PCP
PLANT & CELL PHYSIOLOGY

An Arabidopsis T-DNA Insertion Mutant for Galactokinase (*AtGALK*, At3g06580) Hyperaccumulates Free Galactose and is Insensitive to Exogenous Galactose

Aurélie Egert[1,2], Shaun Peters[1,3], Christelle Guyot[4], Bruno Stieger[4] and Felix Keller[1,*]

[1]Institute of Plant Biology, Molecular Plant Physiology, University of Zürich, Zollikerstrasse 107, CH-8008 Zürich, Switzerland
[2]Division of Molecular and Systems Toxicology, Department of Pharmaceutical Sciences, University of Basel, Klingelbergstrasse 50, CH-4056 Basel, Switzerland
[3]Institute for Plant Biotechnology, Department of Genetics, University of Stellenbosch, Private Bag X1, Matieland, 7602, South Africa
[4]Division of Clinical Pharmacology and Toxicology, University Hospital Zürich, Rämistrasse 100, CH-8091 Zürich, Switzerland
*Corresponding author: E-mail, fkel@botinst.uzh.ch; Fax, +41-44-634-82-04.
(Received December 9, 2011; Accepted March 12, 2012)

Regular Paper

Galactokinase (GALK, EC 2.7.1.6) is a cytosolic enzyme with a wide occurrence across the taxonomic kingdoms. It catalyzes the phosphorylation of α-D-galactose (Gal) to α-D-Gal-1-P. The cytotoxicity of free (unphosphorylated) Gal is well documented in plants and causes marked defects. An Arabidopsis *GALK* (*AtGALK*, At3g06580) was previously identified, cloned and functionally characterized in *Escherichia coli* and was suggested to occur as a single copy gene in Arabidopsis. We identified an *AtGALK* T-DNA insertion mutant (*atgalk*) that (i) is *AtGALK* transcript deficient; (ii) displays no GALK activity in vegetative tissues; and (iii) accumulates Gal up to 6.8 mg g^{-1} FW in vegetative tissues, in contrast to wild-type plants. By constitutively overexpressing the *AtGALK* cDNA, *atgalk* was functionally rescued. Three independent transformed lines showed restored *AtGALK* transcripts and GALK activity and had low leaf Gal concentrations comparable with those observed in wild-type plants. Surprisingly, in vitro grown *atgalk* plants were largely insensitive to the exogenous application of up to 100 mM free Gal, while wild-type plants exhibited sensitivity to low Gal concentrations (10 mM). Furthermore, *atgalk* seedlings retained the capacity for uptake of exogenously supplied Gal (100 mM), accumulating up to 57 mg g^{-1} FW in leaves. Leaves from soil-grown *atgalk* plants that exhibited no growth or morphological defects were used to demonstrate that the accumulating Gal occurred exclusively in the vacuoles of mesophyll protoplasts. Collectively, these findings suggest a novel Gal detoxification pathway that targets free Gal to the vacuole and is active in the *atgalk* mutant background.

Keywords: Arabidopsis • Galactose detoxification • Vacuole.

Abbreviations: DTT, dithiothreitol; Gal, galactose; GALK, galactokinase; MS, Murashige and Skoog; PAD, pulsed amperometric detection; sqPCR, semi-quantitative PCR; TMT, tonoplast monosaccharide transporter; VGT, vacuolar glucose transporter; WSC, water-soluble carbohydrate.

Introduction

Galactokinase (GALK, EC 2.7.1.6) is a cytosolic enzyme with a wide occurrence across the taxonomic kingdoms, ranging from archaea to plants and mammals (Verhees et al. 2002). It catalyzes the first step of the salvage pathway for re-utilization of free galactose (Gal), the MgATP-dependent phosphorylation of α-D-Gal at the C-1 position to α-D-Gal-1-P, and is, therefore, distinctly different from hexokinases, which phosphorylate hexoses at the C-6 position (Granot 2008). Gal-1-P may be further metabolized to glucose-1-P and UDP-glucose either (i) by the Leloir pathway, mainly found in non-plant organisms, involving a Gal-1-P uridylyltransferase (EC 2.7.7.12) and a UDP-galactose 4′-epimerase (EC 5.1.3.2) (Leloir 1951, Frey 1996, Holden et al. 2003); or (ii) by the pyrophosphorylase pathway (mainly found in plants), involving the novel UDP-galactose/UDP-glucose pyrophosphorylase and UDP-galactose 4′-epimerase (Dai et al. 2006, Dai et al. 2011, Kleczkowski et al. 2011).

In plants, GALK enzyme activity has been reported in a number of species and organs/tissues (for a review, see Keller and Pharr 1996). More recently, Arabidopsis *GALK* (*AtGALK*, At3g06580) was identified via the ability of the cDNA to functionally rescue the growth deficiency of a yeast GALK mutant (Δ*gal1*; Kaplan et al. 1997). Further evidence for the identity of *AtGALK* was reported by its ability to restore GALK activity in an *Escherichia coli* mutant deficient in GALK activity (Sherson et al. 1999). Finally, AtGALK was comprehensively biochemically characterized after expression of the cDNA in *E. coli* (Yang et al. 2009). To date this remains the only GALK reported from Arabidopsis.

Gal toxicity in living cells is well established and widespread, ranging from bacteria (Zeng et al. 2010) to humans, where it causes the inherited metabolic disorder, galactosemia (Berry et al. 2006). In plants, a number of early reports have documented deleterious physiological effects in the presence of

Plant Cell Physiol. 53(5): 921–929 (2012) doi:10.1093/pcp/pcs036, available online at www.pcp.oxfordjournals.org

excess Gal, for instance (i) the inhibition of root or shoot growth (Ordin and Bonner 1957, Ferguson et al. 1958, Roberts et al. 1971, Yamamoto et al. 1988, Sherson et al. 2000, Seifert et al. 2002, Rösti et al. 2007); (ii) the inhibition of auxin biosynthesis and translocation (Anker 1974, Krul and Colclasure 1977); and (iii) the induction of leaf senescence (Morre 1968). The exact mechanism of Gal toxicity in plants is largely unknown (Dey 1985).

In this study, we report on the identification and characterization of an *AtGALK* loss-of-function mutant (*atgalk*) that exhibits no GALK activity and contains up to several hundred-fold more Gal in vegetative tissues (leaves and roots) than wild-type plants. Constitutive overexpression of *AtGALK* in the *atgalk* background restored leaf Gal concentrations to those of leaves from wild-type plants. Surprisingly, the excess Gal in *atgalk* plants resulted in no visible growth or morphological defects, and in vitro grown *atgalk* plants were insensitive to the addition of exogenous Gal up to 100 mM. Wild-type plants showed a distinct sensitivity at concentrations as low as 10 mM. We provide evidence for a hitherto unknown detoxification pathway for Gal that potentially becomes active in the *atgalk* mutant background and targets Gal to the vacuoles of *atgalk* plants.

Results

The *atgalk* mutant is *AtGALK* transcript deficient and accumulates free Gal in vegetative tissues

Of the six independent T-DNA insertion lines analyzed, only one homozygous insertion line (*atgalk*, GABI-Kat 489D10; **Fig. 1A**) showed a distinct high free Gal chemotype in vegetative tissues (leaves and roots), in contrast to wild-type plants which contained undetectable or only trace amounts of free Gal in their vegetative tissues (**Fig. 2B**). The wild-type controls were derived from those plants which genotyped as wild-type (wild-type-like) from homozygoty screens of the GABI-Kat 489D10 line. All further experimentation was conducted on these wild-type-like plants (designated wild-type) since they showed responses comparable with Col-0 (data not shown). *AtGALK* transcripts were present in vegetative tissues of wild-type plants, in contrast to the vegetative tissues of *atgalk* plants (**Fig. 1B**). No GALK activity was detected in vegetative tissues of *atgalk* plants, but leaves (0.072 nkat g^{-1} FW) and roots (0.035 nkat g^{-1} FW) of wild-type plants showed clear activity (**Fig. 2A**). HPLC-PAD (pulsed amperometric detection) analysis of water-soluble carbohydrates (WSCs) from vegetative tissues demonstrated that the free Gal concentrations were high in the leaves (6.8 mg g^{-1} FW) and roots (5.8 mg g^{-1} FW) of *atgalk* plants, in contrast to wild-type plants where Gal was absent in leaves and only present at very low concentrations in roots (0.019 mg g^{-1} FW; **Fig. 2B**). The identity and quantity of free Gal in *atgalk* leaves were additionally confirmed by an enzymatic approach using β-D-galactose dehydrogenase (data not shown).

Functional rescue of *atgalk* with AtGALK restores free Gal concentrations to wild-type levels

The *atgalk* mutant was transformed with a construct carrying the *AtGALK* cDNA under control of the 35S promoter (*atgalk*/*35S::AtGALK*). Six independent transformed lines (T_2 generation) genotyped as homozygous for the *atgalk* allele were selected (**Supplementary Fig. S1A**). They showed clear *AtGALK* transcripts and GALK activities in their leaves (**Supplementary Fig. S1B, C**). For further experimentation, three of these six transformed lines were chosen based on their *AtGALK* transcript abundance and GALK activity (**Supplementary Fig. S1B, C**; T_2 generation), and analyzed in the T_3 generation (designated L1, L4 and L5). GALK activity was detected in all three lines, but only between 16 and 71% of the GALK activity was restored, compared with that of leaves of wild-type plants (0.016, 0.0508 and 0.0228 nkat g^{-1} FW for L1, L4 and L5, respectively; **Fig. 3B**). Most importantly, the leaves of all three lines showed a reversion of the high Gal chemotype of *atgalk* to the trace Gal wild-type chemotype (**Fig. 3A**).

In vitro grown *atgalk* seedlings exhibit insensitivity to exogenous free Gal

Seedlings grown on Murashige and Skoog (MS) medium for 7 d were transferred onto MS medium supplemented or not with free Gal (10 and 100 mM, respectively). After a further 7 d, wild-type seedlings showed distinct growth defects on both 10 and 100 mM Gal (**Fig. 4**). Conversely, *atgalk* seedlings appeared little affected (some reduced lateral root formation and rosette size) by either of these Gal concentrations, and growth was similar to that of *atgalk* and wild-type seedlings after 14 d on Gal-free MS medium. Surprisingly, all three T_3 *atgalk*/*35S::AtGALK* lines retained this Gal insensitivity showing comparable growth characteristics to *atgalk* seedlings grown on 10 and 100 mM Gal (**Fig. 4**), despite their functional rescue as described above (**Fig. 3**).

Root uptake mechanisms for free Gal remain intact in the *atgalk* mutant

To determine if the Gal insensitivity displayed in the *atgalk* mutant background was due to cessation of Gal uptake mechanisms, we firstly tested for the transcript abundance of *AtSTP1* (the primary Gal root uptake transporter; Sherson et al. 2000) in the presence or absence of Gal in the roots of wild-type and *atgalk* seedlings exposed to exogenous Gal for 7 d. *AtSTP* transcripts were present in both wild-type and *atgalk* seedlings, with transcript abundance slightly higher in *atgalk* roots exposed to 100 mM exogenous Gal (**Fig. 5A**). Since 100 mM Gal resulted in severe growth retardation in wild-type seedlings (compared with control wild-type seedlings), we chose an exogenous Gal concentration of 10 mM (for wild-type seedlings) which still presented a reduced-growth phenotype but allowed for sufficient vegetative growth for further analyses. *atgalk* seedlings were, however, grown on 100 mM exogenous Gal since

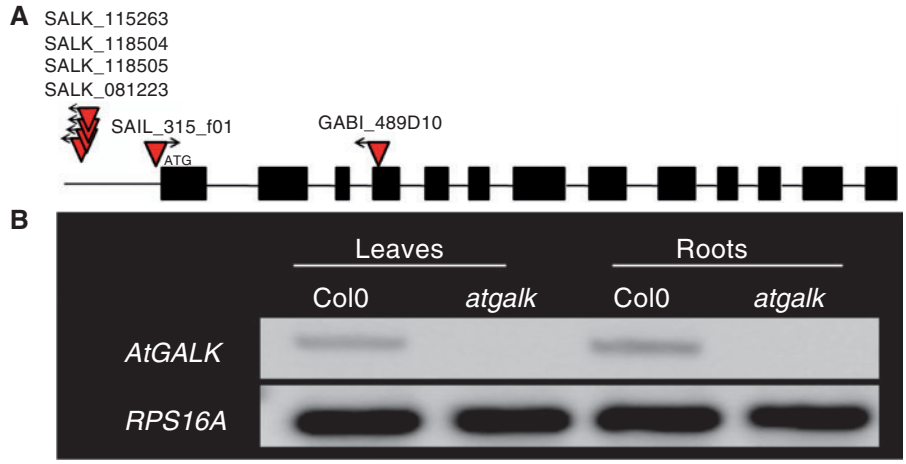

Fig. 1 Analysis of vegetative tissues (leaves and roots) from 6-week-old soil-grown wild-type and *atgalk* plants. (A) T-DNA insertion map of all lines initially tested (red triangles indicate the insertion site). (B) sqPCR of vegetative tissues of the *atgalk* mutant (GABI-Kat 489D10) with *AtGALK* CDS primers. The *RPS16A* gene (At2g09990) was used as a constitutively expressed control. Col0, control plants obtained from homozygoty screens of the GABI-Kat 489D10 line, corresponding to wild-type-like plants.

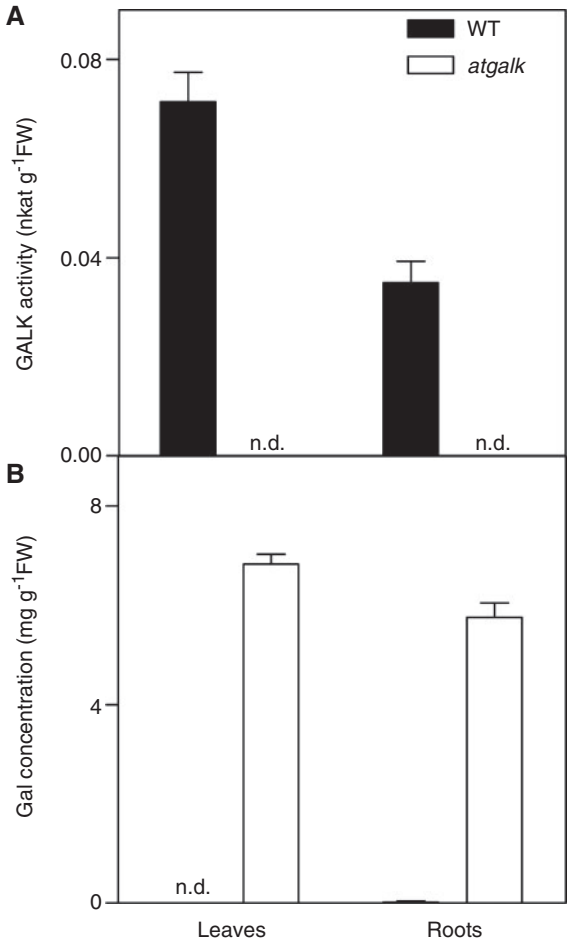

Fig. 2 Analysis of vegetative tissues (leaves and roots) from 6-week-old soil-grown wild-type and *atgalk* plants. (A) GALK activity in vegetative tissues of soil-grown wild-type and *atgalk* plants. (B) Gal concentration in the vegetative tissues of soil-grown wild-type and *atgalk* plants. Data are means ± SE of five replicates. WT, wild-type; n.d., not detected.

they showed no obvious reduced-growth phenotype. We then analyzed the WSC profiles of wild-type and *atgalk* seedlings grown on these exogenous Gal concentrations. In the *atgalk* mutant background, leaves accumulated free Gal up to 57.4 mg g^{-1} FW, while leaves of wild-type plants did not accumulate excess Gal when grown on 10 mM exogenous Gal. The leaves of all three *atgalk*/*35S::AtGALK* lines accumulated some Gal (between 1.3 and 11.4 mg g^{-1} FW), but never to the extent of leaves from *atgalk* seedlings (**Fig. 5B**). In a second approach, we also measured Gal uptake ability using [^{14}C]Gal. Radio-HPLC analysis of [^{14}C]WSCs clearly demonstrated the accumulation of [^{14}C]Gal in the leaves of *atgalk* seedlings grown on 100 mM exogenous Gal, in contrast to the leaves of wild-type seedlings grown on 10 mM exogenous Gal, which did not accumulate any [^{14}C]Gal (**Fig. 5C**). Additionally, the latter did not accumulate the potentially toxic [^{14}C]Gal-1-P as determined by radio-HPLC analysis of leaf [^{14}C]sugar phosphates (data not shown), largely excluding Gal-1-P accumulation as the cause of Gal toxicity in Arabidopsis (Dey 1985).

Free Gal accumulates exclusively in leaf mesophyll vacuoles of soil-grown *atgalk* plants

To determine the subcellular localization of the Gal accumulation observed in leaves of *atgalk* plants, vacuoles were isolated from *atgalk* leaf mesophyll protoplasts. In three independent experiments, the vacuolar fractions were shown to be very pure; no activity of the combined mitochondrial, peroxisomal and cytosolic marker, NADH-malate dehydrogenase, and only traces (1.99%) of Chl present in the protoplasts were detected in the isolated vacuoles (**Table 1**). The distribution of Gal closely paralleled that of the vacuolar marker enzyme, β-*N*-acetylglucosaminidase, allowing the conclusion that the free Gal present in the leaves of the *atgalk* mutant occurs exclusively in the vacuole (**Table 1**).

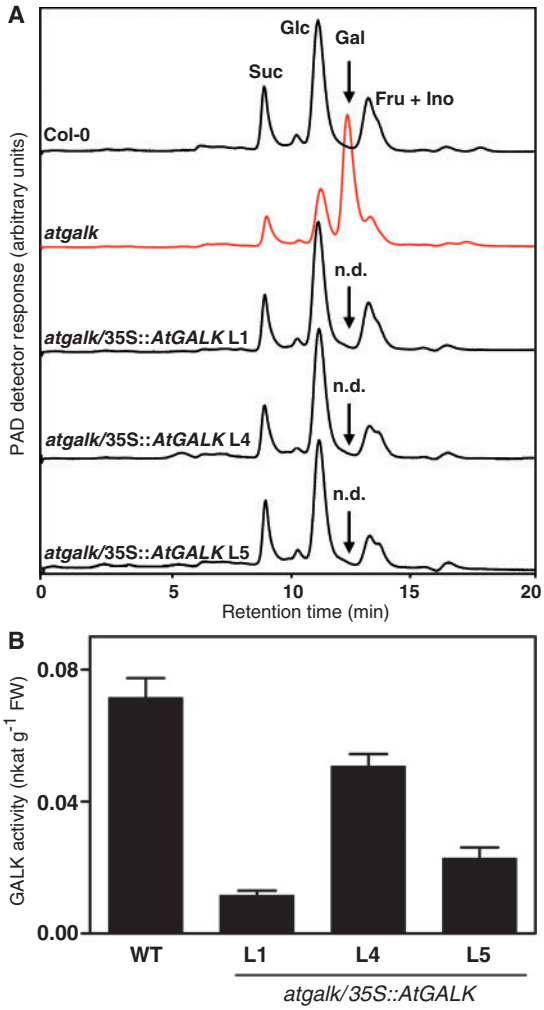

Fig. 3 Analysis of leaves from wild-type and *atgalk/35S::AtGALK* plants (T_3 generation). (A) Representative HPLC traces of total WSCs extracted from the leaves of wild-type, *atgalk* and the three *atgalk/35S::AtGALK* lines, L1, L4 and L5. (B) The *atgalk/35S::AtGALK* lines, L1, L4 and L5 show partially restored GALK activity. Data are means ± SE of six replicates. Ino, *myo*-inositol; n.d., not detected; WT, wild-type.

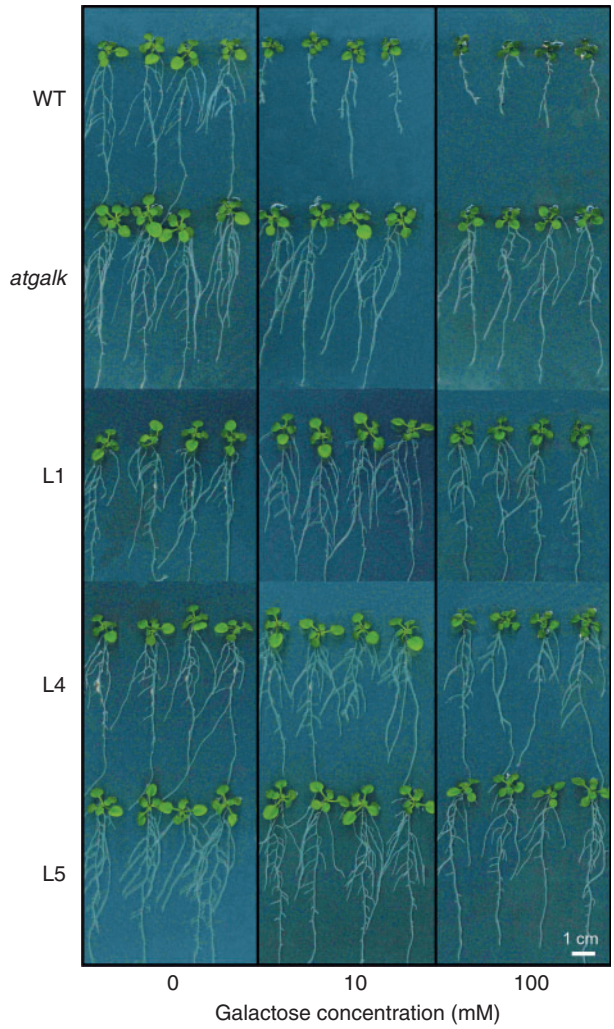

Fig. 4 Representative images of seedlings from wild-type, *atgalk* and *atgalk/35S::AtGALK* lines L1, L4 and L5 grown on half-strength MS medium for 7 d and subsequently transferred to half-strength MS medium supplemented with Gal (10 or 100 mM) for a further 7 d.

Discussion

In this study, we provide clear evidence that the *AtGALK* T-DNA loss-of-function mutant (*atgalk*) hyperaccumulates free Gal in vegetative tissues. Analysis of six independent T-DNA insertion lines identified only one single unique T-DNA loss-of-function mutant (*atgalk*) for *AtGALK* (At3g06580) where both *AtGALK* expression and GALK activity in vegetative tissues (leaves and roots) were completely abolished. In wild-type plants, *AtGALK* transcripts were present in vegetative tissues and a GALK activity was measured. As a consequence of AtGALK loss-of-function, *atgalk* mutant plants hyperaccumulated Gal in vegetative tissues several hundred-fold more than in wild-type plants (**Figs. 2B**, **3A**). Collectively, these observations point to *AtGALK* occurring as a single copy gene in Arabidopsis, as previously suggested (Sherson et al. 1999). Despite free Gal being reported to be cytotoxic to plants (Dey 1985) even at very low concentrations (LD_{50} = 0.7 μM for maize; Roberts et al. 1971), *atgalk* plants showed no obvious morphological or growth defects when grown in soil or in vitro.

Since our initial analyses yielded only a single real loss-of-function mutant as characterized by the absence of GALK activity and hyperaccumulation of Gal in vegetative tissues, *AtGALK* was constitutively overexpressed in the *atgalk* background. Three independent *atgalk/35S::AtGALK* lines were selected where leaf GALK activity was partially restored to the activity in wild-type leaves. Even the lowest activity recovered (16%, L1; **Fig. 3B**) was entirely sufficient to reduce the high free Gal concentrations in the leaves of the mutant to concentrations comparable with those in wild-type leaves (**Fig. 3A**). All three *atgalk/35S::AtGALK* lines were confirmed

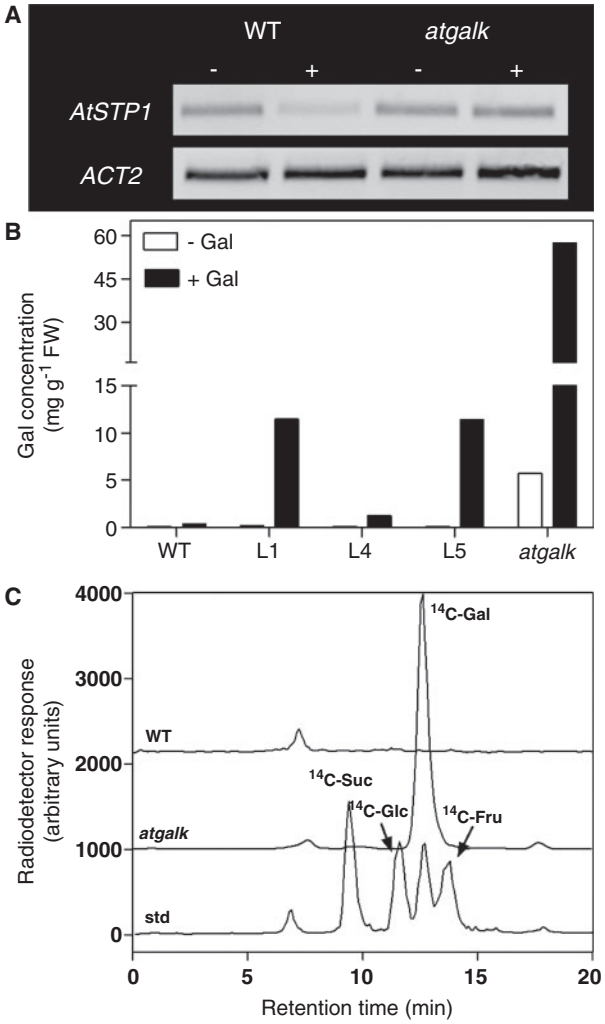

Fig. 5 Two-week-old seedlings grown on half-strength MS medium show uptake (and transport) of Gal from roots to the leaves. (A) sqPCR analyses of *AtSTP1* (At1g11260) transcript abundance in the roots of wild-type (WT) and *atgalk* seedlings grown for 7 d on half-strength MS medium, either without Gal (−) or supplemented with 10 mM (WT, +) or 100 mM (*atgalk*, +) Gal. The *ACTIN2* gene (At3g18780) was used as a constitutively expressed control. (B) Gal concentration in leaves of seedlings exposed to exogenous Gal for 7 d (10 mM for the WT and 100 mM Gal for *atgalk*/*35S:AtGALK* L1, L4, L5 and *atgalk*, respectively). − Gal, without Gal; + Gal, supplemented with Gal. (C) Representative radio-HPLC traces of total WSCs extracted from the leaves of WT and *atgalk* seedlings exposed to [^{14}C]Gal for 7 d. The leaves used in B and C were not in physical contact with the surface of the MS plates. std, [^{14}C]standard containing 0.16 kBq each of [^{14}C]sucrose, [^{14}C]glucose, [^{14}C]Gal and [^{14}C]fructose.

to still genotype as homozygous for the T-DNA insertion in *AtGALK*, demonstrating explicitly that the high Gal concentrations observed in vegetative tissues of *atgalk* were solely due to the absence of AtGALK.

An astonishing finding was that, despite high Gal concentrations in vegetative tissues, in vitro grown *atgalk* seedlings

Table 1 Vacuolar localization of Gal in mesophyll protoplasts isolated from leaves of *atgalk* plants

Substance/enzyme	Protoplasts (μg or nkat ml^{-1})	Percentage in vacuoles
NADH-malate dehydrogenase	12.8 ± 0.8	ND
Chl	256 ± 90	1.99 ± 1.78
β-*N*-acetylglucosaminidase	0.44 ± 0.26	100
Gal	225 ± 31	123 ± 13

Mesophyll protoplasts and vacuoles were isolated from leaves of 6-week-old *atgalk* plants. NADH-malate dehydrogenase activity (mainly indicating contamination by mitochondria, peroxisomes and cytosol) and Chl concentration (indicating contamination by chloroplasts) served as extravacuolar markers. β-*N*-acetylglucosaminidase activity was assumed to be exclusively located in the vacuole. This was confirmed in a control experiment using an additional, well-established vacuolar marker enzyme, α-mannosidase (Keller and Matile 1985; data not shown). Gal was analyzed by HPLC-PAD on a BC-100 column and the data confirmed by enzymatic Gal determination using the β-D-galactose dehydrogenase method. Data are means ± SE of three independent experiments. ND, not detected.

were completely insensitive to exogenous application of Gal (up to 100 mM; **Fig. 4**) and accumulated additional free Gal in the leaves (**Fig. 5**), while wild-type seedlings showed growth defects and severe growth retardation at as low as 10 mM Gal. An identical Gal insensitivity has been previously reported for a T-DNA loss-of-function mutant for the monosaccharide proton symporter *AtSTP1* (At1g11260) which exhibits substrate specificity for mannose and Gal (Sherson et al. 2000). In that study, *atstp1* mutants were also insensitive to exogenous Gal application up to 100 mM. The authors concluded that, since diffusional uptake of hexoses across the plasma membrane is negligible, hexose (Gal) uptake in Arabidopsis seedlings (under normal physiological conditions) is entirely AtSTP dependent. This would effectively explain the Gal insensitivity of the *atstp1* loss-of-function mutant (no Gal uptake when grown on 100 mM Gal). However, such explanations were insufficient when considering the Gal insensitivity of *atgalk* since vegetative tissues already contain up to several hundred-fold more Gal than wild-type plants prior to the addition of exogenous Gal and would be predicted still to contain a functional STP1.

When the transcript abundance of *AtSTP1* was analyzed in the presence or absence of Gal in the roots of wild-type and *atgalk* seedlings exposed to exogenous Gal, it became apparent that the *AtSTP1* transcripts were present in both wild-type and *atgalk* seedlings, with transcript abundance slightly higher in *atgalk* roots exposed to 100 mM free Gal (**Fig. 5A**). Secondly, when roots of in vitro grown seedlings were exposed to exogenous Gal or [^{14}C]Gal, both Gal and [^{14}C]Gal appeared in the leaves of *atgalk* seedlings (**Fig. 5**), confirming that the proposed AtSTP-mediated hexose uptake pathway (Sherson et al. 2000) was still active in the *atgalk* background.

Since *AtGALK* loss-of-function completely abolished GALK activity and led to Gal hyperaccumulation (with no growth defects) and an exogenous Gal-insensitive phenotype, we

believed that a hitherto unknown detoxification pathway for free Gal could explain the Gal insensitivity of *atgalk*. In further experiments, we looked to the most obvious detoxification compartment in the cell, the central vacuole, and asked if the vacuoles of *atgalk* plants accumulated Gal. To this end, mesophyll protoplasts and vacuoles were isolated from leaves of soil-grown *atgalk* plants. Compartmentation analysis of WSCs revealed that *atgalk* vacuoles contained all the free Gal occurring in *atgalk* protoplasts (**Table 1**). This observation would appear to support our argument for a novel free Gal detoxification pathway being activated in the *atgalk* mutant background, allowing the mutant to take up the excess Gal already present in vegetative tissues to the vacuoles. This could also explain the lack of any morphological defects and, possibly, the Gal insensitivity apparent in *atgalk*. Surprisingly, the Gal insensitivity with up to 100 mM exogenous Gal observed in *atgalk* seedlings was retained in the three *atgalk/35S::AtGALK* lines (**Fig. 4**). We assumed that the retention of the Gal insensitivity, despite an apparently complete functional rescue of the *atgalk* mutant background, may imply that the putative Gal detoxification pathway was still active in the *atgalk/35S::AtGALK* lines.

Recent metabolomic approaches have identified some Gal in the vacuoles of soybean (Benkeblia et al. 2007) and barley (Tohge et al. 2011). It would thus appear that vacuolar uptake mechanisms for free Gal in plants are inherent, but, due to the low abundance of free Gal in the cytosol under normal physiological conditions, the absolute concentrations of free Gal in the vacuole are negligible. Under our normal growing conditions (for soil-grown plants) and during routine HPLC-PAD analyses we never observed Gal concentrations exceeding 0.063 mg g^{-1} FW in Arabidopsis wild-type leaves.

The most obvious candidates for a Gal tonoplast transporter would be the tonoplast monosaccharide transporters (TMTs; Wormit et al. 2006, Wingenter et al. 2010, Schulz et al. 2011) or the vacuolar glucose transporters (VGTs; Aluri and Büttner 2007) most of which have been demonstrated to localize to the tonoplast. Apart from VGT1 which showed minimal vacuolar Gal uptake (Aluri and Büttner 2007), neither the TMTs nor VGTs have been tested for their capacity to transport Gal.

In conclusion, we have isolated and characterized a T-DNA loss-of-function mutant for *AtGALK* that (i) shows no GALK activity; (ii) hyperaccumulates free Gal up to several hundred-fold more than wild-type plants; (iii) appears to sequester the free Gal to the vacuoles; and (iv) presents a distinct exogenous Gal-insensitive phenotype. Collectively, these observations led us to speculate that a novel mechanism of detoxification for free Gal became active in the *atgalk* background (artificially high free Gal accumulation). In the future, we will investigate the Gal transport capacity of mesophyll vacuoles isolated from *atgalk* plants to determine if they show an enhanced rate of Gal uptake. Additionally, we will also test candidate genes of tonoplast transporters that may prove to be responsible for Gal uptake into vacuoles.

Materials and Methods

Plant material and growth conditions

Following seed stratification (4°C, 48 h), Arabidopsis (*Arabidopsis thaliana* Col-0) were propagated on soil (Einheitserde, type ED73, Gebr. Patzer GmbH & Co. KG) in a controlled environment chamber (8 h light, 100 µmol photons m^{-2} s^{-1}, 22°C, 16 h dark, 65% relative humidity), unless otherwise stated.

Screening for T-DNA insertion lines

Six independent T-DNA insertion lines (Col-0 background) for *AtGALK* were initially screened (SALK_081223, SALK_115263, SALK_118504, SALK_118505, SAIL_315_F01 and GABI-Kat 489D10; **Fig. 1A**). Genomic DNA was extracted from leaves as previously described (Edwards et al. 1991). Homozygoty was determined by PCR using a combination of gene primers for *AtGALK* and a T-DNA-specific primer (**Supplementary Table S1**). Despite obtaining plants homozygous for the T-DNA insertion in *AtGALK* for all the lines tested, only plants from the GABI-Kat 489D10 line displayed a high Gal chemotype. This line was renamed *atgalk* and used for further experiments.

Enzyme extraction and galactokinase activity assay

Freshly harvested leaf material (200 mg) was ground in 400 µl of chilled extraction buffer [50 mM HEPES-KOH pH 8.0, 2 mM $MnCl_2$, 2 mM $MgCl_2$, 40 mM dithiothreitol (DTT), 1 mM NaEDTA, 2% (w/v) polyvinylpyrrolidone (PVP) K30, 2% (w/v) polyethylene glycol (PEG) 20,000, 2% (w/v) polyvinylpolypyrrolidone (PVPP), 0.1% (v/v) Triton X-100]. Crude extracts were prepared as previously described (Peters et al. 2007, Peters and Keller 2009, Peters et al. 2010) and 200 µl aliquots were centrifuge-desalted (1,700 × *g*, 2 min, 4°C) over Sephadex columns (G25, fine, final bed volume 2 ml). Total GALK activity was measured radiometrically with D-[1-^{14}C]Gal and ATP as the substrates as previously described (Keller and Matile 1985) with minor modifications. The incubation mixture (300 µl) contained 50 µl of desalted extract, 5 mM ATP, 5 mM $MgCl_2$, 10 mM NaF, 1 mM DTT, 50 mM HEPES-KOH pH 7.5, 1 mM D-Gal and 22.2 kBq of D-[1-^{14}C]Gal (specific activity 2,035 MBq $mmol^{-1}$; ARC, ANAWA Trading SA). The assay was conducted for 15 min at 30°C.

Construction of *atgalk/35S::AtGALK* rescued lines

The *AtGALK* cDNA (At3g06580) was obtained as a full-length RIKEN clone (pda 09727, www.brc.riken.jp, Seki et al. 1998, Seki et al. 2002) and was amplified using coding sequence-specific (CDS) primers ($GALK_{fwd}$, 5′-ATGGCGAAACCGGAAGAA GTAT; and $GALK_{rev}$, 5′-TTAGAGGTTGAAGATGGCAGC) with the Expand High Fidelity PCR system (Roche). *AtGALK* was cloned into the pCR8/GW/TOPO vector system (Invitrogen) and subcloned into the GATEWAY destination

vector pMDC32 (Curtis and Grossniklaus 2003) using a conventional LR clonase reaction (Invitrogen). This construct was transformed into *Agrobacterium tumefaciens* (GV3101) by electroporation, using a Genepulser (2.5 kV; 100 Ω; 25 μF; Bio-Rad). Arabidopsis *atgalk* mutant plants were transformed using a floral dip method and hygromycin-resistant plants were selected as previously described (Clough and Bent 1998). T_2 and T_3 transgenic plants were used for analyses.

RNA isolation and semi-quantitative PCR (sqPCR)

Total RNA was extracted from leaves and roots using the RNeasy mini kit (Qiagen), following the manufacturer's instructions. The cDNA templates for sqPCR were obtained by reverse transcription of 1 μg of total RNA with an oligo (dT_{15}) primer and the M-MLV reverse transcriptase (Promega AG) following the manufacturer's instructions. The sqPCR was carried out in 50 μl containing 5 μl of cDNA, 0.5 mM of each dNTP, 0.5 μmol of each primer, 1× PCR buffer and 1.25 U of GoTaq DNA polymerase (Promega), at a primer annealing temperature of 56°C for 24 cycles. The number of cycles chosen for the sqPCR was determined to occur in the linear range of the constitutively expressed *RPS16A* (At2g09990) and *ACTIN2* (*ACT2*, At3g18780) genes. The following primer pairs were used to amplify the corresponding cDNAs: *RPS16A*, $RPS16A_{fwd}$, 5′-GGCGACTCAA CCAGCTACTGA; $RPS16A_{rev}$, 5′-CGGTAACTCTTCTGGTAA CGA); *ACTIN2*, $ACT2_{fwd}$ 5′-ATGGCTGAGGCTGATGATAT and $ACT2_{rev}$ 5′TTAGAAACATTTTCTGTGAACGAT; *GALK*, $GALK_{fwd}$ and $GALK_{rev}$ (see above).

WSC and sugar phosphate extraction and analysis

WSCs were extracted using an ethanol series, as previously described (Peters et al. 2007, Peters and Keller 2009, Peters et al. 2010) with minor modifications. Ground, freeze-dried Arabidopsis leaf material (100 mg) from 6-week-old soil-grown plants was flash-frozen in liquid N_2 and macerated, by hand, using a plastic pestle in a 1.5 ml Eppendorf tube. WSCs were extracted twice (per step) in a three-step sequential process, using 1 ml of 80% (v/v) ethanol, 50% (v/v) ethanol and dH_2O. Extractions were conducted at 80°C for 10 min and the tubes were centrifuged at 15,000 × *g* (5 min, 4°C). WSCs were desalted and analyzed by HPLC-PAD, using a 7.8 × 300 mm BC100 Ca^{2+}/Na^{+}-moderated ion partitioning column (Benson Polymeric; Bachmann et al. 1994, Peters et al. 2007). Sugar phosphates were extracted as described (Sekiguchi et al. 2004) and analyzed by HPLC-PAD, using a 4 × 250 mm CarboPac PA1 column (Dionex) eluted with an NaOH/sodium acetate gradient according to the manufacturer's instructions (Dionex Technical Note 20). [^{14}C]WSCs and [^{14}C]sugar phosphates were detected by separation on a BC100 or CarboPac PA1 column, respectively, coupled to a FLO-ONE radio chromatography detector (Model A-525X; Packard; Peters and Keller 2009). The following [^{14}C]carbohydrates served as standards (0.16 kBq each injected): [U-^{14}C]sucrose, [U-^{14}C]glucose, [1-^{14}C]Gal, [U-^{14}C]fructose and [1-^{14}C]Gal-1-P (from ARC or Hartmann Analytic).

atgalk mesophyll protoplast and vacuole isolation

atgalk mesophyll protoplasts and vacuoles were prepared as previously described (Frelet-Barrand et al. 2008) with modifications. Abraded leaves of 6-week-old *atgalk* plants were incubated with 1% (w/v) Cellulase Y-C and 0.1% (w/v) Pectolyase Y-23 (Seishin Pharmaceutical) for cell wall digestion. The collected protoplasts were purified by centrifugation (2 min at 500 × *g*, followed by 4 min at 1,250 × *g*) in a discontinuous Percoll gradient where resuspended protoplasts (bottom) were sequentially overlayered with 1 vol. of Percoll solution [35% Percoll (v/v), dissolved in MCP solution (0.5 M sorbitol, 1 mM $CaCl_2$, 20 mM MES-KOH pH 6.0)] and 0.5 vol. of MCP solution.

Concentrated protoplasts were recovered from the upper 0/35% Percoll interphase and lysed in 10 vols. of lysis solution described in Frelet-Barrand et al. (2008). Progression of vacuole release was continuously controlled by microscopy, and vacuoles were purified and concentrated by centrifugation (1,400 × *g*, 8 min) using a step gradient as follows: lower phase, 1 vol. of lysate; middle phase, 1 vol. of a 1 : 1 mixture of lysis solution and betaine buffer [0.4 M glycine betaine, 30 mM potassium gluconate, 20 mM HEPES-imidazole pH 7.2, 1 mg ml^{-1} bovine serum albumin (BSA), 1 mM DTT]; and upper phase, 1/3 vol. of betaine buffer. Vacuoles were collected from the interface between the middle and upper phase. For vacuolar yield, the activities of the soluble vacuolar marker enzymes β-*N*-acetyglucosaminidase and α-mannosidase were determined (Keller and Matile 1985). For extravacuolar contamination, the NADH-malate dehydrogenase activity (mainly contamination by mitochondria, peroxisomes and cytosol) and Chl concentration (contamination by chloroplasts) were determined (Schneider and Keller 2009).

Enzymatic Gal quantification

In addition to the HPLC-PAD analyses, free Gal was also quantified enzymatically as described by Keller and Matile (1985) with minor modifications. The enzymatic Gal determination was performed with β-D-galactose dehydrogenase from *Pseudomonas fluorescens* (Roche) in a 96-well microtiter plate (total volume 260 μl), containing 35 μl of extract, 200 μl of 100 mM Tris–HCl pH 8.6, 25 μl of 16.5 mM NAD^{+} and 3 mU of β-D-galactose dehydrogenase. The assay was incubated for 1 h at 37°C and the absorbance was read with a spectrophotometer at 340 nm and compared with a Gal standard curve.

Gal toxicity assay

Surface-sterilized Arabidopsis seeds (wild-type, *atgalk* and *atgalk/35S::AtGALK* lines) were sown onto half-strength MS medium (Murashige and Skoog 1962; Duchefa Biochemie BV) and grown for 7 d in a controlled environment chamber (16 h light, 100 μmol photons m^{-2} s^{-1}, 22°C, 8 h dark, 65% relative humidity). Seedlings were then transferred onto half-strength MS supplemented with Gal (10 and 100 mM, respectively).

After a further 7 d, images of seedlings were captured using a digital camera.

Supplementary data

Supplementary data are available at PCP online.

Funding

This work was supported by the Swiss National Foundation (grant No. 31-116599).

Acknowledgments

We thank Stefan Hörtensteiner for critical reading of the manuscript.

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