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Biosynthesis of nucleotide sugars by a promiscuous UDP-sugar pyrophosphorylase from *Arabidopsis thaliana* (AtUSP)

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ABSTRACT

Nucleotide sugars are activated forms of monosaccharides and key intermediates of carbohydrate metabolism in all organisms. The availability of structurally diverse nucleotide sugars is particularly important for the characterization of glycosyltransferases. Given that limited methods are available for preparation of nucleotide sugars, especially their useful non-natural derivatives, we introduced herein an efficient one-step three-enzyme catalytic system for the synthesis of nucleotide sugars from monosaccharides. In this study, a promiscuous UDP-sugar pyrophosphorylase (USP) from *Arabidopsis thaliana* (AtUSP) was used with a galactokinase from *Streptococcus pneumoniae* TIGR4 (SpGalK) and an inorganic pyrophosphatase (PPase) to effectively synthesize four UDP-sugars. AtUSP has better tolerance for C4-derivatives of Gal-1-P compared to UDP-glucose pyrophosphorylase from *S. pneumoniae* TIGR4 (SpGalU). Besides, the nucleotide substrate specificity and kinetic parameters of AtUSP were systematically studied. AtUSP exhibited considerable activity toward UTP, dUTP and dTTP, the yield of which was 87%, 85% and 84%, respectively. These results provide abundant information for better understanding of the relationship between substrate specificity and structural features of AtUSP.

Nucleotide sugars such as UDP-galactose and UDP-glucose are critical to the survival of all organisms.[1] They are activated forms of monosaccharides and key intermediates of carbohydrate metabolism in both prokaryotes and eukaryotes. In primary metabolism, as the activated donor substrates of Leloir-type glycosyltransferases, nucleotide sugars play an important role in protein glycosylation, the biosynthesis of glycogen, and even the synthesis of cell wall.[2–4] In secondary metabolism, nucleotide sugars are used to synthesize different glycosylated natural products or their derivatives which act as the precursors of many front-line drugs.[5–8] Besides, some nucleotide sugars were immediate activators of $P2Y_{14}$ receptor which promotes the rearrangement of cytoskeleton, change of cell shape, and enhancement of cell migration.[9] With increased attention to biological functions of sugar chains and glycosyltransferases,[10,11] the availability of nucleotide sugars is particularly important to such research.

UDP-sugars can be synthesized by chemical or enzymatic approaches. Recently, various chemical approaches have been reported.[12] Compared with chemical methods, enzymatic approaches are more powerful and in some cases a single transformation can substitute several chemical reactions.[13,14]

In higher plants, sugar nucleotides can be synthesized by both de novo and salvage pathways.[15] For example, UDP-galactose can be synthesized by epimerization of UDP-glucose in de novo pathway. However the low yield and difficulty in separating UDP-galactose from UDP-glucose limit the application of this method. The simplest route for synthesizing UDP-sugars involves the sequential action of monosaccharide kinases and pyrophosphorylases in the salvage pathway.[16] Thus finding highly active and promiscuous monosaccharide kinases and nucleotide sugar pyrophosphorylases becomes a key factor to obtain structurally diverse nucleotide sugars with high yield. Different sourced UDP-galactose pyrophosphorylases had been identified such as UDP-sugar pyrophosphorylases (USPs) from pea (*Pisum sativum* L.) sprouts, *Leishmania major* and *Trypanosoma cruzi*.[17–19] However, the 1-phosphorylation forms of monosaccharides were not easily obtained in vitro. And few non-natural derivatives of monosaccharide-1-P as substrates for USPs have been tested. Recently, Chen et al. cloned a promiscuous UDP-sugar pyrophosphorylase (BLUSP) from *Bifidobacterium longum* strain ATCC55813 and used it together with a monosaccharide 1-kinase to set up a one-pot multi-enzyme system for efficient synthesis of eight UDP-sugars.[20]

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This is the first time to use the USP in preparative scale synthesis. And more importantly, non-natural derivatives of monosaccharide-1-P were tested as substrates for USPs.

UDP-sugar pyrophosphorylase from *A. thaliana* (AtUSP) exhibits housekeeping function in nucleotide sugar metabolism.[21] It was characterized with different natural monosaccharide-1-P substrates.[15,22] The results indicated that AtUSP had relatively broader substrate tolerance compared with other UDP-sugar pyrophosphorylases and its highest activity occurred to GlcA-1-P, Glc-1-P and Gal-1-P. Given that limited methods are available for the preparation of nucleotide sugars, especially their useful non-natural derivatives, we introduced herein an efficient one-step three-enzyme catalytic system using AtUSP for the synthesis of nucleotide sugars from monosaccharides.

In this study, we report the cloning of UDP-sugar pyrophosphorylase from *A. thaliana* (AtUSP) and its application in a one-pot three enzyme system to synthesize four UDP-monosaccharides from simple monosaccharides or their derivatives. Besides, the nucleotide triphosphate substrate specificity of AtUSP was systematically annotated.

In brief, three enzymes were used in the one-pot system to synthesize UDP-monosaccharides including UDP-Gal, UDP-Glc, UDP-6-deoxy-Gal and UDP-4-N_3-Gal. As shown in Figure 1, the first enzyme was a galactokinase cloned from *S. pneumoniae* TIGR4 (SpGalK)[23] to phosphorylate the monosaccharide at C1 position. The second one was a promiscuous USP cloned from *A. thaliana* which catalyzed the reversible formation of UDP-monosaccharide and pyrophosphate from UTP and monosaccharide-1-phosphate. The third one was a commercially available inorganic pyrophosphatase from *Saccharomyces cerevisiae* (PPase) for the hydrolysis of pyrophosphate formed by AtUSP to drive the reaction towards the formation of UDP-monosaccharides.

To achieve a more efficient reaction for UDP-monosaccharide preparation, the optimal reaction conditions were investigated in our research including the optimal pH and temperature, as well as the concentration of Mg^{2+}. AtUSP was firstly overexpressed and purified. As shown in Fig. S1, the recombinant AtUSP had an apparent molecular weight of 70 kDa. The soluble AtUSP protein expression level was about 30 mg/L. The optimal reaction conditions were explored according to the supporting information. At last, the one-pot system has a highest reaction conversion at 45 °C, pH 8.0 and 10 mM $MgCl_2$ with ATP, UTP and Gal as substrates.

Based on the optimum conditions, eight monosaccharides listed in Table 1 were tested as initial substrates to synthesize UDP-monosaccharides using the one-pot three-enzyme system. Four UDP-monosaccharides were successfully synthesized from their corresponding monosaccharides including Gal, Glc, 6-deoxy-Gal and 4-N_3-Gal. As shown in Table 1, the one-pot three-enzyme system provided considerable reaction conversion for the formation of UDP-Gal (**1**, 95%) and UDP-4-N_3-Gal (**5**, 43%). AtUSP showed high activity to Glc-1-P, but we got just 28% conversion for the UDP-Glc (**2**) in our research. This may be attributed to the less SpGalK kinase activity for Glc.[23] Besides, AtUSP was reported to have no activity toward L-Fuc (L-6-deoxy-Gal) by Yoichi,[15] however, we synthesize UDP-D-6-deoxy-Gal (**4**, 29%) by our one-pot system from D-6-deoxy-Gal. This suggests that AtUSP could utilize D-6-deoxy-Gal-1-P. Less than 5% UDP-2-N_3-Gal (**6**) was synthesized in the system to the extent that it can be only detected by MS. If substituting SpGalU (a UTP-glucose-1-phosphate uridylyltransferase from *S. pneumoniae* TIGR4) for AtUSP in the one-pot system, the reaction conversion for UDP-Gal and UDP-Glc is almost the same in the AtUSP system or SpGalU system.[24] But great difference was displayed in the synthesis of **3**, **4**, **5**, **6**, and **7**. For **3**, **4**, **6** and **7**, the SpGalU system got a higher conversion than the AtUSP system. On the contrary, the AtUSP system showed higher yield for **5** than SpGalU system. Considering the structural diversity of the tested monosaccharides, the AtUSP may have better tolerance for C4-derivatives of Gal-1-P (**5**) while the SpGalU for C2- and C6-derivatives of Gal-1-P (**3, 4, 6** and **7**).

To investigate the uridylyltransferase activity of AtUSP on different nucleotide substrates (NTPs) or deoxynucleotide triphosphates (dNTPs), Glc-1-P and nine NTPs or dNTPs were tested as initial substrates (Fig. 2). The 20 μL reaction mixture containing 50 mM Tirs–HCl, pH 8.0, 10 mM Glc-1-P, 10 mM $MgCl_2$, 0.4 μM AtUSP and 10 mM different NTPs or dNTPs (Table 2) was incubated at 45 °C for 1 h. The reactions were finally detected by thin-layer chromatography (TLC, Fig. S6) and capillary electrophoresis (CE). Both dUDP-Gal and dTDP-Glc were synthesized in large scale and purified by Bio-Gel P2 gel filtration chromatography. Identification of the purified dUDP-Gal and dTDP-Glc was based on mass spectrometry (Fig. S7) and NMR spectrometry (Fig. S8). As shown in Table 2, AtUSP presented considerable yields toward UTP, dUTP and dTTP with a conversion of 87%, 85% and 84%, respectively. Surprisingly, among the nine NTPs or dNTPs tested, the UDP-*N*-acetylgalactosamine pyrophosphorylase from *Homo sapiens* (AGX1) could also recognize only UTP, dUTP and dTTP.[25] Amino acid sequence alignment showed that the identity of the two pyrophosphorylase was less than 26%. But BLAST analysis of each pyrophosphorylase suggested that both enzymes belong to the Glyco_tansf_GTA_type superfamily and had the same conserved domains. According to previous reports,[25–28] the hydrogen bonds constructed between the exocyclic oxygen O2 and O4 of the uracil base and Gly110, Gly222 and Gln196 of the AGX1 are crucial for nucleotide-binding and enzyme catalysis. To understand the molecular-level interactions at the binding site of the AtUSP substrates, the primary sequence of AtUSP was blast with other pyrophosphorylase. It appears that three amino acid residues (Gly137, Gln224 and Gly253) in AtUSP interact with nucleotides and are well conserved. The three amino acid residues may act with the uracil base to form hydrogen bonds.

Kinetic parameters of AtUSP toward UTP, dUTP and dTTP were determined with Glc-1-P as the sugar donor. Although there was

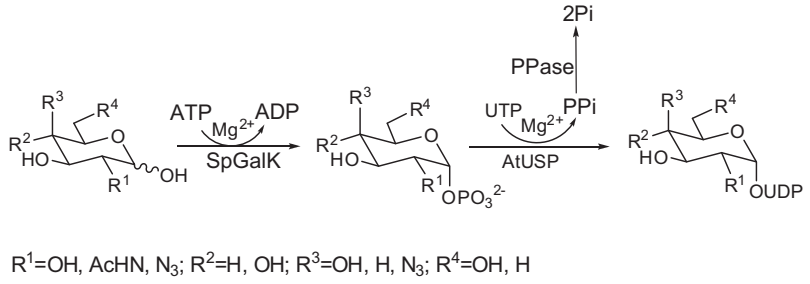
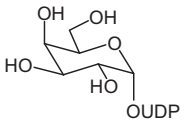
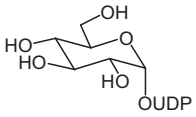
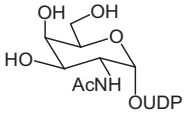
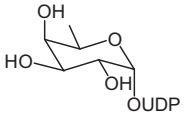
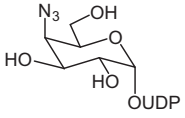
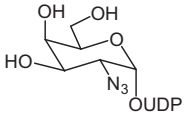
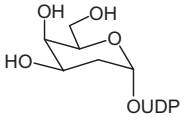
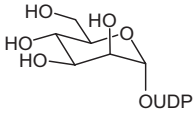


Figure 1. One-pot three enzymes synthesis of UDP-sugars. SpGalK, *S. pneumoniae* TIGR4 galactokinase; AtUSP, *A. thaliana* UDP-sugar pyrophosphorylase; PPase, inorganic pyrophosphatase.

Table 1
Synthesis of UDP-sugars using the one-pot three enzymes system shown in Figure 1

Entry		Producs	Reaction conversion[a] (%)	
			AtUSP	SpGalU
1	UDP-Gal		95	90
2	UDP-Glc		28	26
3	UDP-GalNAc		ND	32
4	UDP-6-deoxy-Gal (UDP-Fuc)		29	68
5	UDP-4-N_3-Gal		43	<5[b]
6	UDP-2-N_3-Gal		<5[b]	78
7	UDP-2-Deo-Gal		ND[c]	15
8	UDP-Man		ND	NT[d]

[a] The ratio of products to monosaccharide.
[b] Only detected by MS.
[c] No products detected by TLC and CE.
[d] Not test.

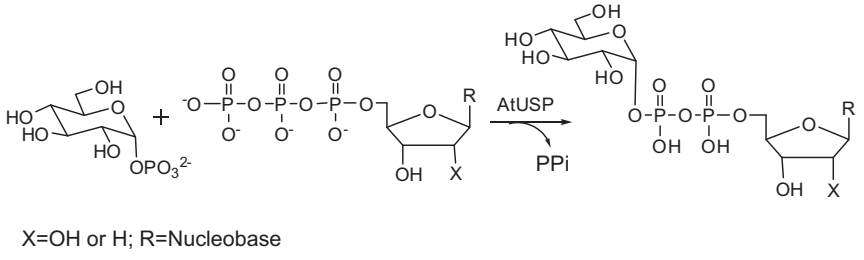
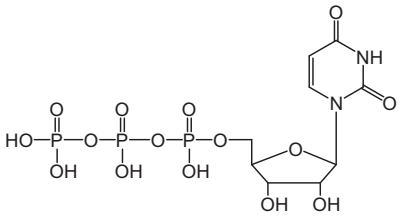
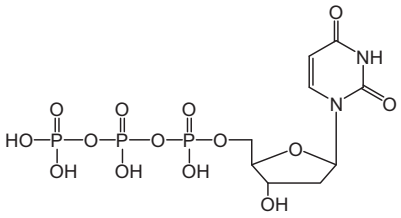
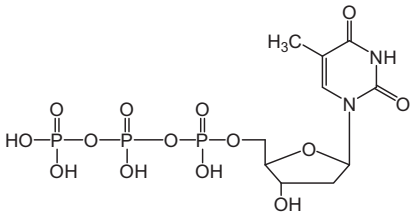
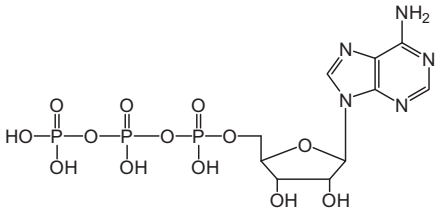
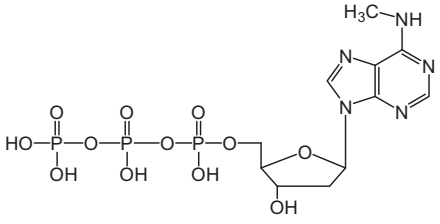
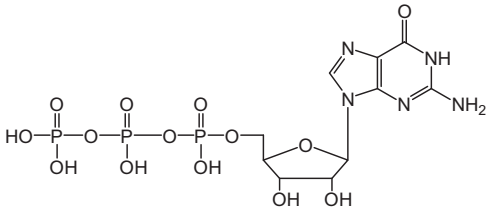
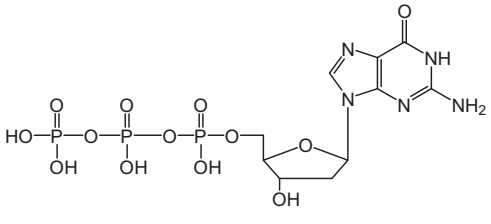


Figure 2. The uridylyltransferase reaction of AtUSP towards NTPs or dNTPs. NTPs, nucleotide triphosphates; dNTPs, deoxynucleotide triphosphates.

little difference in the conversion percentage between the three NTPs or dNTPs, kinetic parameters for them differed greatly. As shown in Table 3, the V_{max} for UTP was the greatest of the three NTPs or dNTPs. It was 1.5 times and 8 times of that for dUTP and dTTP, respectively. But the K_m for UTP was threefold lower than for dTTP. The k_{cat}/K_m for UTP, dUTP and dTTP were 120.54 mM^{-1} s^{-1}, 58.65 mM^{-1} s^{-1} and 3.34 mM^{-1} s^{-1}, respectively.

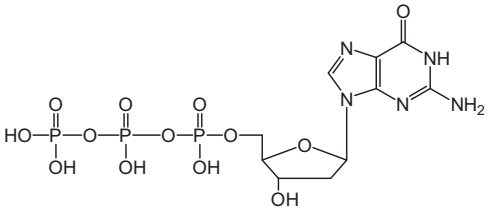
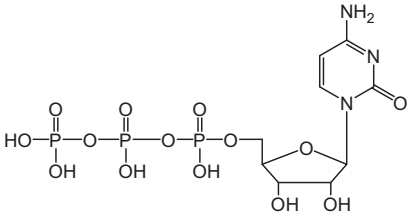
UDP-sugars are the direct substrates of Leloir-type glycosyltransferase to synthesize oligosaccharides involved in adhesion and signaling molecules. The glycan structures on these molecules

Table 2
NTPs or dNTPs substrates specificity study for AtSUP

Entry	Name	Structure	Conversion[a] (%)
11	UTP		87
12	dUTP		85
13	dTTP		84
14	ATP		ND[b]
15	dm^6ATP		ND
16	GTP		ND
17	dGTP		ND

(*continued on next page*)

Table 2 (*continued*)

Entry	Name	Structure	Conversion[a] (%)
18	CTP		ND
19	dCTP		ND

[a] The ratio of products to NTPs or dNTPs.
[b] Not detected by TLC and CE.

Table 3
Kinetic parameters of purified AtUSP towards UTP, dUTP and dTTP

Substrate	V_{max} (mM min^{-1})	K_m (mM)	k_{cat} (S^{-1})	k_{cat}/K_m (mM^{-1} S^{-1})
UTP	8.93	2.88	348.51	120.84
dUTP	5.62	3.74	219.36	58.65
dTTP	1.16	8.14	27.23	3.34

are essential mediators in processes such as protein folding, cell signaling, fertilization, embryogenesis, neuronal development, hormone activity, and the proliferation of cells and their organization into specific tissues.[29] AtUSP is a crucial enzyme in the salvage pathway. Taking advantage of the substrate promiscuity of AtUSP, we have efficiently synthesized UDP-Gal, UDP-Glc, UDP-D-6-deoxy-Gal and UDP-4-N_3-Gal. These UDP-sugars, especially the non-natural UDP-sugars, are potential donor substrates for various glycosyltransferases. And the structure variety of glycoconjugates they synthesized would generate interesting effects on many biological processes. We also illustrated that AtUSP has a finite NTPs or dNTPs substrate tolerance with Glc-1-P as the sugar donor and successfully synthesize dUDP-Gal and dTDP-Glc in mg scale. Amino acid consequence analysis of AtUSP shows that three amino acid residues were conserved compared to other pyrophosphorylases. However, to provide insight into the catalytic mechanism and the molecular basis for substrate selectivity, structural data for the enzyme–substrate complex would be a major issue to be addressed in future work.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.04.090.

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