Sugar Activation for Production of Nucleotide Sugars as Substrates for Glycosyltransferases in Plants

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Abstract: In order to serve as a glycosyl donor, a sugar or a sugar derivative (e.g., GlcA) needs to be “activated” to a highly energetic state of a nucleotide-sugar. This activation requires the involvement of specific enzymes which produce NDP-sugars (or, in one case, NMP-sugar), using NTP or NDP as substrate. The present review provides concise survey of distinct plant nucleotide-sugar pyrophosphorylases (all using NTP as one of the substrates and differing in sugar specificity) as well as nucleotide-sugar phosphorylases and sucrose synthase (all using NDP as one of substrates). The pyrophosphorylases discussed include UGPase, USPase, UAGPase, AGPase, GMPase (VTC1), and FKGP, whereas phosphorylases include ADP-Glc phosphorylase and GDP-Gal phosphorylase (VTC2/VTC5). We also discuss the activation mechanism of 3-deoxy-D-manno-octulosonic acid (Kdo) by CKS, leading to the formation of a unique NMP-linked sugar (CMP-Kdo).

Key words: cell wall formation, glycosyltransferases, nucleotide sugars, sugar activation

INTRODUCTION

Nucleotide sugars serve as substrates for hundreds of glycosyltransferases in all organisms and they can be considered as direct precursors for synthesis of oligo- and polysaccharides, glycoproteins, glycolipids, sulfolipids, and glucosinolates, among other compounds.1,2 For instance, plant cell wall polysaccharides are the most abundant components of biomass in nature, and they are composed of at least 14 different monosaccharides, which are connected by a variety of glycosidic linkages.3 This implies that each of these sugars had to be nucleotide-linked before its addition to a given polysaccharide backbone. The availability of nucleotide sugars as substrates for glycosyltransferase reactions is, thus, a crucial factor regulating cell wall architecture. Almost all glycosyltransferases use nucleotide sugars as substrates. However, there are exceptions, such as enzymes that use lipid-linked sugars as donors (e.g., dolichyl-phosphate-mannose-glycolipid α-mannosyltransferase).4,5

Nucleotide sugars originate by the activity of specific enzymes which “activate” a given sugar by addition of NDP. The nucleotide donor for this reaction is NTP or NDP, and the other substrate is a sugar or sugar-1-P. Once a given nucleotide-sugar is formed, it can then be used by glycosyltransferases which attach the sugar moiety to a given acceptor molecules. The majority of nucleotide sugars have UDP attached to a sugar molecule, the most important being UDP-Glc, a precursor to cellulose (the most abundant organic polymer on Earth) and sucrose (the major transported form of carbon in plants). Other important nucleotide sugars include: ADP-Glc (starch precursor), GDP-sugars (GDP-Man, GDP-Fuc, GDP-Gal, GDP-Glc, and GDP-Gul), and CMP-3-deoxy-D-manno-octulosonic acid (CMP-Kdo), an unusual form of activated sugar.

In this review, we will cover primary mechanisms for production of nucleotide sugars in plants. Other mechanisms, involving interconversions of nucleotide sugars, will be given lesser attention, and they have been covered extensively by others.6–7

SUGAR ACTIVATION REACTIONS

UDP-sugar formation.

There are generally three types of pyrophosphorylases that produce UDP-sugars: UDP-Glc pyrophosphorylase (UGPase), UDP-sugar pyrophosphorylase (USPase) and UDP-N-acetylgalcosamine (UDP-GlcNAc) pyrophosphorylase (UAGPase) (Fig. 1). They differ in substrate specificity, but all three can produce UDP-Glc. These proteins show low or very low identity (below 20%) to each other at the amino acid sequence level, but they share common tertiary structure, with a prominent Rossman fold in the central (catalytic) domain, common details of their active sites, and easily defined N- and C-terminal domains, which may play regulatory functions.8 The pyrophosphorylases carry out fully reversible reactions, with the equilibrium constants (Keq) on the order of ca. 0.14–0.50,9–12 and their kinetics are
consistent with the ordered bi-bi mechanism, where UTP binds first, followed by binding of sugar-1-P, and then PPI is released as first product, followed by UDP-sugar.

Other primary mechanism for production of UDP-Glc is represented by sucroase synthase (SuSy), which uses sucrose and UDP as substrates (Fig. 1).

Both the pyrophosphorylases and SuSy are discussed in more detail below.

I) UGPase (UDP-Glc formation). UGPase (EC 2.7.7.9) catalyzes the reaction where UTP and Glc-1-P are converted to UDP-Glc and PPI (Fig. 1). There are two types of UGPases (UGPase-A and UGPase-B) which carry out the same reaction, but have only ca. 14% identity in their amino acid sequences. Both UGPase-A and UGPase-B utilize Glc-1-P as preferred sugar substrate, but they can also react with Gal-1-P to produce UDP-Gal. The activity with Gal-1-P was also shown for UGPase-A from Giardia, a protozoan pathogen. The crystal structure of UGPase-A, not UGPase-B, was already solved.

Nuclear genomes usually contain two genes for UGPase-A, coding for two isozymes of ca. 51 kDa, and a single gene for UGPase-B, encoding a protein of 99 kDa, of which 8 kDa belong to the N-terminal transit peptide. Expression of UGPase-A genes is regulated by multiple factors, including sugars, light/dark conditions, phosphate deficiency and low temperature. Phosphate deficiency (as well as sulphur deficiency) had also strong effects on upregulation of UGPase-B gene in Chlamydomonas and Arabidopsis. In Arabidopsis, expression of UGPase-B gene is highly correlated with expression of other genes for sulfolipid biosynthesis, which is consistent with UGPase-B role as the mechanism for generation of UDP-Glc, the precursor of the polar head of sulfolipid.

UGPases from plants and Leishmania major (protozoan pathogen) are only active as monomers, whereas yeast and human UGPases are active only as octamers. Plant UGPase-A was found to exist both as active monomers and inactive dimers. In the crystal structure of Arabidopsis UGPase-A1, dimers were composed of monomers oriented in a head-to-tail position, where the access to the monomers’ active sites was blocked. However, in a recent study of sugarcane UGPase-A, using small-angle X-ray scattering analyses, dimers in solution consisted of monomers linked via interaction of their C-terminal domains, which apparently results in conformational changes. The C-terminal of barley UGPase-A was proposed to be important for oligomerization, since its deletion increased an overall UGPase activity and resulted in monomer only formation.

Oligomerization was proposed as the major mechanism regulating UGPase-A activity, with several factors, including hydrophobicity, protein concentration and substrate availability affecting the quaternary structure of the protein. There are, however, other possible regulatory mechanisms, including product inhibition and inhibition by UDP-Xyl. Sugarcane UGPase-A was recently found to be redox regulated in vitro, similar to UGPases-A from protozoan pathogens Entamoeba histolytica and Giardia lamblia. In addition, sugarcane UGPase-A was phosphorylated at Ser419 in vivo in leaves, but not in internodes. Whether this has any regulatory function is unknown at present.

The bulk of UGPase-A activity is located in the cytosol, but some activity is also associated with plasmalemma fraction. The cytosolic location is consistent with the presumed role of UGPase-A as central metabolic step in metabolism of sucrose and other sugars. On the other hand, the plasmalemma-bound UGPase-A activity may be of importance for direct channelling of UDP-Glc for cellulose and/or callose synthesis. In addition, under limiting oxygen concentration (hypoxia), e.g., in nonphotosynthetic tissues, UGPase-A activity is likely linked to that of SuSy and produces UTP and Glc-1-P, which makes metabolism more energy efficient. In seed endosperm (e.g., in barley), UGPase-A activity is positioned directly between SuSy and ADP-Glc pyrophosphorylase (AGPase), resulting in an overall conversion of sucrose to ADP-Glc, the latter the substrate for starch synthesis. In contrast to UGPase-A, the activity of UGPase-B is confined entirely to chloroplasts.
charide contents.\(^{38}\) This is most probably due to high catalytic activity of UGPase-A which, in crude extracts, frequently exceeds that of other enzymes of sugar and starch metabolism.\(^{18}\) However, UGPase-A activity is essential in plants,\(^{8,39}\) and the most pronounced effects in transgenic plants impaired in UGPase-A activity were connected with growth, pollen infertility and seed viability (see below).

Studies on transgenic plants, which overexpressed UGPase-A protein, frequently resulted in significant increases of cellulose content (e.g., \(^{40-43}\)) and/or modifications of primary and secondary metabolism (e.g., \(^{43}\)). However, while overexpression of a given protein may highlight its capability to be involved in a given process, it does not necessarily point to the \textit{in vivo} function of this protein. In this respect, studies using knockouts of specific genes should be most revealing. In rice, knockouts of either UGPase-A\(^{144}\) or -A2\(^{45}\) pointed out to different roles of the two isozymes, with UGPase-A1 involved in the production of pollen callose, whereas UGPase-A2 was linked to pollen starch synthesis, with both proteins essential for formation of viable seeds. In \textit{Arabidopsis}, UGPase-A1 (but apparently not UGPase-A2) was identified as one of the key effectors of pollen infertility and seed viability (see below).\(^{38,39}\) Nevertheless, small-sized homozygous \textit{Arabidopsis} double UGPase-A mutants could have their pollen fertility recovered by growth on media supplemented with UDP-Glc.\(^{39}\)

In contrast to UGPase-A, the role of UGPase-B is well established. The enzyme, localized exclusively in chloroplasts, is an essential step in sulpholipid synthesis, where UDP-Glc production is necessary for formation of sulfoquinovose (SQ), a Glc-derived constituent of sulpholipids. This was elegantly demonstrated using UGPase-B loss-of-function \textit{Arabidopsis} mutants.\(^{16}\)

\textbf{2) USPase (UDP-Gal, UDP-Glc, UDP-GlcA, UDP-Xyl, and UDP-Ara formation).} USPase (EC 2.7.7.64) is present in all plants, including single-celled Chlamydomonas, and in certain protozoan eukaryotic pathogens as well as in some bacteria.\(^{11,47}\) It is absent in animals,\(^{14}\) where UGPase-A was shown to have some activity with various sugar-1-phosphates.\(^{9}\) The enzyme from \textit{Arabidopsis}, pea, soybean, melon, and protozoan pathogens can use a variety of UDP-sugars as substrates (Fig. 1), including Glc-1-P, Gal-1-P, GlcA-1-P, Xyl-1-P, and Ara-1-P.\(^{11,48-54}\) The \(K_m\) values for those substrates vary from ca. 0.1 to 2 mM, with the lowest \(K_m\) values usually found for Glc-1-P, Gal-1-P, and GlcA-1-P.\(^{47}\) USPase is more or less specific for UTP as nucleotide substrate, with activities with ATP, CTP, or GTP less than 10\% of those with UTP.\(^{50}\)

In most cases, plant genomes contain a single gene for USPase, with the possible exception of \textit{Phycocystrella}, a moss, which apparently has three genes coding for this protein.\(^{47}\) USPase gene was found to be expressed in various plant tissues and organs, especially in the pollen\(^{48,50,55}\) and, generally, in flowers of different species.\(^{57}\) In \textit{Arabidopsis}, the USPase gene was strongly upregulated in transgenic plants deficient in UGPase-A activity, which points to a compensatory mechanism by USPase.\(^{58}\)

The USPase gene encodes a protein of ca. 67-70 kDa,\(^{11,49,50}\) The protein is active as monomer and there is no evidence for oligomerization, at least for the protein from \textit{Leishmania major} (a eukaryotic single-celled pathogen).\(^{53}\) Crystal structure of the \textit{Leishmania} USPase revealed generally a similar structure to UGPase-A or UAGPase proteins, with three distinct domains (central domain with prominent Rossmann fold, containing the active site, and flanked by characteristic N- and C-terminal domains).\(^{14}\) The active site of USPase is larger than that in other UDP-sugar producing pyrophosphorylases, probably reflecting relative non-specificity of this enzyme with respect to sugar-1-P as substrate.\(^{8,14}\)

USPase is predominantly a cytosolic enzyme, but it was also found in microsome membranes, as shown by immunoblot studies, and there is evidence for the existence of two isoforms slightly differing in molecular masses, suggesting differential splicing of the gene product or posttranslational modification.\(^{50}\) Being predominantly located in the cytosol, USPase sits at the centre of primary sugar metabolism and it may play a role both in the production of UDP-sugars and in their breakdown to the sugar-1-P and UTP level.\(^{18}\) For instance, similar to UGPase-A, under energy-demanding low O\(_2\) concentration conditions in non-photosynthetic tissues, USPase may work in concert with SuSy, by utilizing SuSy-produced UDP-Glc to make UTP.\(^{57}\)

As producer of several types of UDP-sugars, USPase is expected to have a role in the synthesis of many essential compounds, including different types of pectins and hemicelluloses, glycolipids, and providing UDP-sugars for protein \(N\) - and \(O\)-glycosylation processes. (Table 1). However, overexpression of USPase gene had no effect on phenotype in \textit{Arabidopsis}\(^{48}\) and no homozygous knockout transgenic plants could be obtained. The reason was that the loss-of-function mutation could not be transmitted through pollen.\(^{48,50}\) This was partly overcome by mutant complementation and miRNA approaches.\(^{57,58}\) Overall, one can conclude that USPase is required during vegetative and reproductive growth,\(^{48,55,57}\) being essential for Ara and Xyl recycling\(^{58,59}\) and controlling activities of sugar-1-kinase enzymes, which produce substrates for USPase reaction.\(^{50}\)

\textbf{3) UAGPase (UDP-GlcNAc and UDP-GalNAc formation).} UAGPase (EC 2.7.7.23/2.7.7.83) is responsible for the production of UDP-GlcNAc and UDP-N-acetylgalactosamine (UDP-GalNAc) from GlcNAc-1-P and GalNAc-1-P, respectively (Fig. 1). One of the two isozymes of \textit{Arabidopsis} UAGPase can also produce UDP-Glc from Glc-1-P, but it is probably a minor reaction, since \(K_m\) value for UTP (0.9 mM), when Glc-1-P served as the other substrate, was 3-5 times higher than with GlcNAc-1-P or GalNAc-1-P.\(^{50}\) Plants contain two genes coding for UAGPase, and the two isozymes of \textit{Arabidopsis} UAGPase have molecular masses of ca. 58 kDa each\(^{50}\) and exist as monomers. In contrast, human analogue of UAGPase (AGX1) is a dimer in native conditions, but dissociates to active monomers under assay conditions.\(^{61}\) Protein structures of \textit{Arabidopsis} UAGPase1 and UAGPase2 were homology-modelled using crystal structure of human AGX1 as template, revealing a conserved catalytic fold in the central domain and helped to identify
key conserved motifs.\textsuperscript{60}

Plant extracts contain both UDP-GlcNAc and UDP-D-GlcNAc.\textsuperscript{62} Whereas UDP-GlcNAc can serve as sugar donor for N- and O-linked protein glycosylation in plants (Table 1), the role for UDP-D-GlcNAc is less certain.\textsuperscript{63} However, a Gal-GlcnAc disaccharide was found in rice glutelin, suggesting the presence of a mammalian-like type of Gal-GalNAc disaccharide was found in rice glutelin, although it interconverts UDP-GalNAc and UDP-GlcNAc,\textsuperscript{64} although it UDP-Gal epimerase (UGE) was characterized which can UDP-GlcNAc (UDP-GalNAc) pyrophosphorylase; UXS, UDP-xyl synthase; UAXS, UDP-Api/UDP-xylose synthase; UGAE, UDP-GlcA epimerase; UGDH, UDP-Glc dehydrogenase; UGE, UDP-Gal epimerase; UGEb, bifunctional cytosolic UGE/UXE; UGPase, UDP-Glc pyrophosphorylase; GALT, Gal-1-P uridylyltransferase; GMD-GER, GDP-D-Man dehydratase; GDP-D-Man epimerase; GMPase (VTC1), GDP-D-Man pyrophosphorylase; RG, rhamnogalacturonan; SQ, sulfoquinovose; SuSy, sucrose synthase; UAGPase, URS, UDP-rhamnose synthase; USPase, UDP-sugar pyrophosphorylase; USS, UDP-sulfoquinovose synthase; UXE, UDP-Xyl epimerase (in Table 1).

### Table 1. Origins of nucleotide sugars (NS) by sugar activation and interconversion mechanisms and major uses for the NS-derived sugars.

<table>
<thead>
<tr>
<th>NS</th>
<th>Mechanisms of NS formation</th>
<th>Major use for the NS-derived sugar</th>
</tr>
</thead>
<tbody>
<tr>
<td>UDP-D-Glc</td>
<td>UGPase, USPase, SuSy, UAGPase</td>
<td>UGE, GALT Cellulose, callose, sucrose, mixed-linked glucan, xyloglucan, sulfolipids, glycolipids, protein N-glycosylation</td>
</tr>
<tr>
<td>UDP-D-Gal</td>
<td>UGPase, USPase</td>
<td>UGE, GALT Pectin (RG I, RG II), xyloglucan, galactomannan, galactoglucomannan, glycolipids, protein N- and O-glycosylation</td>
</tr>
<tr>
<td>UDP-D-GlcA</td>
<td>USPase</td>
<td>UGDH, UGAE Pectin (RG II), glucuronarabinobioxylan, glucuronoxylan pectins</td>
</tr>
<tr>
<td>UDP-D-GalA</td>
<td>USPase</td>
<td>UGAE Xylans, xyloglucan, pectin (xylagalacturonan, RG II), protein N-glycosylation</td>
</tr>
<tr>
<td>UDP-D-Xyl</td>
<td>USPase</td>
<td>UXS, UAXS, UXE, UGeb Protein N- and O-glycosylation</td>
</tr>
<tr>
<td>UDP-L-Ara</td>
<td>USPase</td>
<td>UXE, UGeb Pectin (RG I, RG II), xylans, protein O-glycosylation</td>
</tr>
<tr>
<td>UDP-L-Rha</td>
<td>USPase</td>
<td>URS Pectin (RG I, RG II)</td>
</tr>
<tr>
<td>UDP-D-Api</td>
<td>USPase</td>
<td>UAXS Pectin (RG II)</td>
</tr>
<tr>
<td>UDP-D-GlcNAc</td>
<td>UAGPase</td>
<td>UGE Protein N- and O-glycosylation</td>
</tr>
<tr>
<td>UDP-D-GalNAc</td>
<td>UAGPase</td>
<td>UGE Protein O-glycosylation</td>
</tr>
<tr>
<td>UDP-SQ</td>
<td>—</td>
<td>UGS Sulfolipids</td>
</tr>
<tr>
<td>ADP-D-Glc</td>
<td>AGPase, SuSy, AGase</td>
<td>— Starch</td>
</tr>
<tr>
<td>GDP-D-Man</td>
<td>GMGase</td>
<td>GM E L-Ascorbate, mannans, protein N- and O-glycosylation glucuronan</td>
</tr>
<tr>
<td>GDP-D-Gle</td>
<td>VTC2/5, SuSy</td>
<td>— Protein N- and O-glycosylation</td>
</tr>
<tr>
<td>GDP-L-Gal</td>
<td>VTC2/5</td>
<td>GME L-Ascorbate</td>
</tr>
<tr>
<td>GDP-L-Fuc</td>
<td>FKGP</td>
<td>GMD-GER Pectin (RG II), xyloglucan, protein N- and O-glycosylation</td>
</tr>
<tr>
<td>GDP-L-Gal</td>
<td>—</td>
<td>GM E L-Ascorbate</td>
</tr>
<tr>
<td>CMP-Kdo</td>
<td>—</td>
<td>CKS Pectin (RG II)</td>
</tr>
</tbody>
</table>

AGase, ADP-Glc phosphorylase; AGPase, ADP-Glc pyrophosphorylase; CKS, CMP-Kdo synthetase; FKGP, Fuc-kinase/GDP-Fuc pyrophosphorylase; GALT, Gal-1-P uridylyltransferase; GGDH, UDP-D-Man dehydratase; GDP-D-Man epimerase/reductase; GME, GDP-D-Man epimerase; GMPase (VTC1), GDP-D-Man pyrophosphorylase; RG, rhamnogalacturonan; SQ, sulfoquinovose; SuSy, sucrose synthase; UAGPase, URS, UDP-rhamnose synthase; USPase, UDP-sugar pyrophosphorylase; USS, UDP-sulfoquinovose synthase; UXE, UDP-Xyl epimerase (in Golgi); VTC2/5, GDP-L-Gal phosphorylase.

they are osmoticum and sugar-responsive, with Glc and Man effects on SuSy1 gene sensed/ transmitted via a hexokinase-dependent mechanism. Sugar (and osmoticum) effects on SuSy1 are transmitted via a different signaling pathway than sugar effects on UGPase-A gene(s), possibly representing a mechanism where UDP-Glc is assured to be produced even if one of these pathways is blocked or inactive.

SuSy is localized mainly in the cytosol, but there were also several other locations reported, including a SuSy isoenzyme in mitochondria. SuSy was found to bind reversibly to various types of membranes, including plasmalemma and tonoplast. SuSy can undergo phosphorylation, which may affect its solubility and, depending on plant species and presence or absence of sucrose, there may or may not be a direct relationship between SuSy binding to a given membrane and degree of its phosphorylation.

The enzyme is more or less specific with sucrose) to produce either UDP-Glc or ADP-Glc (see below) and SuSy (see above). The best studied ADP-Glc producing mechanism is AGPase (EC 2.7.7.27), which can use to the same extent UDP or ADP as a substrate yielding two different transcripts, as first found for barley AGPase. Similar to UGPase-A and SuSy, genes for AGPase are prone to regulation by sugars and photoperiod, probably reflecting a close link between intracellular sugar contents and starch.

AGPase is active only with Glc-1-P as sugar donor and is activated by 3-phosphoglyceric acid (PGA) and inhibited by inorganic phosphate (Pi), with the respective $K_c$ and $K_i$ values on the order of micromolar. The mode of activation and inhibition is frequently allosteric, as judged by Hill plots; this was especially evident for AGPase from non-photosynthetic tissues (e.g., seed endosperm or potato tubers), but not for leaf AGPase. Leaf AGPase is tightly regulated by the PGA/Pi ratio in chloroplast stroma, with starch levels closely linked to AGPase activity.

The small AGPase subunit is prone to redox regulation, and was shown to exist as dimer under oxidized conditions and as monomer when reduced by a thioredoxin. It has been proposed that stimulation of leaf starch synthesis by sugars is mediated by trehalose-6-P via redox modulation of AGPase. The redox regulation of leaf AGPase, however, may have little or no effect on starch content, and its significance is unclear at present.

AGPase had long been considered to exist only in plastids (see also 100); this view was changed when a major cytosolic isozyme of the enzyme was found in the cereal seed endosperm. The cereal seed AGPase was frequently found to be less sensitive to regulation by PGA and Pi than leaf AGPase. Interestingly, the cytosolic AGPase is also redox regulated, but the modification occurs on the large subunit rather than the small subunit.

In Arabidopsis, AGPase is present in all starch-containing tissues, but also in other locations. Based on studies with AGPase mutants and transgenic plants with modulated AGPase activity, there is no doubt that AGPase is involved in starch biosynthesis. However, whether AGPase or SuSy is the most important mechanism providing ADP-Glc for starch formation in vivo has become a contentious issue [e.g., 74, 75, 123]. More studies are required to resolve the actual contribution of each of these enzymes to the starch pool in both photosynthetic and non-photosynthetic tissues.

2) AGase (ADP-Glc formation). ADP-Glc phosphorylase (AGase, EC 2.7.7.36) is widely distributed in plant tissues, but it has been scarcely studied. The enzyme catalyzes a reversible reaction between Glc-1-P and ADP, producing ADP-Glc and Pi (Fig. 1). AGase is apparently specific for ADP and ADP-Glc as substrates in both directions of the reaction, as found for partially purified enzymes from wheat germ and potato tubers and for purified recombinant AGase from Arabidopsis. Arabidopsis contains one gene for AGase, encoding a protein of ca. 39 kDa. The protein was crystallized and found to form dimers. Based on crystallization data, the AGase reaction is consistent with the ping-pong bi-bi mechanism, where ADP binds first, followed respectively by the release of Pi, binding of Glc-1-P, and release of ADP-Glc.

Even though the AGase reaction is reversible, its equilib-
GDP-sugar formation.

1) **GM Pase (VTC1) (GDP-D-Man formation).** GDP-D-Man is produced from D-Man-1-P by GDP-D-Man pyrophosphatase (GM Pase, EC 2.7.7.13), also called VTC1 (Fig. 1), a ca. 40 kDa protein, which is a key activity in the so-called “L-Gal” pathway as well as “L-Gul” pathway, both pathways leading to L-ascorbate (vitamin C) as the end product. 129,130 Arabidopsis contains three genes encoding GM Pase. 131

Mutants of Arabidopsis and antisense potato plants impaired in GM Pase activity accumulated less ascorbate when compared to wild-type plants. 129,132 The transgenic potato plants showed early senescence, and their leaf cell walls had only ca. 30–50% of Man content when compared to wt plants, whereas the composition of tuber cell walls was unchanged. 129 Studies with transgenic tobacco plants with either decreased or increased activity of GM Pase showed a direct relationship between GM Pase activity and ascorbate content, which could also be linked to plant responses to temperature and oxidative stresses. 133,134 Recently it has been demonstrated that GM Pase is more stable in light than in dark conditions, and that it interacts with CSN5B, a component of the photomorphogenic COP9 signalosome, in dark conditions, and that it interacts with CSN5B, a component of the photomorphogenic COP9 signalosome. 135,136

GMPase activity was shown to be very sensitive to the presence of ammonium (NH₄⁺) in the growth media. 131 Analyses of NH₄⁺-hypersensitive Arabidopsis vtc1 mutants of GM Pase revealed that the plants had defects in protein N-glycosylation in the roots rather than a decreased ascorbate content. The results demonstrated a strong correlation between GM Pase and the degree of defective N-protein glycosylation, 131,136 which could be further linked to pleiotropic alterations in NH₄⁺ metabolism, hormone homeostasis, and nitric oxide signalling. 136

2) **FKGP (GDP-L-Fuc formation).** GDP-L-Fuc is produced by a bifunctional enzyme (FKGP, EC 2.7.1.52/2.7.7.30), combining fucokinase and GDP-L-Fuc pyrophosphorylase activities (Fig. 1). Arabidopsis contains a single gene coding for FKGP, and it encodes a protein with molecular mass of ca. 120 kDa, when analyzed on SDS-PAGE gels. 137 The pyrophosphorylase activity of Arabidopsis FKGP was specific for GTP and L-Fuc as substrates, suggesting that the enzyme produces only GDP-L-Fuc as a nucleotide sugar. The gene was expressed in most cell types, suggesting that salvage reactions for L-Fuc are ubiquitous in plant tissues. Loss-of-function mutants of FKGP accumulated free L-Fuc, but had no visibly altered phenotype. 137 The FKGP enzyme from grape vine was recently obtained as crystals, 138 but details of its protein structure have not been resolved.

3) **VTC2/VTC5 (GDP-L-Gal formation).** GDP-L-Gal is produced from L-Gal-1-P by GDP-L-Gal phosphorylases (VTC2 and VTC5, EC 2.7.7.69) which, contrary to a pyrophosphorylase reaction, use GDP (instead of GTP) as substrate, and produce Pi (instead of PPI) (Fig. 1). The enzymes can also produce GDP-D-Glc (and Pi) from D-Glc-1-P and GDP. 139–141 The enzymatic reactions carried out by VTC2 and VTC5 are reversible; 139,140 however, their equilibrium constants are tilted toward production of L-Gal-1-P (and then toward ascorbate production) rather than GDP-L-Gal. Thus, in vivo, GDP-L-Gal is more likely produced from GDP-D-Man by a specific epimerase 130 (GME) rather than by VTC2/5 reactions (Table 1).

In Arabidopsis, both VTC2 and VTC5 are coded by highly homologous genes, which encode proteins having molecular masses of ca. 50 kDa each. 142–144 The genes’ expression increased at the beginning of the light cycle and was found to be controlled by the circadian clock. 139 Arabidopsis double mutants unable to produce neither VTC2 nor VTC5 proteins showed growth arrest immediately upon germination and subsequently had their cotyledons bleached. Normal growth was restored by supplementation of the double mutant with ascorbate or L-Gal, confirming the key role of those enzymes in ascorbate synthesis. 139 Overexpression of a VTC2/5 gene in tomato, potato, and strawberry plants resulted in up to 2–6-fold increases in ascorbate content in tubers and fruits, indicating that upregulation of GM Pase genes can be used to raise significantly ascorbate concentration in commercially significant edible plants. 145

**CMP-Kdo formation.**

Kdo is an essential component of peptidoglycan/lipid II (RG II) fraction in cell walls. This fraction contains also other unique sugars (or derivatives), such as apiose (Api), aceric acid or 3-deoxy-D-lyxo-2-heptulosonic acid, but details of the activation of the two latter compounds are unknown. Kdo was also found as an intermediate in the putative “lipid A” pathway, which in bacteria is involved in production of lipopolysaccharides. 146 Its activated form is CMP-Kdo produced by CMP-Kdo synthetase (CKS, EC 2.7.7.38) (Fig. 1). The reaction catalyzed by CKS is unique in that the sugar is directly coupled to CMP rather than to a NDP moiety. Plant CKS is thought to originate from Gram-negative eubacteria via horizontal gene transfer. 146 In those bacteria, Kdo is an essential component of the lipopolysaccharides and capsular polysaccharides, both located on cell surface. No genes for CKS were found in yeast or animals. Crystal structure of bacterial CKS was solved. 147

Arabidopsis genome contains one gene for CKS. Purified recombinant protein coded by this gene had molecular mass of ca. 30 kDa and showed CKS activity. 148,149 The recombinant Arabidopsis CKS used CTP as a preferred substrate (with Kdo as second substrate), but it could also react with UTP and dCTP. 149 A homologous gene earlier found in maize also coded for a protein showing CKS activity. 145 Using GFP-CKS construct in transgenic Arabidopsis plants, Kobayashi et al. 149 demonstrated that the protein was localized in mitochondria. This would imply that CMP-Kdo has to be transported from mitochondria to Golgi where Kdo could be inserted into the RG II skeleton. Putative Golgi-associated transporters for CMP-Kdo were identified in...
Arabidopsis and rice (based on their ability to transport CMP-sialic acid), but it is still unknown whether indeed they can recognize CMP-Kdo as their substrate. Similar to Kdo, sialic acid has a 2-keto acid structure, and its activated form contains CMP as the nucleotide part.

Mutation in CKS gene rendered pollen infertile through the inhibition of pollen tube elongation. This is yet another example (see subchapters on UGPase, USPase, and UAGPase) that impairment in sugar activation frequently has its most dramatic effect on fertility of the pollen. The resulting male sterility prevents obtaining homozygous mutants for those proteins, and to a large extent precludes more detailed information on the in vivo importance of those proteins in other processes during plant growth and development.

UDP-SUGAR INTERCONVERSION REACTIONS

Besides the pyrophosphorylases and SuSy reactions, there are several other enzymatic mechanisms for formation of UDP-sugars. They all involve interconversions from one UDP-sugar to another (Table 1), with UDP-Glc frequently as the primary precursor. Examples include UDP-Glc dehydrogenase (EC:1.1.1.22) which produces UDP-GlcA from UDP-Glc; UDP-GlcA epimerase (UGAE, EC 5.1.3.6), interconverting UDP-GlcA and UDP-GalA; UGE (EC 5.1.3.2), which interconverts UDP-Glc and UDP-Gal; and Gal-1-P uridylyltransferase (GALT, EC 2.7.7.12), a key enzyme of the Leloir pathway, which produces UDP-Gal and Glc-1-P from UDP-Glc and Gal-1-P. Several bifunctional cytosolic UGEs with UDP-Xyl 4-epimerase (UXE) activity were also characterized, which are distinct from the UDP-Xyl 4-epimerase localized in the Golgi apparatus. Contents of UDP-sugars may also depend on activity of some isozymes of nucleotide pyrophosphatase/phosphodiesterase family of proteins, which hydrolyze pyrophosphate and phosphodiester bonds of numerous nucleotides and nucleotide sugars (including UDP-Glc).

UDP-Api, UDP-L-Rha, and UDP-SQ are UDP-sugars which apparently are not produced by activation of sugar by linking it with UDP-moiety, but rather originate from other UDP-sugars. UDP-Api is formed by decarboxylation of UDP-GlcA by UDP-Api/UDP-Xyl synthase (UAXS). The other product in the same reaction is UDP-Xyl, which can also be produced by USPase, UXE, bifunctional UGE, and UDP-Xyl synthase (UXS, EC 4.1.1.35), called also UDP-GlcA decarboxylase (Table 1). UDP-L-Rha is produced from UDP-D-Glc by UDP-rhamnose synthase (URS). This protein has UDP-D-glucose 4,6-dehydratase activity carried out by its N-terminal domain, as well as UDP-4-keto-6-deoxy-D-glucose 3,5-epimerase and UDP-4-keto-L-rhamnose 4-keto-reductase activities that are confined to the rest of the protein. All these activities are required for conversion of UDP-Glc to UDP-Rha by URS. Finally, UDP-SQ is produced by UDP-SQ synthase (USS, EC 3.13.1.1), which uses UDP-Glc and sulphite (SO3^-2) as substrates; the produced UDP-SQ serves then as a precursor to sulphur-containing membrane lipids. The UDP-Glc used by USS comes from UGPase-B reaction.

GDP-SUGAR INTERCONVERSION REACTIONS

Besides GMPase, FKGP, and VTC2/5 reactions which activate D-Man, L-Fuc and L-Gal/D-Glc, respectively, by producing the respective GDP-sugars, those and other GDP-linked sugars can also be formed via conversion from existing GDP-sugars (Table 1). Thus, both GDP-L-Gal and GDP-L-Gul can be formed from GDP-D-Man by a specific epimerase (GME), and converted to ascorbate by two distinct pathways. GDP-D-Man can also serve as a precursor to GDP-L-Fuc via combined reactions of a specific dehydratase (GMD) and epimerase/reductase (GER).

PROSPECTS

Efforts to search for new mechanisms producing nucleotide sugars should continue, as plant preparations contain some nucleotide sugars which origins and roles are unknown. These activated sugars include ADP-L-Ara, GDP-L-Ara, ADP-D-Gal, GDP-D-Man, UDP-Fru, and others. Also, some sugar-derived components of plant glycans include Dha (3-deoxy-D-lyxo-2-heptulosonic acid) and aceric acid (3-C-carboxy-5-deoxy-L-xylene), for which their nucleotide-bound forms are not known.

There is also a need to re-examine substrate specificity of enzymes involved in sugar activation, especially pyrophosphorylases, as well as enzymes involved in nucleotide sugar interconversions. Systematic tests with a broad range of sugar-1-phosphates or nucleotide sugars may reveal unexpected specificity, as indeed was the case with UGPase-A and UGPase-B which, in addition to their reactivity with Glc-1-P, were found to use also Gal-1-P as substrate. Information like this would be crucial for elucidation of primary metabolic links for a given glycosyltransferase reaction. This should be linked to further detailed studies on structure/function properties of those enzymes in order to understand roles of crucial amino acids for binding of a given substrate. For bacterial AGPase (from Thermus caldophilus) it was found that mild random mutagenesis (using error-prone DNA polymerase) of its cDNA resulted in altered substrate specificity. From a total of 656 colonies screened, two colonies had UGPase activity, while three had UAGPase activity. Those changes were then linked to specific single substitutions of amino acids within the active site of the enzyme. This indicates that, at least for bacterial pyrophosphorylases, details of their substrate binding and catalysis are similar, and changes in few amino acids can affect their substrate specificity. Active sites of distinct plant pyrophosphorylases also have common architecture, suggesting the possibility for manipulation of their substrate specificity.

No effective inhibitors of any of the UDP-sugar producing pyrophosphorylase are known, so it is impossible to distinguish between their rates in crude extracts when using Glc-1-P or UDP-Glc as a substrate. The use of specific inhibitors represents a useful tool to estimate exact contribution of a given isozyme to an overall rate. When assaying UGPase rates in crude leaf extracts in the presence and absence of specific antibodies against UGPase-A, the overall activity decreased by at least 90% (unpublished data). This suggests that the combined activities of UGPase-B, USPase,
and UAGPase with Glc-1-P as substrate are at most 10% of those of UGPase-A in leaf extracts. Specific inhibitors of UGPase-A, USPase, UAGPase, and CKS would also be useful to study in more detail the in vivo roles of those enzymes, as loss-of-function mutant plants are frequently impaired in their reproductive abilities. However, finding suitable inhibitors that will work in vivo is always risky due to uncertainty of whether the compounds are indeed specific or whether they are uptaken, so they can interact with their targets.

Finally, more studies are required on enzymes involved in GDP-sugar formation (FKGP, GMase, and VTC2/5) as well as CKS, as those enzymes have only been characterized in few instances. The work on GDP-sugar forming enzymes seems particularly important, since GDP-sugars are key intermediates of pathways leading to the formation of L-ascorbate (from GDP-L-Gal), cell wall and glycoproteins (Table 1).

ACKNOWLEDGMENTS

This work was supported, in part, by grants from the Swedish Research Council (Vetenskaprådet).

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