Plant genomes contain genetically encoded isoforms of most nucleotide sugar interconversion enzymes. Here we show that Arabidopsis thaliana has five genes encoding functional UDP-\(\Delta\)-glucose/UDP-\(\Delta\)-galactose 4-epimerase (named UGE1 to UGE5). All A. thaliana UDP-\(\Delta\)-glucose 4-epimerase isoforms are dimeric in solution, maximally active in vitro at 30 – 40 °C, and show good activity between pH 7 and pH 9. In vitro, UGE1, -3, and -5 act independently of externally added NAD\(^+\), whereas cofactor addition stimulates the activity of UGE2 and is particularly important for UGE4 activity. UGE1 and UGE3 are most efficiently inhibited by UDP. The five isoforms display \(K_{\text{m}}\) values between 23 and 128 s\(^{-1}\) and \(K_{\text{m}}\) values between 0.1 and 0.3 mm. This results in enzymatic efficiencies ranging between 97 and 890 mm\(^{-1}\) s\(^{-1}\) for UGE4 = UGE1 < UGE3 < UGE5 < UGE2. The \(K_{\text{m}}\) values, derived from the Haldane relationship, were 0.76 mm for UGE1, 0.56 mm for UGE4, and between 0.13 and 0.23 mm for UGE2, -3, and -5. The expression of UGE isoforms is ubiquitous and displays developmental and cell type-dependent variations. UGE1 and -3 expression patterns globally resemble enzymes involved in carbohydrate catabolism, and UGE2, -4, and -5 expression is more related to carbohydrate biosynthesis. UGE1, -2, and -4 are present in the cytoplasm, whereas UGE4 is additionally enriched close to Golgi stacks. All UGE genes tested complement the \(\text{UGE}^4\text{mad}\) phenotype, confer increased galactose tolerance in planta, and complement the galactose metabolism deficiency in the Saccharomyces cerevisiae gal10 mutant.

We suggest that plant \(\text{UGE}\) isoforms function in different metabolic situations and that enzymatic properties, gene expression pattern, and subcellular localization contribute to the differentiation of isoform function.

The biosynthesis of plant carbohydrates requires specific glycosyltransferases that act on activated sugars, typically uridine diphosphate, adenosine diphosphate, and guanosine diphosphate hexoses and pentoses. In addition to acting as biosynthetic substrates, nucleotide sugars are modified at their glycosyl moieties by nucleotide sugar interconverting enzymes to generate different sugars and are intermediates in the uptake of the free sugars released from the breakdown of nutritional or storage carbohydrates and other sources. The biochemistry and reaction mechanism of many nucleotide sugar interconversion pathways have been reviewed previously (1). cDNAs coding for nucleotide sugar interconverting enzymes have been cloned and recombinant proteins purified, leading the way to x-ray crystallography (2–5). The complete sequencing of entire genomes revealed a surprising over-representation of genes encoding putative isoforms of nucleotide sugar interconversion enzymes in plant genomes (6, 7), but the functional significance of this apparent genetic redundancy remains to be established.

One of the best characterized nucleotide sugar interconversion enzymes is UDP-glucose 4-epimerase (EC 5.1.3.2; UGE), which interconverts UDP-\(\Delta\)-glucose (UDP-Glc) and UDP-\(\Delta\)-galactose (UDP-Gal). The reaction mechanism of UGE is thought to occur via transfer of the 4′-\(\text{OH}\) hydrogen of the sugar to the nicotinamide ring of noncovalently bound \(\text{NAD}^+\), rotation of the resulting 4′-ketopyranose intermediate in the active site, and transfer of the hydride from the nicotinamide ring of \(\text{NADH}\) back to C-4 of the sugar (3). UGE is essential for de novo biosynthesis of UDP-Gal, a precursor for the biosynthesis of numerous different carbohydrates, glycolipids, and glycosides. On the other hand, UGE is also required for the catabolic uptake of galactose into the central metabolism, and therefore UGE deficiency exacerbates galactose toxicity in plants (8) and in yeast (9) and also leads to different forms of human galactosemia (10, 11).

The completely sequenced genome of the model plant Arabidopsis thaliana contains five genes predicted to encode UGE (6, 7); the fully sequenced rice genome contains four putative UGE-encoding genes, and cDNA clones putatively encoding three UGE isoforms were recently isolated from barley (12). Two studies previously addressed the genetic role of individual UGE isoforms in A. thaliana. UGE1 was transgenically overexpressed and suppressed (8). Despite an increase by up to 250% and a decrease to 10% compared with the wild type UGE activity, no alteration in carbohydrate composition was observed. However, tolerance to galactose was correlated to UGE1 expression in various transgenic lines. Loss of function mutations in \(\text{UGE}4\) were found to induce dramatic morphological alterations in roots and correlated with a reduction of cell wall bound galactose without affecting the level of galactolipids (13). Because all UGE isoforms are expressed in roots, the specific effect of \(\text{UGE}4\) loss of function was surprising and prompted the speculation that individual isoforms might be physically associated with specific galactosyltransferases facilitating substrate channeling, a speculation that was also presented in the context of the noncatalytic loops in the structure of Trypanosoma brucei UGE (4). In potato tubers, overexpression of two different UGE isoforms increased cell wall galactose content. This indi-
icates that UGE activity is rate-limiting for the biosynthesis of some cell
wall polymers (14). Interestingly, overexpression of one potato UGE
isoform increased galactose tolerance, whereas overexpression of the
other isoform did not. These observations suggest that UGE isoforms
might perform specific roles in planta. Besides metabolic channeling,
进一步解释了在 bulk UGE
activity and UDP-Gal flux might be found in the differential sensitivities
to control mechanisms such as redox control, feedback inhibition, or
allosteric regulation (reviewed in Ref. 7). Moreover, the possibility has
been discussed of “one-way enzymes,” where the activity and UDP-Gal flux might be found in the differential sensitivities
strate differs substantially from the
other isoform did not. These observations suggest that UGE isoforms
were investigated in public expression data bases and by subcellular
localization studies. Our data suggest that all UGE paralogs are catalyt-
ically active in both directions but appear to have adapted to different
metabolic roles in vivo.

MATERIALS AND METHODS

Cloning and Recombinant Expression of UGEs in Escherichia coli—
UGE4 bacterial expression was described previously (13). Using A. thaliana
wild type (Col-0) seedling total cDNA as template, the coding sequences of
UGE1–3 and -5 were amplified using the following oligonucleotides (from
5’ to 3’): U1/Nde-F, CATATGGGTTCTTCTTGAGACG; U1/Bgl-R,
AGATCCTAAGCTTATTGGAACC; U2/Vsp-F, ATTAAATAAG-
GAGAGTGGTTTGG; U2/Bgl-R, AGATCCTATGAAGAGGAG-
ACATTGGAG; U3/Nde-F, CATATGCTGGTTCTGGACAG; U3/Bgl-R,
AGATCCTAATGCTTATGGGAAAAC; U5/Vsp-F, ATT-
AAATGCTCAAGACATGATAGA; and U5/Bgl-R, AGATC-
TATGAGATGTATTGCGAA, respectively.

PCR products were cloned into pGEM-T (Promega), and individual
clones were sequenced. Correct open reading frames of UGE1 and
UGE3 were released using Ndel and BglII; UGE2 and UGE5 were released
using VspI and BglII and ligated into Ndel- and BglII-di-
gested, gel-purified pET15b (Novagen). Because UGE2 and UGE5
cDNA sequences contain internal Ndel sites, VspI was used for cloning
into pET15. This procedure introduces 1 asparagine residue
between the UGE peptide and the 20-amino acid-long amino-termi-
nal hexahistidine peptide. Recombinant UGE proteins were expressed and affinity-purified as described previously for UGE4
(13). UGE preparations containing 50% glycerol were frozen on dry
ice and kept at −80 °C. After purification, staining with Sypro Ruby
Protein Gel Stain (Molecular Probes, Invitrogen) revealed a certain
degree of unspecific proteins bound to the Hisbind (Novagen) affinity
matrix. The content of recombinant UGE1–5 was 50, 70, 60, 82,
and pGADT7 (Clontech). For functional complementation of the UGE-
deficient yeast mutant, the bait plasmids pGBK7 and pGBK7-UGE1
to-5 were transformed into the gal10 strain by small scale transformation,
and transformants were selected on SD/-Trp-selective medium. For the complementation assay, transformed mutant cells were grown
at 30 °C on SD/-Trp-selective medium and galactose solid medium (1% yeast extract (Duchefa), 2% Bacto-peptone (BD Biosciences), 0.8% galactose (Sigma), 2% Micro agar (Duchefa); w/v). All UGE isoforms were tested for interaction both with themselves and with each other by
yeast two-hybrid assays. Each UGE bait (GBK7-UGE1–5) was
co-transformed with UGE prey (pGAD77-UGE1–5) into the AH109
reporter strain (Clontech), and double transformants were selected on
SD/-Leu/-Trp medium. For yeast two-hybrid interaction assays, dou-
tle transformants were grown on SD/-His/-Trp/-Leu for selection of
activation of the HIS reporter gene and on SD/-Ade/-His/-Trp/
-Leu for selection of activation of the HIS and the ADE reporter genes.

UDP-Gal 4-Epimerase Assay—UGE was assayed as described previ-
ously (8). The standard epimerase reaction was performed in 50 mM
Tris/HCl, pH 7.4, containing 0.1 mM NAD+ and 125 μM 1-acetylated
BSA (Sigma catalog number B 2518) at 25 °C, unless stated otherwise.
To start kinetic measurements, 175 μl of UDP-Gal (Calbiochem catalog number 670111) solution was added to 25 μl of pre-warmed enzyme
solution, and the reaction was stopped after 10 min by adding 25 μl of
1.2 M NaOH. After 5 min at 100 °C and cooling, 25 μl of 1.2 M NaOH was
added. Glucose was quantified in a coupled reaction by adding 100 μl of
a solution containing 5 μg/ml 1-horseradish peroxidase (Sigma catalog number P 8250, 179 units/mg), 100 μg/ml 1-glucose oxidase (Sigma
catalog number 49180, 198 units/mg), and 300 μg/ml 1-o-dianisidine
dihydrochloride (Sigma catalog number D-3252) in 100 mM sodium
phosphate buffer, pH 7.5, for 30 min at room temperature, against a
glucose standard series in a buffer identical to the epimerase buffer. The
reactions were stopped by adding 300 μl of 6 M HCl, and the absorbance
was detected at 540 nm. The linearity of the epimerase reaction was
measured between 5 and 30 min and by varying enzyme concentrations.
Maximal reaction rate and Km values were determined by varying UDP-
Gal concentration between 0.05 and 2.5 mM using best fit to single site
saturaton kinetics in the pharmacology menu of Sigmaplot 8.0 soft-
ware. To determine Km values, UDP-Glc was converted by re-adding fresh
enzyme until there was no further change in the UDP-Glc:UDP-Gal
ratio.

UDP-Glc 4-Epimerase Assay—The velocity of conversion of UDP-
Glc to UDP-Gal was determined at 5 mM UDP-Glc (Sigma catalog num-
er U 4625) in a 50-μl volume of 50 mM Tris/HCl, pH 7.4, containing 0.1
mM NAD+ and 125 μg/ml 1-acetylated BSA (Sigma catalog number B
2518) at 25 °C. The epimerase reaction was stopped after 10 min with
25 μl of 0.3 M HCl, hydrolyzed at 100 °C for 5 min, and neutralized using
a mole equivalent of NaOH. Galactose was quantified in a coupled reac-
tion by adding 50 μl of a solution containing 0.2 units/ml 1-horseradish
peroxidase, 4 units/ml 1-galactose oxidase, and 0.1 mM Amplex Red (all
from Molecular Probes, catalog number A22179), in a 150 mM Tris/
HCl pH 7.2, 3 mM CaCl2 buffer against a galactose standard in a buffer
identical to the epimerase buffer. Because detection of fluorescence
produced unsatisfactorily high variability, the absorbance of the oxi-
dized Amplex Red was detected at 560 nm in 96-well microtiter plates
(Costar 9017) using a Spectramax 340pc plate reader. The absorbance
differential between 200 and 300 s of reaction time typically gave the
best linearity of the reaction. Amplex Red was handled to ensure mini-
mal light exposure. To ensure linearity of the epimerase reaction, a
range of enzyme concentrations was tested. To ensure substrate satu-
ratiom, 5–9 mM UDP-Glc was used. The direct determination of Km

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UDP-Glc at low substrate concentrations did not yield reproducible data because of low signal to noise ratio.

Therefore, the \( K_{m}^{UDP-Glc} \) value was derived from the Haldane relationship shown in Equation 1 (15),

\[
K_{eq} = \frac{[UDP-Gal]_c}{[UDP-Glc]_c} = \frac{K_{m}^{UDP-Glc}}{K_{m}^{UDP-Gal}} \quad (\text{Eq. 1})
\]

using \( K_{eq} \) of 0.33 and the kinetic constants corresponding to the highest rate of UDP-Gal conversion.

Overexpression of UGEs in A. thaliana—UGE2, -3, and -4 were amplified using the following oligonucleotides: UGE2/STA/Xba, TCTAGATGTTGGGAATATTCTGGTGAC-TGTGGAAC; and R1/STA/Xba, TCTAGATGGTTGGGAATATTCTGGTGAC-TGTGGAAC; UGE3/STA/Xba, TCTAGATGGTTGGGAATATTCTGGTGAC-TGTGGAAC; UGE2/STO/Eco, GAATTCTTATGTTGAGTTTGGTGAAAC. The resulting fusion was cloned into pGEM-T (Promega) and sequenced. After release of the pGEMII containing clone. The resulting fusion was cloned into pGREEN0229 (16) and transformed into uge4 mutants as described above.

UGE4-specific Antibodies—UGE4-specific antiserum was raised commercially (BioGenes, Berlin) by immunization of rabbits with a peptide corresponding to the 16 carboxy-terminal amino acid residues of UGE4 conjugated to keyhole limpet hemocyanin. Specific antibodies were subsequently affinity-purified using immobilized peptide. The rhd1–4 mutant, that contains a premature stop codon before the carboxy-terminal half of UGE4 (13), served as control in various assays. In microscopic applications, the antiserum 3605g produced an unspecific signal in cell walls but bound specifically to intracellular UGE4.

Immunogold Transmission Electron Microscopy—Roots of 4-day-old seedlings were excised and were vacuum-infiltrated for 5 min with MES (20 mM). The samples were high pressure frozen, freeze-substituted in acetone containing 0.5% uranyl acetate, and embedded in LR white resin as described previously (18). Ultrathin sections of approximately 80 nm were taken using a Leica UC6 ultramicrotome (Leica, Milton Keynes, UK) and picked up on pyroxylin- and carbon-coated gold grids. For immunogold labeling, grids were incubated on drops of 50 mM glycine/PBS for 15 min and on drops of 0.1% cold water fish skin gelatin, 5–10% normal goat serum, 15 mM Tris-HCl, 0.15 M NaCl. After four 30-min washes two times in water, the grids were contrast-stained with 1% uranyl acetate, and embedded in LR white resin.

For immunogold labeling, grids were incubated on drops of 50 mM glycine/PBS for 15 min followed by drops of pre-prepared Aurion blocking buffer (5% BSA, 0.1% cold water fish skin gelatin, 5–10% normal goat serum, 15 mM NaCl) for 30 min and then equilibrated in 0.1% BSA-C/PBS (Aurion, Netherlands). Grids were incubated with the primary antibody dilution in equilibration buffer overnight at 4 °C, washed 5–6 times in equilibration buffer, and incubated for 3 h with the secondary antibody conjugated to 10 nm gold (BioCell, Agar Scientific Ltd., Essex, UK) diluted 1:50 in equilibration buffer. After four washes in equilibration buffer, 20-min washes three times in PBS and 30-min washes two times in water, the grids were contrast-stained with uranyl acetate and lead citrate before observation in a Jeol 1200 EX transmission electron microscope at 80 kV. Photographs were taken using Kodak electron image film.

Meta-analysis of Publicly Available Expression Data—Data on organ-specific expression were obtained from the GENEVESTIGATOR data base (19) using the Gene Atlas tool. Expression signals for each UGE gene were normalized. Cell type-specific gene expression data in root tips were published previously (20), and spatial representations were queried online. The degree of co-expression between individual genes was queried in the comprehensive systems biology data base (CSB.DB) (21) or by an alternative approach using pre-processing of raw data and discretization of signal changes between time points (22). This method preserves the entire set of genes queried, and its results match well with other algorithms. Experimental details and the
full result of this analysis are available online in the Arabidopsis Systems Interaction data base.

**Gel Filtration** (Size Exclusion) Chromatography—Freshly purified UGE protein (0.1–1.0 mg) was buffer exchanged into gel filtration buffer (20 mM Tris/HCl, pH 7.4, 150 mM NaCl) using an Amicon Ultra-4 centrifugal filter unit with a 10-kDa nominal molecular weight limit (Millipore). The protein solution was separated on a Superdex 200 HR 10/30 gel filtration column (Amersham Biosciences) with a 0.5 mL/min flow rate. At the column outlet the absorption at 280 nm was measured online to detect eluting proteins. The molecular weights corresponding to the protein peaks in the eluate were calculated from the elution volume using a standard curve generated with the following proteins of known molecular weight: β-amylase, 200 kDa; alcohol dehydrogenase, 150 kDa; carbonic anhydrase, 29 kDa; and cytochrome c, 12.4 kDa.

**Dynamic Light Scattering**—The hydrodynamic radius of freshly purified His-tagged UGE protein (80–800 μg/ml) filtered through a 0.1–μm Ultrafree-MC centrifugal filter unit (Millipore) was measured with a DynaPro99 Dynamic Light Scattering instrument (Protein Solutions) at 20 °C. Results were analyzed using the instrument control software Dynamics (Protein Solutions). The molecular weight was calculated from the hydrodynamic radius using the default volume shape hydrodynamic model.

**RESULTS**

UGE Isoforms Are Dimeric and Can Interact Homo- and Heterotypically—All five *A. thaliana* UGE genes are predicted to code for polypeptides of ~40 kDa (Table 1). Dynamic light scattering of all five UGEs indicates molecular masses between 64 and 103 kDa suggesting dimeric quaternary structure (Table 1). In agreement with this interpretation, all UGEs elute as single peaks corresponding to 73–93-kDa proteins in gel filtration experiments (Table 1). Yeast two-hybrid assays detect self-interaction of UGE proteins and reveal interaction between different UGE isoforms (Fig. 1). In this system the affinities of both homotypic and heterotypic interactions are relatively weak, leading to activation of the HIS but not of the ADE reporter genes.

**TABLE 1** Summary of molecular properties of *A. thaliana* UGE isoforms

|       | Calculated mass | Mass, dynamic light scattering (hydrodynamic radius, nm) | Mass, gel chromatography (elution volume, ml) |
|-------|-----------------|--------------------------------------------------------|---------------------------------------------|
| UGE1  | 41.1            | 103 (3.89)                                             | 83 (13.8)                                   |
| UGE2  | 40.3            | 91 (3.74)                                              | 77 (13.9)                                   |
| UGE3  | 40.8            | 98 (3.83)                                              | 73 (14.0)                                   |
| UGE4  | 40.0            | 84 (3.64)                                              | 89 (13.7)                                   |
| UGE5  | 40.2            | 91 (3.74)                                              | 93 (13.6)                                   |

**FIGURE 1.** Yeast two-hybrid interaction assay at low (–His) and high (–His/–Ade) stringency. All combinations of UGE isoforms grew on SD/His–Trp–Leu medium (–His) but did not grow on SD/–Ade/–His/–Trp–Leu medium (–His/–Ade). This shows that all isoforms are able to interact both with themselves and each other in yeast although not very strongly. Combinations of bait (vertically) and prey (horizontally) plasmids are shown. Lanes 1–5 UGE1–5 in bait plasmid or prey plasmid; lane E, empty bait or prey plasmid; lane N, negative control, human lamin C in bait plasmid; lane 4, four times positive control, pGBK7-T7-53 and pGADT7-T.

**FIGURE 2.** Influence of general reaction conditions on the activity of *A. thaliana* UGE isoforms. A, influence of temperature (percentage of activity at optimal temperature). B, influence of buffer and pH (percentage of activity at optimal pH). MES buffer, pH 5–7; Tris/HCl buffer, pH 6.5–9.5. C, activity of UGEs at 40, 160, and 400 mM relative to salt-free control. For all panels: UGE1, ; UGE2, ; UGE3, ; UGE4, ; UGE5, .
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TABLE 2
Effects of metabolites on UGE activity (percentage of control)

| Metabolite              | UGE1 | UGE2 | UGE3 | UGE4 | UGE5 |
|-------------------------|------|------|------|------|------|
| No NAD<sup>+</sup>      | 99%  | 60%  | 101% | 9%   | 98%  |
| NADH (0.01 mM)          | 95%  | 94%  | 102% | 75%  | 90%  |
| NADPH (1 mM)            | 92%  | 94%  | 95%  | 96%  | 93%  |
| NADP (1 mM)             | 87%  | 80%  | 82%  | 88%  | 84%  |
| NADPH (1 mM)            | 85%  | 94%  | 89%  | 92%  | 89%  |
| UDP (1 mM)              | 29%  | 58%  | 35%  | 83%  | 64%  |
| UTP (1 mM)              | 75%  | 87%  | 82%  | 98%  | 95%  |
| GDP (1 mM)              | 88%  | 93%  | 96%  | 109% | 91%  |
| GTP (1 mM)              | 87%  | 90%  | 94%  | 106% | 103% |
| Galactose-1-P (1 mM)    | 72%  | 82%  | 88%  | 107% | 91%  |
| UDP-D-glucuronate (1 mM)| 97%  | 105% | 97%  | 106% | 93%  |
| UDP-D-xylose (1 mM)     | 78%  | 79%  | 79%  | 100% | 94%  |
| UDP-L-arabinose (1 mM)  | 80%  | 83%  | 74%  | 101% | 81%  |
| UDP-D-N-acetylglucosamine (1 mM) | 84% | 90% | 83% | 91% | 77% |
| Control activity (s<sup>-1</sup>) | 18 | 77 | 45 | 16 | 98 |

UGE4 activity is increased almost 10-fold, and UGE2 activity is stimulated 1.7-fold by 0.1 mM NAD<sup>+</sup> (Table 2). Inclusion of 10 μM NADH in general has little effect; however, UGE4 is slightly inhibited. NADPH has a very weak inhibitory effect on all five isoforms. The most marked effect of various nucleotides on UGE activity was observed with UDP, which reduced UGE1 and UGE3 to ~29–35% of control activity. All isoforms are more strongly inhibited by UDP than by the other nucleotides tested. Interestingly, UGE4 was the one isoform that was inhibited least by all nucleotides tested. Likewise, UGE4 was least inhibited by UDP-sugars, whereas the remaining isoforms were inhibited by UDP-D-glucuronic acid (78–94% of tested. Likewise, UGE4 was least inhibited by UDP-sugars, whereas the remaining isoforms were inhibited by UDP-D-glucuronic acid (78–94% of control), UDP-D-xylose (74–83%), UDP-L-arabinose (55–67%), and UDP-D-N-acetylglucosamine (77–90%).

Sensitivity of UGE2 and UGE4 to Dinucleotide Cofactor—Dependence of UGE4 activity on NAD<sup>+</sup> concentration can be described according to single site saturation kinetics, with half-maximal stimulation (K<sub>s</sub>) by NAD<sup>+</sup> at 3.6 μM (Fig. 3A). The affinity between NAD<sup>+</sup> and UGE2 appears to be higher than between NAD<sup>+</sup> and UGE4, as substantial UGE2 activity (60% of the maximal rate) is detected in the absence of added cofactor. Between 0 and 40 μM NAD<sup>+</sup>, there is a steady increase in UGE2 activity. However, the fit with a two-site saturation model is far better (r<sup>2</sup> = 0.72) than with a single-site saturation model (r<sup>2</sup> = 0.17) (Fig. 3B). It is conceivable that one NAD<sup>+</sup>-binding site per UGE2 dimer binds NAD<sup>+</sup> with very high affinity, whereas the second site has lower affinity. This would explain the residual activity of ~50% in the absence of added NAD<sup>+</sup> and the saturation-type kinetics between 50 and 100% of activity by the addition of increasing levels of NAD<sup>+</sup>.

We further investigated the effect of various combinations of NAD<sup>+</sup> and NADH on UGE2 and UGE4 activity (Table 3). UGE2 is inhibited to 56% of control activity by a combination of 100 μM NADH and 10 μM NAD<sup>+</sup>. UGE4 is maximally inhibited by the same combination (100% of controls) but is also inhibited by all other NAD<sup>+</sup>/NADH combinations.

UGE Kinetics—Curve fitting using Sigmaplot indicates that UDP-Gal-Gal-binding sites in all five UGE isoforms behave according to simple Michaelis-Menten kinetics (not shown). The most obvious difference between the five isoforms is their turnover rate (k<sub>cat</sub>). Under standard assay conditions, k<sub>cat</sub><sup>UDP-Gal-Gal</sup> of UGE1 is lowest at 24 s<sup>-1</sup> and that of UGE5 highest at 128 s<sup>-1</sup> (Table 4). Variations of K<sub>cat</sub><sup>UDP-Gal-Gal</sup> between UGE isoforms are less dramatic, ranging from 0.1 to 0.3 mM for UGE2 and UGE4, respectively (Table 4). Both K<sub>cat</sub><sup>UDP-Gal</sup> and K<sub>m</sub><sup>UDP-Gal</sup> values vary slightly between the different enzyme concentrations used. Despite the partial overlap between K<sub>cat</sub><sup>UDP-Gal</sup> of different UGE isoforms assayed at different enzyme concentrations, the enzymatic efficiencies clearly distinguish the isoforms (Table 4). The enzymatic velocities for UDP-Glc determined at high substrate concentrations allow us to estimate K<sub>cat</sub><sup>UDP-Glc</sup>. For UGE2, -3, and -5, this value is 3–3.9-fold lower than K<sub>cat</sub><sup>UDP-Gal</sup>, and for UGE4, it is 1.2-fold lower (Table 5). Notably, K<sub>cat</sub><sup>UDP-Glc</sup> of UGE1 is 1.33-fold higher than K<sub>cat</sub><sup>UDP-Gal</sup>. When the Haldane relationship is applied to the experimentally determined values of the equilibrium ratio, K<sub>cat</sub><sup>UDP-Gal</sup> and K<sub>m</sub><sup>UDP-Gal</sup>, K<sub>cat</sub><sup>UDP-Glc</sup> can be derived K<sub>cat</sub><sup>UDP-Glc</sup>, relatively high values are obtained for UGE1 (0.76 mM) and UGE4 (0.56 mM), whereas the remaining isoforms display higher substrate affinities for UDP-Glc compared with UDP-Gal.
the global fluctuations of UDP-Glc by UGE isoforms involved in carbohydrate biosynthesis to stresses by co-response analysis in CSB.DB (21) and also by using an alternative approach (22). Both approaches consistently indicate that transcript levels of UGE1 and -3 behave in a fundamentally different way to stresses than do UGE2, -4, and -5 transcript levels (Table 6). The same trend is seen when "abiotic stress aboveground organs," "developmental" or "miscellaneous" data sets, which contain fewer genes than the "abiotic stress roots" data set, are used as the matrix. Generally, there is a strong similarity of global expression patterns of UGE1 and -3 on the one hand and of UGE2 and -5 on the other hand (Table 6). The genes encoding enzymes that are likely to be involved in the generation of UDP-Gal from free galactose are the genes coding galactokinase (GalK, AGI code At3g06580) and the A. thaliana galactose-1-phosphate uridylyltransferase homolog (GalT, At5g18200), and these display a stress-regulated expression pattern similar to UGE1 and UGE3 (Fig. 5A). In contrast, carbohydrate biosynthetic enzymes that utilize UDP-Gal as substrate are expressed in a similar fashion to UGE2, -4, and -5. These include xyloglucan-specific galactosyltransferase MUR3 (24) and its close homolog AtGT18, also thought to be involved in xyloglucan galactosylation (25), as well as several genes encoding galactinol synthase family 20 known to encode trehalose-6-phosphate synthase (26) are co-expressed with UGE1 and -3 (Fig. 6), whereas visibility of UGE2:CFP expression in roots is -3 only trehalose-6-phosphate synthase isoforms 9 and 10 are shown in Fig. 5A). This suggests a role for UGE1 and -3 in the reaction from UDP-Gal to UDP-Glc, which is required for the re-metabolization of galactose, released from catabolized carbohydrates.

UGE2,-4, and -5 appear to be involved in the generation of UDP-Gal from UDP-Glc to generate building blocks for carbohydrate biosynthesis (Fig. 5B).

**TEGRIFICAL DESIGN IN THE CYTOPLASM**—Recombining UGE genes encoding fluorescent protein, either at the amino or at the carboxyl terminus of UGE4, restore the wild type root phenotype in uge4 mutant plants, suggesting that fluorescent protein tagging leaves UGEs functional (data not shown). Translational fusions of UGE1, -2, and -4 and fluorescent proteins are detectably expressed in root tips (Fig. 5). Moreover, UGE1:CFP fluorescence is detected in all cell types of hypocotyls, and UGE2:CFP is detected in guard cells in all aboveground organs (data not shown). UGE1:CFP expression is detected in every cell type of root tip, including the root cap (Fig. 6A) and the root epidermis (Fig. 6, C and D), whereas visibility of UGE2:CFP expression in roots is essentially restricted to the root cap (Fig. 6B). UGE4:GFP fluorescence is detected specifically in roots, with particularly strong expression in the elongating epidermis and cortex cells (Fig. 6E). The cell type-specific
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A. thaliana UGE isoforms (13), the fluorescence of all UGE:fluorescent protein fusions suggests subcellular localization in the cytosol (Fig. 6). The diffuse cytoplasmic fluorescence is interspersed with nonfluorescent vacuoles, sometimes forming transvacuolar strands but otherwise unstructured. Both UGE1:CFP and UGE2:CFP display a comparable localization (Fig. 6, A–D). UGE4:GFP localization also appears to be cytosolic; however, many cells in the root elongation zone also contain brightly fluorescent granular structures potentially highlighting specific organelles (Fig. 6E). Comparable structures are not highlighted by the UGE1:CFP construct that is expressed at a similar level in the root epidermis (Fig. 6, C and D). This suggests that despite their overlapping expression in the root epidermis, UGE1:CFP and UGE4:GFP are differentially localized in the cytoplasm.

Antibodies, raised and affinity-purified against the carboxyl terminus of UGE4, bind to the periphery of Golgi stacks (Fig. 7A, arrowhead) and also diffusely to the cytoplasm (Fig. 7A, asterisk). The absence of a signal in sections of uge4 mutants that contain a premature stop codon demonstrates the specificity of the antibody (Fig. 7B). Chemical fixation resulted in comparable antigen distribution (data not shown). In summary, the isoforms UGE1, -2, and -4 are co-localized in the cytosol, but UGE4 is specifically enriched in the periphery of Golgi stacks in the root elongation zone.

**UGE Isoforms Act in Both Directions in Vivo**—To test the action of various UGE isoforms in planta, we expressed UGE1, -2, -3, and -4 as well as GalE from E. coli in the uge4 mutant under the control of the constitutive cauliflower mosaic virus 35S promoter. The loss of function of UGE4 results in dramatically swollen root epidermal cells (Fig. 8A, uge4), probably a result of defective cell wall matrix carbohydrate biosynthesis (13, 27). This phenotype is rescued by transformation of uge4 with a wild type UGE gene (13), as well as with all overexpressed UGE isoforms from A. thaliana and E. coli (Fig. 4A, 35S:UGE uge4). Mutants in UGE4 display a deficiency in fuco-galactosylated xyloglucan and arabino-glactan type II as highlighted by immunocytochemistry using the monoclonal antibodies CCRC-M1 and CCRC-M7, respectively (13). Like the morphological defects of uge4, the chemical cell wall defects are suppressed by overexpressing UGE1 (Fig. 8, B and C).

To test if the various UGE isoforms can act as UDP-D-galactose 4-epimerases in vivo, we made use of the observation that increased UGE activity enhances galactose tolerance (8). Transfer of 4-day-old untransformed wild type and uge4 mutant seedlings to media containing 40 mM D-galactose as the sole carbon source led to the inhibition of lateral root outgrowth (Fig. 8A) and to necrosis in the primary root tip (Fig. 8B). Plants overexpressing UGE1, UGE2, UGE3, UGE4, or GalE do not show inhibition of lateral root outgrowth (Fig. 8A) and root necrosis (Fig. 8B) upon transfer to 40 mM D-galactose. All A. thaliana UGE coding sequences complement the inability of gal10 S. cerevisiae mutants to grow on a medium containing D-galactose as their sole carbon source, thus demonstrating their UDP-D-galactose 4-epimerase activity in vivo (Fig. 9C).

**DISCUSSION**

Dimeric Structure of Arabidopsis UGE Isoforms—X-ray crystallography and other studies suggest that UGEs are dimeric (3, 4, 28), although active monomers and tetramers were observed under certain experi-
mental conditions (29–31). Gel chromatography of *Vicia faba* extract revealed a major UGE activity peak eluting around 78 kDa and two minor UGE peaks of 39 and 159 kDa suggesting an equilibrium of monomer, dimer, and tetramer favoring the dimeric form (32). Using a similar separation matrix to analyze recombinant purified UGEs, we have observed a single dimer peak in each case. This discrepancy could be due to technical reasons. Alternatively, in the previous study, post-translational modifications of the protein or binding to other protein species could have influenced the apparent molecular weight. Here we show that UGE isoforms interact homo- and heterotypically in yeast. Although it is possible that this is an artificial interaction, not occurring under normal expression conditions, the similar strength of homo- and heterotypic two-hybrid interactions is intriguing, as the combinatorial formation of heteromers in planta might dramatically increase the spectrum of potential enzyme properties.

**Biochemical Diversity of A. thaliana UGE Isoforms—NAD⁺ Requirement**—All known nucleotide sugar 4-epimerases belong to the superfamily of small dehydrogenases/reductases and contain an ancestral structural motif required for NAD⁺ binding called the Rossmann fold. The amino acid signature GXGXXXG or GXXGXGG is typically found at the amino terminus of Rossmann fold proteins. NAD⁺ is essential for catalytic activity as it transiently abstracts a hydride ion from the 4'-OH group of the nucleotide sugar to generate a symmetric 4-ketopyranose intermediate (3). Most previous studies reported that plant UGEs acted independently of added cofactor (32–37). However, UGE purified from wheat germ was stimulated 14-fold by the addition of NAD⁺ (0.29 mM) with a \( K_{m, NAD^+} \) of 40 μM (38). Two *A. thaliana* UGE isoforms, UGE2 and UGE4, are stimulated by addition of NAD⁺. Most surprisingly, UGE5, which is more closely related to UGE2 than to UGE1 and -3 in its peptide sequence (not shown), is insensitive to cofactor addition. This suggests that during plant evolution new UGE isoforms have repeatedly evolved and differ in their capacity to respond to cofactor concentration. It also means that it is probably impossible to predict biochemical properties of UGE isoforms in other plant species based simply on their sequence similarity to UGE4 or UGE2. To make such predictions, more extensive biochemical and structural studies of paralogous UGEs in other species are required. The dose dependence of UGE4 activity on NAD⁺ concentration might allow exchange with and in effect the sensing of cytoplasmic NAD⁺ or NADH. Because the reduced form is enzymatically inactive, this might translate the NAD⁺/NADH redox state to the activity of UGE4. Because cofactor redox state is sensitive to anoxia (39), which typically occurs in roots, the biochemical properties of UGE4 would make it particularly well suited for its specific role in root development (13).

**Inhibition by Metabolites**—Among various metabolites, we find that UDP and UDP-1-arabinose most efficiently inhibit individual UGE isoforms. Interestingly, UGE1 and UGE3 are more strongly inhibited by UDP than any of the other isoforms. When three of the six putative isoforms of GAE were characterized in three different laboratories (40–42), it was found that 2 mM UDP inhibits GAE4 (also known as AtGI-
Plant UDP-glucose 4-Epimerase Isoforms

FIGURE 9. UDP-galactose 4-epimerase activity in vivo. A, overexpressed UGEs suppress the toxic effect of 40 mM α-galactose on lateral root formation. Wild type Col-0 does not produce lateral roots on 40 mM α-galactose (group at top left), whereas 35S:UGE-transformed plants form normal root systems (other groups). B, root tips of Col-0 wild type become necrotically discolored after transfer to 40 mM α-galactose-containing medium (left). Overexpression of UGE2 suppresses root tip necrosis (right). C, all five UGE isoforms complement galactose auxotrophy of S. cerevisiae gal10 mutant. To select transformants the strain contains the Trp auxotrophy marker (upper row). Expression of plant and bacterial UGE restores growth on galactose as the sole carbon source (bottom row).

UDP-Glc to UDP-Gal direction, whereas UGE1 and -3 might act in the UDP-Gal to UDP-Glc direction (Fig. 5B). In the case of UGE1, it appears that both kinetic properties on the one hand and regulation at the mRNA level on the other hand have adapted to this role. Apart from variations of kinetic properties and co-regulation of the abundance of sequentially acting enzymes, another factor potentially contributing to the regulation of substrate flux is co-localization of sequentially acting enzymes, a notion most strongly expressed in the concept of metabolic channeling (see e.g. Ref. 44 for a review of metabolic channeling in plants). Here we show that UGE1 and UGE4 are expressed in the elongation zone of roots and that UGE1, -2, and -4 and presumably all other isoforms are in the cytosolic compartment. In contrast to the exclusively diffuse distribution of UGE1, a fraction of UGE4 appears to localize close to the Golgi apparatus as seen by a bright granular distribution of UGE4-GFP fluorescence (Fig. 6E) and the binding of UGE4-specific antibodies to the periphery of Golgi stacks seen in the electron microscope (Fig. 7A). The molecular mechanism and precise topology of this localization are unknown, as two-hybrid screening and protein pull-down experiments using UGE4 as bait have so far been inconclusive. However, it is tempting to speculate that a subfract of UGE4 binds to the cytosolic face of the Golgi membrane where it could interact with UDP-Gal transporters and galactosyltransferases as suggested previously (7). This hypothetical paradigm of metabolic channeling was used previously as one possible explanation for the specific galactosylation defects occurring in uge4 mutant roots (13). The observation that overexpression of UGE1 suppresses the histologically observed galactosylation defects in uge4 could mean that metabolic channeling is not essential for the flux of UDP-Gal into xyloglucan. However, it is also possible that massive overexpression of an enzyme masks the subtleties of metabolic fine regulation. More detailed mis-expression studies and quan-
titative carbohydrate analyses are required to address this question. We conclude that differences between UGE isoforms exist at the level of enzymatic properties, transcript regulation, and subcellular localization. Taken together these properties are consistent with a role for UGE1 and UGE3 in carbohydrate catabolism and a role of UGE2, UGE4 and UGE5 in carbohydrate biosynthesis. The expression and subcellular localization of UGE4 are consistent with its essential genetic role in cell wall galactosylation in roots (7). Via its apparent sensitivity to the NAD$^+/NADH$ ratio, this isoform might also be involved in the alteration of cell wall structure in hypoxically stressed roots (45).

Biological Significance of Diverse UGE Isoforms—One possible explanation of the apparent biochemical redundancy of UGE and other nucleotide sugar interconversion enzymes in plants is the abundance and complexity of plant carbohydrates, in particular their developmental heterogeneity. The differential expression of kinetically diverse isoforms of nucleotide sugar metabolic enzymes might reflect the ability of each cell to fine-tune the activity of glycosyltransferases in response to external stresses. Co-expression and co-localization of sequentially acting enzymes might be necessary to achieve full control strength. Kinetically diverse isoforms might be better adapted to different physiological intracellular environments in different cell types and organs, especially in organisms devoid of systemic homeostasis.

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