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Cloning of Glucuronokinase from *Arabidopsis thaliana*, the Last Missing Enzyme of the *myo*-Inositol Oxygenase Pathway to Nucleotide Sugars*

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Nucleotide sugars are building blocks for carbohydrate polymers in plant cell walls. They are synthesized from sugar-1-phosphates or epimerized as nucleotide sugars. The main precursor for primary cell walls is UDP-glucuronic acid, which can be synthesized via two independent pathways. One starts with the ring cleavage of *myo*-inositol into glucuronic acid, which requires a glucuronokinase and a pyrophosphorylase for activation into UDP-glucuronate. Here we report on the purification of glucuronokinase from *Lilium* pollen. A 40-kDa protein was purified combining six chromatographic steps and peptides were *de novo* sequenced. This allowed the cloning of the gene from *Arabidopsis thaliana* and the expression of the recombinant protein in *Escherichia coli* for biochemical characterization. Glucuronokinase is a novel member of the GHMP-kinase superfamily having an unique substrate specificity for D-glucuronic acid with a K_m of 0.7 mM. It requires ATP as phosphate donor (K_m 0.56 mM). In *Arabidopsis,* the gene is expressed in all plant tissues with a preference for pollen. Genes for glucuronokinase are present in (all) plants, some algae, and a few bacteria as well as in some lower animals.

Growing plant cells are surrounded by a rigid cell wall composed of a network of different carbohydrate polymers and glycoproteins. During development an extensive increase in cell wall material is necessary. The primary cell wall is composed of high amounts of cellulose, pectins, hemicelluloses, and glycoproteins (1). Most polymers are synthesized in the Golgi apparatus by using nucleotide sugars as glycosyl donors. In plants nucleotide sugars, especially UDP and GDP sugars, can be synthesized *de novo* from UDP-glucose and GDP-mannose as starting substrate (2). UDP-glucuronic acid (UDP-GlcA) derived from UDP-glucose (UDP-Glc) provides about 50% of the cell wall biomass and represents a key metabolite for the nucleotide sugar interconversion pathways (2–4) (Fig. 1). The enzyme UDP-glucose-dehydrogenase (UDP-α-D-glucose: NAD^+ oxidoreductase; EC 1.1.1.22; UGD)[2] (5) catalyzes the oxidation of UDP-glucose into UDP-GlcA with concomitant reduction of NAD^+. The product of this reaction can be further converted by an epimerase into UDP-galacturonic acid acting as an important progenitor for pectic polymers (6, 7) or it can be decarboxylated to UDP-xylose (8, 9). Further epimerization of UDP-xylose is essential for the production of UDP-arabinose (10). Early studies by Loewus *et al.* (11) pointed out the existence of a second pathway beside the biosynthesis of UDP-GlcA in plants, which is called the *myo*-inositol oxygenation pathway. Within this, *myo*-inositol is converted into GlcA by ring cleavage. GlcA has to be activated to UDP-GlcA via an intermediate step, the synthesis of GlcA-1-phosphate by glucuronokinase (GlcAK) (12, 13).

Additionally, in many plants a supplementary "salvage" pathway exists, which enables cells to convert free sugars into NDP sugars via phosphorylated intermediates allocated by the successive work of sugar kinases and nucleotide sugar pyrophosphorylases. These salvage pathways presumably play a primary role in the recycling of sugars derived from the conversion of macromolecules like polysaccharides, proteoglycans, and galactolipids, which are substituted with sugar components. The existence of different salvage pathways for D-galactose, D-galacturonic acid, D-glucuronic acid, L-fucose, and L-arabinose (14) was reinforced by the ability of plant cells or tissues to incorporate exogenous labeled sugar into polysaccharides (14–16). This concept is supported by the *in vitro* identification of different sugar kinases, necessary for the phosphorylation reaction (17–19). Sugar-1-kinases principally catalyze the transfer of the γ-phosphoryl group of a NTP to a monosaccharide resulting in the formation of NDP and a monosaccharide-1-phosphate. Up to now sugar-1-kinases were only identified for monomers of arabinose (20), galactose (21), fucose (22), and galacturonic acid (23). The importance of these kinases within the salvage pathways was investigated in work by Dolezal *et al.* (15) who identified the L-arabinose-sensitive mutant *ara1-1* of *Arabidopsis* showing a 90% decrease in arabinose kinase activity (ARA1, EC 2.7.1.46). This leads to a reduced uptake of exogenously applied arabinose due to a block in the arabinose salvage pathway (20). An important contribution to understand the regulation of the tight network of cell wall biosynthesis is the identification of the last unknown enzyme within the *myo*-inositol pathway, the glucuronokinase (EC 2.7.1.43) (GlcAK), which catalyzes the reaction as D-glucuronic acid + ATP → D-glucuronic acid-1-phosphate + ADP. Leibowitz *et al.* (24)

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[2] The abbreviations used are: UGD, UDP-glucose dehydrogenase; GlcAK, glucuronokinase; UDP-GlcA, UDP-D-glucuronic acid; UDP-Glc, UDP-D-glucose; MIOX, *myo*-inositol oxygenase; USP, UDP-sugar pyrophosphorylase; HPLC, high pressure liquid chromatography; MOPS, 4-morpholinepropanesulfonic acid.

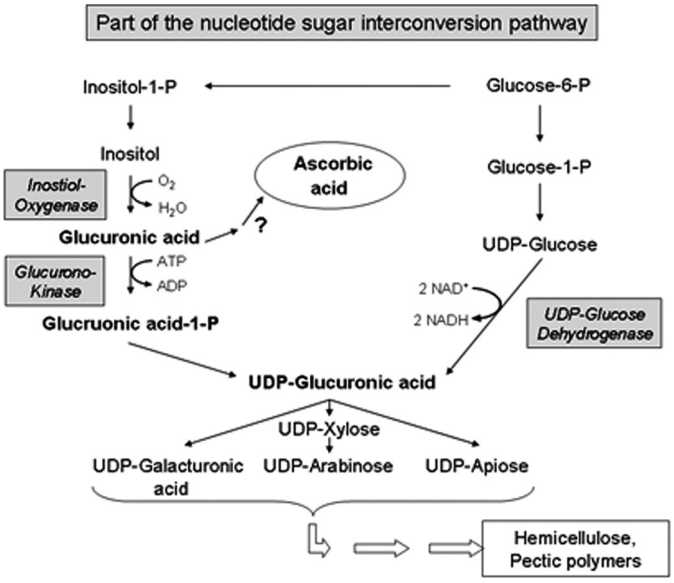


FIGURE 1. **Part of the nucleotide sugar interconversion pathway.** Two different pathways leading to the production of UDP-glucuronic acid are well known in plants. The nucleotide sugar oxidation pathway with the enzyme UDP-glucose dehydrogenase is predominant. Glucuronokinase is part of the second pathway starting with *myo*-inositol oxygenase. Produced UDP-glucuronic acid is an important precursor for the production of other nucleotide sugars, necessary for the build up of hemicellulose and pectic polymers. A pathway leading into ascorbic acid in plants is still under discussion.

partially purified this kinase from the pollen of *Lilium longiflorum*, but so far no gene could be identified in any organism for the enzyme until now.

Here, we report on the purification of GlcAK from *L. longiflorum* and characterization and cloning of GlcAK from *Arabidopsis thaliana*. This enzyme is a novel sugar-1-kinase belonging to the family of GHMP kinases (galacto- (EC 2.7.1.6), homoserine (EC 2.7.1.39), mevalonate (EC 2.7.1.36), and phosphomevalonate (EC 2.7.4.2) kinases).

EXPERIMENTAL PROCEDURES

Materials—Bulbs of *L. longiflorum* were purchased from BulbsOnaWire (Alkmaar, The Netherlands) and plants were grown outside in the botanical garden. Anthers with pollen were harvested, dried using silica gel with indicator (Roth, Karlsruhe, Germany), and stored at −20 °C for at least a year without any decrease in enzyme activity.

Protein Determination—Protein concentration was determined by the method of Bradford with bovine serum albumin as reference protein.

Protein Separation—Purity of collected fractions during different purification steps was analyzed by SDS-PAGE on silver-stained gels according to the protocol of Chevallet *et al.* (25). Bands excised for peptide sequencing were stained with colloidal Coomassie (26).

Enzyme Purification—All enzyme purification steps for LlGlcAK were carried out at 4 °C. The crude enzyme was prepared by homogenizing about 15 g of Easter lily pollen (*L. longiflorum*) in an ice-cold motorized potter homogenizer with 80 ml of pollen extraction buffer (10 mM HEPES-KOH, pH 7.4, 1 mM dithiothreitol, 1 mM EDTA, and 0.5 mM phenylmethylsulfonyl fluoride). After 30 min of extraction, the crude extract was clarified by centrifugation at 15,000 × *g* for 30 min and the homogenate filtered through two layers of Miracloth (Calbiochem/Merck, Darmstadt, Germany).

The crude enzyme fraction was further fractionated by the addition of crystalline ammonium sulfate (giving 20% saturation), which was added to a total volume of 81 ml of enzyme solution. Precipitated proteins were removed by centrifugation at 15,000 × *g* for 30 min. 82 ml of this supernatant was brought to 45% saturation, and precipitated proteins were collected by centrifugation at 15,000 × *g* for 30 min and resuspended in 15 ml of 50 mM Tris-Cl buffer, pH 8.0. Desalting the crude enzyme solution was achieved by dialysis (molecular mass cut-off, 12 kDa) against 2 liters of the same buffer. Three buffer changes were necessary for optimal dialysis. Desalted fractions were concentrated to a final volume of 5 ml using an Amicon centrifugation device (molecular mass cut-off, 10 kDa) (Millipore, Vienna, Austria).

The dialyzed and concentrated enzyme solution was adsorbed onto a 5.5 × 1.3-cm inner diameter (MoBiTec GmbH, Goettingen, Germany) DEAE-Sepharose CL-4B column (GE Healthcare) equilibrated with buffer containing 50 mM Tris-Cl, pH 8.0. The anion exchange column was loaded with the enzyme extract and unbound proteins were removed by washing with the same buffer. Elution was performed using a linear gradient of 0–500 mM NaCl in 50 mM Tris-Cl buffer, pH 8.0 (total volume, 66 ml). Fractions (1.2 ml) were collected and assayed for GlcAK enzyme activity using the coupled HPLC assay.

Active fractions were pooled and buffer was exchanged for 10 mM HEPES, pH 7.4, containing 1 mM dithiothreitol, brought to 1 M KCl and applied onto a 4.0 × 1.0-cm inner diameter (MoBiTec GmbH, Goettingen, Germany) butyl-S-Sepharose 6 Fast Flow (GE Healthcare, Vienna, Austria) column, equilibrated with buffer containing 10 mM HEPES, 1 mM dithiothreitol, 1 M KCl, pH 7.4. The column was eluted with a linear gradient of 1 to 0 M KCl in the buffer (total volume, 40 ml). Fractions were screened for enzyme activity and all active fractions were combined, concentrated by ultrafiltration, and dialyzed against a 10 mM HEPES buffer, pH 7.4, with 1 mM dithiothreitol.

The desalted sample was then applied on a 4.0 × 1.0-cm inner diameter (MoBiTec GmbH, Goettingen, Germany) blue dextran-agarose (Sigma) column equilibrated with the same HEPES buffer and proteins were eluted with a linear gradient of 0–100 mM KCl (total volume, 42 ml). Active fractions were collected, concentrated, and dialyzed against 50 mM Tris-Cl, pH 8.0, buffer using an Amicon centrifugation device.

Dialyzed sample at a final volume of 3 ml was then applied to a 0.5 × 5.0-cm inner diameter MonoQ 5/50 GL column (GE Healthcare), equilibrated with 50 mM Tris-Cl, pH 8.0, and eluted with a linear gradient of 0–600 mM NaCl (total volume, 25 ml). After desalting and concentration of the active fractions with an Amicon centrifugation device, this purification step was repeated using slightly different conditions. Proteins were eluted with a linear gradient of 0–450 mM NaCl (total volume, 25 ml) and positive fractions were combined and dialyzed against a 50 mM Tris-Cl, pH 8.0, buffer using an Amicon centrifugation device.

The concentrated proteins (final volume, 1 ml) were loaded onto a 0.5 × 5.0-cm inner diameter Toyopearl DEAE-5 PW column (Tosho Bioscience GmbH, Stuttgart, Germany) equilibrated with 50 mM Tris-Cl buffer and eluted with a linear gradient of 0–550 mM NaCl (total volume, 20 ml). Positive fractions were concentrated to a final volume of 100 μl using a Microcon centrifugation device (molecular mass cut-off, 10 kDa) (Millipore) and stored at −20 °C.

Peptide Sequencing—Samples with LlGlcAK activity from the last purification step were separated on SDS-PAGE, stained with colloidal Coomassie, and protein bands were excised from the gel. For *de novo* peptide sequencing samples were analyzed at OMX GmbH (Wessling, Germany).

cDNA Cloning—Total RNA was extracted from flowers of *A. thaliana*. Flowers were frozen in liquid nitrogen, homogenized with a rocker mill, and extracted with the NucleoSpin RNA Plant extraction kit (Macherey-Nagel GmbH, Düren, Germany) according to the manufacturer's instructions. Single strand cDNA was synthesized from 1 μg of total flower RNA using the RevertAid First Strand cDNA synthesis kit (Fermentas, Vienna, Austria) according to the manufacturer's instructions. For cloning the glucuronokinase gene from *A. thaliana* two primers with restriction sites were designed, Fwd-1 with SacI (5′-GGGAGCTCAAACGCGTCAGGGAAGAGAAG-3′) and Rev-1 with HindIII (5′-CCGGAAGCTTTAAGGTCT-GAATGTC-3′) based on the cDNA sequence of *Arabidopsis*. PCR was performed with Phusion High-Fidelity DNA polymerase (New England Biolabs) using Fwd-1 and Rev-1 primers and single-stranded cDNA as template under the following conditions: 7 s denaturing at 98 °C, 20 s annealing at 51 °C, 40 s elongation at 72 °C, for 35 cycles. The amplified cDNA sequence was restricted with SacI and HindIII and cloned into expression vector pQE 30 (Qiagen).

Expression and Purification of Recombinant Enzyme—The pQE30-AtGlcAK construct was transfected into the XL-1 strain of *Escherichia coli* (Stratagene) and cells were routinely grown in 2× YT medium (400 ml of medium in a 2-liter flask) containing 100 μg ml^{-1} of ampicillin at 37 °C to A_{600} between 0.6 and 1.0 under vigorous shaking. *E. coli* culture was cooled to 20 °C and the expression of recombinant protein was induced by addition of 0.5 mM isopropyl 1-thio-β-D-galactopyranoside. The protein was expressed at 20 °C under vigorous shaking for a further 20 h. All purification steps were performed at 4 °C. Before harvesting, cells were cooled to 4 °C for 30 min. After centrifugation the pellet was resuspended in lysis buffer (10 mM sodium phosphate buffer, pH 8.0, containing 300 mM NaCl, 10 mM imidazole). Cells were lysed by the addition of 200 μg ml^{-1} lysozyme from chicken egg (Roche Applied Science) and frozen for 30 min at −20 °C. While thawing on ice for 30 min under gently shaking, 2.5 units ml^{-1} of benzonase (Merck Biosciences GmbH, Bad Soden, Germany) and 0.2 mM phenylmethylsulfonyl fluoride were added. Bacterial cells were additionally disrupted by sonication. Insoluble residues were removed by centrifugation for 5 min at 13,000 × *g*. The clarified supernatant was applied to a 4.0 × 1.0-cm inner diameter (MoBiTec GmbH, Goettingen, Germany) nickel-nitrilotriacetic acid-agarose column (Qiagen), equilibrated with starting buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, and 20 mM imidazole). The column was washed with 6 volumes of starting buffer and bound protein was eluted with 4 volumes of the same buffer containing 250 mM imidazole. Protein solutions were desalted via dialysis (molecular mass cut off 10 kDa) against a 50 mM Tris-Cl buffer, pH 7.5, with two buffer changes. Storage was performed at −80 °C in 50 mM Tris-Cl buffer, pH 7.5, containing 50 mM KCl and 20% (v/v) glycerol. Purified recombinant AtGlcAK was analyzed for purity (Fig. 4), specific activity, and substrate specificity.

HPLC Enzyme Assay—GlcAK HPLC assays were carried out in 1.5-ml reaction tubes. The reaction mixture contained 50 mM MOPS-KOH, pH 7.5, 2 mM $MgCl_2$, 1 mM ATP, 2 mM GlcA, and 0.01 unit of recombinant AtGlcAK (1 unit = 1 μmol of product min^{-1}) in a final volume of 0.2 ml. Reaction mixtures were incubated for 20 min at 30 °C in a water bath. The reactions were stopped by heating the assay tubes for 5 min at 95 °C in a bench top heater and afterward allowed to cool on ice. Assay mixtures were centrifuged for 5 min at 13,000 × *g*, supernatants were diluted 2.5-fold and transferred into a HPLC autosampler vial. Fifteen μl of diluted enzyme assay was injected for analysis. Determination of biochemical data like pH and temperature optimum of the recombinant *Arabidopsis* glucuronokinase was performed by measurement of produced ADP during the kinase reaction with this HPLC method.

Detection of UDP-GlcA by HPLC Analysis—After each purification step of *L. longiflorum* GlcAK, fractions were tested with the coupled enzyme assay using UDP-sugar pyrophosphorylase (USP) from *Pisum sativum* as coupling enzyme. This assay was described elsewhere (27).

Photometrical Enzyme Assay—For determination of enzyme kinetics a photometrical kinase assay based on ATP consumption was used. The reaction mixture contained 80 mM MOPS-KOH, pH 7.5, 10 mM $MgCl_2$, 25 mM KCl, 500 μM NADH, 1 mM phosphoenolpyruvic acid, 1 mM ATP, 1 mM GlcA, 1 unit of lactate dehydrogenase, 0.7 units of pyruvate kinase, and 5 milliunits of GlcAK in a final volume of 0.2 ml. Measurement was performed in 96-well microplates (Greiner BioOne, Kremsmünster, Austria) at a wavelength of 340 nm for 30 min.

RESULTS

Purification of a Glucuronokinase from L. longiflorum—According to a work of Dickinson (28) who found the highest GlcAK activity in pollen of *L. longiflorum* compared with other plant tissues, we decided to use Lily pollen as the source for purification of this sugar kinase. Initial attempts to use the easily available pollen from rape, collected by bees, were unsuccessful. Purification of LlGlcAK was achieved by a six-step procedure starting with differential ammonium sulfate fractionation of the crude extract. The purification scheme is shown in Fig. 2. After each chromatographic step of the LlGlcAK purification, the enzyme activity of the fractions was determined and active fractions were pooled, desalted, and concentrated if necessary and applied to the next chromatography column. The purity of positive fractions was monitored by SDS-PAGE. The combination of anion-exchange chromatography, affinity chromatography with blue dextran, and hydrophobic interaction resulted in a ~688-fold purification in a yield of 0.07% (Table 1). The enzyme preparation with two major bands

having an apparent molecular mass of ~40 kDa on SDS-PAGE (Fig. 2) was used for protein sequencing. Both distinct protein bands were excised from the gel for *de novo* sequencing.

Protein Sequencing—Two tryptic peptides were sequenced from the upper band (marked by an *asterisk* in Fig. 2), whereas sequencing of the second band failed because of a limiting amount of the sample. Both peptides (VGFLGNPSDVYFGR (mass 1526.76) and WLDGDEFLLSSMEDLAK (mass 1967.77)) derived from sequencing the marked band matched two highly similar putative unknown proteins from *Arabidopsis*. The proteins contain two PFAM domain signatures pointing to the superfamily of GHMP-kinases, a family that also contains other sugar-1-kinases. Additionally a small conserved nucleotide binding site, found in several kinases, is present (Fig. 3).

The two *Arabidopsis* sequences (At3g01640; At5g14470) are aligned and annotated in Fig. 3*A* using the bioinformatics data base from TAIR (29). Furthermore, the positions of the sequenced peptides from *Lilium* are indicated. BLAST homology searches in GenBank™ revealed no other homologs in *Arabidopis* except for a very weak similarity to fucose kinase (At1g01220; $p = 9$ E-5). In contrast, highly similar sequences to the *Arabidopsis* GlcAK were found in all plant species including the moss *Physcomitrella*. Furthermore, related sequences were found in several algae, a few bacteria, and some (basal) animals (amphibians, fishes, cnidarians) but not in mammals. An unrooted distance tree is shown in Fig. 3*B* clearly separating the GlcAK sequences from plants, algae, animals, and bacteria. The protein is assumed to be localized in the cytosol as no transmembrane spanning domains and transit peptides are predicted (iPSORT software).

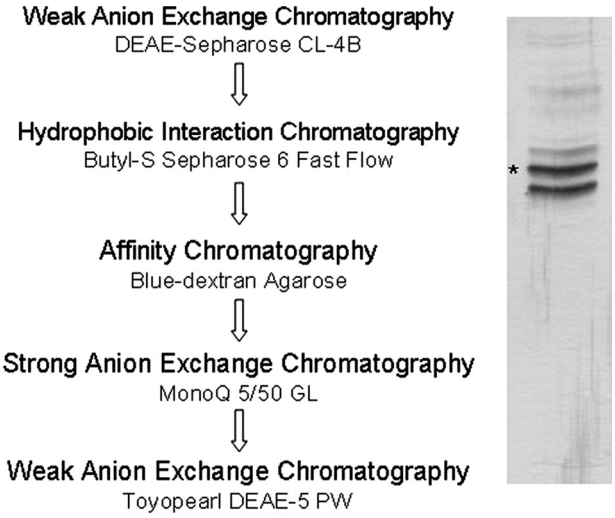


FIGURE 2. **Scheme of chromatographic purification steps of *Lilium* glucuronokinase.** Different steps used for LIGlcAK purification are listed in chronological order. Fractions with LIGlcAK activity obtained from each purification step were pooled and applied to the subsequent chromatography column. A combination of all listed chromatographies resulted in a preparation with two distinct bands. Both were subjected to *de novo* peptide sequencing. The band identified as GlcAK is indicated with a *black asterisk*, sequencing of the second band failed because of a limitation in sample material.

To confirm the function of the identified protein we expressed the full-length open reading frame of At3g01640, encoding 362 amino acids with a molecular mass of about 40 kDa and a calculated isoelectric point of 5.4 in *E. coli* using the pQE30 vector with a His_6 tag. This sequence was chosen from the two candidate genes on the basis of a far higher expression in *Arabidopsis* (>15-fold compared with gene At5g14470), in particular in pollen, and the fact that the isoform At5g14470 was most likely not correctly predicted and annotated in the GenBank data base.

After induction of the *E. coli* culture with isopropyl 1-thio-β-D-galactopyranoside, 4.75 mg of the recombinant *Arabidopsis* protein per liter of culture with a specific activity of 20.8 units mg were produced. SDS-PAGE of the purified soluble recombinant enzyme is shown in Fig. 4*B*.

Characteristics of Enzyme—GlcAK catalyzes the conversion of GlcA acid into GlcA-1-phosphate. We used UDP-sugar pyrophophorylase as a coupling enzyme (27) to convert the GlcA-1-phosphate into UDP-GlcA (Fig. 4*A*), which can be easily detected with UV (260 nm) on HPLC. A typical chromatogram with appropriate controls is shown in Fig. 4*C, 1*. The recombinant enzyme of *A. thaliana* catalyzes the phosphorylation of GlcA yielding UDP-GlcA (*4*) in the coupled enzyme assay. The product is dependent on the presence of ATP (see *5*, Fig. 4*C, 3*) and GlcA (Fig. 4*C, 2*) and identical with the commercially available reference compound UDP-GlcA (Fig. 4*C, 4*) (Sigma) or UDP-GlcA produced by recombinant UDP-glucose dehydrogenase (30). Concomitant, the intermediates ADP (*2*) and UDP (*3*) accumulate linearly with UDP-GlcA product formation. These experiments confirm that the purified enzyme indeed is a GlcAK. The enzyme activity of AtGlcAK1 was also shown in two other variants of the enzyme assays not relying on the coupling enzyme UDP-sugar pyrophosphorylase. One assay measures the formation of ADP linked to a decrease of NADH by the well established pyruvate kinase/lactate deydrogenase system (31). The second assay directly measures the substrate-dependent formation of ADP in the assay by HPLC (data not

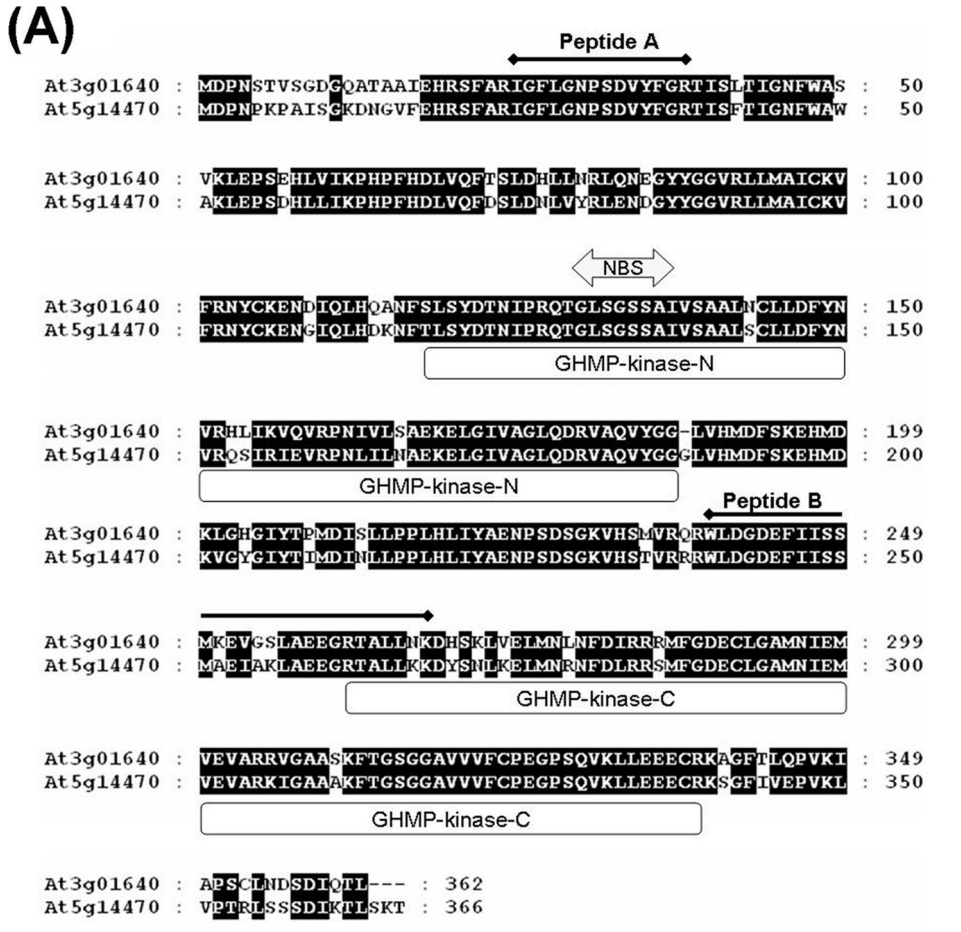
TABLE 1

Purification of glucuronokinase from *L. longiflorum*

Standard HPLC enzyme assays were performed for determination of glucuronokinase activity.

	Total protein	Total activity	Specific activity	Purification	Yield
	mg	*units*	*units/mg protein*	*-fold*	*%*
Crude extracts[a]	1450	37.5	0.026	1.0	100
20–45% Ammonium sulfate	540	16.3	0.030	1.2	43.4
DEAE-Sepharose CL-4B	25.7	6.79	0.264	10.2	18.1
Butyl-S Sepharose 6 Fast Flow	16.4	2.67	0.163	6.27	7.12
Blue-dextran agarose	3.47	1.18	0.340	13.1	3.15
First MonoQ 5/50 GL	0.091	0.34	3.74	143.8	0.91
Second MonoQ 5/50 GL	0.012	0.07	5.83	224.2	0.19
Toyopearl DEAE-5-PW	0.0014	0.025	17.9	688.5	0.07

[a] Crude extract was prepared from 15 g of *Lilium* pollen.



shown). All assays clearly show a GlcAK activity of the recombinant *Arabidopsis* protein.

Characterization of AtGlcAK was determined at different pH values ranging from pH 4.0 to 9.5 dependent on the buffer used (Fig. 5*A*), using ADP formation as the detection method. Almost no activity was measured at pH 4.0, whereas the maximum was reached at pH 7.5 with a far lower activity above pH 8. Utilization of Tris-Cl buffer, pH 7.5, under standard conditions showed a 75% decrease of enzyme activity compared with MOPS-KOH buffer at the same pH 7.5.

AtGlcAK showed kinase activity within a temperature range between 10 and 60 °C (Fig. 5*B*). The optimum was located around 35 °C. More than 90% of the enzyme activity was lost at 55 °C.

For most kinases divalent cations are necessary for substrate conversion. The effect of different monovalent, divalent, and trivalent metal ions was tested (Table 2) and ADP production was monitored during enzyme assay by HPLC measurement. Two mM of the corresponding ions were applied and values were calculated referenced to the magnesia used as the divalent cation in the standard assay. AtGlcAK requires Mg^{2+} but also other divalent cations, like Mn^{2+} and Co^{2+}, were able to substitute for magnesium. Little substrate turnover was also measured with trivalent but none with monovalent cations.

Substrate Specificity—For determination of substrate specificity toward monosaccharide-1-phosphate synthesis standard enzyme assays with ATP and different sugar substrates were performed. Monosaccharides (2 mM) like D-glucose, D-xylose, L-arabinose, D-galactose, and D-galacturonic acid were used and the increase of ADP due to ATP consumption during the kinase reaction was measured (Table 3). The *Arabidopsis* enzyme only catalyzed the conversion of D-GlcA into its corresponding sugar-1-phosphate but failed to utilize any other monosaccharide tested in the

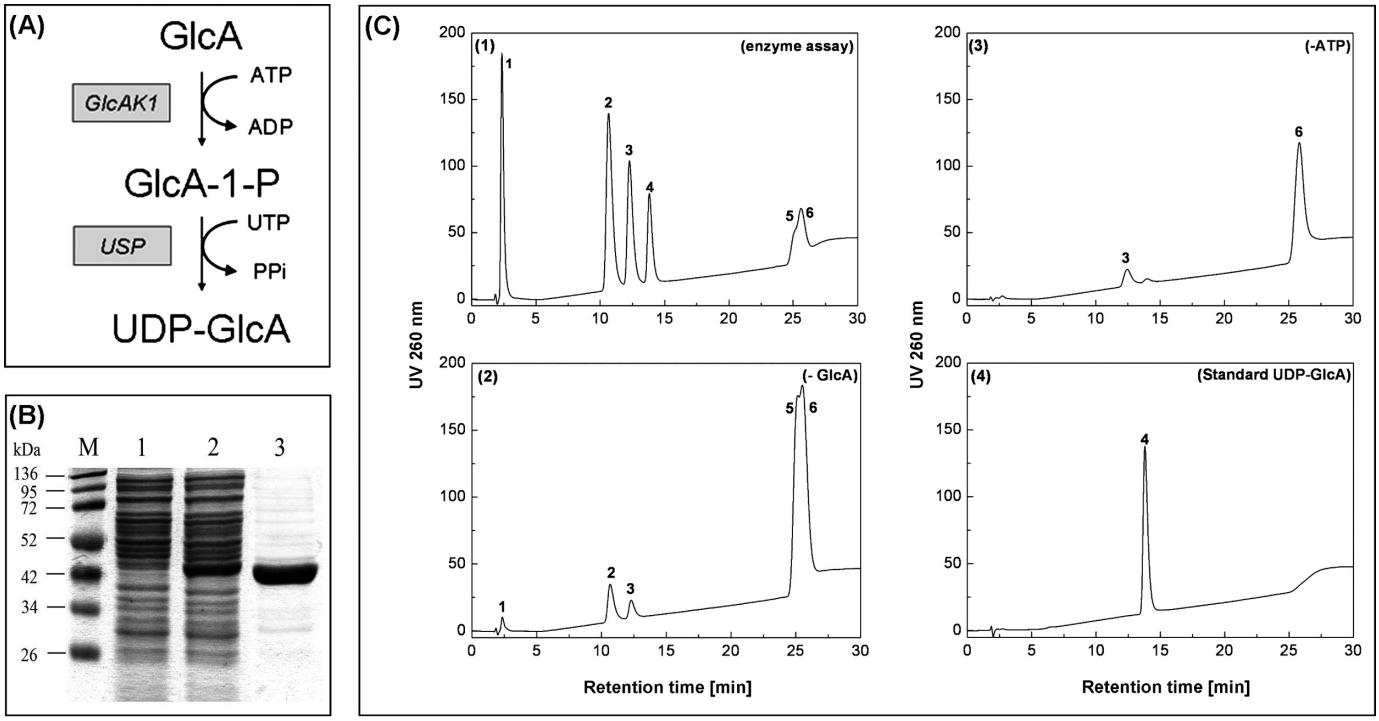


FIGURE 4. **Scheme of coupled HPLC enzyme assay, expression of recombinant AtGlcAK, and HPLC analysis of reaction products.** *A*, representation of the coupled HPLC enzyme assay that uses UDP-sugar pyrophosphorylase as coupling enzyme for the conversion of GlcA-1-P, catalyzed by AtGlcAK, into UDP-GlcA. *B*, different fractions during purification of the recombinant *Arabidopsis* GlcAK with nickel-nitrilotriacetic acid-agarose were analyzed on SDS-PAGE. A specific activity of the recombinant AtGlcAK was determined with 7.5 units/mg. *Lane M*, molecular mass marker; *lane 1*, lysate of *E. coli* before isopropyl 1-thio-β-D-galactopyranoside induction; *lane 2*, lysate of *E. coli* 20 h after isopropyl 1-thio-β-D-galactopyranoside induction; *lane 3,* AtGlcAK purified on chelating column. *C*, HPLC chromatograms of coupled enzyme reaction (complete assay *C, 1*), control reactions (*C*, *2* (minus GlcA) and *C*, *3* (minus ATP)), and UDP-GlcA standard (*C*, *4*) are shown. Numbered peaks are as follows: *1*, AMP; *2*, ADP; *3*, UDP; *4*, UDP-GlcA; *5*, ATP; *6*, UTP.

assays, indicating a very high substrate specificity of AtGlcAK for the sugar D-GlcA.

We also tested different nucleotide tri- and diphosphates as phosphate donors in the kinase reaction. For this we used UTP, CTP, GTP, UDP, and ADP. Apart from ATP none of the nucleotides was able to phosphorylate GlcA.

Kinetic analyses were performed for different substrate concentrations and activities of the enzyme were measured with a photometer assay based on the consumption of ATP. Data obtained from our measurements and published reference values of other sugar kinases from *A. thaliana* and *L. longiflorum* are summarized in Table 4. Substrate saturation curves for GlcA followed a hyperbolic curve according to a Michaelis-Menten kinetic (Fig. 6*A*). The K_m value of the enzyme for AtGlcA in a substrate range of 0.01 to 1.4 mM was calculated as 697 μM by curve regression analysis (SigmaPlot 9.0 software). The enzyme kinetic for ATP shows a sigmoidal curve from which a K_m value for ATP of 555 μM was estimated (Fig. 6*B*). This kind of graph is normally indicative of allosteric enzymes with positive substrate cooperativity. Determination of kinetic parameters for ATP were done according to the Hill equation. The Hill coefficient was calculated as the basis of our data corresponds to a value of 1.54.

Expression Pattern—GlcAK is part of a biosynthetic route to UDP-GlcA, in which the first irreversible step is catalyzed by the enzyme *myo*-inositol oxygenase (MIOX). We were therefore interested in comparing the expression patterns of AtGlcAK and those of the preceding MIOX and the subsequent UDP-sugar pyrophosphorylase. The microarray dataset available in Genevestigator (32) clearly shows quite a good correlation between the levels of *MIOX*, *GlcAK*, and *USP* gene expression in *A. thaliana,* all peaking in pollen tissue (Fig. 7).

DISCUSSION

Here we describe the cloning and biochemical characterization of *A. thaliana* GlcAK, a new member of the GHMP (galacto-, homoserine, mevalonate, and phosphomevalonate) kinases superfamily. Roughly 600 unique protein sequences belong to this superfamily, acting on diverse substrates (33). The structure of eight different members is

FIGURE 3. **Sequence alignment of amino acids and phylogenetic analysis of AtGlcAK.** *A*, *Arabidopsis* sequences of both isoforms (At3g01640; At5g14470 (manually extended) were aligned with the pairwise method using ClustalW. Amino acid residues were numbered beginning at the start methionine. Identical and conservative substituted amino acids are highlighted in *black*. The positions of the sequenced peptides from *Lilium* are indicated as well as typical GHMP-kinase domains (*NBS,* nucleotide binding site). *B*, phylogenetic relationships of both isoforms of GlcAK in higher plants, algae, bacteria, and animals analyzed using ClustalW. Accession numbers are listed for different organisms as followed: Os11g11060 and Os11g0217300 from rice; *Vitis vinifera,* CAO14683; *Sorghum bicolour,* EES15489; *Zea mays,* ACG36196; *Populus trichocarpa,* XM_002298517; *Physcomitrella patens* subsp. *patens*, EDQ81053; *Chlamydomonas reinhardtii,* EDP07430; *Micromonas pusilla*, EEH56491; *Ostreococcus lucimarinus,* ABO96425; *Xenopus tropicalis,* AAI55522; *Danio rerio,* AAI54512; *Nematostella vectensis*, EDO43506; *Solibacter usitatus* Ellin6076, ABJ87639; *Dyadobacter fermentans* DSM 18053, ACT95827*; Verrucomicrobium spinosum* DSM 4136, ZP_02929783. The *bar* indicates 0.1 substitutions per site.

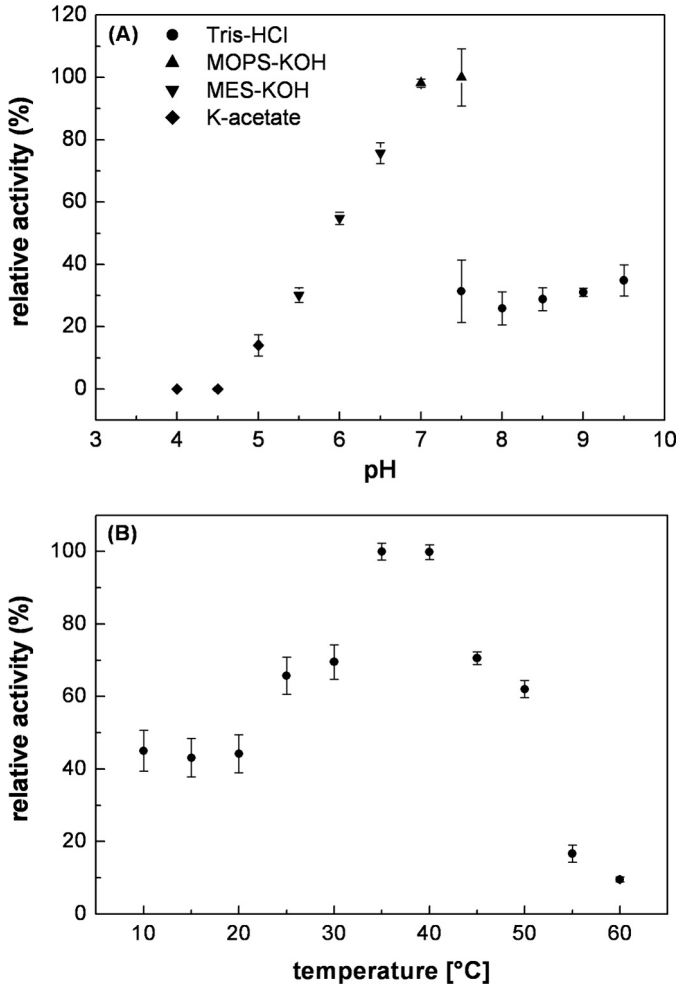


FIGURE 5. **The effect of different pH values and temperature on the activity of AtGlcAK.** *A*, the activity of *Arabidopsis* GlcAK was measured with buffers (50 mM) at different pH values. *B*, the temperature curve results from incubation of the enzyme assay at different temperatures. For both measurements standard assays were performed and the ADP amount produced during the reaction was analyzed by HPLC. All data are averages of triplicate assays.

TABLE 2

Metal ion requirement of AtGlcAK for phosphorylation of D-GlcA

Enzyme assay was performed using D-GlcA and ATP as substrates under standard assay conditions. Activity of the recombinant *Arabidopsis* enzyme is quantified as percent referred to the standard reaction assay with 2 mM Mg^{2+}. Values represent the average (±S.D.) of three independent assays.

Metal ion (2 mM)	Relative activity
	%
None	0.0 ± 0.0
Mg^{2+}	100.0 ± 7.0
Mn^{2+}	94.2 ± 3.2
Ca^{2+}	44.6 ± 4.9
Cd^{2+}	26.7 ± 5.0
Zn^{2+}	7.7 ± 1.3
Cu^{2+}	2.2 ± 0.8
Co^{2+}	83.2 ± 4.0
Ba^{2+}	0.0 ± 0.0
K^{+}	0.0 ± 0.0
Li^{+}	4.1 ± 1.1
Al^{3+}	5.2 ± 0.7
Fe^{3+}	10.2 ± 2.2

found in the Protein Data Bank and a common feature of them is a very low sequence identity located somewhere between 10 and 20% but they all share similarities in their three-dimensional structure. Thus the structure is sustained, whereas amino acid residues have been allowed to change (34).

TABLE 3

Substrate specificity of AtGlcAK

Enzymatic activity of the enzyme was measured by ADP detection with HPLC. Standard conditions were used and the reaction was allowed to proceed in the presence of 2 mM of various monosaccharides. Enzyme activity is expressed as percentage of those for the standard reaction assay with GlcA. Values are averages of three independent assays (±S.D.).

Substrate (2 mM)	Relative activity
	%
D-Glucuronic acid	100 ± 2.8
D-Glucose	ND[a]
D-Xylose	ND
L-Arabinose	ND
D-Galactose	ND
D-Galacturonic acid	ND

[a] ND, not detectable; detection limit 0.1% of the standard assay with D-glucuronic acid.

AtGlcAK is a novel plant sugar kinase able to phosphorylate D-GlcA to D-GlcA-1-phosphate in the presence of ATP. It is the last missing enzyme in the *myo*-inositol oxygenation pathway to UDP-GlcA (11), which was not known at the molecular level so far. BLAST analysis of GenBank clearly reveals that no other proteins beside the two AtGlcAK sequences are present in this plant genome. We are surprised that the recently discovered D-galacturonic acid kinase (23), although acting on a very similar substrate, shares almost no sequence identity with GlcAK. This underlines the previous observations that the structure but not sequence conservation was retained in the evolution of GHMP kinases (34). In contrast to these findings the sequences of GlcAKs from other species are highly conversed between different phyla suggesting an evolutionary old enzyme sequence maintained over a long period of time.

The biochemical property of *Arabidopsis* GlcAK indicates a unique substrate specificity for D-GlcA. None of the similar substrates (*e.g.* glucose or galacturonic acid) are phosphorylated at a detectable rate. This is surprising, as the K_m of GlcAK from *Arabidopsis* for GlcA is relatively high (0.697 mM), similar to the previously reported value for the partially purified *Lilium* enzyme (0.6 mM) (24). This suggests that the enzyme is operating under nonsaturating conditions as the concentration of GlcA in plants is rather low.

GlcAK likely has several functions in plants. The most obvious is the catalysis of D-GlcA within the MIOX pathway to UDP-GlcA. The Loewus group (35) showed by precursor feeding experiments that germinating *Lilium* pollen readily convert *myo*-inositol into pectic polymers of the pollen tube cell wall. Moreover, during pollen tube germination a high amount of cell wall material must be provided. HPLC analyses of the style secreted mucilage of *L. longiflorum* showed a very high amount of *myo*-inositol.[3] In the work of Schneider *et al.* (36) a *myo*-inositol-specific uptake transporter of *A. thaliana* was identified in pollen tubes. This suggests that *myo*-inositol secreted by the style can be taken up by pollen through the *myo*-inositol transporter and metabolized via the MIOX pathway into UDP-GlcA providing enough precursors necessary for the build up of different cell wall components.

[3] S. Endres and R. Tenhaken, unpublished results.

TABLE 4

Enzyme kinetics *of Arabidopsis* glucuronokinase

Kinetic measurements were performed for GlcA (0.01–1.4 mM) and ATP (0.01–1.2 mM) under standard conditions for 15 min. Each value is a mean of three independent measurements and K_m and V_{max} were calculated using SigmaPlot 9.0 for best fit curve. Published kinetics of other sugar kinases from *L. longiflorum* (24) and *A. thaliana* (22, 23) are shown for comparison.

	K_m (sugar)	$K_m/S_{0.5}$[a] (ATP)	V_{max} (sugar)	V_{max} (ATP)	k_{cat} (sugar)	k_{cat} (ATP)	R_S[b] (ATP)
	μM	μM	*nmol min*$^{-1}$ μg^{-1}	*nmol min*$^{-1}$ μg^{-1}	s^{-1}	s^{-1}	
AtGlcAK1	697	555	12.2	12.6	8.3	8.5	1.54
LlGlcAK1	620	1900	NA[c]	NA	NA	NA	
AtGalAK	70.8	195	12	6	NA	NA	
AtGalK	701	701	42	39.4	NA	NA	
rAtFKGP	1000	450	NA	NA	0.34	0.38	

[a] $S_{0.5}$ is part of the Hill equation and is similar to K_m. It represents the substrate concentration giving a velocity equal to half-maximal velocity.
[b] R_s represents the Hill coefficient indicative for the cooperativity of a curve.
[c] NA, data not available.

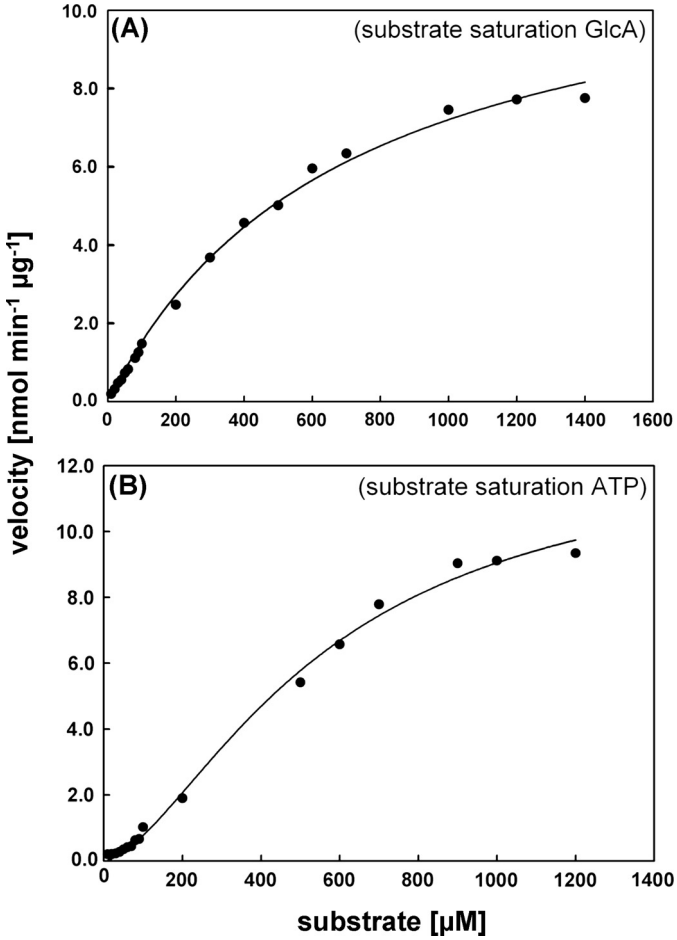


FIGURE 6. **Substrate saturation curves for GlcA and ATP.** For both substrates Michaelis-Menten curves are shown. Activity of AtGlcAK was measured with varying concentrations of D-GlcA (0.01–1.4 mM) and ATP (0.01–1.2 mM) under standard conditions for 15 min. Each value was calculated from three independent measurements done by the photometrical ATP assay. K_m values for GlcA and ATP (697 and 555 μM) and V_{max} (12.2 and 12.6) were calculated using SigmaPlot 9.0 for curve fitting.

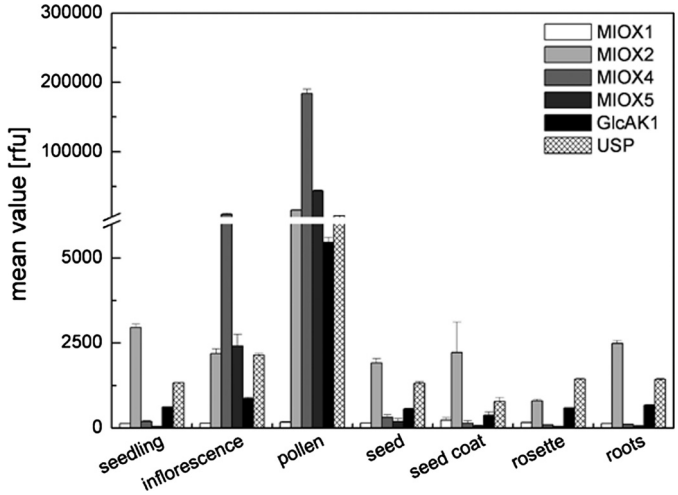


FIGURE 7. **Comparison of expression data for *MIOX* isoforms, *GlcAK* and *USP*.** Expression data of the four *MIOX* isoforms, *GlcAK* and *USP* in different tissues of *A. thaliana* were compared using Genevestigator (31). All enzymes are located within the *myo*-inositol pathway.

Although *MIOX* and *GlcAK* genes are highly expressed in pollen the pathway is clearly not restricted to these specialized cells. Kanter *et al.* (37) showed in *MIOX* knock-out plants a strongly reduced incorporation of *myo*-inositol into seedling cell walls, which must be dependent on the GlcAK enzyme.

A second function of GlcAK may be a role in a salvage pathway for GlcA. Plant growth requires the remodeling of primary cell wall components that may liberate GlcA from recycling of pectic polymers or arbinogalactan proteins. In addition, GlcA may be released from UDP-GlcA by the accidental activity of hydrolases. Given that UDP-GlcA is the quantitatively most important precursor for primary cell walls, providing indirectly, for instance, half of the cell wall biomass of *Arabidopsis* leaves, the release of some amounts of hydrolyzed GlcA seems possible. In this scenario the GlcAK will be essential to recycle GlcA into UDP-GlcA. A role in cell wall turnover was recently also suggested for galacturonic acid kinase (23). GalA is not directly synthesized in plants but may be derived from hydrolysis of pectic polymers.

With the identification of GlcAK the pathway for the generation of most major sugar-1-phosphates is now known. The data support a model of a parallel biosynthetic network for nucleotide sugar production, mainly interconverted at the level of nucleotide sugars but backed up by a salvage pathway starting with a group of sugar-1-kinases.

The GlcAK described in this paper channels GlcA into a pathway to UDP-GlcA, which is an important sugar donor for plant cell wall polymers, and proteoglycans like hyaluronic acid and glucuronosyltransferases mainly in animals. On the other hand GlcA is the precursor for ascorbic acid in animals when converted into gluonate before final oxidation (38). Ascorbic acid is an important antioxidant and protein cofactor in plants as well as in animals and became a vitamin C for human and a few other mammals when they lost a functional gene for L-gu-

lono-1,4-lactone oxidase (38). As a consequence these animals need to take up ascorbic acid with the plant food. Why did the *GlcAK* gene disappear in animals above amphibians? A consequence of a lost GlcAK enzyme is a redirection of GlcA into the ascorbic acid pool, which may ensure a steady production of this critical cellular metabolite. In this scenario UDP-GlcA for proteoglycans is produced by UDP-glucose dehydrogenase, thereby uncoupling the two pathways. Whether GlcA is also a precursor for ascorbic acid in plants is discussed controversially (38) but compelling evidence is still missing (39). Up to now no report has measured the availability of GlcA plants but feeding of labeled methyl-GlcA to *Arabidopsis* cell cultures strongly increased the ascorbic acid synthesis (40). The metabolite GlcA would be a prerequisite for the possible further conversion into ascorbic acid. Isolation of plant mutants in *GlcAK* will help to elucidate this controversial plant pathway. We will explore this possibility in the future by generating *GlcAK* knock-out mutants.

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Metabolism and Bioenergetics:

Cloning of Glucuronokinase from Arabidopsis thaliana, the Last Missing Enzyme of the *myo*-Inositol Oxygenase Pathway to Nucleotide Sugars

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