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GDUs stimulate amino acid export

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RESEARCH AREA

Bioenergetics and photosynthesis
TITLE

Stimulation of non-selective amino acid export by Glutamine Dumper proteins

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FOOTNOTES

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Phloem and xylem transport of amino acids involves two steps: export from one cell type to the apoplasm, and subsequent import into adjacent cells. High affinity import is mediated by proton/amino acid co-transporters, while the mechanism of export remains unclear. Enhanced expression of the plant-specific type I membrane protein GDU1 has previously been shown to induce secretion of glutamine from hydathodes, and increased amino acid content in leaf apoplasm and xylem sap. In the present work, tolerance to low concentrations of amino acids and transport analyses using radiolabeled amino acids demonstrate that net amino acid uptake is reduced in the glutamine-secreting GDU1-over-expressor gdu1-1D. The net uptake rate of phenylalanine decreased over time, and amino acid net efflux was increased in gdu1-1D compared to the wild type, indicating increased amino acid export from cells. Independence of the export from proton gradients and ATP suggests that over-expression of GDU1 affects a passive export system. Each of the seven Arabidopsis GDU genes led to similar phenotypes, including increased efflux of a wide spectrum of amino acids. Differences in expression profile and functional properties suggested that the GDU genes fulfill different roles in roots, vasculature and reproductive organs. Taken together, the GDUs appear to stimulate amino acid export by activating non-selective amino acid facilitators.
INTRODUCTION

Minerals and organic solutes cycle through the vascular conduits of higher plants (Marschner et al., 1996; Marschner et al., 1997). Cycling requires multiple transport steps from apoplasm to cytosol (cellular import) and from cytosol to apoplasm (cellular export). Cellular import is typically mediated by proton co-transporters able to import solutes against a concentration gradient, e.g. for acquisition of amino acids from the rhizosphere (Hirner et al., 2006; Lee et al., 2007; Svennerstam et al., 2008), for phloem loading (Koch et al., 2003; Lalonde et al., 2003), or for import from the xylem or into developing embryos (Zhang et al., 2007). Export is required for xylem loading (Schobert and Komor, 1990; Gaymard et al., 1998; Takano et al., 2002), efflux into the rhizosphere (Jaeger et al., 1999), and for the transfer of assimilates from leaf cells to the apoplasm before phloem loading (Lalonde et al., 2003), or from the seed coat into the apoplasm to supply developing embryos (Zhang et al., 2007).

Transport of metabolites across membranes is typically mediated by membrane proteins specific for a solute or a class of solutes. During the past two decades, numerous transporters of amino acids belonging to the ATF1 and APC families have been isolated from plants. Several ATF1 members were shown to function as amino acid/proton co-transporters that are characterized by low amino acid selectivity (Rentsch et al., 2007). While the amino acid import process is well characterized at both the physiological level (e.g. Kinraide, 1981; Schobert and Komor, 1987) and molecular level (Li and Bush, 1990; Näsholm et al., 2009), the mechanism of amino acid export from plant cells is still elusive. The physiology of the export has been addressed by a small number of studies that showed that export is independent of the proton-motive force or other source of energization (Jones and Darrah, 1994; De Jong et al., 1997). The identification of cellular exporters has been difficult, since yeast complementation assays that rely on auxotrophies may be unsuitable for identifying exporters, and export assays are more challenging in the context of screens (Pilot & Frommer, unpublished). Recently a putative amino acid exporter BAT1 has been isolated (Dundar and Bush, 2009). BAT1 shares weak sequence similarities with fungal GABA transporters and with CAT amino acid transporters of the APC family (Su et al., 2004). Unlike the APC-member proton-coupled amino acid importers, BAT1 mediates amino acid efflux (Glu and Lys) when expressed in yeast. Interestingly yeast assays also showed that BAT1
mediates Ala and Arg uptake, suggesting that BAT1 may function as a facilitative uniporter (Dundar and Bush, 2009). RT-PCR assays and microarray analyses suggested that BAT1 is expressed at high levels in all plant organs, especially the vasculature (Dundar, 2009).

Previous work had shown that the activation-tagged gdu1-1D mutant secretes glutamine from the hydathodes and is characterized by increased amino acid content in apoplasms and xylem sap (Pilot et al., 2004). Mutant gdu1-1D seedlings were tolerant to amino acids supplied at concentrations that are toxic to the wild type (Pratelli and Pilot, 2007a). The increased tolerance could result either from induction of a detoxification mechanism (Voll et al., 2004), or from reduced net amino acid uptake by roots (Lee et al., 2007). The features of the Gdu1 phenotype are consistent with an increased amino acid efflux.

The work presented here aimed at testing the hypothesis of GDU1 being an activator of amino acid efflux, as well as evaluating the functional role of the GDU paralogs. Amino acid transport in gdu1-1D was studied by uptake and efflux analyses using radiolabeled compounds. The effect of the over-expression of the GDU paralogs on amino acid content and transport was examined. The results show that the seven GDU proteins are able to increase amino acid export by plant cells.

RESULTS

Increased tolerance of gdu1-1D to amino acids

A sensitive, quantitative assay was developed to assess tolerance of plants to toxic levels of amino acid. In contrast to previous systems that determined survival of seedlings at high external amino acid supply (10 mmol L⁻¹; Pratelli and Pilot, 2007a), the new assay measures root growth at relatively low amino acid concentrations. To test the contribution of transport to amino acid tolerance in gdu1-1D, root growth was determined using the lowest amino acid concentration found to inhibit wild type root growth (less than 5 mmol L⁻¹). Length of wild type roots was decreased by 80-95% on media containing Val, Ser, Thr, Phe, Leu, Ile, His, Arg or Gly. By comparison, gdu1-1D roots showed only 0-40% root growth inhibition (Fig. 1, upper panel). Tolerance of gdu1-1D to multiple amino acids synthesized from different metabolic pathways, suggested a reduction of net amino acid uptake (i.e. amounts imported minus amounts exported), rather than elevated detoxification capacity.
Reduced net amino acid accumulation in *gdu1-1D*

Uptake capacities of various radiolabeled compounds were compared in *gdu1-1D* and wild type. Phe uptake was reduced by 55%, while glucose and methyl-ammonium uptakes were unaffected or slightly increased. Uptake of other amino acids was also reduced in *gdu1-1D*: 50-60% for Arg, Pro and Gly and 20% for His and Glu (Fig. 2A and 2B). Reduced uptake of Phe, Arg, His and Gly correlated with the tolerance of *gdu1-1D* to these toxic amino acids (Pro and Glu were not toxic to the wild type; Fig. 1).

Based on the large reduction of uptake and its effect in the tolerance assay, Phe was chosen for more detailed analyses. The uptake rate of radiolabeled Phe by wild type plants followed a biphasic curve over the range 0.01 - 5 mmol L\(^{-1}\). Treatment with the protonophore carbonyl cyanide 3-chloro-phenylhydrazone (CCCP) inhibited the high-affinity component (hyperbolic curve) revealing the activity of the low affinity component (linear curve; Fig. 2C). Phe uptake was analyzed at three concentrations, each concentration revealing a different contribution of the high-affinity transport to the total uptake (80, 60 or 40%). After 2 min, Phe accumulated at the same level in *gdu1-1D* as in wild type (Fig. 2D), suggesting that the initial import capacity of Phe is similar. Phe uptake in wild type followed linear kinetics, corresponding to a constant uptake rate, as described for other amino acids (Schobert and Komor, 1987; Heremans et al., 1997; Hirner et al., 2006). On the contrary, the uptake rate in *gdu1-1D* decreased over time, leading to a lower Phe accumulation in *gdu1-1D* plants after 10 min (Fig. 2D, middle and right panel).

A decrease in uptake rates over time could result from: (1) feedback-inhibition of the uptake by accumulation of Phe, or (2) increased amino acid export in *gdu1-1D*. When plants were pre-treated with 1 mM Phe prior to uptake analyses, little difference in the time course of Phe accumulation for both wild type and mutant was observed (Fig. 2E). This finding argues against a contribution of cytosolic Phe accumulation in inhibiting its own uptake. The unchanged initial Phe uptake together with the unchanged uptake kinetics after Phe pre-treatment favor the second hypothesis, i.e. an increase in amino export as a result of *GDU1* over-expression.

Increased amino acid efflux from *gdu1-1D*

To further resolve Phe export capacity, seedlings were preloaded with \(^{14}\)C\)Phe and the net efflux (i.e. export minus import) of radiotracer into the medium was measured. Thin layer chromatography analysis of the medium proved that the radiolabeled compound released by
the plants was Phe (Supplemental Figure S1A). The amount of Phe released into the medium was higher in gdu1-1D than wild type plants (150 and 64 nmol mg DW\(^{-1}\) respectively; Table I). In gdu1-1D, released Phe amount corresponded also to a higher fraction of the total radioactivity taken up, defined as the sum of Phe amounts left in the plant and released into the medium (~60% in gdu1-1D versus ~15% in wild type). Similar experiments showed that efflux of radiolabeled His and Glu were also increased in gdu1-1D, but not the efflux of radiolabeled glucose (Supplemental Figure S1B).

It has been proposed that a fraction of the amino acids exported from roots is re-imported by high-affinity transporters, controlling amino acid net efflux (Schobert and Komor, 1987; Jones and Darrah, 1993). The contribution of re-import activity to amino acid net efflux was assessed using the lht1-1 mutant, carrying a TDNA insertion in the LHT1 gene, which encodes a dominant high affinity amino acid importer (Hirner et al., 2006, Svennerstam et al., 2007). LHT1 contributed for about 75% of Phe uptake when supplied with 10 μM Phe (Phe uptake was 5.6 in lht1-1 versus 20.8 nmol mg DW\(^{-1}\) in the wild type; Table I), but for about 25% when supplied with 100 μM or 1 mM Phe (Table I, Supplemental Figure S1C). The decreased import of lht1-1 was expected to lead to a decreased Phe re-import and hence an increase in Phe net efflux. Surprisingly, the fraction of labeled Phe exported after pre-loading with 10 μM or 1 mM Phe was comparable in lht1-1 and wild type (~15%, Table I), suggesting that Phe net efflux is not controlled by the high-affinity import system. The comparable Phe efflux from gdu1-1D and gdu1-1D / lht1-1 double mutants further supported the hypothesis that Phe efflux is independent from LHT1-mediated import (Table I). The reductions in Phe uptake caused by the lht1-1 and gdu1-1D mutations were additive (Table I and Supplemental Figure S1C), as expected if reduced import is cumulated with increased export.

To evaluate the selectivity of amino acid efflux, amino acid content in plants and conditioned medium (medium from plants grown for two days) was analyzed. Amino acid content was nearly identical or even decreased in gdu1-1D plants compared to the wild type plants (e.g. Pro, Tyr, Val, Met, Ile, Lys and Leu; Table II). In contrast, gdu1-1D growth medium contained about four times as much amino acids as for the wild type (Table II). The content in most amino acids was increased in the medium, and Asn and Gln accounted for about 75% of the total increase, suggesting that the export mechanism stimulated by GDU1 over-expression is non-selective, but shows a preference for Asn and Gln.
Inhibitors as tools for dissecting GDU1-mediated efflux

One may speculate that in- and export may be differentially sensitive to inhibitors, specifically to protonophores. Net Phe efflux was thus measured in the presence of uncouplers (CCCP and 2,4-dinitrophenol [DNP]), or inhibitors of ATP hydrolysis (orthovanadate [VO$_4$]) and proton pumps (diethylstilbestrol [DES]), shown to inhibit proton-coupled high-affinity amino acid import (Jones and Darrah, 1993). Uptake of 5 mM Phe in presence of the inhibitors was decreased by up to 48% in the wild type (CCC treatment) and 22% in gdu1-1D (DNP treatment; Fig. 3A). Independent of the conditions, Phe net efflux was always higher in gdu1-1D (Fig. 3B), suggesting that the GDU1-stimulated export mechanism is independent of ATP and proton gradient across the membrane, which is characteristic of a passive uniport system.

Over-expression of GDU paralogs

The Arabidopsis genome encodes six GDU1-like proteins (called thereafter GDUs) that may share functions similar to that of GDU1. Five T-DNA insertions in the GDU genes caused more than 95% reduction in the content of the corresponding mRNA. Homozygous plants did not present any morphological abnormality when grown on soil and no change in Phe uptake or efflux was observed (data not shown). Sequence conservation amongst the GDUs (Pratelli and Pilot, 2006; Pratelli and Pilot, 2007b) and the absence of an obvious phenotype for the knock-out lines may suggest that GDU proteins are functionally redundant.

To test whether all GDUs elicit amino acid efflux, each of the seven genes was expressed under control of the CaMV 35S promoter (35Sp), leading to a 100- to 50,000-fold increase in mRNA levels relative to their endogenous levels in wild type. For each construct, six to eight transformant lines were selected that segregated 3:1 for the kanamycin resistance marker, and whose kanamycin resistant offspring was phenotypically identical. GDU mRNA accumulation, rosette diameter and amino acid content were determined. Several lines containing the 35Sp-GDU2, 35Sp-GDU3 and 35Sp-GDU4 constructs also over-accumulated GDU1, GDU3 or GDU6 mRNAs (circled symbols in Supplemental Figures S2 and S3). No other GDU gene was over-expressed in the GDU1, GDU5, GDU6 and GDU7 over-expressors. The reason for induction of other GDU genes is not clear at present, but it probably hints at complex regulations of the expression of the GDUs. Lines showing over-accumulation of the targeted GDU mRNA and not any other GDU were used for further
characterization, and were named GDU1-OE to GDU7-OE thereafter (Supplemental Table S1).

Except for GDU4 and GDU7, plant size decreased with mRNA accumulation of the GDU genes (Fig. 4A; Supplemental Figure S2), and free amino acid content of all the GDU-OEs increased in correlation with the intensity of the over-expression (Supplemental Figure S3), similar to what was observed with GDU1 over-expression (Pilot et al., 2004). HPLC analyses from two independent experiments showed that the content in nearly all free amino acids was increased in the GDU-OEs, except for Asp and Glu (Fig. 4B; Supplemental Table S2). The effects of the over-expression of the GDUs on plant size, amino acid content suggest that all the GDUs have similar functional properties, probably targeting a common mechanism for amino acid efflux.

**GDU expression affects amino acid export**

Amino acid tolerance of one GDU-OE line per gene was assessed using the root growth assay described previously. Root length was almost identical for the over-expressors and wild type grown on Cys (Fig. 5), Met and Trp (data not shown). Except for GDU7, the over-expression of the GDUs led to a tolerance to most of the toxic amino acids (Fig. 5, upper panel), similar as found in the case of gdu1-1D.

Phe net uptake was reduced for all GDU-OEs compared to the control (Fig. 6A). Phe accumulation kinetics in two GDU7-OE lines was nearly linear, with an uptake rate ~10% lower than the control. Radioactivity accumulation kinetics measured for two lines over-expressing the five other genes was characterized by a decrease in the uptake rate over time, as observed for gdu1-1D (Supplemental Figure S4). Amino acid efflux analyses showed that a higher fraction of the incorporated radioactivity is exported by the GDU-OEs (~35-50%), compared to the control (~12%), except for GDU7-OE (~17%, Fig. 6B), indicating that Phe export is increased in almost all GDU-OEs.

The culture medium in which the GDU-OEs had been grown for two days contained 2 to 60 times more amino acids compared to control plants grown in the same conditions. Gln and Asn accounted for ~50-70% of the increase (Supplemental Table S3). The amino acid profiles were similar for all GDU-OEs, and different from the control, especially concerning the amino acids Asp, Glu, Asn, and Gln (Supplemental Figure S5). Amino acid content of the plants employed for this in vitro assay was slightly increased (by ~30 to 50%) for GDU3-, GDU4-, GDU5-, GDU6- and GDU7-OEs but not for GDU1-OE and GDU2-OE (Supplemental Table
S4), while all GDU-OEs exhibited elevated amino acid content when grown on soil (Fig. 4B). The increased release of amino acids into the growth medium suggested that similar non-selective amino acid export systems are stimulated by over-expression of any of the seven GDUs.

The GDU genes display specific expression patterns that overlap partially in the vascular tissues

Different roles of the GDUs in the plant could be revealed by specific expression patterns for each of the genes. Analysis of the expression of the GDUs genes showed that the mRNA accumulation levels varied greatly, from 12 ng GDE for GDU6 to 1.9 μg GDE for GDU3, in agreement with an analysis of Arabidopsis gene expression using tiling arrays (Table III; Laubinger et al., 2008). Both RT-PCR and tiling array showed that all GDUs were mainly expressed in roots and stems (Table III). To analyze cell specificity of the expression, stable transformants expressing GDU promoter-GUS fusions were generated. Promoter activity of all GDUs was detected in the vascular tissues of the roots (Fig. 7A), and, except for GDU6, in vascular tissues of leaves and stems (Fig. 7B, C). While the expression patterns overlapped in the vasculature, each GDU gene showed expression specificity for other cell types (see Supplemental Table S5, Supplemental Text1 and Supplemental Figure S6). For instance, GDU5 promoter drove GUS activity in guard cells, GDU3 promoter was active in anthers (Fig. 7D), and GDU4 and GDU7 promoters were the only ones to be active in the minor veins of the leaves, suggesting that the differences in localization of the GDU proteins could account for specific roles in different tissues.

DISCUSSION

Over-expression of the GDU genes stimulates amino acid efflux

Plant cell growth is sensitive to high levels of exogenously supplied amino acids (Heremans and Jacobs, 1994; Bonner et al., 1996; Voll et al., 2004), due to feedback inhibition of amino acid biosynthesis pathways (Less and Galili, 2008). Amino acid resistant mutants have been found to express feedback-insensitive enzymes (Mourad and King, 1995; Li and Last, 1996; Heremans and Jacobs, 1997), show reduced amino acid uptake activity
(Bright et al., 1983; Heremans et al., 1997; Lee et al., 2007), or display a deregulation of amino acid metabolism like the pig1-1 mutant (Voll et al., 2004). In analogy to pig1-1, tolerance of gdu1-1D to exogenously supplied amino acids was proposed to result from a perturbation in amino acid metabolism (Pratelli and Pilot, 2007a). The elevated amino acid content of gdu1-1D apoplas (root xylem sap and leaf apoplasm wash fluid) also suggested an increased net efflux of amino acids from the cells (Pilot et al., 2004).

Phe, Arg, Pro, Gly, Glu and His net uptake was lower in gdu1-1D compared to wild type while time course analyses indicated that the high affinity uptake capacity of Phe is identical in gdu1-1D and the wild type (Fig. 2D). Direct determination of amino acid efflux and analysis of amino acid content in the growth medium showed that amino acid export is enhanced in gdu1-1D. The observed decrease in Phe uptake rate shown in Fig. 2D can then be explained as follows. At the beginning of the uptake experiment, Phe is more concentrated in the medium than in of the cytosol (1 mM Phe in the medium and < 0.5 mM Phe in the cytosol; Farré et al., 2001). Proton-coupled uptake systems concentrate Phe in the cytosol. When cytosolic concentration of Phe exceeds the external concentration, Phe diffuses out of the cell along the concentration gradient via the passive export system enhanced in gdu1-1D. This futile cycle of successive import and export would result in a decreased net uptake rate. Lower amino acid uptake has been shown to lead to amino acid tolerance (Bright et al., 1983; Heremans et al., 1997; Lee et al., 2007). A decreased amino acid uptake (observed for Phe, Arg, Gly, Pro, Glu and His) consequent to an increased amino acid export would result in the amino acid tolerance described in Fig. 1. Enhanced amino export would also explain increased amino acid in the xylem, apoplasm and secretion of glutamine at the hydathodes of gdu1-1D.

The over-expression of the GDU genes using the CaMV 35S promoter led to plants that were phenotypically similar to the activation-tagged mutant gdu1-1D. The GDU-OEs displayed increased free leaf amino acid content when grown on soil (Fig. 4B), enhanced amino acid tolerance, decreased Phe uptake rate over time, and increased amino acid export into the medium. All the GDU-OEs exported a similar set of amino acids, different from the wild type (Supplemental Figure S5). All GDUs have similar functional properties and are able to increase amino acid export upon over-expression. The weaker phenotype of the GDU7-OEs (Fig. 4 and 6), is possibly related to the sequence divergence of GDU7 from the other genes (Pratelli and Pilot, 2007b).
Mechanisms of amino acid efflux in plants

Tolerance of *gdu1-1D* to 11 out of 14 toxic amino acids (Fig. 1; Pratelli and Pilot, 2007a), reduction of Pro and Glu uptake and increased release into the medium of most amino acids (Table II) suggested that at least 16 amino acids are substrates of the export mechanism enhanced by the *GDU1* over-expression. The transport appeared not specific to any amino acid but seemed to be more efficient with Gln and Asn. The observed similarity of the amino acid composition of apoplasm and cytosol further indicate that the amino acid export system is poorly selective (Lohaus et al., 1995; Pilot et al., 2004; Hirner et al., 2006).

The *GDU1*-stimulated amino acid export mechanism was not dependent on the proton gradient or ATP hydrolysis (Fig. 3). Consequently, vesicular trafficking and ABC transporters, requiring ATP for energization, and putative amino acid/proton antiporters are not candidates for the export mechanism. Another possibility would be a facilitator, which allows passive transport of amino acids along the concentration gradient. The increased activity of a facilitator in *gdu1-1D* is expected to lead to increased Phe uptake when external Phe concentration is higher than in the cytosol (e.g. 5 mmol l⁻¹, Fig. 3A), which was not observed. The *GDU1*-stimulated amino acid export then appears to be mediated by a system able to transport amino acids only towards the outside of the cell. Such a system and the corresponding export mechanism still remain to be identified in plants.

Amino acid exporters from other organisms have been found to be (i) highly selective, like the vesicular GABA and glutamate exporters of neurons (McIntire et al., 1997; Takamori et al., 2000); (ii) selective for a class of amino acids, such as the amino acid exporters from microbes (Eggeling and Sahm, 2003); or (iii) poorly selective, as AQR1, an amino acid exporter cloned from yeast (Velasco et al., 2004). The cloning and the characterization of the transporter BAT1 has recently shed light on plant amino acid export systems (Dundar and Bush, 2009). Amino acid tolerance and uptake experiments have suggested that BAT1 can both import and export amino acids when expressed in yeast, and might be poorly selective for amino acids. BAT1 is expressed at high levels throughout the plants, especially in vascular tissues (Dundar, 2009), compatible with an important role in amino acid export from cells.

Because of its expression and functional properties, BAT1 would be a candidate for the export system stimulated by *GDU1* over-expression. By analogy to the effects of subunits of animal transporters, *GDU1* would modulate the trafficking and/or the functional properties of BAT1. Mammalian heteromeric amino acid transporters require the activity of a 1-TM protein.
for correct targeting to the plasma membrane (Palacin and Kanai, 2004; Verrey et al., 2004).

Transporter functional properties have been also shown to be modified by regulatory subunits. For instance, association of β-subunits to potassium channels can modify the opening properties of the complex (McCormack et al., 1999). In this context, GDU1 could be an accessory subunit of BAT1, controlling its cellular trafficking and/or functional properties: upon GDU1 over-expression BAT1 could accumulate, or be more active at the plasma membrane and function as an exporting-only amino acid transporter. This hypothesis could be tested by analyzing the phenotype of a gdu1-1D / bat1 double mutant, or the trafficking of BAT1 protein in a gdu1-1D background.

**Differences in the role of the GDU genes**

The absence of an apparent phenotype of the GDU knock-out mutants, the overlapping expression patterns and the overall similar phenotype of the GDU over-expressors suggest functional redundancy among the GDU proteins. GDU proteins are expressed in regions where amino acid transport is known to occur. Phloem has been shown to unload in seeds at the chalaza/funiculus region (Stadler et al., 2005), where GDU1 and GDU2 are expressed (Supplemental Figure S6). Amino acid efflux has been detected from roots (Schobert and Komor, 1987; Jones and Darrah, 1993; Phillips et al., 2004), and it has been suggested that efflux takes place mainly at the root tip, where GDU2 and GDU3 are expressed. Amino acids constitutively exported from roots are recovered by active transporters (Schobert and Komor, 1987; Phillips et al., 2004). A similar efflux / import cycle is also suspected to occur along the root stele, where high affinity amino acid importers (Okumoto et al., 2002; Okumoto et al., 2004) and the GDU proteins are expressed. GDU genes could also be involved in apoplastic transport of amino acids from one cell to another, necessary for xylem loading and phloem re-loading (Schobert and Komor, 1990; Lalonde et al., 2003).

The phenotypes of the GDU-OEs are not identical in every aspect. Several differences were noted for the amount of secretion crystals on the leaves (Fig. 4A), and the magnitude of the size reduction and amino acid content upon over-accumulation of the GDU mRNAs (Supplemental Figure S2 and S3). In addition to the specificities of expression patterns (Supplemental Figure S6 and Supplemental Table S5), the phenotypical differences suggest that the GDU proteins play different roles in the plant and are endowed with similar but not identical functional properties.
Conclusion

Uptake of radio-labeled amino acids by gdu1-1D was reduced and gdu1-1D plants excreted more amino acids in the growth medium than the wild type, indicating an increased amino acid export. The over-expression of the GDU1-like proteins from Arabidopsis led to amino acid tolerance and increased amino acid export, suggesting that the GDU proteins have a similar function. Differential expression of the seven GDU genes suggests specific roles in amino acid export in different cell types. The data presented here provide direct evidence that the over-expression of the GDU genes specifically stimulates amino acid export, and that they potentially act as regulators of amino acid exporters. It is expected that the study of the GDUs and interacting proteins will shed light on the poorly understood export mechanisms in plants.

MATERIAL AND METHODS

Plant growth and transformation

Arabidopsis plants (ecotype Columbia-7) were grown in soil (Floragard type B without clay from Floradur, Germany) in the greenhouse or in a growth room (16 h light, 120 µmol m⁻² s⁻¹, at 23°C) and were watered from below. Fertilizer was applied once at the time of bolting.

Constructs were introduced into plants using Agrobacterium tumefaciens GV3101 (pMP90) and the floral dip method (Clough and Bent, 1998). Transgenic plant selection and segregation analyses for kanamycin resistance were performed in vitro on Murashige and Skoog (MS) medium (Phytotechnology laboratories, Shawnee Mission, KS) containing 50 µg mL⁻¹ kanamycin, 1% (w/v) sucrose and 0.7% (w/v) agar (16 h light, 120 µmol.m⁻².s⁻¹ at 22°C).

For amino acid tolerance experiments, surface sterilized seeds were sown using 0.1% (w/v) agar on half-strength MS medium containing 0.5 % (w/v) sucrose, 0.5 g L⁻¹ MES pH 5.7, 0.8% (w/v) agar and various concentrations of amino acids. Plants were grown vertically for 10 days in a growth cabinet (16 h light, 22°C and 80 µmol m⁻² s⁻¹). Plates were scanned and root lengths were calculated using imageJ (http://rsbweb.nih.gov/ij/).
Expression analysis

Total RNA extraction and Northern blotting were performed as described previously (Pratelli and Pilot, 2006). Over-accumulated mRNAs were quantitated by reference to a dot blot. For this purpose, amounts from 2 to 500 amol of PCR fragments corresponding to the full coding sequence of the GDU genes were dotted on a nylon membrane using 0.4 N NaOH. These membranes were hybridized at the same time and in the same hybridization bottle as the membranes used for the RNA gel blots. Signals were quantitated with the Typhoon 9400 scanner and ImageQuant software (GE Healthcare, USA). Pixel intensities on RNA gel blots were converted in amol DNA using the standard curve obtained with the dot blots. Probes corresponded to the coding sequence of the GDU genes.

For real time RT-PCR, RNA (10 µg) was treated with DNAse I (Invitrogen, Carlsbad, CA) for 30 min at room temperature and precipitated in the presence of ethanol. After solubilization in 10 µL water, 2.5 micrograms of RNA were reverse-transcribed using the SuperScript® III Reverse Transcriptase (Invitrogen) in a 10 µL reaction. Real time PCR was performed on 5 µL of a 50-fold dilution of the reverse transcription product using the Lightcycler 480 SYBR Green I Master mix and detected with a Roche 480 Lightcycler (Roche Applied Science, Indianapolis, IN). Threshold cycle values were determined by the Roche Lightcycler 480 SW 1.5 software. Amplification efficiencies were determined using amounts from 2.5 pg to 2.5 ng genomic DNA as template, and used for absolute quantitation of mRNA levels. Absolute quantities of mRNA are given in Genomic DNA Equivalent (GDE), corresponding to the amount of genomic DNA that would be needed as template to get the same threshold cycle as the reverse transcription template. Sequences of the oligonucleotides used as primers for quantitative PCR are available upon request.

Constructs

pPTkan3 and pUTkan binary vectors are derivatives of pJHA212K (Yoo et al., 2005). The promoterless pUTkan contains, in this order, a multiple cloning site (MCS), the E. coli β-glucuronidase coding sequence and the Rubisco terminator from pea. pPTkan3 contains the CaMV 35S promoter, a MCS and the Rubisco terminator from pea. Promoters of the GDU genes (between 1300 and 3050 bp) were amplified by PCR (Pfx accuprime, Invitrogen) from genomic DNA and cloned into pUTkan using restriction enzymes. DNA fragments starting at the start codon and ending close to the poly-A tail of the GDU mRNAs were amplified by PCR as above and cloned into pPTkan3. All amplified fragments were sequenced after cloning to
ensure the absence of any error. Cloning strategies, primer sequences and resulting plasmids are available upon request.

**Localization of gene expression**

Histochemical staining of GUS activity was performed on *in vitro*- and soil-grown plants as described (Lagarde et al., 1996). Histochemical staining reactions were performed at 37°C for time ranging from 2 to 24 h, depending on the organ and the studied gene. After staining, and clearing in ethanol, stems were embedded in 4% (w/v) agarose and cut into thin sections by hand. For each gene, the localization of the staining was studied on about 20 independent plants. Histochemical staining of plant organs was more precisely investigated in parallel on six lines representative for the most frequently observed staining pattern and showing a similar expression level.

**Amino acid quantitation**

Tissues were frozen in liquid nitrogen, freeze-dried and ground with a 5-mm steel ball in a tissue-lyzer (Qiagen, Hilden, Germany). Amino acids were extracted from the dry powder by 500 μL 80% (v/v) ethanol at 80°C for 30 min. The pellets obtained by centrifugation at 16,000 g were extracted a second time in the same way. The supernatants were pooled and dried under vacuum.

Total amino acid content was determined by reaction with ninhydrine. Dried samples were solubilized in 250 μL 20% ethanol. After centrifugation, 30 μL of the supernatants were mixed with 200 μL of ninhydrine reagent (3.34 M propionic acid, 2.1 N NaOH, 50% (v/v) 2-ethoxy-ethanol, 2% (w/v) ninhydrine) and 100 μL 0.1% (w/v) ascorbic acid. The mixture was heated for 10 min at 95°C, cooled for 5 min on ice and the OD 570 was determined after addition of 500 μL 60% (v/v) ethanol. Reactions with increasing amounts of glycine were used to establish a standard curve.

For determination of amino acid content in plants, extracts were prepared as above. For determination of amino acids present in the medium, growth solutions were filtered (0.2 μm pore size) and dried under vacuum. Amino acids were then derivatized with the fluorophore 6-aminoquinolyl-N-hydroxysuccimidyl carbamate (AccQ Taq, Waters Corp, Milford, MA) and the amino acid derivatives were separated at a flow rate of 1 mL min⁻¹ at 37°C on a Dionex Summit HPLC system essentially as described (van Wandelien and Cohen, 1997) using the
eluents A (140 mM sodium acetate, pH 6, 7 mM triethanolamine), B (acetonitrile) and C (water), and detected by fluorescence (excitation at 300 nm, detection at 400 nm).

**Amino acid transport analysis in plantlets**

Arabidopsis plantlets were grown for 7 days (16h light, 80 120 µmol m⁻² s⁻¹ at 21°C) on solid (0.7% agar) half strength MS medium containing 1% (w/v) sucrose, pH 5.7. Plates contained 50 µg mL⁻¹ kanamycin for the selection of plants harboring the 35Sp-GDU constructs or the 35Sp-GFP construct, used as control. About six plants were immersed into 3 mL half strength MS medium containing 1% (w/v) sucrose, pH 5.7, and grown in a 12-well plate under gentle shaking (40 rpm) for 4-5 days with the same light regime. One hour before transport analyses, plants were transferred to a 24-well plate, into 1 mL of the same medium and were shaken for one hour at room temperature under incandescent light for acclimatization to the uptake conditions. The solution was replaced with fresh medium containing between 3.7 and 7.4 kBq [U⁻¹⁴C] radio-labeled substrate (GE Healthcare) and unlabeled substrate supplied at the concentrations indicated in the figures. Plantlets were washed three times with 5 mL 0.2 mM CaSO₄. For efflux analyses, plants were let take up the radiolabeled substrate for 10 min, then rinsed three times with 0.2 mM CaSO₄ and transferred to 1 mL half strength MS medium without sucrose, pH 5.7. Radioactivity released in the medium was quantitated by scintillation counting of the solution, after addition of 5 mL scintillation cocktail (Ultima Gold XR, Perkin Elmer, Waltham, MA). Plants were then dried for three hours at 70°C, weighed and digested over-night in 1 mL 7% NaClO. Radioactivity in the samples was measured by scintillation counting several hours after addition of 5 mL scintillation cocktail. For treatments with proton gradient and ATP hydrolysis inhibitors, pre-incubation (40 min), uptake and efflux were performed in presence of 100 µM CCCP, DNP or DES (stock solutions at 40 mmol l⁻¹ in DMSO), or 1 mM VO₄ (stock solution at 100 mmol l⁻¹ in water).

For thin layer chromatography (TLC) analyses of amino acid extracts, the efflux medium (half strength MS medium, pH 5.7, without any sucrose) was dried under vacuum. Metabolites were solubilized in 30 µL 70% ethanol and 15 µL were loaded on TLC silica plates GF (Analtech, Newark, DE), one microliter at a time. The plates were developed for 2 h at room temperature with a 1-butanol/acetic acid/water (3/1/1) mix. After drying under air flow, the plates were placed for 7 days against a storage phosphor screen. The screen was then scanned by a Typhoon 9400 scanner.
Accession numbers

The locus numbers of the GDU genes are: GDU1, At4g31730; GDU2, At4g25760; GDU3, At5g57685; GDU4, At2g24762; GDU5, At5g24920; GDU6, At3g30725; GDU7, At5g38770.

Supplemental data

The following materials are available in the online version of this article:

Supplemental Figure S1. Analysis of Phe accumulation and export in wild type, gdu1-1D and lht1-1.

Supplemental Figure S2. Correlation between the mRNA levels and the size of plants over-expressing the GDU genes.

Supplemental Figure S3. Correlation between the mRNA levels and the free amino acid content of plants over-expressing the GDU genes.

Supplemental Figure S4. Time course analysis of Phe accumulation in wild type and GDU over-expressing plants.

Supplemental Figure S5. Amino acid content of plants grown in liquid culture.

Supplemental Figure S6. Localization of the activity of the GDU promoters in the organs of Arabidopsis.

Supplemental Table S1. Summary of the GDU over-expressing lines from Supplemental Figures S3 and S4 chosen for further analyses.

Supplemental Table S2. Free amino acid accumulation in leaves of greenhouse grown plants over-expressing GDU1, GDU2, GDU3, GDU4, GDU5 and GDU6.

Supplemental Table S3. Amino acid content of medium from GDU-OEs and the control, grown in liquid medium.

Supplemental Table S4. Amino acid profiles of plants from GDU-OEs and the control, grown in liquid medium.

Supplemental Table S5. Summary of the localization of the promoter activity of the GDU genes.

Supplemental Text S1. Analysis of the expression pattern of the GDU genes.
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FIGURE LEGENDS

**Figure 1.** *gdu1-1D* root growth is tolerant to exogenously supplied toxic amino acids.

Wild type (WT) and *gdu1-1D* (*gdu*) plants were grown vertically for 10 days on solid medium containing the amino acid indicated above each picture (the concentration in mmol L$^{-1}$ is indicated in brackets). Pro, Gln, Glu, Asn, Asp and Ala had no effect on root growth, while Lys, Trp, Cys and Met inhibited equally growth of wild type and *gdu1-1D* roots; Val, Ser, Thr, Phe, Leu, Ile, His, Arg and Gly inhibited strongly wild type but not *gdu1-1D* root growth.

**Figure 2.** Analyses of the uptake of radiolabeled [$^{14}$C]-compounds by *gdu1-1D* and wild type plants.

(A) Accumulation of Phe, Glu, His, methyl-ammonium (MetNH$_3^+$) and Glucose (Gluc) after 10 min (supplied at 1 mmol L$^{-1}$) in whole plantlets. Wild type: light grey bars; *gdu1-1D*: dark grey bars. Mean ± SE of three biological replicates. *significantly different from the WT (t-test, P<0.02).

(B) Accumulation of Phe, Arg, Pro and Gly (supplied at 1 mmol L$^{-1}$) after 1 h in plants. Mean ± SE of three biological replicates. *significantly different from the WT (t-test, P<0.01).

(C) Concentration dependence of Phe uptake into wild type plantlets treated with dimethyl-sulfoxide (DMSO, closed squares) or CCCP (100 μM, open diamonds). CCCP inhibits the proton gradient-dependent high affinity uptake system, and reveals the activity of the low-affinity uptake system. The difference between the uptake of the DMSO- and the CCCP-treated plants corresponds to activity of the high-affinity amino acid uptake system, indicated by a broken line. Plants were let taking up Phe for 15 min before counting the amount of absorbed radioactivity. Mean ± SE of three biological replicates.

(D) Time course kinetics of the uptake of Phe supplied at a concentration of 50, 500 or 5000 μmol L$^{-1}$ in wild type (open squares) and *gdu1-1D* (closed triangles) in plants. Wild type data points were fitted by a line. Mean ± SE of three biological replicates.

(E) Effect of Phe pre-treatment to time course analysis of Phe uptake. Wild type (WT, squares) and *gdu1-1D* (triangles) were treated (dotted lines, open symbols) or not (solid lines, closed symbols) with 1 mM Phe for 30 min prior to uptake analysis performed in presence of 0.1 mM Phe. Mean ± SE of three biological replicates.
**Figure 3.** Amino acid export sensitivity to inhibitors of the proton gradient or ATP hydrolysis.

Wild type (WT) and *gdu1-1D* plants were pre-treated with DMSO, 100 μM CCCP, 100 μM DNP, 1 mM VO₄, and 100 μM DES. The same amount of DMSO was present in each sample. Uptake was performed for 10 min in presence of 5 mM Phe; plants were let release radioactivity for 10 min in the same medium without Phe.

**(A)** Total Phe taken up.

**(B)** Percentage of Phe present in the medium after the efflux experiment, expressed as a percentage of the total Phe taken up.

Mean ± SE of three biological replicates. Significantly different from the wild type (t-test): * P<0.05; ** P<0.01.

**Figure 4.** Size and amino acid profiling of plants over-expressing the *GDU* genes.

**(A)** Rosette of a representative plant from one of the two over-expressing line used in the present study (see Supplemental Table S1). The amount of secreted crystals at the margin of the leaves was lower for *GDU2-, GDU3-, GDU5- and GDU6-OEs* than for *GDU1-OEs*. Bar: 1 cm.

**(B)** One *GDU-OE* line per gene (see supplemental Table S1) was selected on kanamycin containing medium for seven days, transferred to soil and grown for three more weeks. Leaves from eight plants were pooled and freeze-dried. The amino acids were extracted and quantitated by HPLC. CTR: plants expressing GFP under the control of the 35S promoter. Increases in the content in Pro, Gln, Ser and Asn accounted for 50-65% of the total augmentation in free amino acid content, while His and Thr contents were increased the most (5-14 and 2-19 fold respectively).

**Figure 5.** Tolerance of the seven *GDU* over-expressors towards exogenously supplied amino acids.

The *GDU-OEs* were grown vertically for 10 days on solid MS medium supplemented with 5 mM Ala, Asn, Asp, Gln, Glu, Gly, Pro; 3 mM Ser; 2.5 mM Arg, Cys, His, Ile, Thr, Val; 1 mM Phe; or 0.25 mM Leu, Lys. Means ± SE of the length of 5 to 10 roots.
**Figure 6.** Analysis of the Phe uptake and efflux by the *GDU* over-expressors.

(A) Seven-day old plantlets were assayed for uptake of 1 mM Phe (light grey bars) or 0.1 mM Phe (dark grey bars) for one hour. Phe accumulation is expressed as a percentage of wild type uptake (15.1 ± 1.1 and 2.9 ± 0.5 nmol mgDW⁻¹ for 1 and 0.1 mM Phe, respectively). Bars represent the SE of three biological replicates. *Significantly different from the wild type (t-test, P<0.05).

(B) Analysis of Phe efflux for the *GDU* over-expressing lines, performed as described in Table I. Amounts of Phe remaining in plants or excreted into the medium were calculated from the respective amounts of radioactivity. The number above each bar represents the percentage of incorporated radioactivity that was released into the medium. Bars represent the SE of three biological replicates. * Percentage significantly different from the wild type (t-test, P<0.05).

Uptake and efflux analyses were performed for two over-expressor lines per *GDU* gene with similar results, only one of which is presented for clarity and simplicity.

**Figure 7.** Localization of the activity of the *GDU* promoters in Arabidopsis organs.

GUS activity was revealed by histochemical staining of plants expressing GUS under the control of the *GDU* promoters.

(A) Root stele, *GDU1* promoter. Similar staining was obtained for all other *GDUs*.

(B) Leaves, *GDU5* promoter. Similar staining was obtained for *GDU1, GDU2* and *GDU3*.

(C) Stem cross section, *GDU3* promoter. Similar staining was obtained for *GDU1* and *GDU4*.

(D) Flowers, *GDU3* promoter.
### Table I

Amounts of Phe taken up by the wild type, *lht1-1*, *gdu1-1D* and the corresponding double mutant, and subsequently released in the medium. After incubation with Phe supplied at the indicated concentration for 10 min, plantlets were quickly rinsed and placed in fresh medium for 1 hour. Total Phe taken up was estimated by adding the amounts of radioactivity in the medium (efflux) and left in the plants after efflux analyses.

|                | 1 mM Total<sup>a</sup> | Efflux<sup>a</sup> | %<sup>b</sup> | 10 μM Total<sup>a</sup> | Efflux<sup>a</sup> | %<sup>b</sup> |
|----------------|-----------------------|-------------------|-------------|----------------------|-------------------|-------------|
| **WT**         | 456 ± 45              | 64 ± 6            | 14 ± 2      | 20.8 ± 3.9           | 1.9 ± 0.3         | 9 ± 1       |
| **gdu1-1D**    | 255 ± 15*             | 150 ± 16*         | 59 ± 3**    | 3.2 ± 0.1*           | 1.8 ± 0.1         | 55 ± 5**    |
| **lht1-1**     | 354 ± 22              | 62 ± 5            | 18 ± 0      | 5.6 ± 0.6*           | 0.6 ± 0.1*        | 12 ± 1      |
| **gdu1-1D / lht1-1** | 239 ± 28*             | 135 ± 17**        | 56 ± 1**    | 2.7 ± 0.2*           | 1.3 ± 0           | 50 ± 4**    |

<sup>a</sup>nmol mg Chlorophyll<sup>-1</sup>. <sup>b</sup>Percentage of Phe present in the medium relative to total Phe taken up. Mean ± SE of three biological replicates. Significantly different from the wild type (t-test, ** P<0.02, * P<0.05).
Table II. Amino acid amounts in whole plantlets and released in the growth medium.

Plants were grown for six days in modified MS medium containing no ammonium. Amino acids were quantitated by HPLC three days after transfer into fresh MS medium without ammonium.

| Plant Medium | WT | gdu1-1D | WT | gdu1-1D |
|--------------|----|---------|----|---------|
| Amount \(^\text{a} \) | %  | Amount \(^\text{a} \) | %  | Amount \(^\text{a} \) | %  |
| Asp 1699 ± 188 | 9.6 | 1735 ± 272 | 10.8 | 41.9 ± 15.4 | 13.6 | 76.1 ± 15.8 | 4.1 |
| Glu 5024 ± 637 | 28.3 | 4860 ± 811 | 30.1 | 39.4 ± 18.2 | 12.8 | 119.7 ± 28.2 | 6.5 |
| Asn 776 ± 100 | 4.4 | 660 ± 109 | 4.1 | 15.6 ± 9.4 | 5.1 | 210.3 ± 36.8* | 11.4 |
| Ser 2030 ± 176 | 11.4 | 1970 ± 273 | 12.2 | 13 ± 3.7 | 4.2 | 95.7 ± 17.4* | 5.2 |
| Gln 2174 ± 364 | 12.3 | 2031 ± 295 | 12.6 | 72.3 ± 44.4 | 23.5 | 939.2 ± 114.8** | 50.7 |
| Gly 1720 ± 647 | 9.7 | 1696 ± 481 | 10.5 | 17.9 ± 4.9 | 5.8 | 62.8 ± 10.6 | 3.4 |
| His 91 ± 8 | 0.5 | 80 ± 14 | 0.5 | 5.1 ± 2.2 | 1.7 | 58.9 ± 11.2* | 3.2 |
| Thr 149 ± 40 | 0.8 | 80 ± 27 | 0.5 | 41.6 ± 17.5 | 13.5 | 25.2 ± 9.3 | 1.4 |
| Ala 2738 ± 331 | 15.4 | 2164 ± 399 | 13.4 | 47.1 ± 16.5 | 15.3 | 105.3 ± 27 | 5.7 |
| Pro 407 ± 52 | 2.3 | 284 ± 73 | 1.8 | 4.8 ± 1.1 | 1.5 | 36.8 ± 7.2 | 2 |
| Tyr 259 ± 56 | 1.5 | 185 ± 21 | 1.1 | 0.2 ± 0.1 | 0.1 | 4.2 ± 0.8* | 0.2 |
| Val 265 ± 70 | 1.5 | 170 ± 30 | 1.1 | 6.2 ± 2.2 | 2 | 68.3 ± 9* | 3.7 |
| Met 13 ± 2 | 0.1 | 7 ± 3 | 0 | 0 ± 0 | 0 | 3 ± 0.6* | 0.2 |
| Ile 109 ± 42 | 0.6 | 45 ± 10 | 0.3 | 0.8 ± 0.6 | 0.3 | 16.9 ± 4.1 | 0.9 |
| Lys 68 ± 25 | 0.4 | 37 ± 9 | 0.2 | 0.2 ± 0.1 | 0.1 | 5.6 ± 2.1 | 0.3 |
| Leu 128 ± 51 | 0.7 | 62 ± 14 | 0.4 | 0.8 ± 0.3 | 0.3 | 12.8 ± 3 | 0.7 |
| Phe 88 ± 20 | 0.5 | 58 ± 12 | 0.4 | 0.8 ± 0.4 | 0.3 | 10.2 ± 1.7* | 0.6 |
| Total 17738 ± 1583 | 100 | 16125 ± 2266 | 100 | 307.6 ± 125 | 100 | 1851.1 ± 278.9* | 100 |

\(^{a}\)nmol mg Chlorophyll\(^{-1}\). Mean ± SE of four biological replicates. Significantly different from the wild type (t-test): * P<0.05; ** P<0.01
**Table III.** Absolute mRNA levels of the *GDU* genes in various plant organs. *GDU* mRNA abundance was determined by quantitative RT-PCR. Tiling array data are from Laubinger et al., 2008). Unless otherwise mentioned, organs were harvested from 6-week old plants.

|                  | GDU1  | GDU2  | GDU3  | GDU4  | GDU5  | GDU6  | GDU7  |
|------------------|-------|-------|-------|-------|-------|-------|-------|
| Roots            | 246\(^a\) | 33    | 1239  | 123   | 81    | 12    | 180   |
| 2-week old leaves| 15    | 35    | 404   | 395   | 64    | 1     | 22    |
| 4-week old leaves| 4     | 3     | 288   | 285   | 33    | 0.5   | 9     |
| Not senescing leaves | 14  | 5     | 513   | 174   | 49    | 0     | 5     |
| Senescing leaves | 9     | 4     | 1783  | 126   | 72    | 0     | 1     |
| Caulinary leaves | 6     | 2     | 126   | 27    | 13    | 0.2   | 2     |
| 4-week old rosette core | 76  | 49    | 1893  | 944   | 84    | 4     | 50    |
| Stems            | 86    | 13    | 1642  | 700   | 96    | 0.1   | 30    |
| Flowers          | 10    | 18    | 562   | 94    | 4     | 0.4   | 18    |
| Young siliques   | 9     | 2     | 120   | 66    | 30    | 0.1   | 64    |
| Old Siliques     | 1     | 0     | 7     | 6     | 3     | 0     | 0.4   |
| RT-PCR maximum   | 14\(^b\) | 2     | 100   | 39    | 5     | 1     | 10    |
| RT-PCR average   | 6     | 1     | 100   | 25    | 6     | 0.2   | 5     |
| Tiling array maximum | 20\(^b\) | 10    | 100   | 50    | 28    | 1     | 10    |
| Tiling array average | 14  | 10    | 100   | 45    | 26    | 1     | 9     |

\(^a\)ng genomic DNA equivalent per 250 ng total RNA extracted (see Methods). \(^b\)mRNA content expressed as percentage of *GDU3* transcript levels.
Figure 1. *gdu1-1D* root growth is tolerant to exogenously supplied toxic amino acids.

Wild type (WT) and *gdu1-1D* (*gdu*) plants were grown vertically for 10 days on solid medium containing the amino acid indicated above each picture (the concentration in mmol L$^{-1}$ is indicated in brackets). Pro, Gln, Glu, Asn, Asp and Ala had no effect on root growth, while Lys, Trp, Cys and Met inhibited equally growth of wild type and *gdu1-1D* roots; Val, Ser, Thr, Phe, Leu, Ile, His, Arg and Gly inhibited strongly wild type but not *gdu1-1D* root growth.
Figure 2. Analyses of the uptake of radiolabeled [{}^{14}C]-compounds by gdu1-1D and wild type plants.

(A) Accumulation of Phe, Glu, His, methyl-ammonium (MetNH_{3}^{+}) and Glucose (Gluc) after 10 min (supplied at 1 mmol L^{-1}) in whole plantlets. Wild type: light grey bars; gdu1-1D: dark grey bars. Mean ± SE of three biological replicates. *significantly different from the WT (t-test, P<0.02).

(B) Accumulation of Phe, Arg, Pro and Gly (supplied at 1 mmol L^{-1}) after 1 h in plants. Mean ± SE of three biological replicates. *significantly different from the WT (t-test, P<0.01).

(C) Concentration dependence of Phe uptake into wild type plantlets treated with dimethyl-sulfoxide (DMSO, closed squares) or CCCP (100 μM, open diamonds). CCCP inhibits the proton gradient-dependent high affinity uptake system, and reveals the activity of the low-affinity uptake system. The difference between the uptake of the DMSO- and the CCCP-treated plants corresponds to activity of the high-affinity amino acid uptake system, indicated by a broken line. Plants were let taking up Phe for 15 min before counting the amount of absorbed radioactivity. Mean ± SE of three biological replicates.

(D) Time course kinetics of the uptake of Phe supplied at a concentration of 50, 500 or 5000 μmol L^{-1} in wild type (open squares) and gdu1-1D (closed triangles) in plants. Wild type data points were fitted by a line. Mean ± SE of three biological replicates.

Effect of Phe pre-treatment to time course analysis of Phe uptake. Wild type (WT, squares) and gdu1-1D (triangles) were treated (dotted lines, open symbols) or not (solid lines, closed symbols) with 1 mM Phe for 30 min prior to uptake analysis performed in presence of 0.1 mM Phe. Mean ± SE of three biological replicates.
Figure 3. Amino acid export sensitivity to inhibitors of the proton gradient or ATP hydrolysis.

Wild type (WT) and gdu1-1D plants were pre-treated with DMSO, 100 μM CCCP, 100 μM DNP, 1 mM VO₄ and 100 μM DES. The same amount of DMSO was present in each sample. Uptake was performed for 10 min in presence of 5 mM Phe; plants were let release radioactivity for 10 min in the same medium without Phe.

(A) Total Phe taken up.

(B) Percentage of Phe present in the medium after the efflux experiment, expressed as a percentage of the total Phe taken up.

Mean ± SE of three biological replicates. Significantly different from the wild type (t-test): * P<0.05; ** P<0.01.
Figure 4. Size and amino acid profiling of plants over-expressing the GDU genes.

(A) Rosette of a representative plant from one of the two over-expressing line used in the present study (see Supplemental Table S1). The amount of secreted crystals at the margin of the leaves was lower for GDU2-, GDU3-, GDU5- and GDU6-OEs than for GDU1-OEs. Bar: 1 cm.

(B) One GDU-OE line per gene (see supplemental Table S1) was selected on kanamycin containing medium for seven days, transferred to soil and grown for three more weeks. Leaves from eight plants were pooled and freeze-dried. These amino acids were extracted and quantitated by HPLC. CTR: plants expressing GFP under the control of the 35S promoter. Increases in the content in Pro, Gln, Ser and Asn accounted for 50-65% of the total augmentation in free amino acid content, while His and Thr contents were increased the most (5-14 and 2-19 fold respectively).
Figure 5. Tolerance of the seven GDU over-expressors towards exogenously supplied amino acids.

The GDU-OEs were grown vertically for 10 days on solid MS medium supplemented with 5 mM Ala, Asn, Asp, Gln, Glu, Gly, Pro; 3 mM Ser; 2.5 mM Arg, Cys, His, Ile, Thr, Val; 1 mM Phe; or 0.25 mM Leu, Lys. Means ± SE of the length of 5 to 10 roots.
Figure 6. Analysis of the Phe uptake and efflux by the GDU over-expressors.

(A) Seven-day old plantlets were assayed for uptake of 1 mM Phe (light grey bars) or 0.1 mM Phe (dark grey bars) for one hour. Phe accumulation is expressed as a percentage of wild type uptake (15.1 ± 1.1 and 2.9 ± 0.5 nmol mgDW⁻¹ for 1 and 0.1 mM Phe, respectively). Bars represent the SE of three biological replicates. *Significantly different from the wild type (t-test, P<0.05).

(B) Analysis of Phe efflux for the GDU over-expressing lines, performed as described in Table I. Amounts of Phe remaining in plants or excreted into the medium were calculated from the respective amounts of radioactivity. The number above each bar represents the percentage of incorporated radioactivity that was released into the medium. Bars represent the SE of three biological replicates. * Percentage significantly different from the wild type (t-test, P<0.05).

Uptake and efflux analyses were performed for two over-expressor lines per GDU gene with similar results, only one of which is presented for clarity and simplicity.
Figure 7. Localization of the activity of the GDU promoters in Arabidopsis organs.
GUS activity was revealed by histochemical staining of plants expressing GUS under the control of the GDU promoters.

(A) Root stele, GDU1 promoter. Similar staining was obtained for all other GDUs.
(B) Leaves, GDU5 promoter. Similar staining was obtained for GDU1, GDU2 and GDU3.
(C) Stem cross section, GDU3 promoter. Similar staining was obtained for GDU1 and GDU4.
(D) Flowers, GDU3 promoter.