopentaose as an acceptor. A mutant in these three genes showed less β-1,4-galactan content, whereas the

overexpression of GALS1 increased the β-1,4-galactan content by 50%, suggesting that these three proteins participate in the synthesis of the galactan side chains of rhamnogalacturonan 1 (RG1; Liwanag et al. 2012). Other evidence suggests that the arabinan side chain in RG1 is synthesized by two members of the GT47 family, ARAD1 and ARAD2. These putative GTs are proteins located in the Golgi apparatus and Föster resonance energy transfer experiments suggest that they are part of a complex. Furthermore, genetic evidence shows that mutants in ARAD1 have lower levels of arabinan content (Harholt et al. 2006, 2012). Therefore, the evidence indicates that the lateral chains of hemicelluloses and pectins are formed by type II membrane-bound GTs. A summary of the GTs described in these sections, including their function and insertion into the membrane (type II or multiple spanning domains) is described in Table I.

Nucleotide sugars, building blocks of the plant cell wall, are made in various subcellular compartments

Nucleotide sugars are universal donors for the formation of glycoconjugates, and because they serve as donor substrates for GTs that incorporate them into appropriate glycan acceptors, are key molecules for the biosynthesis of all polysaccharides present in cell walls. With the large diversity of sugars present in noncellulosic polysaccharides, it is expected that a variety of nucleotide sugars exist in plants, and hence, 16 nucleotide sugars have been identified in plants in the form of UDP-sugars, GDP-sugars and cytidine monophosphate (CMP)-sugars (Bar-Peled and O'Neill 2011). UDP-sugars are mainly derived from UDP-Glc through a series of interconversion reactions, many of which occur in the cytosol. These include enzymes that interconvert UDP-Glc into UDP-galactose (Gal) (Dormann and Benning 1998; Seifert et al. 2002), UDP-rhamnose (Rha) (Usadel, Kuschinsky, et al. 2004; Western et al. 2004; Oka et al. 2007) and UDP-GlcA. UDP-GlcA is the precursor for the formation of UDP-Xyl (Harper and Bar-Peled 2002; Pattathil et al. 2005), which in turn can also be transformed into UDP-Apiose (UDP-Api) (Molhoj et al. 2003). Therefore, the pathway that goes through UDP-GlcA plays a central role in noncellulosic polysaccharide synthesis (Reboul et al. 2011). UDP-GlcA also serves as a substrate for the glucuronate epimerases (GAEs), Golgi located enzymes able to convert UDP-GlcA into UDP-GalA (Gu and Bar-Peled 2004; Molhoj et al. 2004; Usadel, Schluter, et al. 2004). In addition, UDP-GlcA can be utilized both in the cytosol and in the Golgi lumen to form UDP-Xyl by a decarboxylation reaction catalyzed by UDP-Xyl synthases (UXSs) (Harper and Bar-Peled 2002; Pattahil et al. 2005). Furthermore, UDP-Xyl present in the Golgi also serves as substrate for the enzyme UDP-Xyl epimerase (UXE), another Golgi membrane-bound protein (Burget et al. 2003), to form UDP-arabinose pyranose (Ara*p*). However, the substrate for most arabinosyltransferases is UDP-arabinose furanose (Ara*f*), and a mutase [UDP-Ara mutase (UAM)] is responsible for this interconversion (Konishi et al. 2007, 2011; Rautengarten et al. 2011). Interestingly, UAM is a protein with no transmembrane domain, although it is commonly found attached to the cytosolic face of the Golgi membrane. All these reactions take place in two compartments (the cytosol and the Golgi lumen), posing a question about topological issues related to the interconversion of UDP-GlcA, an issue that we will now address.

GDP-Man is the source of mannose for the synthesis of glycoproteins, polysaccharides and ascorbic acid in plants (Bar-Peled and O'Neill 2011). It is the starting substrate for the de novo pathway, from which other GDP-sugars are formed. VTC1 is the enzyme necessary for converting α-D-Man-1-P and GTP to GDP-Man and pyrophosphate (Conklin et al. 2000; Barth et al. 2010). GDP-L-Fuc and GDP-L-Gal are formed through interconversion reactions in the cytosol (Reiter and Vanzin 2001; Reiter 2008).

The eight-carbon sugar 3-deoxy-Dmanno-2-octulosonic acid (Kdo) is an important component in the structure of RG2. The synthesis of CMP-Kdo, its activated form, is first catalyzed by the enzymes *AtkdsA1* and *AtkdsA2* forming Kdo-8-P (Brabetz et al. 2000; Delmas et al. 2003). A CMP-Kdo synthase then catalyzes transfer of the cytidylyl group from cytidine triphosphate to Kdo, yielding CMP-Kdo (Royo et al. 2000). Single mutants of the genes *AtkdsA1* and *AtkdsA2* had no obvious phenotype, but attempts to obtain the double mutant were unsuccessful because the haploid pollen grains of the *atkdsa1/atkdsa2* double mutant are unable to elongate a proper pollen tube (Delmas et al. 2008; Bar-Peled and O'Neill 2011).

Synthesis of these substrates is crucial for synthesizing all the polymers present in the cell wall, but equally important is that these substrates reach the Golgi apparatus lumen where many type II membrane protein GTs have their active site. Consequently, the various subcellular localizations of enzymes involved in the biosynthesis of nucleotide sugars and GTs pose a topological issue for polysaccharides synthesis.

Nucleotide sugar transporters are fundamental for overcoming the topological issues associated with nucleotide sugar interconversion and polysaccharide biosynthesis

Biochemical experiments provided the first evidence that nucleotide sugars are transported through the plant Golgi membrane (Munoz et al. 1996; Wulff et al. 2000; Norambuena et al. 2002). Proteins involved in this process are known as nucleotide sugar transporters (NSTs), originally described in animal cells, but present throughout all analyzed eukaryotes (Abeijon et al. 1989; Hirschberg et al. 1998; Puglielli and Hirschberg. 1999; Caffaro and Hirschberg 2006; Caffaro et al. 2006; Liu et al. 2010). The NSTs are hydrophobic proteins (300–350 amino acids) containing 6–10 transmembrane domains. Most NSTs are located in the Golgi apparatus, which is expected because this is the main organelle where the biosynthesis of glycoconjugates and polysaccharides takes place (Reyes and Orellana 2008). Nevertheless, some have been found to be localized in the ER (Reyes et al. 2006, 2010; Niemann et al. 2015). Because NSTs transport a nucleotide sugar across the membrane into the Golgi apparatus and export a nucleoside monophosphate from the lumen of the organelle, they are antiporters (Figure 1). Given the massive amount and heterogeneity of polysaccharides present in the cell wall and the large number of GTs and wide range of nucleotide sugars used in the biosynthesis of polysaccharides and glycoconjugates, it is likely that the Golgi apparatus contains a significant number of these transporters in its membrane. The first Golgi-localized NSTs characterized in plants were Golgi nucleotide sugar transporter 1 (GONST1) and AtUTr1, a GDP-Man and UDP-Gal/UDP-Glc transporter, respectively (Baldwin et al. 2001; Norambuena et al. 2002). A number of other NSTs have been identified since then, and today, more than 40 putative NST encoding genes have been bioinformatically identified in *Arabidopsis thaliana* (Rautengarten et al. 2014). All NSTs belong to the NSTs/triose

Table I. Glycosyltranferases involved in the synthesis of noncellulosic polysaccharides described in the text

Activity	Name	Gene ID	CAZy	Topology	Ref	Comment
Hemicelluloses						
Xylan						
Xylan backbone synthesis β(1→4)-xylan synthase	IRX10	At1g27440	GT47	Type II-GT	Wu et al. (2009), Urbanowicz et al. (2014), Jensen et al. (2014), Zeng et al. (2016)	IRX10 and IRX10L have β(1→4)-xylan activity.
Xylan backbone synthesis β(1→4)-xylan synthase	IRX10L	At1g27440	GT47	Type II-GT	Wu et al. (2009), Urbanowicz et al. (2014), Mortimer et al. (2015)	IRX10 and IRX10L have β(1→4)-xylan activity. IRX10L participate in the primary cell wall xylan.
Xylan backbone synthesis	IRX9	At2g37090	GT43	Type II-GT	Brown et al. (2007), Lee et al. (2007), Wu et al. (2010), Zeng et al. (2016)	No β(1→4)-xylan activity detected, but is needed for xylan synthesis. Form multiprotein complexes along with IRX10 and IRX14.
Xylan backbone synthesis	IRX9L	At1g27600	GT43	Type II-GT	Brown et al. (2007), Wu et al. (2010), Mortimer et al. (2015)	No β(1→4)-xylan activity detected, participate in primary cell wall synthesis.
Xylan backbone synthesis	IRX14	At2g37090	GT43	Type II-GT	Brown et al. (2007), Lee et al. (2007), Wu et al. (2010), Mortimer et al. (2015), Zeng et al. (2016)	No β(1→4)-xylan activity detected, but is needed for xylan synthesis. Form multiprotein complexes along IRX10 and IRX9.
Xylan backbone synthesis	IRX14L	At5g67230	GT43	Type II-GT	Wu et al. (2010), Keppler and Showalter (2010)	
Xylan glucuronylation α(1→2)-GlcAT	GUX1	At3g18660	GT8	Type II-GT	Mortimer et al. (2010), Bromley et al. (2013)	GUX1 and GUX2 are enzymes responsible for secondary cell wall xylan glucuronosylation. GUX1 participate in glucuronosylation of the denominated xylan major domain.
Xylan glucuronylation α(1→2)-GlcAT	GUX2	At4g33330	GT8	Type II-GT	Mortimer et al. (2010), Bromley et al. (2013)	GUX2 participate in the glucuronosylation of the referred xylan minor domain.
α-(1→3)-Arabinosyltransferase	–	TaXAT1 TaXAT2	GT61	Type II-GT	Anders et al. (2012)	Heterologous expression of two wheat arabinosyltransferases (GT61 family) in *Arabidopsis* lead to xylan arabinosylation.
Mannan						
β(1→4)-Mannan synthase	CSLA9	At5g03760	GT2	MTD GT active site facing Golgi Lumen	Liepman et al. (2005), Davis et al. (2010)	
Mannan synthesis?	CSLD2	At5g16910	GT2	MTD GT	Verhertbruggen et al. (2011), Yin et al. (2011)	Overexpression along with CSLD3 in tobacco increase GDP-Man transferase activity.
Mannan synthesis? β-Glucan synthesis	CSLD3	At3g03050	GT2	MTD GT, reported to be locate in the Golgi apparatus and the plasma membrane	Verhertbruggen et al. (2011), Yin et al. (2011), Park et al. (2011)	Over-expression along with CSLD3 in tobacco increase GDP-Man transferase activity. A role in β-glucan synthesis has been reported in root hairs cells.
Mannan synthesis?	CSLD5	At1g02730	GT2	MTD GT	Verhertbruggen et al. (2011), Yin et al. (2011)	Over-expression led to increased GDP-Man transferase activity.
Xyloglucan						
Xyloglucan synthesis β(1→4)-glucan synthase	CSLC4	At3g28180	GT2	MTD GT Active site facing cytosol	Cocuron et al. (2007), Davis et al. (2010)	
α(1→6)-FucT	AtFUT1/ MUR2	At2g03220	GT37	Type II-GT	Perrin et al. (1999), Vanzin et al. (2002), Chevalier et al. (2010)	Located in the medial/trans cisternae.
β(1→2)-GalT	MUR3	At2g20370	GT47	Type II-GT	Madson et al. (2003), Chevalier et al. (2010), Kong et al. (2015)	Specific for the third Xyl in the XXXG repeating unit. Located in the media cisternae.
α(1→6)-XylT	XXT1	At3g62720	GT34	Type II-GT	Chevalier et al. (2010), Faik et al. (2002)	XylT is located in the cis/medial cisternae.
α(1→6)-XylT	XXT2	At4g02500	GT34	Type II-GT	Chevalier et al. (2010), Faik et al. (2002)	

Pectin						
Homogalacturonan						
HG α-(1→4)GalAT	GAUT1	At3g61130	GT8	Type II-GT	Sterling et al. (2006), Atmodjo et al. (2011)	GAUT1:GAUT7 complex is the catalytic core of an HG: GalAT complex.
HG synthesis	GAUT7	At2g38650	GT8	Type II-GT	Atmodjo et al. (2011)	GAUT7 has no HG:GalAT activity, but is needed for the formation of GAUT1:GAUT7 core.
RG1						
α(1→5)Arabinosyltransferase?	ARAD1	At2g35100	GT47	Type II-GT	Harholt et al. (2006)	Putative arabinosyltransferase, mutants show decreased levels of arabinose (Ara) in leaves and stems.
α(1→5)Arabinosyltransferase?	ARAD2	At5g44930	GT47	Type II-GT	Harholt et al. (2006, 2012)	Putative arabinosyltransferase. ARAD1 and ARAD2 localize in the same Golgi compartment.
β-1,4-Galactan	GALS1	At2g33570	GT92	Type II-GT	Liwanag et al. (2012)	GALS1 could transfer galactose (Gal) residues from UDP-Gal onto β-1,4-galactopentaose
β-1,4-Galactan?	GALS2	At5g44670	GT92	Type II-GT	Liwanag et al. (2012)	
β-1,4-Galactan?	GALS3	At4g20170	GT92	Type II-GT	Liwanag et al. (2012)	

Abbreviations: GT, glycosyltransferase; MTD, multitransmembrane domain; CAZy, carbohydrate active enzymes.

phosphate translocator (TPT) gene family. In *A. thaliana*, 51 genes have been identified and clustered into six clades, where clades I–III, V and VI contain NSTs, and clade IV contains TPTs (Rautengarten et al. 2014). Clades I–III contain NSTs that show transport activities for UDP-Gal, UDP-Rha, UDP-Xyl, UDP-Glc and GDP-Man (Table II), whereas no activity for NSTs in clades V and VI has been reported to date. To determine whether this number of genes encoding for NSTs is common among plant genomes and whether they have orthologs in other plant species, we looked for genes of the NST/TPT gene family in *Oryza sativa* (rice) because its genome is well assembled and annotated; in addition, there is increasing information about its cell wall. We identified 36 genes of the NST/TPT gene family, of which 31 encode NSTs. Interestingly, all rice putative NSTs clustered with their *Arabidopsis* orthologs, covering all clades (Figure 2). However, the incorporation of the rice sequences led to changes in clade III in such manner that this was split into two clades leading to the creation of a new clade (clade VII). This likely occurred because the distribution observed for clade III, using exclusively *A. thaliana* sequences, was supported by a low bootstrap value, probably associated with only a few sequences (Rautengarten et al. 2014). The addition of rice NST sequences allows a better distribution of sequences into clades with a higher bootstrap value; this agrees with the determined in vitro activity of the sequences clustered in the new clades. Indeed, the new distribution separates the GDP-Man transporters from the remaining NSTs, but orthologs with the same in vitro activity retained their association (Figure 2).

The biochemical characterization and specificity of plant NSTs has been difficult to address because the Golgi apparatus in plants is involved in the transport of a range of nucleotide sugars, and the unavailability of all nucleotide sugar substrates used to carry out biochemical assays is an issue. Radiolabeled substrates are commonly utilized, but many of them are not available in this form. This problem has been nicely solved by Rautengarten et al. (2014) who designed a nonradiolabeled assay that combines the use of proteoliposomes with HPLC separation of the nucleotide sugars, which are detected by mass spectrometry. The current information regarding NSTs chracterized in Arabidopsis is summarised in Table II. The functional characterization of NSTs is leading to associations between their substrate specificities and their distributions in the phylogenetic tree among clades and subclades. One example of this is the UDP-Rha/UDP-Gal transporter (URGT) subclade in clade I (Figure 2), which contains six genes involved in the transport of UDP-Rha and UDP-Gal (Rautengarten et al. 2014). Another clade 1 subclade, containing three genes, is involved in the transport of UDP-Xyl (Ebert et al. 2015). Of the GONST transporters of clade VII, GONST1 was initially characterized as a GDP-Man transporter (Baldwin et al. 2001), and recently, Mortimer et al. (2013) showed that it is able to transport the four GDP-sugars [GDP-Man, GDP-Fuc, GDP-Glc and GDP-Gal], being GDP-Man the main substrate. Another example of a similar transport linked to closely related NSTs sequences is observed in the transporters AtUTr1 and AtUTr3, grouped in clade II.

NSTs and their roles in the biosynthesis of plant cell wall polysaccharides

Based on the information provided above, it becomes clear that translocation of nucleotide sugars by NSTs is a key step for the synthesis of noncellulosic polysaccharides in the Golgi apparatus. Therefore, changes leading to alterations in NST functions might

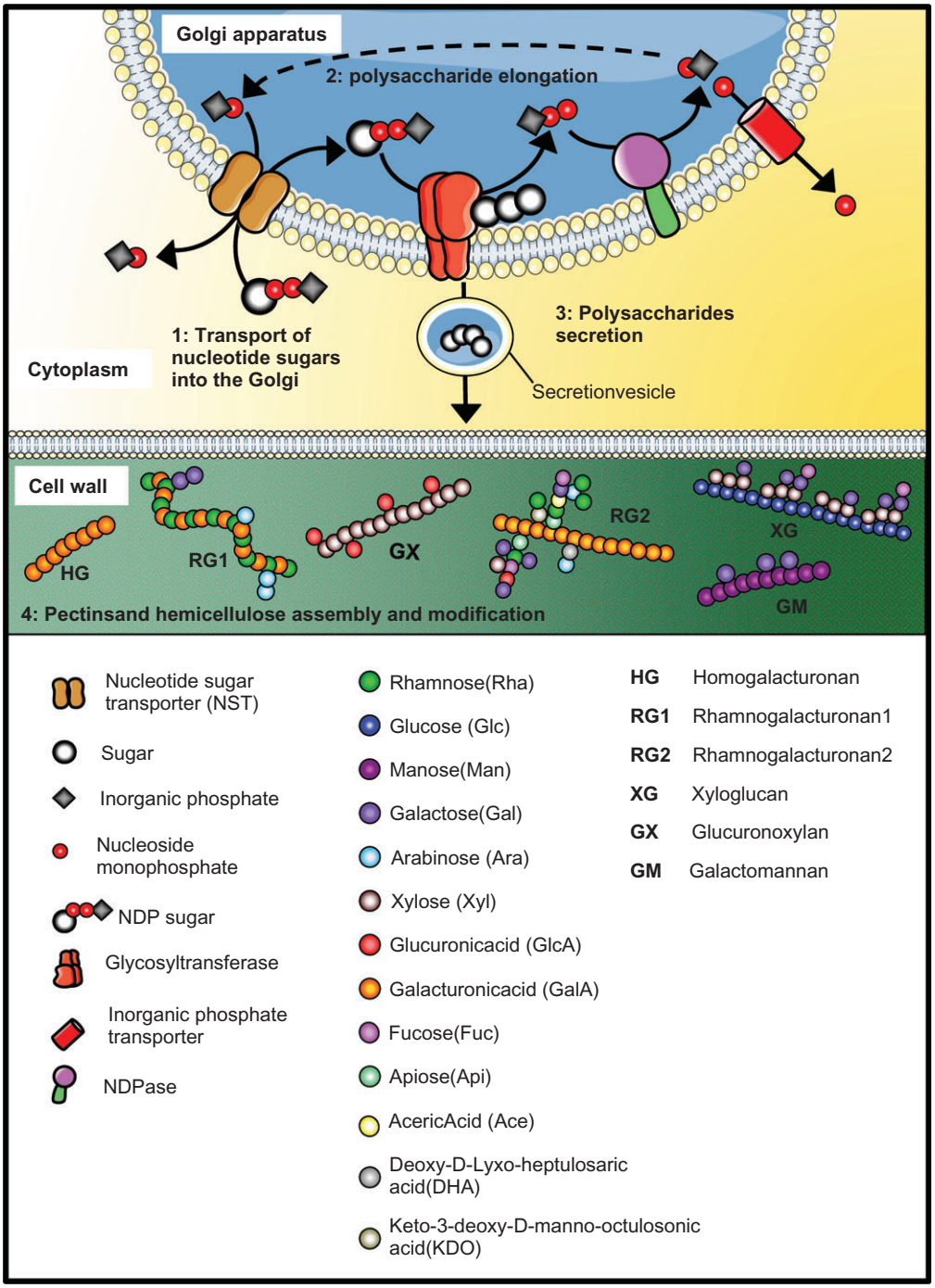

Fig. 1. Role of NSTs in noncellulosic polysaccharide biosynthesis. Nucleotide sugars are the building blocks of polysaccharide biosynthesis. They are mostly synthesized in the cytosol but type II GTs have their catalytic domain facing the lumen of the organelle; therefore they need to be transported by NSTs (1). In the Golgi lumen, GTs transfer the glycosyl residue from a nucleotide sugar (NDP-sugar) onto the growing polysaccharide (2). The polysaccharides chains are then secreted into the apoplast (3) and assembled into the cell wall (4). NSTs work as antiporters and the transport of NDP-sugar into the Golgi is coupled to the export of a nucleoside monophosphate. When the sugar is transferred by a GT onto the nascent polymer, NDP is released and hydrolyzed to NMP and inorganic phosphate (Pi) by a luminal NDPase. Finally, the excess of Pi is exported to the cytosol by Pi transporters in order to restore the balance in the Golgi apparatus.

affect cell wall structure. To date, NSTs with transport activities for several nucleotide sugars have been identified (Table II), and their contributions to the biosynthesis of cell wall polysaccharides have been addressed by searches for the cell wall phenotypes in *Arabidopsis* NST mutants.

Studies of *URGT* mutants show that *urgt2*, a URGT highly expressed during seed development, is associated with lower levels of Rha in mucilage. While *urgt2* plants contain less Rha, the phenotype is not as strong as seen in the *mum4/rhm2* mutant, an enzyme involved in UDP-Rha synthesis. This observation suggests that other

Table II. NSTs characterized in Arabidopsis

Gene name	Gene ID	Subcellular localization	In vitro substrate (major, minor)	K_m (μM; major, minor substrate)	Pectin and hemicellulose modifications	Biological function	Reference
UXT1	At2g28315	Golgi, ER	UDP-Xyl, UDP-Ara*p*	39 (3), ≥200 mM	*uxt1* have less Xyl and GlcA: GX modification	ND	Ebert et al. (2015)
UXT2	At2g30460	Golgi	UDP-Xyl, UDP-Ara*p*	40 (4), ≥200 mM	ND	ND	Ebert et al. (2015)
UXT3	At1g06890	Golgi	UDP-Xyl, UDP-Ara*p*	58 (9), ≥200 mM	ND	ND	Ebert et al. (2015)
URGT1/UDP-GalT1	At1g76670	Golgi	UDP-Rha, UDP-Gal	87 (12), 90 (22)	Less galactose content in *urgt1*. Overexpressor lines have modifications in RG1 side chains	ND	Rautengarten et al. (2014), Bakker et al. (2008)
URGT2	At1g21070	Golgi	UDP-Rha, UDP-Gal	61 (11) , 68 (16)	*urgt2* have Less Rha, GalA, Gal and Xyl: RG1 modification in pectin from seed mucilage	Participate in seed coat mucilage synthesis	Rautengarten et al. (2014)
URGT3	At5g42420	Golgi	UDP-Rha, UDP-Gal	40 (7), 149 (37)	ND	ND	Rautengarten et al. (2014)
URGT4/ AtNST-KT	At4g39390	Golgi	UDP-Rha, UDP-Gal	37 (8), 77 (19)	ND	ND	Rollwitz et al. (2006), Rautengarten et al. (2014)
URGT5	At4g09810	Golgi	UDP-Rha, UDP-Gal	17 (5), 177 (53)	ND	ND	Rautengarten et al. (2014)
URGT6	At4g34020	Golgi	UDP-Rha, UDP-Gal	33 (8), 196 (96)	ND	ND	Rautengarten et al. (2014)
AtUTr1	At2g02810	ER	UDP-Glc	ND	ND	Pollen and embryo sac development, ER quality control	Norambuena et al. (2002), Reyes et al. (2006, 2010)
AtUTr2	At4g23010	Golgi	UDP-Gal	ND	ND	ND	Norambuena et al. (2005)
AtUTr3	At1g14360	Golgi, ER	UDP-Glc, UDP-Gal	6.4	ND	Pollen and embryo sac development, ER quality control	Norambuena et al. (2002), Reyes et al. (2010)
AtUTr7	At4g31600	Golgi	UDP-Gal, UDP-Glc	ND	*atutr7* show modified labeling with JIM5 (recognize HG poorly methylesterified)	Lateral root and root-hair morphology	Handford et al. (2012)
UDP–GalT2	At1g21070	ND	UDP-Gal	ND	ND	ND	Bakker et al. (2008)
GONST1	At2g13650	Golgi	GDP-Man, GDP-Glc, GDP-Fuc and GDP-L-Gal	26 (2) for GDP-Man	ND	Plant growth and development, salicilic acid signaling	Baldwin et al. (2001), Colleoni et al. (2010), Mortimer et al. (2013)
GONST2	At1g07290	ND	GDP-Man	ND	ND	No transport activity determined. Yeast *vrg4* mutant complementation, suggesting GDP-Man transport activity	Handford et al. (2004)
GONST3	At1g76340	ND	GDP-Man	ND	ND		Handford et al. (2004)
GONST4	At5g19980	Golgi	GDP-Man	ND	ND		Handford et al. (2004)
GONST5	At1g21870	Golgi	GDP-Man	ND	ND		Handford et al. (2004)
ROCK1	At5g65000	ER	UDP-GlcNAc and UDP-GalNAc	ND	ND	Regulation of shoot apical meristem activity	Niemann et al. (2015)
CMP-Sia[a]	At5g41760	ND	CMP-Sia	ND	ND	ND	Bakker et al. (2008)

ND, not determined.

[a]CMP-sialic acid is not present in plants but the study was performed in vitro using CMP-sialic acid.

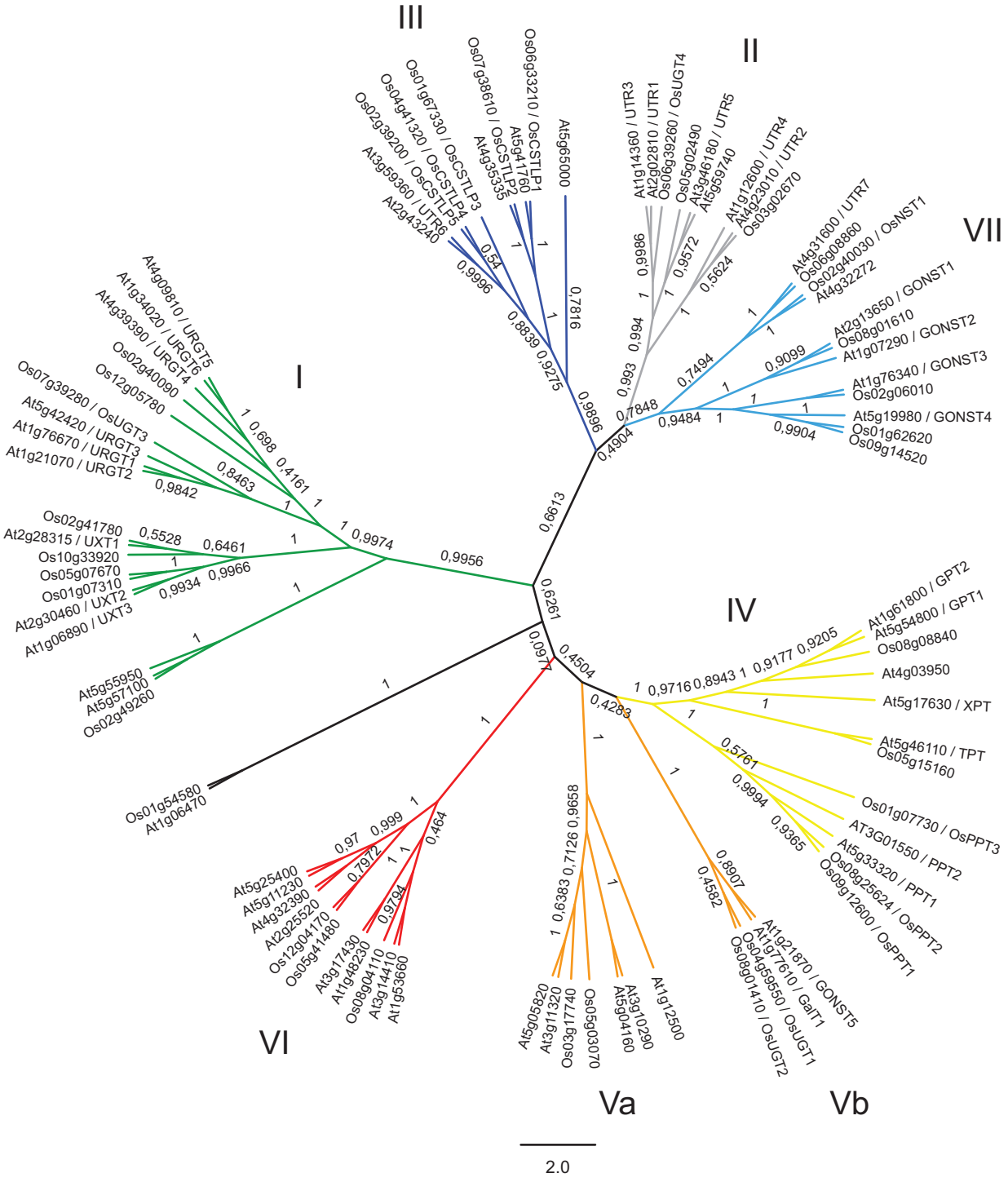

Fig. 2. Phylogenetic tree of the *A. thaliana* and *O. sativa* NST/TPT superfamily. Full-length amino acid sequences (51 from *A. thaliana* and 36 from *O. sativa*) were aligned using MUSCLE, and the phylogenetic tree was generated using Molecular Evolutionary Genetics Analysis (MEGA) 7 and visualized by FigTree 1.4.2. Clades were assigned following the nomenclature used by Rautengarten et al. (2014).

URGTs should be participating in mucilage synthesis. In addition, RG1 is a polymer whose backbone is made by repeats of the disaccharide Rha-GalA. Interestingly, mucilage from *urgt2* plants also shows less GalA. Since URGT2 does not transport UDP-GalA, it is likely that lower levels of Rha impair the biosynthesis of the RGI backbone, leading to the lower level of GalA. Intriguingly, only small changes in the content of Gal could be observed, suggesting that URGT2 is not primarily involved in the transport of UDP-Gal *in planta*. On the other hand, mutants in URGT1 do not exhibit a decrease in Rha, but instead, showed a decrease in Gal. Furthermore, plants over-expressing *URGT1* exhibited higher levels of Gal (Rautengarten et al. 2014). URGT1 and URGT2 are

highly similar at the protein level, and their in vitro activities are quite similar (Table II). Both utilize UDP-Rha and UDP-Gal, and the K_m values for these substrates are in similar ranges. However, their functions in vivo regarding cell wall composition are astonishingly different in that *urgt1* mutants exhibit changes in Gal and not Rha, and *urgt2* mutants exhibit changes in Rha and not in Gal. These results indicate that in vitro data are important for determining the intrinsic activity of the protein; however, it also suggests that NSTs undergo additional levels of regulation in how they deliver the substrates for biosynthesis of the various polysaccharides in vivo. One possibility is the formation of protein complexes, which might channel the substrates into the biosynthesis of specific polysaccharides; however, this hypothesis remains to be tested.

Considering that two transporters with similar transport activities in vitro exhibit different cell wall phenotypes, different mechanisms for regulating their functions in vivo are suggested. Because the biosynthesis of plant cell wall polysaccharides requires the concerted action of nucleotide sugar interconversion enzymes, NSTs and GTs (Reiter and Vanzin 2001), it is attractive to think that these differences are the result of formation of potential complexes of these transporters along with GTs. To test this hypothesis, protein–protein interaction experiments could be conducted and may give us some insight into the roles that these transporters play in vivo. In addition, it is necessary to study mutants comprising other members of the URGT family; this would help gain a better understanding of the roles of these transporters *in planta*.

UDP-Xyl is a nucleotide sugar that can be synthesized both in the cytosol and the Golgi lumen by the decarboxylation of UDP-GlcA in a reaction catalyzed by UXS. There are several UXSs, and some are Golgi localized, whereas others are cytosolic (Harper and Bar-Peled 2002; Pattahil et al. 2005; Atmodjo et al. 2013). It is not yet clear why UDP-Xyl is made both in the cytosol and the Golgi apparatus. It is a key substrate needed for the synthesis of xylan and xyloglucan. In addition, it is essential for the synthesis of UDP-Ara*p*, which is then transformed into UDP-Ara*f*, the substrate needed for the synthesis of side chains containing arabinose (Ara), present in polysaccharides and arabinogalactan proteins (Figure 3).

Ebert et al. (2015) characterized a set of genes, *UXT1-3*, that belong to clade I, subclade C, and found that they transport UDP-Xyl in vitro, supporting the fact that UDP-Xyl formed in the cytosol can be used in reactions that take place in the Golgi. Mutants in *UXT1* showed decreased Xyl in noncellulosic polysaccharides; more detailed analyses, using antibodies, indicated that xylan was affected in two mutant alleles of *UXT1*. Interestingly, a decrease in GlcA content was observed in stems. Because UXT1 does not transport UDP-GlcA, this decrease is likely the result of impairment in the biosynthesis of GX. No significant changes were observed in xyloglucan when analyzed by oligosaccharides mass profiling, and no differences in protein glycosylation were observed in the analysis of *N*-glycosylated proteins in the *uxt* mutants. The authors proposed that UXT1 could be associated with the xylan biosynthetic machinery, explaining the absence of phenotype in other Xyl-containing polysaccharides such as xyloglucan and xylogalacturonan. *UXT2* and *UXT3* mutants showed no changes in cell wall composition. The double mutant also showed no obvious phenotype, and therefore, the contribution of these two transporters to the synthesis of the cell wall remains uncertain.

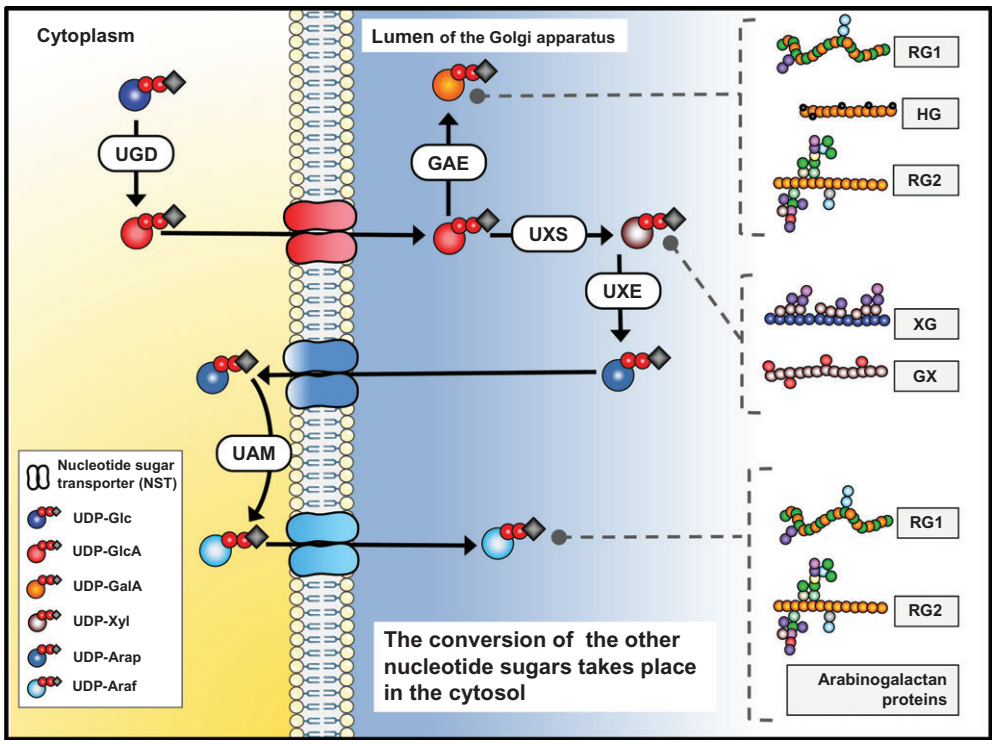

Fig. 3. Topology of the UDP-GlcA interconversion reactions into UDP-GalA, UDP-Xyl and UDP-Ara. Overview of the topology of reactions occurring during the interconversion reactions of UDP-GlcA. UDP-GlcA is synthesized in the cytosol and then is transported into the Golgi lumen. Once there, UDP-GlcA can be converted to UDP-GalA, UDP- Xyl and UDP-Ara*p* by GAE, UXSs and UXE, respectively. The UDP-Ara*p* formed in the Golgi lumen, needs to be exported to the cytosol to be transformed into UDP-Ara*f* (the common substrate of Arabinosyltransferases), by the UAM, a cytosolic enzyme. The proteins involved in the transport of these nucleotide sugars are putative NSTs not yet identified.

Identification of NSTs with transport activities for many substrates remains elusive. As described above, the incorporation of UDP-GlcA into the Golgi lumen seems to be a key step for the synthesis of pectin and hemicellulose. Pectic polysaccharides are GalA-enriched polysaccharides; the substrate UDP-GalA is produced by epimerization of UDP-GlcA by GAEs located in the Golgi (Molhoj et al. 2004; Usadel, Schluter, et al. 2004; Gu and Bar-Peled 2004) (Figure 3). This suggests that incorporating UDP-GlcA into the Golgi is essential for pectin biosynthesis. An alternative salvage pathway for UDP-GalA synthesis could exist in plants because the heterologous expression of a gene encoding GalA kinase is able to produce GalA-1-P in vitro (Yang et al. 2009). The GalA-1-P could then be converted to UDP-GalA by a UDP-sugar pyrophosphorylase with broad specificity toward monosaccharide-1-phosphate (Kotake et al. 2004), also known as SLOPPY. However, the contribution of this salvage pathway to plant cell wall biosynthesis and the amounts of GalA that are recycled to UDP-GalA in vivo are not known (Bar-Peled and O'Neill 2011), but the existence of a transporter able to transport UDP-GalA is suggested.

UDP-GlcA inside the Golgi also serves as a substrate for UXSs. These enzymes contribute to the pool of Golgi UDP-Xyl, and therefore to the synthesis of UDP-Ara*p* that occurs inside the organelle. Although UDP-Ara*p* is synthesized in the Golgi lumen, Ara exists predominantly in the furanose form (Ara*f*) in most plant glycans. The mutases responsible for interconversion of UDP-Ara*p* into UDP-Ara*f* correspond to a group of cytosolic proteins that tend to associate with the Golgi membrane (De Pino et al. 2007; Konishi et al. 2007; Rautengarten et al. 2011). This suggests that UDP-Ara*p* is exported from the Golgi to the cytosolic face by a UDP-Ara*p* transporter, converted into UDP-Ara*f*, and then incorporated into the Golgi lumen by a UDP-Ara*f* transporter (Figure 3). To confirm this hypothesis, transporters with these activities must be identified.

Kdo is present only in the RG2 polysaccharide structure (Figure 1). The incorporation of CMP-Kdo into the Golgi apparatus has not been described, but an NST able to transport CMP-sialic acid was identified in *A. thaliana* (Bakker et al. 2008). Because sialic acid has not been detected in plants, they proposed that the substrate for this transporter, At5g41760, *in planta*, could be CMP-Kdo. There is no biochemical or genetic evidence for this transporter to be involved in CMP-Kdo transport, but it would be interesting to evaluate its role in RG2 synthesis.

UDP-Api is also an important substrate for RG2 synthesis, forming parts of lateral side chains A and B (Atmodjo et al. 2013). UDP-Api synthesis is predicted to occur in the cytosol via the bi-functional AXS enzyme capable of converting UDP-GlcA to UDP-Xyl and UDP-Api in a 2:1 ratio (Guyett et al. 2009). Api residues in RG2 are critical for plant growth and development; silencing AXS1 in *Nicotiana benthamiana* led to plants with severe developmental phenotypes directly related to the reduction of Api in RG2 (Ahn et al. 2006). However, Api is also present in a number of secondary metabolites (Picmanova and Moller 2016); thus, it is important to keep in mind that modulating Api content would affect not only the structure of cell wall polysaccharides. Therefore, identifying UDP-Api transporters and mutants with impairments in their expression and function could give us more clues regarding the role of NSTs in the synthesis cell wall polysaccharides, RG2 in particular.

Another nucleotide sugar important for RG2 biosynthesis is GDP-Fuc, which is also important for xyloglucan biosynthesis. Because GONST1 reportedly transports all GDP-sugars, but its role was related to mannosylation of glycosphingolipids and not synthesis of any polysaccharide (Mortimer et al. 2013), it is therefore likely that other members of the GONST family may be involved in the transport of this substrate.

Conclusions and perspectives

In this review, we focused on the available evidence of the role of NSTs in the biosynthesis of noncellulosic polysaccharides (Table II); however, a number of NSTs, whose substrate specificities are not known, remain. Therefore, it is not yet possible to address their roles in cell wall biosynthesis. None of the NSTs within clades V and VI (Figure 2) are characterized as yet and they might be the transporters for those UDP-sugars not yet reported. Fortunately, the set-up of a new approach for addressing NSTs' functions in vitro is leading us to a more comprehensive view of the biochemical features of these transporters. However, to get a better understanding of their contributions to cell wall biosynthesis in vivo, mutants for single NSTs genes and multiple knock-outs must be identified. These, along with gene overexpression lines, assessed for changes in cell wall composition, should shed light on the roles of NSTs *in vivo*.

To date, most of the information regarding the function of NSTs has been obtained from Arabidopsis. Even though NSTs from other plant species, such as rice and *Vitis* have been characterized (Song et al. 2011; Zhang et al. 2011; Utz and Handford 2015), our knowledge about NSTs contributions to the formation of cell wall structures in species other than the Arabidopsis model remains scarce. Furthermore, the use of NSTs as tools to engineer the plant cell wall is something yet to be tested. Certainly, these are challenging topics that remain to be addressed.

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Conflict of interest statement

None declared.

Abbreviations

Api, apiose; Ara, arabinose; Ara*f*, arabinose furanose; Ara*p*, arabinose pyranose; CESA, cellulose synthase; CMP, cytidine monophosphate; CSL, cellulose synthase-like; ER, endoplasmic reticulum; Fuc, fucose; GAE, glucuronate epimerase; Gal, galactose; GalA, galacturonic acid; GDP, guanidine diphosphate; GlcA, glucuronic acid; GT, glycosyltransferase; GX, glucuronoxylan; HG, homogalacturonan; Kdo, 3-deoxy-D-manno-2-octulosonic acid; Man, mannose; NST, nucleotide sugar transporter; RG1, rhamnogalacturonan 1; RG2, rhamnogalacturonan 2; Rha, rhamnose; UAM, UDP-Ara mutase; UDP, uridine diphosphate; URGT, UDP-Rha/UDP-Gal transporter; Xyl, xylose.

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