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REVIEW PAPER

Nucleotide-sugar metabolism in plants: the legacy of Luis F. Leloir

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Abstract

This review commemorates the 50th anniversary of the Nobel Prize in Chemistry awarded to Luis F. Leloir 'for his discovery of sugar-nucleotides and their role in the biosynthesis of carbohydrates'. He and his co-workers discovered that activated forms of simple sugars, such as UDP-glucose and UDP-galactose, are essential intermediates in the interconversion of sugars. They elucidated the biosynthetic pathways for sucrose and starch, which are the major end-products of photosynthesis, and for trehalose. Trehalose 6-phosphate, the intermediate of trehalose biosynthesis that they discovered, is now a molecule of great interest due to its function as a sugar signalling metabolite that regulates many aspects of plant metabolism and development. The work of the Leloir group also opened the doors to an understanding of the biosynthesis of cellulose and other structural cell wall polysaccharides (hemicelluloses and pectins), and ascorbic acid (vitamin C). Nucleotide-sugars also serve as sugar donors for a myriad of glycosyltransferases that conjugate sugars to other molecules, including lipids, phytohormones, secondary metabolites, and proteins, thereby modifying their biological activity. In this review, we highlight the diversity of nucleotide-sugars and their functions in plants, in recognition of Leloir's rich and enduring legacy to plant science.

Keywords: ADP-glucose, cellulose, GDP-mannose, Luis Federico Leloir, starch, sucrose, trehalose, UDP-glucose.

Introduction

Nucleotide-sugars are critical intermediates in the biosynthetic pathways of the complex carbohydrates that dominate plant metabolism. Sucrose (a disaccharide) is the major product of photosynthesis and the most widely transported sugar in vascular plants (Lunn, 2016), starch (a polysaccharide) is the most common storage reserve (Smith and Zeeman, 2020), and up to 70% of total plant biomass is comprised of cellulose and other structural cell wall polysaccharides (Jacobsen and Wyman, 2000). Our understanding of the biosynthesis of all of these carbohydrates is based on the seminal discovery of UDP-Glc (Fig. 1) and other nucleotide-sugars by Luis F. Leloir and his colleagues, for which he was awarded the 1970 Nobel Prize in Chemistry (Box 1). This review of plant nucleotide-sugar metabolism marks the 50th anniversary of this award and celebrates Leloir's legacy to plant biology.

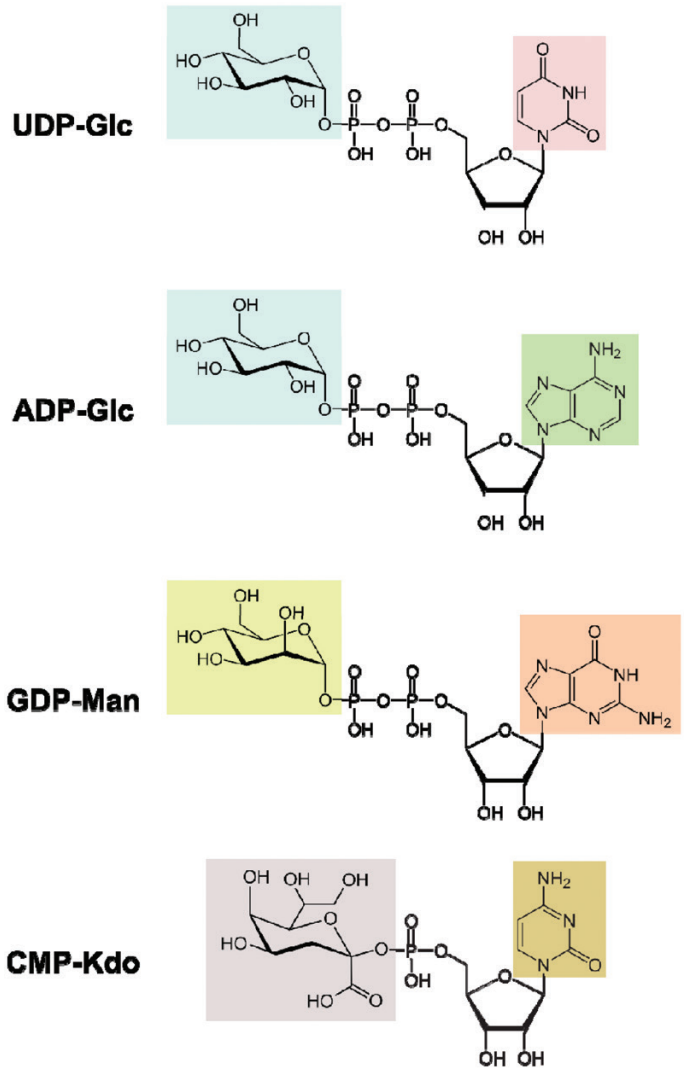


Fig. 1. Representative sugar-nucleotides. Colour shades are as follows: pink, uracil; green, adenine; orange, guanine; brown, cytidine; blue, glucose; yellow, mannose; violet, 3-deoxy-D-manno-2-octulosonate.

Synthesis and utilization of UDP-glucose

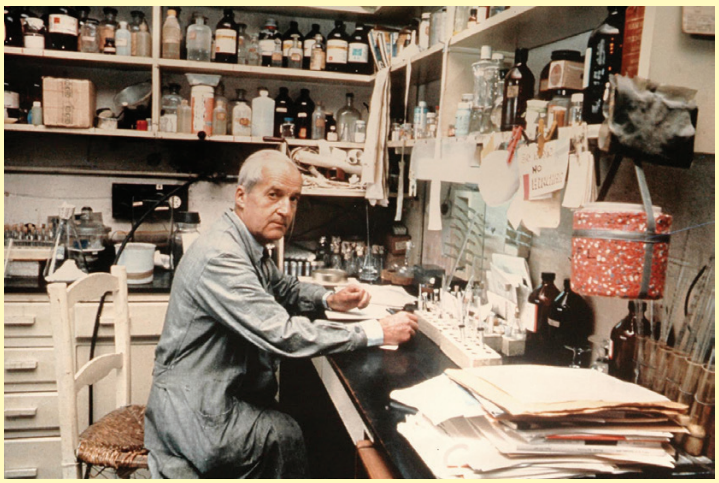
UDP-Glc was the first nucleotide-sugar discovered by the Leloir group, and plays a central role in the biosynthetic pathways of sucrose, the lifeblood of vascular plants, and of the main structural cell wall polysaccharides. UDP-Glc is synthesized from glucose 1-phosphate (Glc1P) and UTP in a reversible reaction catalysed by UDP-Glc pyrophosphorylase (UDP-Glc PPase; Fig. 2). The pyrophosphorolysis of UDP-Glc using *Zwischenferment* preparations (an obsolete term used to describe a preparation containing glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase from yeast) was first described by Munch-Petersen *et al.* (1953), whereas the first comprehensive characterization of a plant UDP-Glc PPase was reported by Turner and Turner (1958) using pea seeds. Arabidopsis (*Arabidopsis thaliana*) has three genes (*UGP1–UGP3*) coding for this enzyme (Meng *et al.*, 2009; Okazaki *et al.*, 2009). *UGP1* and *UGP2* encode cytosolic isoforms, accounting for at least 85% of total activity, whereas *UGP3* encodes a minor chloroplastic isoform that contributes 10–15% of total activity in leaf extracts (Okazaki *et al.*, 2009). The *UGP1* and *UGP2* genes differ in their spatio-temporal expression patterns, with *UGP1* encoding the predominant isoform in leaves, where sucrose is synthesized (Meng *et al.*, 2009). Double *ugp1 ugp2* mutants are viable and retain ~15% of wild-type activity. In addition to the chloroplastic (UGP3) isoform, a non-specific UDP-sugar pyrophosphorylase (UDP-sugar PPase; Fig. 2) accounts for some of the residual UDP-Glc PPase activity in *ugp1 ugp2* mutants, and appears to provide sufficient capacity to produce UDP-Glc in the cytosol for sucrose synthesis (Decker and Kleczkowski, 2019). UDP-Glc PPase shows similar catalytic efficiencies to Glc1P and galactose 1-phosphate (Gal1P) *in vitro* (Kotake *et al.*, 2007; Minen *et al.*, 2020), and plays a key role in the so-called 'salvage pathway', which incorporates monosaccharides generated from the turnover of cell wall polysaccharides into the NDP-sugars pool (Bar-Peled and O'Neill, 2011). The fate of UDP-Glc depends on where it is made in the plant. In source leaves, much of the UDP-Glc is consumed by sucrose synthesis (Szecowka *et al.*, 2013), whereas in growing tissues it is the direct substrate for synthesis of cellulose and hemicelluloses, and a precursor for other nucleotide-sugars that are needed for synthesis of non-cellulosic cell wall polysaccharides (Chen *et al.*, 2013; Ishihara *et al.*, 2017). In chloroplasts, UDP-Glc is also needed for the synthesis of sulfolipids (Essigmann *et al.*, 1998; Sanda *et al.*, 2001).

Sucrose

Sucrose (α-D-glucopyranosyl-1,2-β-D-fructofuranoside) is a non-reducing disaccharide that is synthesized in the cytosol in a two-step pathway. In the first reaction, the glucosyl moiety of UDP-Glc is transferred to fructose 6-phosphate (Fru6P) by sucrose-6′-phosphate synthase (SPS) to produce sucrose 6′-phosphate (Leloir and Cardini, 1955), and then this is hydrolysed by a specific sucrose-6′-phosphate phosphatase (SPP) to release sucrose (Hawker and Hatch, 1966). This pathway was discovered by the Leloir group working on wheatgerm (Leloir and Cardini, 1955). Thermodynamically, the SPS reaction is potentially reversible, but the forward reaction is strongly favoured *in vivo* by the irreversible hydrolysis of sucrose 6′-phosphate by SPP (Lunn and ap Rees, 1990). The substrates for sucrose synthesis, UDP-Glc and Fru6P, are produced from triose-phosphates that are exported from the chloroplasts, via the triose-phosphate translocator, in exchange for orthophosphate (Pi), which is released in the cytosol by the synthesis of sucrose (Stitt *et al.*, 2010). A complex network of regulatory mechanisms, including allosteric regulation and reversible phosphorylation of SPS, coordinates the rate of sucrose

Box 1. Luis Federico Leloir (1906–1987)—Nobel Laureate in Chemistry 1970

Born in Paris in 1906 to Argentinean parents, his widowed mother took him home to Argentina in 1908, where he grew up and received most of his early education. After graduating from the University of Buenos Aires with a medical degree in 1932, he decided not to pursue a career in clinical medicine, studying instead for a PhD under the supervision of Bernardo A. Houssay (Nobel Laureate in Physiology/Medicine 1947, shared with Carl and Gerty Cori) at the Institute of Physiology in the University of Buenos Aires Medical School. Leloir's PhD work on the role of adrenals in carbohydrate metabolism won the prize for the best thesis in 1934. He then spent a year in England working with Frederick Gowland Hopkins (Nobel Laureate in Physiology/Medicine 1929) in the Biochemical Laboratory at the University of Cambridge, before returning to Argentina to work with J.M. Muñoz on fatty acid oxidation (Leloir and Muñoz, 1939). Their group discovered a peptide hormone, angiotensin (then called hypertensin), that plays a major role in hypertension (Menendez *et al.*, 1943). From 1943 to 1945, Leloir was based in the USA, working with Carl and Gerti Cori at the Washington University Medical School (St. Louis, MO) and later with David E. Green at the Columbia University Medical School (NY). In 1945, he returned to Argentina to work with Houssay, before becoming the founding director of the Institute of Biochemical Research of the Campomar Foundation in 1947. There, he switched his research focus to carbohydrate metabolism, leading to the discovery of UDP-Glc and its role as an intermediate in the conversion of galactose1-phosphate (Gal1P) to glucose 1-phosphate (Caputto *et al.*, 1950). This not only led to an understanding of galactosaemia, a rare hereditary disorder that results in toxic accumulation of galactose or Gal1P, but also opened the doors to the discovery of other nucleoside diphosphate (NDP)-sugars that play such a central role in the carbohydrate metabolism of all living organisms. In the following years, Leloir and his colleagues elucidated the biosynthetic pathways of two important disaccharides—trehalose (Leloir and Cabib, 1953) and sucrose (Cardini *et al.*, 1955; Leloir and Cardini, 1955)—as well as the main storage carbohydrates in animals and plants, namely glycogen (Leloir and Cardini, 1957; Leloir *et al.*, 1959) and starch (Leloir *et al.*, 1961; Recondo and Leloir, 1961; Espada, 1962; Recondo *et al.*, 1963). They also identified other NDP-sugars, such as UDP-GlcNAc (Cabib *et al.*, 1953), UDP-Xyl, and GDP-Man (Cabib and Leloir, 1954), and their roles as intermediates in the synthesis of structural polysaccharides in plant and algal cell walls, including cellulose (Elbein *et al.*, 1964), xylans (Feingold *et al.*, 1959), callose (Feingold *et al.*, 1958), and paramylon (Goldemberg and Marechal, 1963). In 1970, Leloir was awarded the Nobel Prize in Chemistry for 'his discovery of sugar-nucleotides and their role in the biosynthesis of carbohydrates' (www.nobelprize.org), and he received many other honours and awards before his death in 1987.



Leloir en su laboratorio de Campomar Vuelta de Obligado.
Fundacion Instituto Leloir. Repositorio Institucional CONICET Digital http://hdl.handle.net/11336/121889

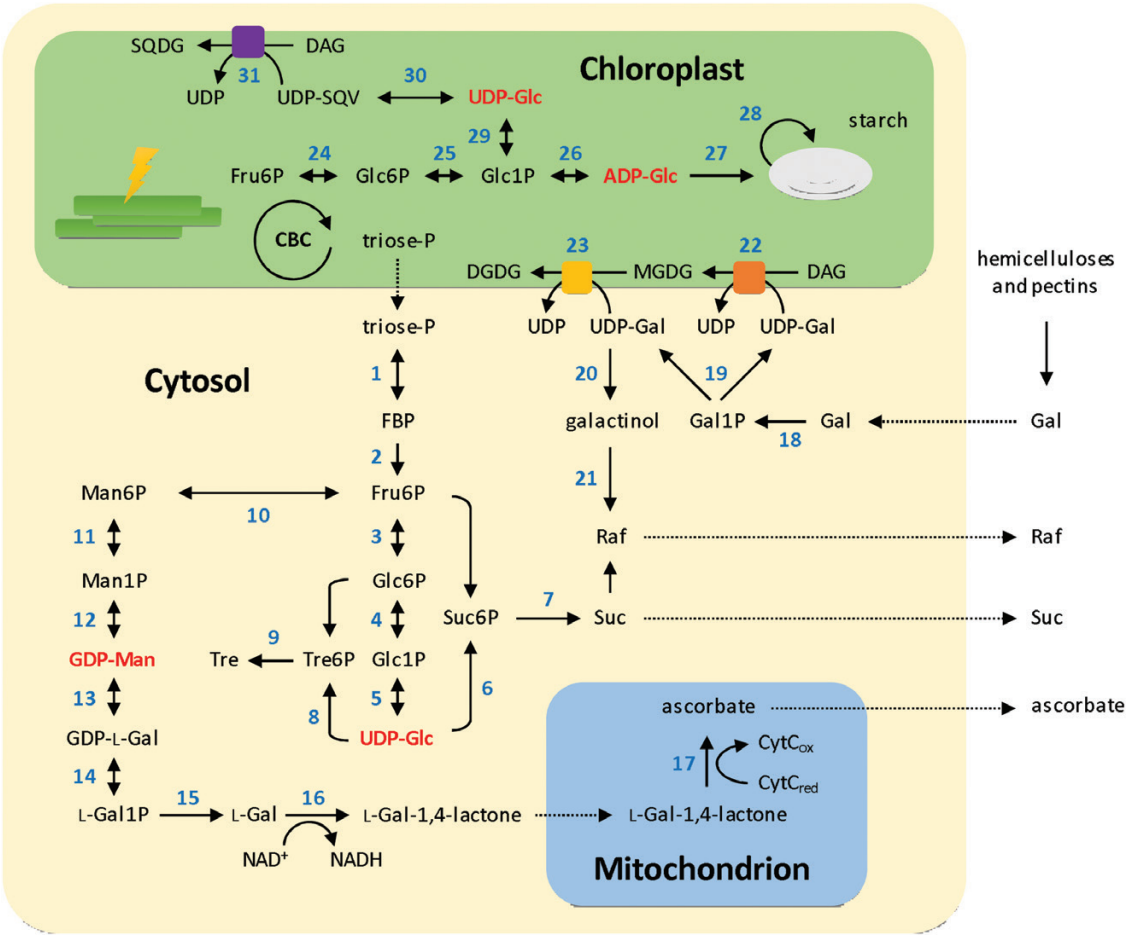


Fig. 2. Simplified scheme of the reactions producing NDP-sugars for the synthesis of sucrose, raffinose, ascorbate, starch, and galactolipids. Enzymes were numbered in blue as follows: 1, cytosolic aldolase (EC 4.1.2.13); 2, cytosolic FBPase (3.1.3.11); 3, cytosolic phosphoglucose isomerase (EC 5.3.1.9); 4, cytosolic phosphoglucomutase (EC 5.4.2.2); 5, cytosolic UDP-Glc PPase (EC 2.7.7.9); 6, SPS (EC 2.4.1.14); 7, SPP (EC 3.1.3.24); 8, TPS (EC 2.4.1.15); 9, TPP (EC 3.1.3.12); 10, Man6P isomerase (EC 5.3.1.8); 11, phosphomannomutase (EC 5.4.2.8); 12, GDP-Man PPase (EC 2.7.7.13); 13, GDP-Man 3,5-epimerase (EC 5.1.3.18); 14, GDP-L-Gal phosphorylase (EC 2.7.7.69); 15, L-Gal1P phosphatase (EC 3.1.3.92); 16, L-Gal dehydrogenase (EC 1.1.1.316), 17, L-galactono-1,4-lactone dehydrogenase (EC 1.3.2.3); 18, galactokinase (EC 2.7.1.6); 19, UDP-sugar PPase (EC 2.7.7.64); 20, galactinol synthase (EC 2.3.1.123); 21, Raf synthase (EC 2.4.1.82); 22, MGDG synthase (EC 2.4.1.46; yellow square); 23, DGDG synthase (EC 2.4.1.241; orange square); 24, plastidic phosphoglucose isomerase (EC 5.3.1.9); 25, plastidic phosphoglucomutase (EC 5.4.2.2); 26, ADP-Glc PPase (EC 2.7.7.27); 27, starch synthase (EC 2.4.1.21); 28, branching enzyme (EC 2.4.1.18); 29, plastidic UDP-Glc PPase (EC 2.7.7.9); 30, UDP-SQV synthase (EC 3.13.1.1); 31, SQDG synthase (EC 2.4.1.–; violet square). CBC, Calvin–Benson cycle. Dashed lines indicate transport of metabolites across membranes. The structures of NDP-sugars labelled in red are detailed in Fig. 1.

synthesis in the cytosol with the rates of CO_2 fixation and starch synthesis in the chloroplasts, and with sucrose export (Stitt *et al.*, 2010).

Vascular plants transport various sugars and sugar alcohols (e.g. sorbitol and mannitol), with the major transport sugar differing between species, but sucrose is by far the most common and probably the only universal transport sugar (Ziegler, 1975). In some species, such as poplar, sucrose produced in the mesophyll cells of the leaves is loaded into the phloem by symplastic (via plasmodesmata) pathways (Zhang *et al.*, 2014). In other species, such as Arabidopsis, phloem loading occurs via an apoplastic pathway, involving movement of sucrose across the plasmalemma into the apoplast via SWEET (SUGARS WILL EVENTUALLY BE EXPORTED TRANSPORTER) efflux carriers and then active uptake from the apoplast into the phloem companion cell–sieve element complex by SUT sucrose–H^+ symporters (Chen *et al.*, 2015). In cucurbits and some other families, raffinose-family oligosaccharides (RFOs) are transported alongside sucrose (Haritatos *et al.*, 1996). These are synthesized in specialized intermediary cells by sequential addition of galactosyl moieties from galactinol to sucrose, generating raffinose (trisaccharide), stachyose (tetrasaccharide), verbascose (pentasaccharide), and higher order RFOs (Fig. 2; Beebe and Turgeon, 1992). Galactinol is synthesized from UDP-Gal, which is also the substrate for the synthesis of galactolipids (see below).

After loading into the phloem in the leaves, sucrose is transported in the sieve elements to non-photosynthetic parts of the plant. There, it is unloaded from the phloem to provide carbon and energy for growth, and for the synthesis of storage reserves in organs such as seeds, fruits, tubers, and bulbs. Depending on the species, organ, and developmental stage, sucrose can be unloaded directly into heterotrophic cells via plasmodesmata (symplastic unloading). Alternatively, it can be released into the apoplast and then either taken up via sucrose transporters or hydrolysed by cell wall invertases, producing glucose and fructose that enter the cells via monosaccharide transporters (Winter and Huber, 2000; Koch, 2004). There are two routes for sucrose catabolism in heterotrophic cells: (i) hydrolysis to glucose and fructose by intracellular, especially cytosolic and vacuolar, invertases; or (ii) cleavage by sucrose synthase to yield UDP-Glc and fructose (Lunn, 2016). In growing tissues, UDP-Glc produced by sucrose synthase can be used directly to synthesize cellulose and other cell wall polysaccharides, while glucose and fructose require phosphorylation by hexokinase/fructokinase and isomerization to Glc1P before synthesis of UDP-Glc by UDP-Glc PPase (Fig. 3; Verbančič *et al.*, 2018). UDP-Glc PPase can also operate in the reverse direction to convert UDP-Glc, produced by sucrose synthase, to hexose-phosphates. These can be channelled to glycolysis, the oxidative pentose-phosphate pathway, and the tricarboxylic acid cycle to provide energy (ATP) and reducing equivalents (NADPH and NADH) for biosynthesis and maintenance, or be converted into storage compounds such as starch. In line with this, Long *et al.* (2017) reported that the *flo8* rice mutant (with 33% of the UDP-Glc PPase activity measured in wild-type plants) showed a decreased content of starch, which in turn displayed different structural and physical properties.

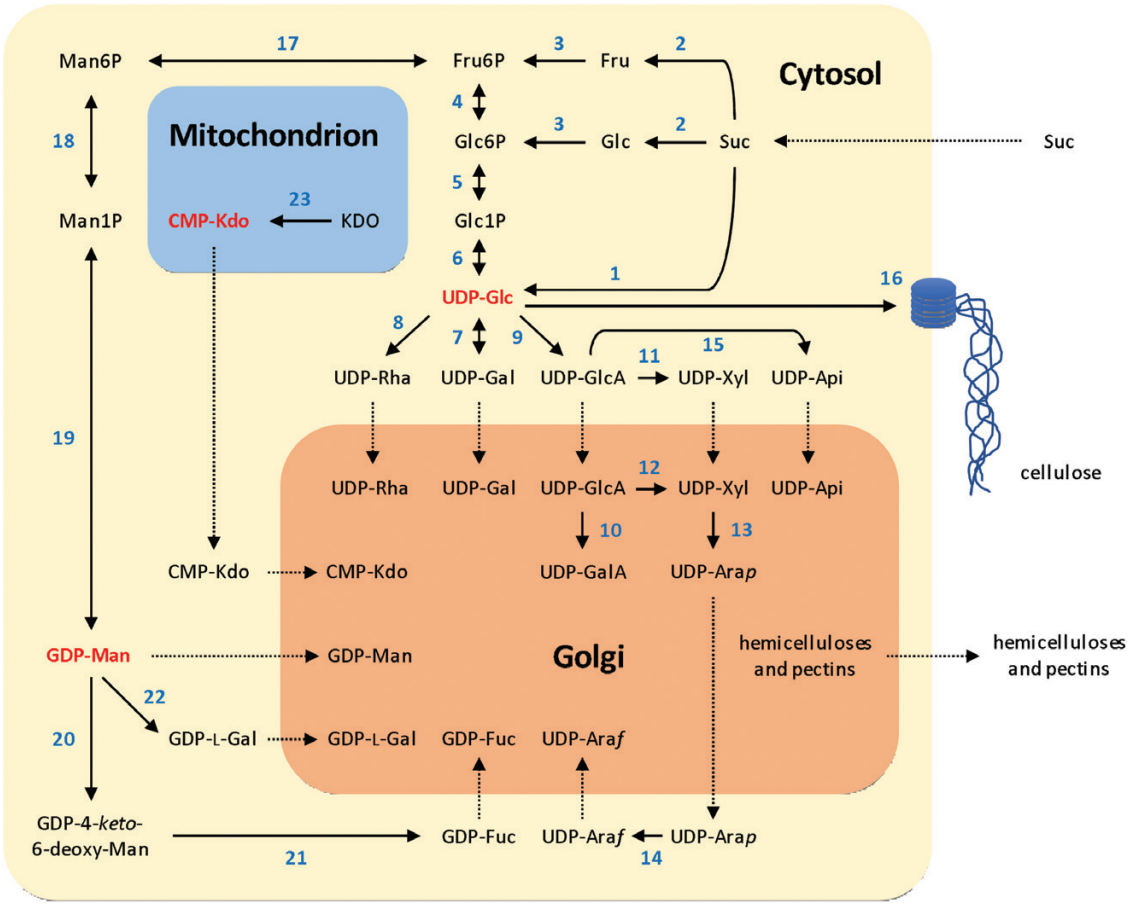


Fig. 3. Simplified scheme of the reactions producing NDP-sugars for the synthesis of cellulose, hemicelluloses, and pectins. Enzymes were numbered in blue as follows: 1, sucrose synthase (EC 2.4.1.13); 2, cytosolic invertase (EC 3.2.1.26); 3, hexokinase (EC 2.7.1.1); 4, cytosolic phosphoglucose isomerase (EC 5.3.1.9); 5, cytosolic phosphoglucomutase (EC 5.4.2.2); 6, UDP-Glc PPase (EC 2.7.7.9); 7, UDP-Glc 4-epimerase (EC 5.1.3.2); 8, RHM (EC 4.2.1.76/1.1.1.133); 9, UDP-Glc dehydrogenase (EC 1.1.1.22); 10, UDP-GlcA 4-epimerase (EC 5.1.3.6); 11, cytosolic UXS (4.1.1.35); 12, Golgi-located UXS (4.1.1.35); 13, UDP-Xyl 4-epimerase (EC 5.1.3.5); 14, UDP-Ara mutase (EC 5.4.99.30); 15, UAXS (4.1.1.35); 16, CESA (EC 2.4.1.12); 17, Man6P isomerase (EC 5.3.1.8); 18, phosphomannomutase (EC 5.4.2.8); 19, GDP-Man PPase (EC 2.7.7.13); 20, MUR1 (EC 4.2.1.47); 21, GER1 (EC 1.1.1.271); 22, GDP-Man 3,5-epimerase (EC 5.1.3.18); 23, CMP-Kdo synthetase (2.7.7.38). Dashed lines indicate transport of metabolites across membranes. The structures of NDP-sugars labelled in red are detailed in Fig. 1.

Trehalose

Trehalose (α-D-glucopyranosyl-1,1-α-D-glucopyranose) is the only other non-reducing disaccharide, along with sucrose, that is commonly found in nature, being present in bacteria, archaea, and eukaryotes, except for vertebrates (Elbein, 1974; Kandler and Hopf, 1980). In fungi and invertebrates, trehalose has similar functions to sucrose in plants: osmolyte, carbon reserve, transport sugar, and stress protectant. It can be fairly abundant in non-vascular plants and some lycophytes (e.g. *Selaginella lepidophylla*; Anselmino and Gilg, 1913), but most flowering plants have only very low amounts of trehalose, except for some desiccation-tolerant 'resurrection' plants (Drennan *et al.*, 1993; Iturriaga *et al.*, 2000; Carillo *et al.*, 2013). In plants and many other organisms, trehalose is synthesized in a two-step pathway via a phosphorylated intermediate: trehalose 6-phosphate (Tre6P). First, Tre6P synthase (TPS) catalyses the synthesis of Tre6P from UDP-Glc and Glc6P, and then a specific Tre6P phosphatase (TPP) dephosphorylates Tre6P to trehalose (Fig. 2). This pathway has obvious similarities to the synthesis of sucrose, and was also first described by the Leloir group (Cabib and Leloir, 1958). The major TPS in Arabidopsis, TPS1, is an essential multidomain protein (Eastmond *et al.*, 2002). In addition to the catalytic glucosyltransferase domain, it has non-catalytic N- and C-terminal domains that are important for targeting the protein to the nucleus and for catalytic fidelity, and are potentially involved in post-translational regulation of the enzyme (Fichtner *et al.*, 2020*b*).

The identification of *TPS* and *TPP* genes in Arabidopsis (Blazquez *et al.*, 1998; Vogel *et al.*, 1998) overturned a common perception that trehalose metabolism is absent from most flowering plants, and subsequent genome surveys pointed to the pathway being universal across plant species (Avonce *et al.*, 2006; Lunn, 2007; Lunn *et al.*, 2014). It was also observed that perturbation of the trehalose biosynthetic pathway in plants can lead to severe developmental defects (Goddijn *et al.*, 1997; Romero *et al.*, 1997; Pilon-Smits *et al.*, 1998; Eastmond *et al.*, 2002), which are linked to changes in the levels of Tre6P, rather than trehalose itself (Schluepmann *et al.*, 2003). Tre6P has a dual function as a signal and negative feedback regulator of sucrose levels in plants (Lunn *et al.*, 2006; Yadav *et al.*, 2014; Figueroa and Lunn, 2016). In source leaves, it regulates the production of sucrose, to match demand from sink tissues, by modulating photoassimilate partitioning during the day (Figueroa *et al.*, 2016) and the breakdown of transitory starch reserves at night (Martins *et al.*, 2013; dos Anjos *et al.*, 2018). In sink tissues, Tre6P regulates the utilization of sucrose for growth and accumulation of storage reserves, in part via inhibition of the SUCROSE-NON-FERMENTING1-RELATED-KINASE1 (SnRK1) protein kinase (Schluepmann *et al.*, 2003; Zhang *et al.*, 2009; Zhai *et al.*, 2018; Baena-González and Lunn, 2020). Developmental decisions that increase future demand for sucrose, such as flowering (Wahl *et al.*, 2013), the juvenile to adult transition in leaves (Ponnu *et al.*, 2020), and shoot branching (Fichtner *et al.*, 2017, 2020*a*), are also linked to the sucrose status of the plant by Tre6P (Fichtner and Lunn, 2021).

Cellulose

Cellulose is a linear β-1,4-polyglucan and is the major polysaccharide in plant cell walls, and therefore the most abundant biopolymer on earth (Allen *et al.*, 2021). Cellulose polymers are bundled into microfibrils that are linked, via hydrogen bonding, to non-cellulosic polysaccharides, forming the core structural element of plant cell walls to confer strength and rigidity (Cosgrove, 2005). The first demonstration of cellulose synthesis *in vitro* used a cell-free extract from *Acetobacter xylinum* and UDP-Glc as substrate (Glaser, 1957), but it was subsequently proposed that GDP-Glc played that role (Elbein *et al.*, 1964). This debate was finally resolved by labelling studies showing that UDP-Glc is the true substrate for cellulose synthesis in cotton fibres (Carpita and Delmer, 1981; Delmer, 1983).

Cellulose is produced by cellulose synthase (CESA) complexes (Fig. 3), located in the plasma membrane (Paredez *et al.*, 2006). In vascular plants, these heteromeric complexes consist of three different CESA isoforms, with 36 subunits in total arranged in a rosette formation, along with accessory proteins that guide the movement of CESA complexes along microtubules and potentially regulate cellulose production (Somerville, 2006). Arabidopsis has 10 *CESA* genes. *CESA1*, *CESA3*, and *CESA6* encode core components of the CESA complexes that are responsible for primary cell wall cellulose synthesis in most tissues, with CESA6 being replaced by other subunits in specific tissues (Persson et al., 2005, 2007). CESA4, CESA7, and CESA8 are responsible for secondary cell wall cellulose synthesis (Turner and Somerville, 1997; Taylor *et al.*, 2003). There is evidence that sucrose synthase partially co-localizes with CESA complexes at the plasma membrane, potentially delivering UDP-Glc directly to the CESA complexes for cellulose synthesis (Koch, 2004). However, sucrose synthase appears not to be essential for cellulose synthase as Arabidopsis *sus1 sus2 sus3 sus4* mutants, lacking the four main isoforms of sucrose synthase, have wild-type levels of cellulose (Barratt *et al.*, 2009). Presumably, UDP-Glc is adequately supplied by UDP-Glc PPase in the quadruple *sus* mutants.

NDP-sugars for the synthesis of hemicelluloses and pectins

In addition to cellulose, plant cell walls contain other polysaccharides, mainly hemicelluloses (xyloglucans, xylans, and mannans) and various types of pectins [e.g. homogalacturonan, xylogalacturonan, rhamnogalacturonan-I (RG-I) and RG-II; Carpita *et al.*, 2015]. All of these are synthesized intracellularly from NDP-sugars in the Golgi apparatus and then transported to the apoplast by exocytosis for incorporation into the cell wall (Cosgrove, 2005; Rautengarten *et al.*, 2016; Zhao *et al.*, 2018). UDP-Glc is the starting point for the synthesis of most NDP-sugars, with the main exceptions being GDP-mannose (GDP-Man; Fig. 1) and GDP-fucose (GDP-Fuc), which are made from mannose 1-phosphate (Man1P). Almost

all NDP-sugars are synthesized in the cytosol and then translocated into the Golgi lumen, except for UDP-GalA and UDP-Ara, which are synthesized within the Golgi lumen (Seifert, 2004; Orellana *et al.*, 2016; Temple *et al.*, 2016), as described in the following sections. Another exception is the activation of 3-deoxy-D-manno-2-octulosonate (Kdo), a specific component of RG-II, by CMP-Kdo synthetase. This reaction occurs in the mitochondria (Kobayashi *et al.*, 2011) and produces CMP-Kdo (Fig. 1), which is then transported to the Golgi lumen by a CMP-sialic acid transporter-like protein (Fig. 3; Takashima *et al.*, 2009).

UDP-Glc is the direct substrate for the synthesis of UDP-Rha, UDP-Gal, and UDP-GlcA. UDP-Rha is synthesized by the trifunctional enzyme RHM (Fig. 3), which is thought to catalyse the conversion of UDP-Glc to UDP-Rha in three steps, namely dehydration, epimerization, and reduction (Oka *et al.*, 2007). UDP-Gal can be synthesized by UDP-Glc 4-epimerase (Fig. 3), which is part of the Leloir pathway (Leloir, 1951). Arabidopsis has five genes coding for UDP-Glc 4-epimerase, which are partially redundant. Isoforms 2 and 4 provide UDP-Gal for the synthesis of cell wall components (Rösti *et al.*, 2007). Alternatively, UDP-Gal can be synthesized by UDP-sugar PPase (see above). Mutation of the only gene coding for this enzyme in Arabidopsis leads to sterile plants, due to abnormal pollen production (Schnurr *et al.*, 2006). Plants seem to lack (or have limited activity of) Gal1P uridylyltransferase (GALT; EC 2.7.7.12), which is also part of the Leloir pathway (Leloir, 1951). Thus, UDP-sugar PPase has a dual role: removal of Gal1P (which is considered to be toxic for cell metabolism) and, at the same time, production of UDP-Gal for glycosylation reactions (Decker and Kleczkowski, 2019).

UDP-GlcA is made in the cytosol by oxidation of UDP-Glc by UDP-Glc dehydrogenase. UDP-GlcA is then transported to the Golgi lumen, where it is converted into UDP-GalA by UDP-GlcA 4-epimerase (Fig. 3; Gu and Bar-Peled, 2004; Seifert, 2004; Sharples and Fry, 2007). UDP-GlcA is also used for the synthesis of UDP-Xyl by UDP-GlcA decarboxylase, also known as UDP-Xyl synthase (UXS). Arabidopsis has multiple *UXS* genes, encoding soluble and membrane-bound isoforms, located in the cytosol and the Golgi lumen, respectively (Fig. 3; Harper and Bar-Peled, 2002; Pattathil *et al.*, 2005). Cytosolic UDP-Xyl is transported to the Golgi apparatus by a family of specific transporters, known as UXTs. Arabidopsis *uxt1 uxt2 uxt3* mutants showed altered xylan content and structure (Zhao *et al.*, 2018), indicating that UDP-Xyl made in the cytosol plays a key role in xylan synthesis.

UDP-Ara is produced from UDP-Xyl by UDP-Xyl 4-epimerase, a reaction that takes place in the Golgi lumen (Burget *et al.*, 2003). The first product is UDP-Ara in the pyranose form (UDP-Ara*p*), which is then transported to the cytosol for conversion into the furanose form (UDP-Ara*f*) by UDP-Ara mutase (Konishi *et al.*, 2007). The product of this reaction is transported back to the Golgi apparatus by a specific transporter (Fig. 3; Rautengarten *et al.*, 2017). Alternatively, UDP-Ara*p* can be synthesized in the cytosol, via the salvage pathway (Geserick and Tenhaken, 2013) or by a cytosolic UDP-Xyl 4-epimerase (Kotake *et al.*, 2009). UDP-apiose (UDP-Api) is synthesized in the cytosol from UDP-GlcA by a decarboxylase different from UXS, known as UDP-Api/UDP-Xyl synthase (UAXS). Unlike UXS, Arabidopsis UAXS is not specific and produces both UDP-Api and UDP-Xyl in a ratio close to 1 (Mølhøj *et al.*, 2003). The synthesis of UDP-Api involves decarboxylation of the precursor and contraction of the sugar ring, from the pyranose to the furanose form, which is also catalysed by UAXS (Fig. 3; Savino *et al.*, 2019).

The synthesis of GDP-Man and GDP-Fuc also occurs in the cytosol, but through a different route. Fru6P is converted into Man6P and then Man1P by the sequential action of Man6P isomerase and phosphomannomutase. Man1P and GTP are the substrates of GDP-Man pyrophosphorylase, which produces GDP-Man (Seifert, 2004; Sharples and Fry, 2007). The latter can be translocated to the Golgi lumen or used in the cytosol to generate GDP-Fuc by the sequential action of GDP-Man 4,6-dehydratase (MUR1) and a bifunctional enzyme with 3,5-epimerase and 4-reductase activities named GER1 (Bonin *et al.*, 1997; Bonin and Reiter, 2000); GDP-Fuc is then transported to the Golgi lumen (Fig. 3; Rautengarten *et al.*, 2016). GDP-Man is also used in the cytosol by GDP-Man 3,5-epimerase (GME) to produce GDP-L-Gal (Fig. 3), a key intermediate for the synthesis of RG-II and ascorbic acid (see below). RG-II is a complex polysaccharide, comprising a plethora of sugars (including L-Gal), which in turn can be methylated and/or acetylated (Carpita *et al.*, 2015). Two molecules of RG-II are usually cross-linked by borate through Api residues, producing a structure that is important for the tensile strength of the cell wall. Silencing of the two *GME* genes in tomato plants led to reduced content of L-Gal in RG-II and decreased capacity of RG-II to perform *in muro* cross-linking, indicating that this is a crucial process for normal plant growth and development (Voxeur *et al.*, 2011).

Hemicelluloses and pectins are synthesized by sequential addition of sugar moieties from NDP-sugars to elongate the sugar backbones, with additional sugars being added singly or in groups along the sugar backbone. A large family of glycosyltransferases catalyses these reactions with different substrate specificities (Bar-Peled and O'Neill, 2011). Together, cellulose and the other cell wall polysaccharides constitute a huge proportion of global biomass. Thus, these pathways not only illustrate the enormous diversity of nucleotide-sugar metabolism in plants, but also represent one of the quantitatively most important metabolic outputs on the planet (Allen *et al.*, 2021).

ADP-glucose: the pathway of starch synthesis

In addition to structural polysaccharides, nucleotide-sugars are the substrates for synthesis of the most important storage polysaccharide in plants—starch. This polymer is an insoluble,

semi-crystalline material comprised of two α-1,4-linked glucans, amylose and amylopectin, which differ in chain length and degree of branching (Smith and Zeeman, 2020). Glycogen is a soluble, highly branched α-1,4-linked glucan that serves as a storage reserve in animals, fungi, and bacteria. In animals, glycogen is synthesized from UDP-Glc, but Leloir's group discovered that the substrate for starch synthesis is not UDP-Glc, but ADP-Glc (Fig. 1; Recondo and Leloir, 1961; Recondo *et al.*, 1963). Bacterial glycogen is also synthesized from ADP-Glc (Shen *et al.*, 1964). ADP-Glc is unusual in two respects: it is only present in prokaryotes (including cyanobacteria) and photosynthetic eukaryotes, including red and green algae and land plants (Ballicora *et al.*, 2003, 2004; Smith and Zeeman, 2020). It is also one of the only ADP-sugars found in nature, with the only other examples being ADP-Gal and ADP-Fru, both of which are rare and of unknown function (Bar-Peled and O'Neill, 2011; Kleczkowski and Decker, 2015). In plants, ADP-Glc is exclusively used for starch synthesis (Ballicora *et al.*, 2004), whereas some bacteria also use it as the glucosyl donor for the synthesis of trehalose and sucrose by TPS and SPS, respectively (Porchia and Salerno, 1996; Lunn *et al.*, 2003; Cumino *et al.*, 2007; Asencion Diez *et al.*, 2015, 2017). Plant sucrose synthases can also use ADP-Glc as a substrate *in vitro*, and bacterial forms of this enzyme from *Thermosynechococcus elongatus* and *Nitrosomonas europaea* preferentially use ADP-Glc (Figueroa *et al.*, 2013*a*; Wu *et al.*, 2015).

In green algae and plant leaves, starch is synthesized in the chloroplasts, starting with Fru6P that is withdrawn from the Calvin–Benson cycle, and converted to Glc6P and then Glc1P by phosphoglucose isomerase and phosphoglucomutase (Fig. 2). ADP-Glc pyrophosphorylase (ADP-Glc PPase) then catalyses the conversion of Glc1P and ATP to ADP-Glc and inorganic pyrophosphate (PPi). The latter is hydrolysed by pyrophosphatase to drive the reversible ADP-Glc PPase reaction in the direction of ADP-Glc synthesis (Stitt *et al.*, 2010). This is the first committed step in the pathway of starch synthesis and the major site for regulation of flux through the pathway, via allosteric activation by 3-phosphoglycerate and inhibition by Pi (Ballicora *et al.*, 2003, 2004; Boehlein *et al.*, 2010; Figueroa *et al.*, 2011, 2013*b*). ADP-Glc PPase is also subject to redox modulation by thioredoxins (Fu *et al.*, 1998; Ballicora *et al.*, 2000; Tiessen *et al.*, 2002; Hendriks *et al.*, 2003; Tuncel *et al.*, 2014) and to reversible protein phosphorylation (Yu *et al.*, 2019; Ferrero *et al.*, 2020). Redox regulation of ADP-Glc PPase appears to play a relatively minor role in regulating the leaf enzyme (Hädrich *et al.*, 2012; Mugford *et al.*, 2014). The amylose component of starch granules is synthesized from ADP-Glc by granule-bound starch synthase, while the more complex and highly branched amylopectin polymer requires the activity of several soluble starch synthase (SS1–SS4), as well as starch branching enzymes (SBEI and SBEII) and debranching enzymes (isoamylase 1 and 2; Smith and Zeeman, 2020).

In non-photosynthetic tissues, starch is made and stored in specialized plastids called amyloplasts (Smith and Zeeman, 2020). In most species, ADP-Glc is synthesized within the amyloplasts by ADP-Glc PPase, using hexose-phosphates (chiefly Glc6P) imported from the cytosol via glucose-phosphate transporters in the amyloplast envelope (Hill and Smith, 1991). However, in the endosperm of cereals (e.g. maize, wheat, and barley) and wild grasses, ADP-Glc is also synthesized by cytosolic isoforms of ADP-Glc PPase (Denyer *et al.*, 1996; Beckles *et al.*, 2001; Burton *et al.*, 2002; Johnson *et al.*, 2003) and then imported into the amyloplasts via BRITTLE1-type adenylate transporters (Shannon *et al.*, 1998).

Other metabolic pathways that use nucleotide-sugars

The biosynthetic pathways for sucrose, starch, and cell wall polysaccharides represent the major fluxes involving nucleotide-sugars in plants, but are not the only ones. In this section, we highlight some other pathways that also depend on NDP-sugars as intermediates.

Galactolipid synthesis

Monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) are galactolipids that constitute ~80% of the lipid content of chloroplast thylakoid membranes (Dormann and Benning, 2002; Li and Yu, 2018; Rocha *et al.*, 2018; Fujii *et al.*, 2019). MGDG is mainly produced in the inner membrane, whereas DGDG is exclusively synthesized in the outer membrane of the chloroplast envelope (Rocha *et al.*, 2018). MGDG is synthesized by transferring the galactosyl moiety of UDP-Gal to diacylglycerol by MGDG synthase (Dormann and Benning, 2002; Moellering and Benning, 2011; Rocha *et al.*, 2018). DGDG is synthesized in a similar reaction, with the transfer of the galactosyl moiety of UDP-Gal to MGDG by DGDG synthase (Fig. 2). DGDG can also be made by galactolipid galactosyltransferase, which transfers the galactosyl moiety from one molecule of MGDG onto another. This reaction is independent of UDP-Gal, and the enzyme can also use DGDG as a second substrate to generate tri- and tetra-galactosyl derivatives, which modify membrane properties during adaptation to freezing stress (Moellering and Benning, 2011; Rocha *et al.*, 2018). In plants, UDP-Gal is synthesized from UDP-Glc by UDP-Glc 4-epimerase, especially under abiotic stress (Dormann and Benning, 2002; Rösti *et al.*, 2007; Li *et al.*, 2011; Wang *et al.*, 2015; Abdula *et al.*, 2016). UDP-Gal can also be made by UDP-sugar PPase, using UTP and Gal1P as substrates, although its contribution to galactolipid synthesis in chloroplasts of wild-type plants is unclear. The enzyme appears to play an essential role in salvaging sugars during the turnover of cell walls (Fig. 2), primarily in reproductive and non-photosynthetic tissues (Kotake *et al.*, 2007; Geserick and Tenhaken, 2013).

Sulfolipid synthesis

Sulfoquinovosyl diacylglycerol (SQDG) is a type of sulfolipid that is present in the chloroplast membranes of plants and in photosynthetic bacteria. The sulfur-containing headgroup of this sulfolipid is derived from UDP-Glc, which is synthesized by the chloroplastic UDP-Glc PPase, encoded by the *UGP3* gene (Okazaki *et al.*, 2009). UDP-Glc is then converted to UDP-sulfoquinovose (UDP-SQV) by UDP-SQV synthase, using sulfite (SO_3^-) as the sulfur donor (Essigmann *et al.*, 1998; Sanda *et al.*, 2001). The sulfoquinovose moiety of UDP-SQV is then transferred to diacylglycerol by a glycosyltransferase, SQDG synthase, to form SQDG (Fig. 2; Yu *et al.*, 2002).

Ascorbic acid synthesis

Ascorbic acid (vitamin C) has various functions in plants, but its primary role is as a free radical scavenger, thereby contributing to redox homeostasis (Smirnoff, 2018; Paciolla *et al.*, 2019). Unlike most animals, humans are unable to synthesize ascorbic acid, and plants provide the majority of this essential vitamin in the human diet. There are multiple pathways for the synthesis of ascorbic acid, and different groups of organisms use distinct ways (Gallie, 2013; Smirnoff, 2018; Paciolla *et al.*, 2019). The main route for ascorbic acid production in plants is known as the Smirnoff–Wheeler pathway, and involves two nucleotide sugars: GDP-Man and GDP-L-Gal (Smirnoff *et al.*, 2001). The latter is particularly noteworthy as a rare example of a naturally occurring nucleotide-sugar containing the L-enantiomer of the sugar moiety. The first step in the pathway is the synthesis of GDP-Man from Man1P by GDP-Man pyrophosphorylase. GDP-Man is then converted by GDP-Man 3,5-epimerase to GDP-L-Gal (Voxeur *et al.*, 2011), which is cleaved by GDP-L-Gal phosphorylase to release L-Gal1P in the first committed step in the pathway. Expression of Arabidopsis GDP-L-Gal phosphorylase is repressed by a *cis*-acting upstream ORF (uORF) under high ascorbate concentration (Laing *et al.*, 2015). This finding allowed the manipulation of ascorbate levels in economically important species. Genome editing of the homologous uORF by CRISPR/Cas9 [clustered regularly interspaced palindromic repeats (CRISPR)/CRISPR-associated protein 9] technology increased ascorbic acid levels in lettuce (Zhang *et al.*, 2018) and tomato plants (Li *et al.*, 2018). A specific phosphatase (encoded by the *VTC4* gene) hydrolyses L-Gal1P to L-Gal, which is then successively oxidized by L-Gal dehydrogenase and L-galactono-1,4-lactone dehydrogenase to ascorbic acid (Fig. 3). The Arabidopsis *vtc4* mutant showed decreased content of ascorbic acid (50% of the wild-type levels) and increased amounts of L-Gal in cell wall polysaccharides. These results confirmed that L-Gal1P phosphatase is involved in ascorbic acid biosynthesis, but also suggested that L-Gal1P could be hydrolysed by other phosphatases, such as the inositol/*myo*-inositol monophosphatases (Conklin *et al.*, 2006). All the Smirnoff–Wheeler pathway enzymes are localized in the cytosol, except for the final dehydrogenase, which is a mitochondrial protein (Smirnoff *et al.*, 2001; Gallie, 2013; Smirnoff, 2018; Paciolla *et al.*, 2019).

Glycosylation of phytohormones, secondary metabolites, and proteins

Nucleotide-sugars are also important as sugar donors for glycosylation of many types of molecules. There is such a vast range of these reactions in plants, many of which have not yet been characterized; thus we cannot cover these in detail, so we highlight just a few examples to illustrate the diversity of molecules that can be glycosylated using NDP-sugars. The glycosyltransferases that are responsible for such glycosylation reactions have been classified into different families according to their reaction mechanism (retaining or inverting), substrate(s), protein domain structure, and other features, and the Carbohydrate-Active enZYmes database (Lombard *et al.*, 2014) provides a comprehensive repository of information about these enzymes.

The major auxin in plants, indole-3-acetic acid, is inactivated by sequential glycosylation reactions. The initial step is a glucosylation reaction using UDP-Glc as substrate, followed by displacement of the glucose moiety of the glucoside by *myo*-inositol, and then further conjugation with either galactose (from UDP-Gal) or L-Ara (from UDP-L-Ara*p*; Corcuera *et al.*, 1982). Plants produce a myriad of secondary metabolites, many of which need to be conjugated with sugars for their biological function. For example, many phenylpropanoids (e.g. flavonoids) and isoprenoids (e.g. saponins) are maintained in a soluble, biologically active form by glycosylation, with NDP-sugars serving as the sugar donors (Saito *et al.*, 2013). Glycosylation can also be an important mechanism for sequestering reactive secondary metabolites and toxic xenobiotics in vacuoles to prevent damage to other cellular components. These molecules can be activated by de-glycosylation, to serve as defence compounds, if cells become damaged when the plant is under attack from pathogens or herbivores (Le Roy *et al.*, 2016).

Many secreted (extracellular) proteins are co-translationally glycosylated in the endoplasmic reticulum, by attachment of oligosaccharides synthesized from NDP-sugars. Asparagine *N*-glycosylation starts with *en bloc* transfer of pre-assembled oligosaccharides containing Glc, Man, and GlcNAc to a nascent polypeptide at the consensus sequence Asn-X-Ser/Thr, where X represents any amino acid, except proline (Nagashima *et al.*, 2018). This reaction is catalysed by the oligosaccharyltransferase complex, which is highly conserved among eukaryotes and is associated with ribosomes in a 1:1 ratio (Jeong *et al.*, 2018). The Arabidopsis *stt3a* mutant (lacking a subunit of the oligosaccharyltransferase complex) showed sensitivity to salt and osmotic stress, while the *stt3a stt3b* double mutant is non-viable due to gametophyte lethality (Koiwa

et al., 2003). Properly folded proteins are translocated to the Golgi apparatus, whereas those that do not adopt their native conformation are degraded within the endoplasmic reticulum. Glycosidases and glycosyltransferases located in the Golgi apparatus are involved in the maturation of the oligosaccharides bound to *N*-glycosylated proteins (Nagashima *et al.*, 2018). Among the multiple *N*-glycosylated proteins that are secreted to the apoplast, KORRIGAN1 provides a link between *N*-glycosylation, cell wall biosynthesis, and abiotic stress tolerance (Nagashima *et al.*, 2020). KORRIGAN1 (a membrane-anchored, *N*-glycosylated, endo-β-1,4-glucanase) interacts with the CESA complex and facilitates cellulose synthesis, either by increasing the amount of non-crystalline cellulose or by preventing the formation of cellulose aggregates (Nagashima *et al.*, 2018).

The cell wall is primarily composed of polysaccharides, but it also contains structural proteins, named after the most abundant amino acids: hydroxyproline-rich glycoproteins (HRGP), proline-rich proteins, and glycine-rich proteins (Carpita *et al.*, 2015). Extensin is an HRGP that contains repeating Ser–$(Pro)_4$ and Tyr–Val–Tyr sequences. The Pro residues are first hydroxylated by prolyl 4-hydroxylases to hydroxyproline (Hyp) and then *O*-glycosylated with up to four Ara residues on each Hyp, while the Tyr–Val–Tyr motif has been implicated in extensin cross-linking (Borassi *et al.*, 2016). Disruption of the genes encoding different isoforms of prolyl 4-hydroxylases, arabinosyl transferases (involved in the transfer of Ara residues to extensin Hyp residues), and extensins consistently blocked root hair elongation in Arabidopsis (Velasquez *et al.*, 2011). A second type of HRGP are arabinogalactan proteins (AGPs), which are heavily *O*-glycosylated (~95% carbohydrate content) proteoglycans located in the apoplast. AGPs contain highly branched galactans decorated with Ara residues and they often contain other sugars, such as GlcA and Rha (Carpita *et al.*, 2015). Recently, Lopez-Hernandez *et al.* (2020) showed that the *glcat14a/b/d* and *glcat14a/b/e* Arabidopsis mutants (lacking particular β-glucuronyltransferases) had multiple developmental defects and perturbed propagation of the calcium waves in roots, suggesting that AGPs might be important for binding and releasing cell surface apoplastic calcium.

Conclusion

The seminal discovery of nucleotide-sugars by Leloir and his colleagues opened the doors to a molecular understanding of complex carbohydrate biosynthesis in plants. They elucidated the biosynthetic pathway of sucrose, the lifeblood of vascular plants, and another disaccharide, trehalose. The phosphorylated intermediate of trehalose synthesis that they discovered, Tre6P, has become a molecule of great interest in recent years, following its recognition as a signal metabolite that is essential for normal plant growth and development. The Leloir group also identified ADP-Glc as the substrate for the synthesis of starch, the most common storage reserve in plants and the major source of calories in the human diet. Their work also laid the essential foundations for understanding how cellulose and other structural cell wall polysaccharides are produced, as well as the myriad of glycosylation reactions that play crucial roles in phytohormone signalling and the biological activity of glycolipids, secondary metabolites, and glycoproteins.

Seventy years after the discovery of nucleotide-sugars, their metabolism in plants remains a highly active area of research. As we have outlined above, the major pathways of nucleotide-sugar metabolism and their subcellular compartmentation have now largely been elucidated, and the genes encoding the enzymes involved have been identified. However, there are still some gaps in our knowledge of nucleotide-sugar transport that remain to be resolved. The field is now turning towards understanding how these pathways are regulated. A particularly important question is to understand how the pathways for synthesis of different cell wall components, which take place in different subcellular compartments, are coordinated to generate the characteristic cell wall composition in a given species. Furthermore, we also need to know how these processes are regulated in specific cell types, whose function requires a specialized cell wall structure, and how cell wall composition is modified in response to biotic (e.g. pathogen attack) and abiotic stresses, including mechanical stress. Such knowledge will open the doors to biotechnological engineering of plant cell wall composition, to improve disease resistance and stress tolerance, and generate novel biomaterials. Another exciting area of nucleotide-sugar metabolism is focused on their role in signalling, directly as potential signal molecules themselves, and indirectly in their capacity to modify the biological activity of other molecules (e.g. phytohormones, lipids, and secondary metabolites), as well as proteins and other structural components of the plant cells.

The awarding of the 1970 Nobel Prize in Chemistry to Luis F. Leloir was a fitting tribute to a remarkable man who made so many important contributions to our understanding of carbohydrate metabolism in both plants and animals. We honour the memory of a great scientist who bequeathed such a rich and enduring legacy to plant science.

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