Characterization and expression of Arabidopsis UDP-sugar pyrophosphorylase

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Received 28 November 2005
Available online 17 May 2006

Abstract

At5g52560, a homolog of pea (Pisum sativum) UDP-sugar pyrophosphorylase (PsUSP) was functionally annotated by expression in Escherichia coli and subsequent characterization of substrate specificity and kinetic properties. Arabidopsis contains a single USP gene (AtUSP) and evaluation of gene databases suggests that USP is unique to plants. The 69 kDa AtUSP gene product exhibited high activity with Glc-1-P, GlcA-1-P and Gal-1-P, but low activity with GlcNAc-1-P, Fuc-1-P, Man-1-P, inositol-1-P or Glc-6-P. AtUSP was activated by magnesium and preferred UTP as co-substrate. Apparent \(K_m\) values for GlcA-1-P, Glc-1-P and UTP were 0.13 mM, 0.42 mM and 0.14 mM, respectively. In the reverse direction (pyrophosphorolysis), the apparent \(K_m\) values for UDP-GlcA, UDP-Glc and pyrophosphate were 0.56 mM, 0.72 mM and 0.15 mM, respectively. USP enzyme activity (UDP-GlcA \(\rightarrow\) GlcA-1-P) was detected in Arabidopsis tissues with highest activity found in the inflorescence. As determined by semi-quantitative RT-PCR, AtUSP transcript is widely expressed with high levels detected in the inflorescence. To evaluate tissue-specific expression of AtUSP, histochemical GUS staining of plants transformed with AtUSPprom:GUS constructs was performed. In 7-day-old seedlings, GUS staining was detected in cotyledons, trichomes and vascular tissues of the primary root. In the inflorescence of older plants, high levels of GUS staining were detected in cauline leaves, the epidermis of the stem and in pollen. In silico analysis of AtUSP expression in developing pollen indicates that transcript levels increase as development proceeds from the uninucleate to the tricellular stage. The results suggest that AtUSP plays an important role in pollen development in Arabidopsis.

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Keywords: Arabidopsis thaliana; Cell wall; Pyrophosphorylase; UDP-glucuronic acid; UDP-glucose; UDP-sugar pyrophosphorylase

1. Introduction

Plant cell walls consist of cellulose, matrix polysaccharides (pectin and hemicellulose), phenolics and glycoproteins [3,8]. Matrix polysaccharides make up more than 60% of the primary cell wall of Arabidopsis leaves [36]. The amount and composition of cell wall polysaccharides influence the quality of food and fiber produced by crop plants. For example, the pectin content of forage cell walls is important in determining nutritional value for ruminants [29]. The tools of molecular biology can be used to develop transgenic plants that exhibit desirable modifications in the amount and composition of cell wall matrix polysaccharides. However, progress in this area is limited by lack of understanding of key enzymes regulating pectin and hemicellulose biosynthesis.

UDP-GlcA is a major precursor for nucleotide sugars (UDP-GalA, UDP-Xyl, UDP-Ara) that are incorporated into pectin and hemicellulose [12]. Despite its importance in matrix polysaccharide biosynthesis, the regulation of UDP-GlcA synthesis is poorly understood. As seen in Fig. 1, UDP-GlcA can be synthesized by two distinct pathways in plants: (1) the nucleotide sugar oxidation (NSO) pathway where UDP-Glc dehydrogenase catalyzes the oxidation of UDP-Glc, or (2) the myo-inositol oxidation (MIO) pathway which involves reactions cat-

Abbreviations: Ara, arabinose; Fuc, fucose; Gal, galactose; Glc, glucose; GlcA, glucuronic acid; GlcNAc, N-acetylglucosamine; GUS, β-glucuronidase; Man, mannose; PPI, pyrophosphate; UDP, uridine diphosphate; USP, UDP-sugar pyrophosphorylase; Xyl, xylene.

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Recently, Kotake et al. [22] purified and characterized a broad substrate UDP-sugar pyrophosphorylase (USP) from pea. A comparison of various monosaccharide-1-P substrates indicated that the highest activity occurred with Glc-1-P, GlcA-1-P, Gal-1-P and Ara-1-P. The recombinant PsUSP was also characterized and found to have the same properties as the purified enzyme. Kotake et al. [22] proposed that PsUSP plays an important role in the salvage pathway for the synthesis of nucleotide sugars. In this pathway, monosaccharides released during degradation of glycoconjugates and polysaccharides are recycled back into the UDP-sugar pool. Homologs of PsUSP were identified in rice (Oryza sativa L.) (AK064009) and Arabidopsis (At5g52560) [22].

The objectives of this study were to: (1) functionally annotate the putative USP from Arabidopsis (AtUSP), including characterization of kinetic parameters with substrates, and (2) investigate its expression in various tissues of Arabidopsis. Recombinant AtUSP exhibits similarities, but also significant differences compared to recombinant PsUSP [22]. Both transcripts and enzyme activity of AtUSP are widely expressed in Arabidopsis. Histochemical GUS staining of inflorescences of plants transformed with a AtUSPprom:GUS construct indicated high expression of AtUSP in pollen. The possibility that AtUSP serves as UDP-GlcA pyrophosphorylase, the terminal enzyme in the MIO pathway, is discussed.

2. Results

2.1. Functional annotation of AtUSP

Kotake et al. [22] identified At5g52560 as a homolog of the broad substrate UDP-sugar pyrophosphorylase (PsUSP) characterized in pea. PsUSP and the putative Arabidopsis UDP-sugar pyrophosphorylase, AtUSP (At5g52560), share 80% identity (nucleotide) and the predicted proteins share 77% identity and 88% similarity [22]. AtUSP has an open reading frame of 1842 bp that encodes a predicted protein of 614 amino acids. Sequence analysis using TargetP [11] predicted that AtUSP is a cytosolic protein because an identifiable target sequence was not found.

To functionally annotate At5g52560, the coding region was cloned by RT-PCR, inserted into pET28, expressed in Escherichia coli and purified. His-tag affinity purification of putative AtUSP yielded a single peptide that migrated at approximately 70 kDa on SDS-PAGE (Fig. 2, lane 1). The N-terminal 17 amino acids which included the poly-His motif were cleaved from the fusion peptide using thrombin. The size of the thrombin-cleaved, purified putative AtUSP was 69 kDa, approximately 70 kDa on SDS-PAGE (Fig. 2, lane 1). The N-terminal 17 amino acids which included the poly-His motif were cleaved from the fusion peptide using thrombin. The size of the thrombin-cleaved, purified putative AtUSP was 69 kDa. The recombinant PsUSP was also characterized and found to have the same properties as the purified enzyme. Kotake et al. [22] proposed that PsUSP plays an important role in the salvage pathway for the synthesis of nucleotide sugars. In this pathway, monosaccharides released during degradation of glycoconjugates and polysaccharides are recycled back into the UDP-sugar pool. Homologs of PsUSP were identified in rice (Oryza sativa L.) (AK064009) and Arabidopsis (At5g52560) [22].

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TAPS (pH 8.0), 1 mM MgCl$_2$ and 1 mM DTT stabilized the enzyme for several days at 4 °C.

Putative AtUSP was assayed in the forward direction with various sugar phosphates (Table 1). High levels of activity were measured with Glc-1-P, GlcA-1-P or Gal-1-P as substrates. In contrast, GlcNAc-1-P, Fuc-1-P, Man-1-P, inositol-1-P and Glc-6-P exhibited very low activity. Currently, the AtUSP mRNA sequences available through GenBank are annotated as an unknown protein (AF360236, AY040035) or as a gene with sequence similarities with the pea PsUSP (NM124635). The results presented here functionally annotate AtUSP as a broad substrate UDP-sugar pyrophosphorylase and confirm that AtUSP is a homolog of PsUSP.

Activity with the pentose sugars Xyl-1-P and Ara-1-P was not examined because they are not commercially available. Instead, we examined the ability of UDP-Xyl, UDP-Ara and UDP-Glc to serve as competitive inhibitors of the pyrophosphorylase of UDP-GlcA (Table 2). Addition of 1 mM UDP-Xyl to a standard reaction mixture containing 1 mM UDP-GlcA reduced UDP-GlcA pyrophosphorylase activity by 5%, addition of 1 mM UDP-Ara reduced activity by 13%, and addition of 1 mM UDP-Glc reduced activity by 23%. If two sugars are equally effective as substrates, each would potentially reduce activity for the other by 50% in this assay. We also examined whether 1 mM UDP-Xyl, UDP-Ara or UDP-GlcA were competitive inhibitors of the pyrophosphorylase of UDP-Glc. Addition of 1 mM UDP-Xyl to a standard reaction mixture containing 1 mM UDP-Glc reduced UDP-Glc pyrophosphorylase activity by 17%, addition of UDP-Ara reduced activity by 22%, and addition of UDP-GlcA reduced activity by 75%. From this data, we can conclude that UDP-GlcA is a more competitive inhibitor of UDP-Glc pyrophosphorylase activity than UDP-Glc is of UDP-GlcA pyrophosphorylase activity. Our results also show that AtUSP exhibits lower affinity for UDP-Ara and UDP-Xyl compared with UDP-GlcA or UDP-Glc, and that affinity is higher for UDP-Ara than for UDP-Xyl. Based on the substrates examined, our results indicate that the preferred substrates of AtUSP are GlcA-1-P (forward reaction) and UDP-GlcA (reverse reaction).

A comparison of nucleotide triphosphate preference indicated that UDP-GlcA pyrophosphorylase activity was specific for UTP (Table 3). Activity in the presence of other nucleotide triphosphates (ATP, CTP or GTP) was less than 10% of that measured in the presence of UTP. Similar to other pyrophosphorylases, AtUSP activity required divalent cations. No activity was detected when 20 mM EDTA was added to the assay. When assayed at 2 mM, magnesium and manganese resulted in equivalent activation; no activity was detected in the presence of calcium (2 mM) (data not shown). Effects of temperature on enzyme activity were evaluated over the range of 15-55 °C. Maximum activity was obtained at 45 °C, with a rapid drop in activity at higher temperatures (data not shown).

Enzymatic activity for the forward reaction (UDP-GlcA formation) was measured using [14C]Glc-1-P or [14C]GlcA-1-P as substrate. Values are means ± SE (N = 3).

<table>
<thead>
<tr>
<th>Added competing substrate</th>
<th>UDP-GlcA pyrophosphorylase activity</th>
<th>%</th>
<th>UDP-Glc pyrophosphorylase activity</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>52.4 ± 2.5</td>
<td>100</td>
<td>57.8 ± 1.9</td>
<td>100</td>
</tr>
<tr>
<td>UDP-Glc</td>
<td>40.4 ± 2.0</td>
<td>77</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>UDP-GlcA</td>
<td>–</td>
<td>–</td>
<td>14.6 ± 3.5</td>
<td>25</td>
</tr>
<tr>
<td>UDP-Xyl</td>
<td>49.6 ± 0.8</td>
<td>95</td>
<td>47.8 ± 3.4</td>
<td>83</td>
</tr>
<tr>
<td>UDP-Ara</td>
<td>45.6 ± 4.2</td>
<td>87</td>
<td>45.2 ± 0.7</td>
<td>78</td>
</tr>
</tbody>
</table>

a Activity was determined in the reverse direction by measuring [14C]Glc-1-P or [14C]GlcA-1-P production in a reaction containing 5 mM NaPPI, 2 mM MgCl$_2$, 1 mM UDP-[14C]Glc or UDP-[14C]GlcA plus 1 mM UDP-sugar (when added). Values are means ± SE (N = 3).
Table 3
Relative activity of AtUSP with various nucleotide triphosphates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>UTP</td>
<td>100</td>
</tr>
<tr>
<td>ATP</td>
<td>5.3 ± 2.1</td>
</tr>
<tr>
<td>CTP</td>
<td>5.1 ± 2.0</td>
</tr>
<tr>
<td>GTP</td>
<td>8.8 ± 2.6</td>
</tr>
</tbody>
</table>

Specific activity was determined in the forward direction by detection of PPi produced in the presence of 1 mM GlcA-1-P, 2 mM MgCl₂ and 1 mM of the indicated nucleotide. Values are means ± SE (N = 3).

Kinetic parameters of AtUSP were determined in both the forward and reverse directions for GlcA-1-P and Glc-1-P (Table 4). Although \( V_{\text{max}} \) for the forward reaction was greater with Glc-1-P compared to GlcA-1-P, the \( K_m \) for GlcA-1-P was three-fold lower than for Glc-1-P. In contrast, the affinity for UTP was only slightly greater with GlcA-1-P as substrate compared to Glc-1-P. The \( k_{\text{cat}}/K_m \) values for GlcA-1-P and Glc-1-P were \( 3, 1 \times 10^5 \, \text{s}^{-1} \, \text{M}^{-1} \) and \( 1.6 \times 10^5 \, \text{s}^{-1} \, \text{M}^{-1} \), respectively, indicating that the catalytic efficiency of USP was greater with GlcA-1-P as substrate compared to Glc-1-P. In the reverse direction, the \( K_m \) for UDP-GlcA was 0.56 mM while the \( K_m \) for UDP-Glc was 0.72 mM. These results are in agreement with the substrate competition experiments (Table 2) that indicated higher affinity for UDP-GlcA compared to UDP-Glc.

2.2. USP Homologs

AtUSP appears to be a single gene in Arabidopsis as no obvious homologs are present in the genome. A search of both genomic and expressed sequences indicated that more than 20 plant species have putative AtUSP homologs that are 70 to 80% identical and 80 to 95% similar. Although USP has been identified in both dicots and monocots, we found no obvious AtUSP homologs in fungi, bacteria, archaea or metazoa.

AtUSP is only moderately similar to other members of the Arabidopsis pyrophosphorylase family. The most closely related Arabidopsis genes are the two putative UDP-GlcNAc pyrophosphorylases (At1g31070, At2g35020) with 42% similar and 26% identical amino acids. The region of homology with UDP-GlcNAc pyrophosphorylases covers the N-terminal 355 residues of both peptides. AtUSP is less closely related to the two putative UDP-Glc pyrophosphorylases (At3g03250, At5g17310) with 35% similar and 23% identical amino acids, respectively. Homology with the Arabidopsis UDP-Glc pyrophosphorylases begins approximately 50 residues downstream of the N-terminus of At5g2560 and extends to aa 403. The C-terminal third of AtUSP has no similarity to any annotated pyrophosphorylase in GenBank but contains a 64 aa motif shared with microtubule-associated protein MAP65-1a (GenBank NP_567756).

2.3. USP enzyme activity and transcript levels

USP enzyme activity was measured in the reverse direction (UDP-GlcA → GlcA-1-P) in crude extracts of Arabidopsis seedlings, the rosettes of 2 to 5-wk-old plants and the inflorescences of 35-day-old plants (Table 5). USP activity was present in extracts of all tissues tested, but highest activity was detected in the inflorescence. In these same tissues, AtUSP transcript levels were quantified by semi-quantitative RT-PCR. AtUSP transcripts were present in all tissues tested and were most abundant in rosettes of 21-day-old plants and the inflorescences of 35-day-old plants (Table 5).

2.4. AtUSP promoter activity and in silico analysis

To determine tissue-specific activity of the USP promoter, we amplified 2 kb of sequence upstream of the start codon in AtUSP and cloned it into pBI101.1, a promoterless-GUS construct. Two independent transformants were used for the analysis.

Table 4
Kinetic parameters for forward and reverse reactions of AtUSP for UDP-GlcA and UDP-Glc pyrophosphorylase activity

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Substrate A</th>
<th>Substrate B</th>
<th>( V_{\text{max}} ) , nmol min(^{-1})</th>
<th>( K_m ) , mM</th>
<th>( K_B ) , mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward</td>
<td>GlcA-1-P</td>
<td>UTP</td>
<td>3.47 ± 0.18</td>
<td>0.13 ± 0.02</td>
<td>0.14 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>Glc-1-P</td>
<td>UTP</td>
<td>5.76 ± 0.38</td>
<td>0.42 ± 0.05</td>
<td>0.19 ± 0.02</td>
</tr>
<tr>
<td>Reverse</td>
<td>UDP-GlcA</td>
<td>PPi</td>
<td>4.25 ± 0.17</td>
<td>0.56 ± 0.12</td>
<td>0.15 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>UDP-Glc</td>
<td>PPi</td>
<td>4.95 ± 0.53</td>
<td>0.72 ± 0.17</td>
<td>0.16 ± 0.07</td>
</tr>
</tbody>
</table>

*\( a \) Kinetic parameters for AtUSP in the forward direction (formation of UDP-sugars) were measured at five concentrations of each substrate and the Michaelis constants were calculated by non-linear regression. Rates in the reverse direction were determined with saturating conditions for one substrate (1 mM PPi, 2 mM UDP-GlcA, or 2 mM UDP-Glc). The data was fitted to the Michaelis-Menten equation using non-linear regression. Values for both the forward and reverse reactions are means ± SE (N = 3).

*\( b \) \( K_m \) value for substrate A.

*\( c \) \( K_B \) value for substrate B.
lysis of promoter activity. Histochemical GUS staining of segregating progeny from the two individuals revealed that in 7-day-old seedlings, GUS activity was present in cotyledons, trichomes of primary leaves and the vascular tissues within the basal portion of the root (Fig. 3A–D). Rosettes at 14 days exhibited GUS activity primarily in the trichomes of leaves and the vascular system of the petiole (Fig. 3E). Staining in rosettes of flowering plants was similar to that observed in 14-day-old plants (data not shown).

GUS expression was also observed in young stems of the inflorescence (Fig. 4A). As revealed in cross-section, GUS activity in stems was most intense in the epidermis with fainter staining also observed in xylem and phloem (Fig. 4B). The most intense GUS staining of the inflorescence occurred in cauline leaves, sepals, and anthers (Fig. 4A, C). In stamens, staining was limited to individual pollen grains (Fig. 4C, inset). The staining pattern that was observed is consistent with in silico expression data obtained through Genevestigator [37].

Fig. 5 summarizes the pattern of AtUSP transcript levels found in various tissues. AtUSP transcript is present in every tissue tested, with the highest signal intensity observed in stem and stamen (Fig. 5). Because histochemical GUS staining showed intense staining of pollen within the anther (Fig. 4C, inset), we analyzed in silico transcript levels within developing pollen. AtUSP transcript levels increased during pollen development, peaking at the tricellular stage (Fig. 6).

3. Discussion

By functional annotation of AtUSP (At5g52560), we confirmed that the gene is a homolog of PsUSP [22]. Both genes encode broad substrate pyrophosphorylases that are similar in many respects. Both AtUSP and PsUSP exhibit: (1) high activity with GlcA-1-P, Glc-1-P and Gal-1-P, but little or no activity with GlcNAc-1-P, Man-1-P, Fuc-1-P, or Glc-6-P, (2) a temperature optimum of approximately 45 °C, and (3) similar \( K_m \) values for Glc-1-P. However, there are significant differences between the two pyrophosphorylases. The relative in vitro activities of AtUSP are Glc-1-P > GlcA-1-P > Gal-1-P (Table 1). In contrast, the relative activities for PsUSP are Gal-1-P > GlcA-1-P > Glc-1-P > GlcA-1-P [22]. Other differences between AtUSP and PsUSP are the \( K_m \) values for GlcA-1-P and UTP. The \( K_m \) value for GlcA-1-P is significantly lower for AtUSP (0.13 mM) compared to PsUSP (0.48 mM). For UTP, the \( K_m \) values are...
0.14 mM and 0.048 mM for AtUSP and PsUSP, respectively. The pH optimum for AtUSP and PsUSP also differ. PsUSP exhibits maximum activity between pH 6.5–7.5 while AtUSP exhibits maximum activity between pH 7.5–8.5 which is similar to plant UDP-Glc pyrophosphorylase [24,31].

The function of USP in plants is not clearly defined. On the basis of its broad substrate specificity, Kotake et al. [22] proposed that PsUSP plays a key role in the salvage pathway for synthesis of nucleotide sugars. The broad specificity of Arabidopsis USP for monosaccharide-1-P sugars (Table 1) and its wide expression in various tissues (Table 5, Figs. 3 and 4) are consistent with this role. In the salvage pathway, monosaccharides released during hydrolytic reactions involving polysaccharides and other glycoconjugates (glycoproteins, glycolipids) are converted to nucleotide sugars by a two-step process involving: (1) C-1 sugar kinase and (2) nucleotide diphosphate (NDP) sugar pyrophosphorylase [12]. Although the reaction catalyzed by NDP-pyrophosphorylase is reversible, it is believed to function in the forward direction (NDP-sugar formation) because of the high levels of pyrophosphatase found in plant cells [12]. There is evidence that the salvage pathway plays a role in recycling monosaccharides released from polysaccharides during cell wall synthesis and turnover [8,14,15]. Pulse-chase studies of cell wall polysaccharides indicate turnover of Ara and Gal [14–16].

Our results, based on UDP-sugar competition studies for the reverse reaction (pyrophosphorolysis) (Table 2), show that AtUSP exhibited lower affinity for UDP-Xyl compared to UDP-GlcA or UDP-Glc. For the forward reaction, PsUSP exhibited low activity and affinity for Xyl-1-P compared to GlcA-1-P or Glc-1-P [22]. Kotake et al. [22] proposed that USP may play a role in recycling Xyl via the salvage pathway. However, evidence in the literature suggests that this may not occur because xylose kinase is not found in plants [7,8,15]. Instead, Xyl is isomerized to xylulose and its carbons recycled via the pentose phosphate pathway [7,8,12].
In addition to its role in the salvage pathway for synthesis of nucleotide sugars, it is also possible that USP serves as UDP-GlcA pyrophosphorylase (EC 2.7.7.44), the terminal enzyme of the MIO pathway (Fig. 1). The gene for UDP-GlcA pyrophosphorylase has not been identified. The high activity and affinity of AtUSP for GlcA-1-P is consistent with this role. Based on determination of $K_m$ values for GlcA-1-P and Glc-1-P, and competition studies comparing the effects of UDP-sugars on the reverse reaction (pyrophosphorolysis), it appears that GlcA-1-P/UDP-GlcA are the preferred substrates for the forward and reverse reactions of AtUSP, respectively.

It is not clear whether UDP-GlcA pyrophosphorylase activity characterized in earlier research [10,13,18,27,28,35] with semi-purified fractions represents the activity of USP which exhibits broad substrate specificity, a substrate specific UDP-GlcA pyrophosphorylase (EC 2.7.7.44), or a combination of both enzymes. In these earlier studies, the enzyme was not purified and substrate specificity was not thoroughly examined. The most purified fractions exhibiting UDP-GlcA pyrophosphorylase activity were obtained from barley seedlings (80-fold purification) [27] and common cattail pollen (600-fold purification) [18]. The barley UDP-GlcA pyrophosphorylase activity characterized by Roberts [27] cannot be attributed to USP because the enzyme had an estimated molecular wt of 35 kDa and did not cross-react with UDP-Glc. However, the UDP-GlcA pyrophosphorylase characterized from common cattail pollen exhibited properties similar to AtUSP [18]. The common cattail enzyme has a molecular wt of approximately 71 kDa and an apparent $K_m$ value for GlcA-1-P that is in close agreement with values observed for AtUSP.

The expression of USP has not been previously characterized. Our results indicate that USP enzyme activity (UDP-GlcA $\rightarrow$ GlcA-1-P) and transcript are widely expressed throughout development in Arabidopsis tissues. Both enzyme activity and transcript levels showed high expression in the inflorescence. Although high levels of USP transcript and enzyme activity were measured in the inflorescence, we cannot be certain that the enzyme activity measured solely represents the activity of the USP gene product. It is possible that the extracts in which enzyme activity was measured contain other pyrophosphorylases capable of catalyzing the conversion of UDP-GlcA to GlcA-1-P.

Histochemical analysis of plants transformed with AtUSP-prom:GUS demonstrated high expression in specific tissues, including epidermis, trichomes, cotyledons and vascular tissues. High levels of GUS expression were also detected in pollen of lily [10,28]. Other evidence that the MIO pathway may be active in pollen is the finding that one of the myo-inositol oxidase genes (MIOX 4) is highly expressed in Arabidopsis pollen [20]. These authors concluded that the MIO pathway may play a major role in the biosynthesis of UDP-GlcA in Arabidopsis pollen. Further research will be needed to define the role of USP in the salvage pathway for nucleotide sugar synthesis and its possible contribution to de novo synthesis of UDP-GlcA via the MIO pathway. The availability of Arabidopsis knock-out lines for USP and the fact that Arabidopsis contains a single USP gene will allow for research to better define the role of AtUSP in pollen and other plant tissues.

4. Materials and methods

4.1. Chemicals

Radiolabeled substrates, $[^{14}C]$UDP-GlcA (1.16 × 10$^{10}$ Bq mmol$^{-1}$) and $[^{14}C]$UDP-Glc (1.12 × 10$^{10}$ Bq mmol$^{-1}$) were purchased from MP Biomedicals (Irvine, CA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

4.2. Plant growth conditions

For experiments conducted with 7-day-old seedlings of Arabidopsis thaliana (ecotype Columbia), sterilized seed were grown on solidified medium [MS Salts, 1% (w/v) sucrose, 3.5 g l$^{-1}$ Phytagel, pH 5.7]. For all other experiments, Arabidopsis (ecotype Columbia or transgenic plants) was grown on Sunshine Mix LP5 (SunGro Horticulture, Bellevue, WA). Plants were grown under a 24 h photoperiod at 21 ± 2 °C.

4.3. Cloning, expression and purification of AtUSP

The coding region corresponding to the At5g52560 locus was cloned using RT-PCR. Gene specific primers were selected from the 5′- and 3′-untranslated regions of expressed sequence contig TC211316 <http://www.tigr.org> (5′-TTGTTAAAGAGCCTTCTTCA-3′, 5′-AATGTCAAAAGA TACCTT-3′). Total RNA from whole plants was used as the template for cDNA synthesis with the 3′ gene-specific primer and AMV reverse transcriptase (Promega, Madison, WI). Nucleotides and enzymes were removed after each step by washing on spin columns (Qiagen, Valencia, CA). The coding
sequence was amplified from the cDNA using Fpx DNA polymerase (Invitrogen, Carlsbad, CA) which yielded a product of approximately 2.5 kb. This product was incubated with dATP and Taq polymerase to add terminal overhangs, then ligated into pGEM-T Easy (Promega).

The coding region of AtUSP was cloned into expression vector pET28b (Novagen, San Diego, CA) and transformed into E. coli strain BL21pLysS. Cultures were grown in LB medium at 37 °C to an optical density of 0.6, then induced by addition of 1 mM isopropyl-β-D-thiogalactoside. After induction, the cultures were incubated at 22 °C for 5 h. Cells were harvested by centrifugation (8,000g, 15 min) and resuspended in 1/20 culture volume of lysis buffer [0.4 M NaCl, 100 mM HEPES, 10 mM imidazole, 5 mM DTT (pH 7.5)] with 2 units ml⁻¹ DNase (Sigma-Aldrich), and then frozen at −20 °C. Uninduced cultures or control cultures with an empty vector gave no detectable UDP-GlcA pyrophosphorylase activity.

AtUSP was purified from the soluble cytoplasmic protein fraction by binding to His-Link affinity resin (Promega) according to the manufacturer’s instructions. AtUSP was eluted with a buffer containing 500 mM imidazole, 10 mM HEPES (pH 7.5) and 1 mM DTT. The fraction was then desalted on a PD-10 column (Amersham Biosciences, Piscataway, NJ) equilibrated with buffer [20% (w/v) sucrose, 50 mM TAPS (pH 8.0), 1 mM MgCl₂, 1 mM DTT]. At this point no other proteins could be detected by silver staining a SDS-PAGE gel. After purification, the poly-His tag was removed by thrombin cleavage for 16 h at 4 °C with 1 unit thrombin per mg AtUSP. Thrombin was removed by affinity chromatography according to the manufacturer’s instructions (Novagen). Protein concentrations were determined using the method of Bradford [6] with BSA as the standard.

4.4. USP assays and kinetic analyses

USP activity was measured in both the forward direction (conversion of GlcA-1-P to UDP-GlcA) and in the reverse direction (conversion of [¹⁴C]UDP-GlcA to [¹⁴C]GlcA-1-P). For the forward reaction involving the conversion of GlcA-1-P to UDP-GlcA, Pi formation was measured after hydrolysis of PPi by pyrophosphatase. The assay medium contained 10 mM TAPS (pH 8.0), 2 mM MgCl₂, 1.67 nkat ml⁻¹ inorganic pyrophosphatase, 1 mM UTP and 1 mM sugar-phosphate substrate. Inorganic pyrophosphatase activity was in excess of that needed to completely hydrolyze PPi produced in the assay. The reaction was started by addition of recombinant enzyme (100 ng) in a final reaction volume of 0.5 ml. After incubating 10 min at 30 °C, formation of Pi was detected by the method of Aoyama et al. [1]. An equal volume of 3% (w/v) ammonium molybdate and 0.1 volume of 1% (w/v) ascorbic acid, both made up in 200 mM Na acetate buffer (pH 4.0), was added. Color was allowed to develop for 30 min at room temperature and absorbance at 720 nm was measured. A standard curve prepared in assay buffer gave a linear reaction over the range of 0-250 µM Pi.

USP activity in the reverse direction was determined by measuring the formation of [¹⁴C]GlcA-1-P from UDP-[¹⁴C]GlcA and inorganic pyrophosphate using a modified protocol of Szumilo et al. [34]. The reaction mixtures contained the following components in a final volume of 50 µl: 100 mM TAPS (pH 8.0), 2 mM MgCl₂, 2 mM UDP-[¹⁴C]GlcA (1.85 × 10⁷ Bq mmol⁻¹), 5 mM sodium pyrophosphate and purified recombinant enzyme. After a 10 min incubation at 30 °C, the reactions were terminated by adding 0.5 ml 5% (v/v) TCA. Nucleotides were adsorbed on charcoal by adding 0.3 ml ENVI-Carb 120/400 (Sigma-Aldrich, St. Louis, MO) (150 mg ml⁻¹ in water). The suspension was shaken for 2 min and the charcoal pelleted by centrifugation (16,000g, 2 min). The supernatant was removed and saved, and the charcoal was washed with 1 ml water. The supernatants were combined and 1.0 ml was added to 15 ml EcoLume for scintillation counting. Blank controls were run simultaneously and contained all components with the exception of sodium pyrophosphate.

The formation of [¹⁴C]GlcA-1-P in the reverse assay was verified by separating the reaction products on cellulose TLC plates. The reverse assay was stopped by adding 2 µl 0.5 M EDTA, and 2 µl of the reaction mixture was applied to a Celullose 300 flexible-backed TLC plate (Selecto Scientific). The plate was developed with n-butanol:acetic acid:water (3:5:2:5:1.5:5:1) [26]. The standard lanes, containing 20 nmol of either UDP-GlcA or GlcA-1-P, were sprayed with an acidic molybdate spray (1% ammonium molybdate, 3% perchloric acid and 0.1N HCl) to detect phosphate esters [4,5]. The corresponding bands from the reaction lanes were cut out and placed in 7 ml scintillation vials with 0.2 ml water. After vortexing, 5 ml of EcoLume was added for scintillation counting.

The non-radioactive forward assay (GlcA-1-P → UDP-GlcA) described above was modified for determination of kinetic parameters. Inorganic pyrophosphatase was omitted from the assay medium. After the 10 min incubation at 30 °C with 100 ng of purified AtUSP, the reaction was stopped by boiling for 2.5 min. The samples were then placed on ice to cool. Inorganic pyrophosphatase [50 µl of 16.7 nkat ml⁻¹ in 50% (v/v) glycerol] was added and the samples were incubated for 10 min at 30 °C to cleave Pi. Phosphate was measured as described above. \( K_m \) and \( V_{max} \) for GlcA-1-P, Glc-1-P and UTP were determined by varying the concentration of UTP from 0.025 to 0.5 mM at each of five fixed concentrations of GlcA-1-P or Glc-1-P (0.1 to 2.0 mM). The data was fitted to an equation describing a two substrate ping pong mechanism using non-linear regression software (GrafFit 3.0, Erithacus Software). \( K_m \) and \( V_{max} \) for UDP-GlcA and UDP-Glc were determined by varying the concentration of UDP-sugar from 0.1 to 2 mM at a fixed concentration of NaPPi (1 mM). \( K_m \) and \( V_{max} \) for PiPPi were determined by varying the concentration of PiPPi from 0.1 to 1 mM at a fixed concentration of either UDP-GlcA or UDP-Glc (2 mM). The data was fitted to the Michaelis-Menten equation using non-linear regression software (GrafFit 3.0). The concentrations of fixed substrates were empirically determined to be saturating.
4.5. Determination of USP activity in plant extracts

Approximately 1 g fresh wt of tissue was ground with sand using a mortar and pestle in 5 ml extraction buffer [50 mM Tris-Cl (pH 8.0), 1 mM MgCl₂, 0.5 M KCl, 0.1% (v/v) Triton X-100, 0.07% (v/v) β-mercaptoethanol, 0.5 mM phenylmethanesulfonfyl fluoride] and then filtered through cheesecloth and Miracloth. Extracts were centrifuged at 12,000g for 10 min at 4 °C. After filtering through Miracloth, 2.5 ml of supernatant was loaded onto a PD-10 column (Amersham Pharmacia Biotech, Piscataway, NJ) pre-equilibrated in desalting buffer [50 mM Tris-Cl (pH 8.0), 1 mM MgCl₂, 10% (v/v) glycerol, 0.07% (v/v) β-mercaptoethanol]. Eluate was collected after 3.5 ml desalting buffer was added to the column. Desalted crude extracts (20 μl) were used in the USP reverse assay (UDP-[^14]C)GlcA → [^14]C GcA-1-P). Assay conditions were the same as described above except the reaction time was 30 min and the nucleotide binding step was modified. Reactions were stopped with 0.5 ml 5% (w/v) TCA and 0.3 ml ENVI-Carb 120/400 (150 mg ml⁻¹ in water) was added. After 2 min of mixing, the entire mixture was loaded onto a 2 ml, 0.2 μm nylon filter spin unit (Chrom Tech, Inc., AppleValley, MN). After a 1 min spin at 1000g, 0.5 ml was counted in 10 ml Ecolume.

4.6. RNA expression analysis

Total RNA was extracted from 100 to 150 mg of tissue using Trizol reagent (Invitrogen, Carlsbad, CA) according to manufacturer’s protocol and then treated with DNase at 37 °C for 30 min in a 200 μl reaction containing 5 units ml⁻¹ RNasin (Promega), 200 units ml⁻¹ DNase (Promega), 50 mM Tris (pH 7.8), 10 mM MgCl₂ and 1 mM DTT. The RNA was extracted from the DNase reaction with phenol:chloroform (1:1, v/v), then ethanol precipitated prior to use in the RT reaction. For each sample, reverse transcription was carried out in a 20 μl reaction using 1 μg of total RNA and Invitrogen Reverse Transcriptase (Promega) according to the manufacturer’s instructions. The reaction was then diluted with 20 μl of water to give a final volume of 40 μl of cDNA. Multiplex PCR reactions were conducted with 2 μl of the diluted cDNA in a volume of 20 μl. The reactions were carried out according to the QuantumRNA 18S Internal Standards Kit (Ambion, Austin, TX) using the Quantum 18S Universal standards (4:6 ratio of 18Sprimers:18Scompeitimers) to amplify a 315 bp fragment of 18S ribosomal DNA, and two primers (5′-GGAAAGCTTTTG GAGGAATTAG-3′ and 5′-GACACCGCCCTTTGGAAGAA-3′) designed to amplify a 450 bp fragment of AtUSP. The resulting PCR reactions were run on a 2% agarose gel, stained with ethidium bromide and the bands were quantified using the BioRad Doc 2000 gel documentation system with Quantity One (version 4.1.1) software from BioRad (Hercules, CA).

In silico expression analysis was performed at https://www.genevestigator.ethz.ch/ using Gene Atlas [37]. For the Arabidopsis organ profile, only wild-type (Col-0) data sets on ATH1: 22 k arrays were chosen. Developing pollen arrays were only available from RNA isolated from the Landsberg erecta ecotype. Data is current as of November, 2005.

4.7. Histochemical GUS analysis

To determine tissue-specific expression of AtUSP, 2.0 kb immediately upstream of the At5g52560 start codon was amplified with USPpromL (5′-GCTCTAGATTATCTTGGAAAGACGTCTGTTACCC-3′) and USPpromR (5′-GCTCTAGAAGAGGCTCTTTAACAAGGAAATG-3′) primers using wild-type (Col) genomic DNA with Expand High Fidelity PCR System (Roche Applied Sciences, Germany). The gel-purified PCR product (Qiagen) was digested with XhoI and ligated into a promoterless-GUS contract (pBI101.1) resulting in the transformation vector pJS1. Agrobacterium (GV3101) harboring pJS1 were used to transform wild-type (Col) Arabidopsis plants according to Clough and Bent [9]. Two independent transformation events were used in the analysis of promoter activity. Kanamycin-resistant seedlings were selected on Germination Medium (MS salts, 1% (w/v) sucrose, 3.5 g l⁻¹ Phytagel, 75 mg l⁻¹ kanamycin, pH 5.6) after 10 days. Histochemical GUS assays were performed as described [19]. Assay buffer was vacuum infiltrated into tissues and allowed to incubate at 37 °C overnight. Tissues were cleared in 70% ethanol.

Acknowledgments

We thank Ann Chaptman and Renee Schirmer for technical assistance.

References
