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ORIGINAL ARTICLE

Uptake and conversion of D-amino acids in *Arabidopsis thaliana*

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Abstract The D-enantiomers of proteinogenic amino acids fulfill essential functions in bacteria, fungi and animals. Just in the plant kingdom, the metabolism and role of D-amino acids (D-AAs) still remains unclear, although plants have to cope with significant amounts of these compounds from microbial decay in the rhizosphere. To fill this gap of knowledge, we tested the inhibitory effects of D-AAs on plant growth and established a method to quantitate 16 out of 19 proteinogenic amino acids and their D-enantiomers in plant tissue extracts. Therefore, the amino acids in the extracts were derivatized with Marfey's reagent and separated by HPLC–MS. We used two ecotypes (Col-0 and C24) and a mutant (*lht1*) of the model plant *Arabidopsis thaliana* to determine the influence and fate of exogenously applied D-AAs. All of them were found in high concentrations in the plant extracts after application, even in *lht1*, which points to additional transporters facilitating the import of D-AAs. The addition of particular amino acids (D-Trp, D-Phe, D-Met and D-His) led to the accumulation of the corresponding L-amino acid. In almost all cases, the application of a D-AA resulted in the accumulation of D-Ala and D-Glu. The presented results indicate that soil borne D-AAs can actively be taken up and metabolized via central metabolic routes.

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Introduction

Proteinogenic amino acids are indispensable in all kingdoms of life, but also their D-enantiomers fulfill partially essential functions in a variety of organisms. The discovery and description of the first D-amino acids in organisms were made in the early 1950s in the body fluids of insects and molluscs (Auclair and Patton 1950; Izumiya et al. 1957). Later they have also been detected in mammalian tissues, where D-Asp and D-Ser attracted special interest. D-Asp could be detected in invertebrates and vertebrates as well and was shown to influence the nervous and the endocrine system (for a review, see D'Aniello 2007). Furthermore, D-Asp appears in many proteins of elderly patients as a result of inversion within a peptide chain (Fujii 2005). D-Ser has been found in substantial amounts in the brain and is thought to play an important role in the central nervous system by modulating the *N*-methyl-D-aspartate (NMDA) glutamate receptor (Fuchs et al. 2005). Apart from these and other examples in animals, D-AAs fulfill essential functions in the life cycle of microbes. Microbial bioactive macrocyclic peptides often contain D-amino acids (Grünewald and Marahiel 2005). The most prominent function of D-amino acids in bacteria is their contribution to the peptidoglycan of the bacterial cell wall. It consists of linear glycan chains interlinked by short peptides which regularly contain D-Ala and D-Glu (van Heijenoort 2001).

In contrast to the knowledge about this class of substances in microbes and animals, there is only little known about them in plants. The occurrence of malonylated conjugates of D-Trp has been reported in a variety of plant species in the early 1960s (Zenk and Scherf 1963, 1964). Beside *N*-acylated derivatives of other D-amino acids (for an early summary, see Robinson 1976) also D-alanyl dipeptides were found in numerous plants (Kullman et al. 1999; Manabe 1992; Rozan et al. 2001). The free forms of D-amino acids have also been detected in different tissues of gymnosperms and angiosperms in the range of about 0.2–8% relative to the corresponding L-amino acids (L-AAs) (Brückner and Westhauser 1994, 2003). Although their existence is a well documented fact, the source of the free D-AAs in plants is still a matter of debate. One possibility to explain their appearance in plant tissues is by uptake from soil. The rhizosphere represents a major source of D-AAs for plants, where D-AAs like D-Ala or D-Asp can be found in amounts of several milligrams per kilogram of soil (Brodowski et al. 2004). D-AAs in soil mostly originate from microorganisms in the rhizosphere. They are known to produce D-AAs and to release them after death and autolysis together with L-AAs into the soil. From there, it is assumed that on the one-hand side this amino acid pool is taken up by other organisms and on the other L-AAs are chemically racemized in a time and temperature-dependent manner (Amelung 2003; Amelung and Zhang 2001). It was long debated if and how plants import D-AAs by their roots. Final evidence for root uptake of exogenously supplied D-AAs was given by the characterization of the amino acid transporter AtLHT1 from *Arabidopsis thaliana* (Svennerstam et al. 2007; Forsum et al. 2008).

The determination of D-AAs from plants grown on synthetic media indicates that not all of these amino acids in plant tissues can be soil borne (Brückner and Westhauser 2003; Funakoshi et al. 2008). Chemical conversion of amino acids by pH-independent Maillard reaction in the presence of sugars would be an explanation for these findings (Brückner et al. 2001). Another major source for these amino acids would be the enzymatic racemization by amino acid racemases. It is the only known biosynthetic way, where D-AAs are produced de novo from the corresponding L-AAs. Generally, amino acid racemases are classified into pyridoxal phosphate (PLP)-dependent types, like alanine and serine racemases, and PLP-independent types, where glutamate and aspartate racemases belong to (Yoshimura and Esaki 2003). The activities of such enzymes had been reported quite early in plants (for a summary, see Robinson 1976), but just recently the first racemase of plant origin, AtSR1, a serine racemase from *Arabidopsis thaliana*, could be identified and characterized on molecular level (Fujitani et al. 2006). Later the orthologs of this gene could be characterized from monocotyledonous species (Fujitani et al. 2007), but the molecular nature of other plant amino acid racemases remains to be unravelled.

Although uptake and formation of D-AAs in plants are long documented facts almost nothing is known about their functions. One of the most debated questions in this respect is, if and how plants utilize D-AAs as nitrogen sources. A major argument against such an utilization is the toxicity of several D-AAs on plant growth (Erikson et al. 2004; Forsum et al. 2008). Furthermore, it had been argued that plants have a low capacity to degrade D-AAs, which causes also their toxicity (Näsholm et al. 2009). But recently, two genes and their products have been identified from different plants which shed a different light on this question. First, in maize the first D-AA oxidase from plants was identified. Homologous genes were also found in the genomes of rice and *Arabidopsis*. Accompanying biochemical studies revealed also D-AA oxidase activity for the gene product and expression of the gene was strongly induced by D-Ala (Gholizadeh and Kohnehrouz 2009). Second, a D-AA aminotransferase (D-AAT) was characterized in *A. thaliana*, which was shown to be functional. The discovery of this gene delivered a possible explanation for the conversion of exogenously supplied D-Asp to D-Ala and D-Glu (Funakoshi et al. 2008). Taken together these and other findings indicate that an active uptake of D-AAs and their metabolization can take place in plants. Nevertheless the question remains if these amino acids are utilized as nitrogen sources, if they fulfill other needs or if they are unwanted by-products.

As one approach to characterize the metabolism of D-AAs in plants we tested 18 D-AAs as growth inhibitors on two ecotypes of *A. thaliana* (Col-0 and C24) and the amino acid transporter mutant *lht1-1*. In addition, we measured 16 D- and L-AAs from extracts of these plants after application of 2 mM D-AAs. The measurements were done using a novel HPLC–MS procedure after derivatization of the amino acids with a variant of the Marfey's reagent, which bases on L-valine amide instead of L-alanine amide (Brückner and Keller-Hoehl 1990). Our results indicate that uptake of D-AAs is facilitated by several transporters and that racemization and transamination to D-Ala and D-Glu are central steps in the metabolization of D-AAs in plants.

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana wild type plants (ecotypes Col-0 and C24) and an amino acid transporter mutant *lht1-1* (Hirner et al. 2006) were grown in growth chambers (16 h light,

22°C). Germination of sterile seeds took place in 96-well microtiter plates with one seedling per well in 200–250 µl of autoclaved medium containing half-strength Murashige and Skoog (MS) basal salts (0.5 × MS: 825 mg/l NH_4NO_3, 3.1 mg/l H_3BO_3, 166.1 mg/l $CaCl_2$, 0.0125 mg/l $CoCl_2 \times 6H_2O$, 0.0125 mg/l $CuSO_4 \times 5H_2O$, 18.63 mg/l Na_2-EDTA; 13.9 mg/l $FeSO_4 \times 7H_2O$, 90.35 mg/l $MgSO_4$, 8.45 mg/l $MnSO_4 \times H_2O$, 0.125 mg/l $Na_2MoO_4 \times 2H_2O$, 0.415 mg/l KI, 950 mg/l KNO_3, 85 mg/l KH_2PO_4, 4.3 mg/l $ZnSO_4 \times 7H_2O$; Murashige and Skoog 1962) with 1% sucrose (pH 5.8).

For growth inhibition tests in the first row (8 wells) of a microtiter plate 10 mM of a particular D-AA in medium was prepared. This solution was serially diluted 1:1 with 0.5 × MS in each row. As a result 11 rows of 0.5 × MS with D-AAs in concentrations from 10 to 10,000 µM were produced. The last row just contained medium without any D-AA supplement as control. Then seeds were sown out, plates were sealed with parafilm and stratified at 4°C for 48–96 h. Seeds were germinated for 15 days and afterwards growth was phenotypically recorded. When more than half of the plants in a row displayed affected growth compared to the control row, this concentration was counted as inhibitory.

For the measurement of amino acids plants were sown and grown as described above. Just the addition of D-AAs to a final concentration of 2 mM took place after 16 days of germination for 20 h. Afterwards seedlings were taken out of the medium, thoroughly washed in distilled water and then frozen in liquid nitrogen.

Amino acid extraction from plant material and derivatization

To extract amino acids out of the plants four seedlings were ground in an Eppendorf cap. Afterwards 100 mM Tris–HCl, pH 8, was given to the plant material and amino acids were extracted for 1 h on ice with occasional vortexing. Finally, cell debris and other insoluble parts of the extracts were removed by centrifugation. Soluble protein concentrations of the solutions were determined with the Roti-Quant reagent (Carl Roth GmbH, Karlsruhe, Germany) according to the manufacturer's protocol.

The derivatization of the amino acids in the plant extracts was generally performed according to Brückner and Keller-Hoehl (1990) using N^{α}-(2, 4-dinitro-5-fluorophenyl)-L-valine amide (Sigma–Aldrich, Germany) as Marfey's reagent. For the derivatization, 100 µl of the extract was mixed with 200 µl of 1% Marfey's reagent in acetone and 40 µl of 1 M $NaHCO_3$. After incubation for 1 h at 37°C, 20 µl of 2 M HCl was added and the solution was adjusted to 600 µl with methanol for further separation and quantitation of amino acids.

Determination of D- and L-AAs in plant extracts

For calibration purposes the following amino acids were purchased from Sigma–Aldrich: D-Asp (98%), D-Val (99%), D-Phe (99+%), D-Glu (99+%), D-Asn (99%), D-Leu (97%), D-Trp (99+%), D-Ile (98%), D-Ala (99+%), D-Thr (99%), D-Met (99+%), D-Ser (98%), L-Asp (98+%), L-Val (99%), L-Phe (99%), L-Glu (99%), L-Asn (99%), L-Pro (99+%), L-Leu (97%), L-Trp (99%), L-Ile (99%), and L-Ala (99%). The amino acids D-Arg (>99%), D-Glu (>98.5%), D-Pro (≥99%), D-His (≥99%), L-Arg (≥99.5%), L-Gln (≥99.5%), L-His (≥99.5%), L-Met (≥99%), and L-Ser (≥99%) were purchased from Fluka (Buchs, Switzerland). As internal standard (*R*)-(-)-2-phenylglycine (99%, Sigma–Aldrich, Steinheim, Germany) was used. Acetonitrile HPLC Gradient Grade (Roth, Karlsruhe, Germany), formic acid (98%, Sigma–Aldrich, Steinheim, Germany), and purified water from a Milli-Q Synthesis A10 system (Millipore, Schwalbach, Germany) were used for the LC eluent. Nitrogen, with a purity of 99.9999% (Linde, Germany), was used as collision gas.

An Agilent Technologies 1200 Series LC (Waldbronn, Germany) coupled to an Agilent Technologies 6410 Triple Quad LC/MS operating in positive electrospray ionization mode (ESI) was used for the analysis. The chromatographic column was a Zorbax Eclipse SB-C18 (4.6 × 150 mm × 1.8 µm) with an Eclipse SB-C18 (4.6 × 12.5 mm × 5 µm) guard column. The column temperature was held at 70°C, the initial flow rate was 0.9 ml, and the injection volume was 5 µl. Solvent A consisted of 0.1% formic acid in H_2O and solvent B was acetonitrile. The mobile phase composition started at 90:10 (v, v) and changed according to Table 1. A representative chromatogram of such an LC run is given in Fig. 1.

The mass spectrometer was operated at a capillary voltage of 4,000 V, a fragmentor voltage of 120 V, a nebulizer pressure of 50 psi, a nitrogen gas flow of 10 L/min, and a gas temperature of 350°C. The total run time of 25 min was divided into seven time segments to achieve maximum sensitivity. The precursor and product ions, along with optimized collision energy, are shown in

Table 1 LC gradient

Time (min)	Flow (mL/min)	Solvent A (%)	Solvent B (%)
0	0.9	90	10
12	0.9	60	40
16	0.9	56	44
21	0.9	56	44
21.1	1	0	100
23	1	0	100
23.1	0.9	90	10

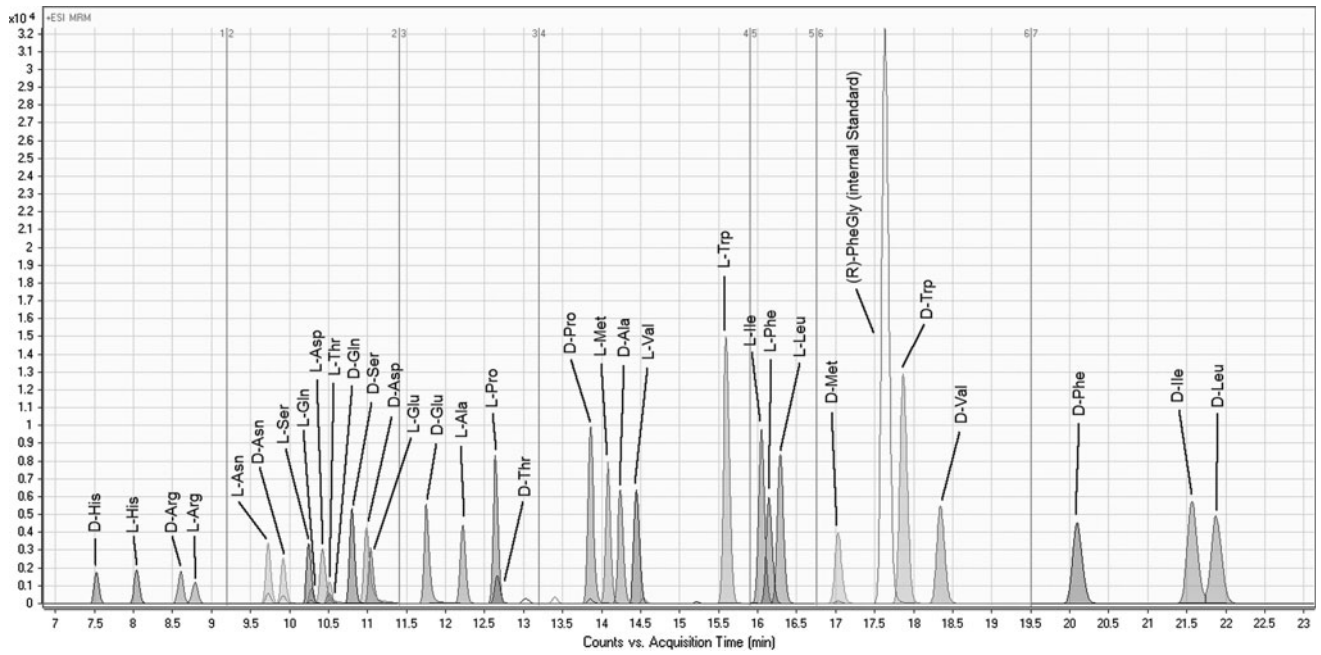

Fig. 1 Overlaid multiple reaction monitoring (MRM) chromatogram of diastereomers of amino acids resulting from a representative standard composed of L- and D-amino acids (each 125 pmol), and the internal standard (*R*)-(-)-phenylglycine (1.25 nmol) derivatized with 1-fluoro-2,4-dinitrophenyl-5-L-valine amide (FDNP-L-Val-NH_2)

Table S1. The vacuum in the collision cell was adjusted between 2.75 and 3.00×10^{-5} torr with nitrogen as collision gas.

Calibration of the assay was calculated using quadratic regression analysis, 1/*x* weighting of the calibration points, and no forcing through the origin. For each AA, a seven-point calibration curve (5, 10, 25, 50, 125, 250, and 500 µmol/l) was created; the internal standard concentration was 250 µmol/l. Examples for calibration graphs are shown in the Figs. S1–S4 together with transition plots of all measured AAs (Figs. S5–S7). Equations and correlations of all calibration plots as well as limits of quantitation (LOQ) are given in Table S2. The LOQ for each AA was established by entering a signal-to-noise ratio (SNR) of 10 into the method analysis. All LOQs were between 1.25 and 5 µmol/l.

Results

Growth suppression of D-AAs on Col-0 and *lht1*

It has been shown before that growth of *Arabidopsis* plants is inhibited by millimolar concentrations of D-alanine and D-serine, but not by D-Ile or D-Val (Erikson et al. 2004). Thus one of the first questions was to determine, which D-AAs cause growth inhibition at similar concentrations. Therefore, 18 different D-AAs were tested in concentrations of 10–10,000 µM on their effect on the growth of Col-0 and *lht1*. As displayed in Fig. 2 only D-Ala, D-Arg, D-His, D-Ser, D-Trp and D-Tyr inhibited the growth of Col-0 in submillimolar concentrations with D-Tyr showing the strongest effect at 78 µM. In contrast, there were also four other D-AAs which affected seedling growth just at 10 mM. Another one, D-Ile, was not effective even at the highest tested concentration (10 mM) and, therefore, has been excluded from the diagram. The results for C24 were identical (data not shown).

LHT1 seems to be a major facilitator for amino acid uptake from soil (Forsum et al. 2008; Hirner et al. 2006; Svennerstam et al. 2007). A mutant of this gene, *lht1-1*,

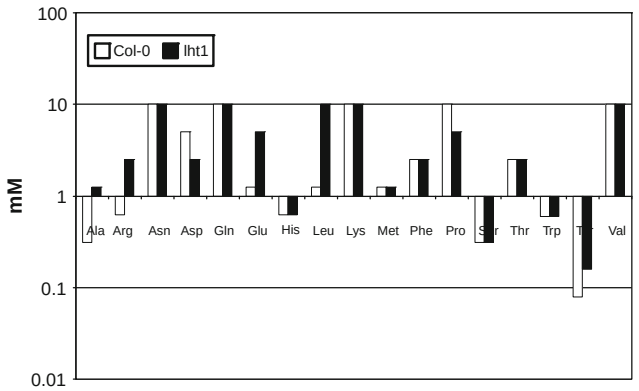

Fig. 2 Inhibitory concentrations (mM) of the tested D-AAs on seedling growth of Col-0 (*white bars*) and *lht1-1* (*black bars*). The procedure of this assay is described in "Materials and methods". D-Ile has been excluded from the diagram, because even the highest concentration (10 mM) did not affect seedling growth

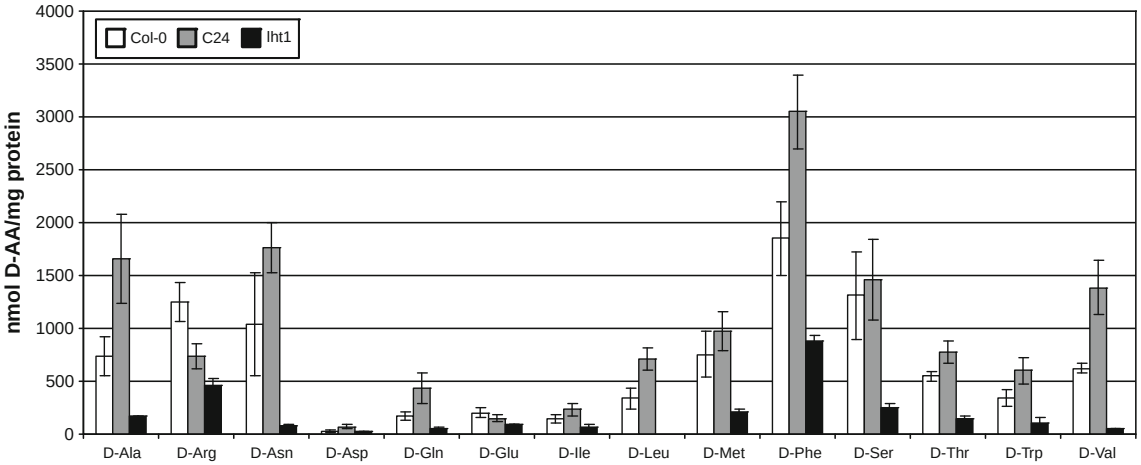

Fig. 3 Accumulation of particular D-AAs in the plants after 20 h of growth in medium supplemented with this D-AA. In the figure, the concentration of the measured D-AAs in the plant extracts of Col-0 (*white bars*), C24 (*gray bars*) and *lht1-1* (*black bars*) are given in nmol of the D-AA per mg protein of the protein extract. *Bars* represent the average of measurements (±SD) from three samples with four plants in each sample. Measurements of D-His and D-Pro have been excluded from this diagram because all values exceeded the limit of quantitation

displayed reduced sensitivity against particular D-AAs (e.g., D-Ala, D-Glu and D-Leu) in comparison to the corresponding wild type Col-0 (Fig. 2). In these cases, growth inhibition was achieved with two to fourfold higher concentrations of the respective D-AAs in comparison to the control plants. It is remarkable in this diagram that the inhibitory concentration of all other tested D-AAs remained mostly unchanged (e.g., D-His, D-Ser, D-Trp and D-Tyr), which points to additional import proteins or mechanisms for D-AAs apart from LHT1.

Uptake of D-AAs by the different *A. thaliana* lines

In the course of amino acid measurements in plant extracts one important question concerned the import of D-AAs from the medium. The import of D-AAs into plants is a major prerequisite for investigation of their conversion when they are exogenously supplied. In contrast to the untreated control plants, where only D-Ser of all analyzed D-AAs with 0.8–1.8 nmol/mg protein was above the detection level (data not shown), all D-AAs could also be detected in the plant extracts after their addition to the medium. In Fig. 3, it can be seen that the D-AAs in all the analyzed plant lines accumulated from 22 nmol/mg Protein (D-Asp in *lht1*) to up to more than 3,000 nmol/mg Protein (D-Phe in C24). As a general trend in this figure it can also be seen that the majority of the supplied D-AAs accumulates to several fold lower extent in the transporter mutant *lht1* compared to the corresponding ecotype Col-0. Nevertheless, it can also be stated that the import of none of the analyzed D-AAs in *lht1* was completely abolished (Table S3). Furthermore, the lower content of AAs in *lht1* in comparison to reference wild types was not restricted to the D-AAs. The contents of L-AAs in the unchallenged control plants of *lht1* were also lower than in the wild type strains. The measurement of L-AAs in the same plants challenged with D-AAs show an overall comparable lower content of corresponding L-AAs in *lht1-1*, too (Table S4). These changes in contents of D-and L-AAs do not seem to occur evenly in the mutant. The ratio of D-/L-AA differs for particular AAs between *lht1* and its reference phenotype Col-0, whereas for others they stay the same (Table S5). The changes in L-AA content and in D-/L-AA ratio after application of exogenous D-AAs in *lht1* in comparison to wild type plants point to further changes in this mutant apart from import of D-AAs.

Racemization of particular D-AAs in the plants

After the observation of root uptake of D-AAs the question about their metabolization has to be solved. Theoretically, there are different possibilities to remove D-AAs from a plant. One among them is racemization into the corresponding proteinogenic L-AA. It has been mentioned above that amino acid racemase activities have been detected in plants quite early (Robinson 1976), but if these enzymes contribute to the metabolization of D-AAs and to which extent still remains an open question.

Measurements of L-AAs in the plant extracts did not reveal any significant changes after supplementation of most D-AAs in all three analyzed lines. Just when D-His, D-Phe, D-Trp and D-Met were added to the medium the content of the corresponding L-AAs raised significantly in all analyzed lines compared to the unchallenged control plants. The lowest increase with 2.1 to 2.2-fold change of L-His was recorded after addition of D-His (Fig. 4a),

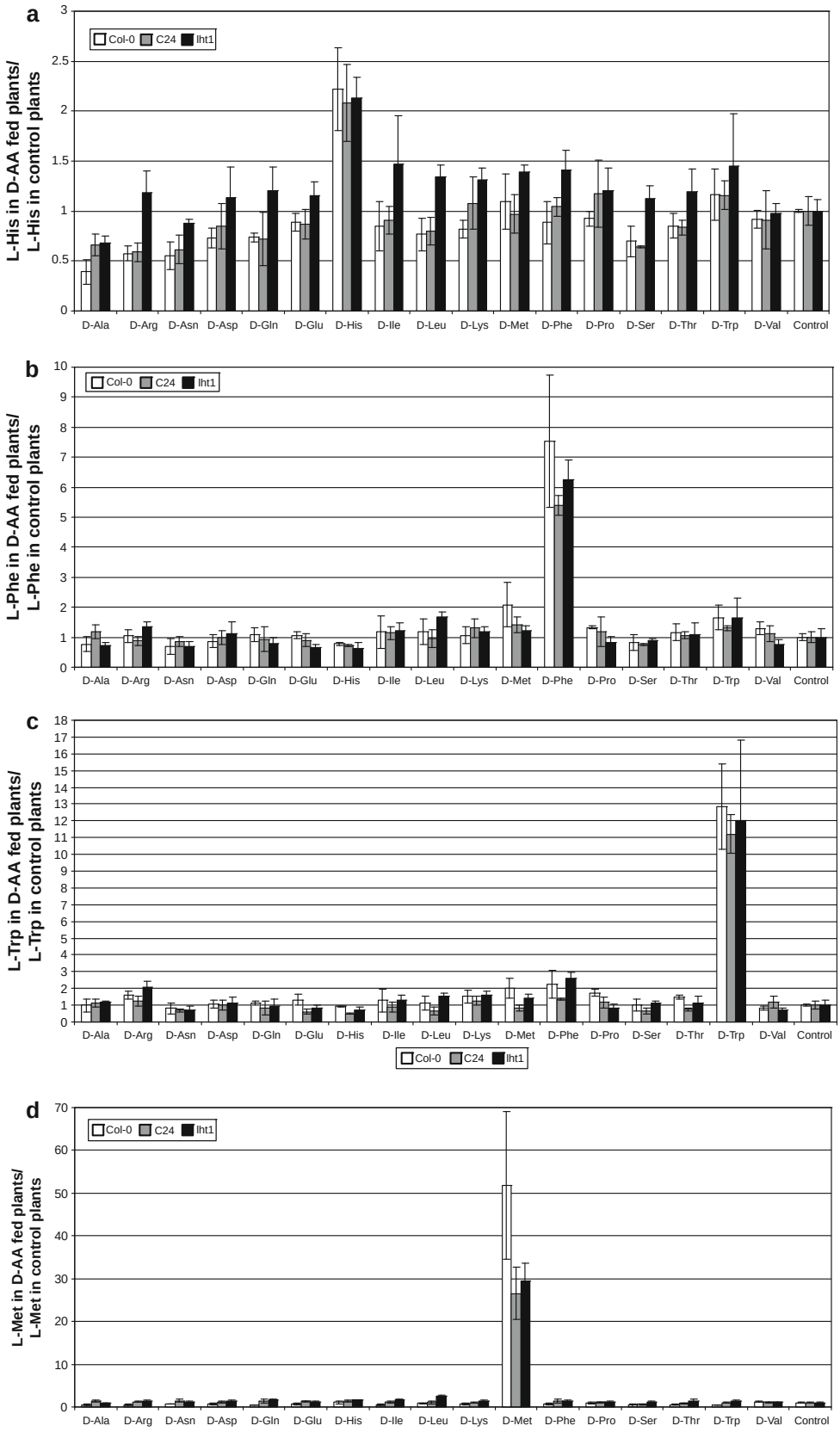

Fig. 4 Alteration of the content of **a** L-His, **b** L-Phe, **c** L-Trp and **d** L-Met in plants after 20 h of growth in medium supplemented with different D-AAs compared to control plants grown in medium without supplementation. In the figure, the ratio of particular L-AAs in plants grown in medium with D-AAs in comparison to unsupplemented medium is given. The D-AAs in the abscissa denote the amino acids applied to the medium. *Bars* represent the average of measurements (±SD) from three samples with four plants in each sample. The analyzed lines were the same as in Fig. 2

Table 2 Change of L-AA concentration (compared to untreated control plants) after addition of the corresponding D-enantiomer

AA	Col-0		*lht1-1*		C24	
	Change factor[a]	P^b	Change factor[a]	P^b	Change factor[a]	P^b
L-Ala	0.26 ± 0.05	>0.001	0.43 ± 0.04	>0.1	0.65 ± 0.13	>0.1
L-Trp	12.86 ± 2.55	>0.05	12.05 ± 4.75	>0.1	11.20 ± 1.15	>0.01
L-Phe	7.54 ± 2.19	>0.05	6.26 ± 0.66	>0.01	5.40 ± 0.32	>0.01
L-Asp	0.70 ± 0.10	n. s.	1.49 ± 0.27	n. s.	0.67 ± 0.22	n. s.
L-Met	51.87 ± 17.22	>0.1	29.52 ± 4.04	>0.05	26.59 ± 6.12	>0.05
L-Val	1.17 ± 0.10	n. s.	1.01 ± 0.06	n. s.	1.10 ± 0.27	n. s.
L-Arg	0.43 ± 0.09	>0.01	0.68 ± 0.18	n. s.	0.42 ± 0.10	>0.05
L-Glu	0.74 ± 0.13	n. s.	0.70 ± 0.04	n. s.	0.63 ± 0.24	n. s.
L-His	2.22 ± 0.41	>0.1	2.13 ± 0.21	>0.05	2.08 ± 0.39	>0.05
L-Ile	1.87 ± 0.05	>0.01	1.46 ± 0.34	n. s.	1.69 ± 0.40	n. s.
L-Thr	0.71 ± 0.06	>0.01	1.22 ± 0.22	n. s.	0.89 ± 0.07	n. s.
L-Pro	1.17 ± 0.08	n. s.	0.91 ± 0.16	n. s.	1.17 ± 0.18	n. s.

n. s. Not significant

[a] Ratio of particular L-AAs in plants grown in D-AA supplemented to unsupplemented media (±SD)

[b] Probability values determined with Student's *t* test

followed by 5.4 to 7.5-fold increase of L-Phe (Fig. 4b) and 11.2 to 12.9-fold increase of L-Trp (Fig. 4c) after addition of their D-enantiomers. The highest increase was observed for methionine, where after addition of D-Met 26.6 to 51.9-fold of the corresponding L-enantiomer could be detected (Fig. 4d). All these increases were observed in all analyzed lines in a significant manner (Table 2). It is remarkable that the mentioned increases of L-AAs after supplementation of the corresponding D-AA did not differ significantly between *lht1* and the corresponding wild type Col-0 and the second reference wild type C24 (Table 2). Furthermore, a significant decline of L-Ala values down to one-fourth was observed in all lines after addition of D-Ala. It has to be assumed that D-Ala affects the biosynthesis of L-Ala at applied concentrations. Furthermore, these data indicate that the plant alanine racemase plays a rather neglectable role in the equilibrium of alanine enantiomers.

Most of exogenously supplied D-AAs are converted to D-Ala and D-Glu

With the discovery of the first eukaryotic D-AAT from *Arabidopsis thaliana* it was also observed that addition of D-Asp to the medium led to an increase of the cellular D-Ala and D-Glu level. Biochemical characterization of the D-AAT, which is probably responsible for this effect, revealed that almost all D-AAs could act as substrates of this enzyme (Funakoshi et al. 2008). This relatively broad substrate specificity implies also a production of D-Ala and D-Glu in the plants after supplementation of the medium with other D-AAs than D-Asp.

Figure 5a, b supports this hypothesis. In the control plants, without supplementation of D-AAs, no D-Ala or D-Glu could be detected. In contrast, after supplementation of any of the tested D-AAs, D-Ala (except after addition of D-Pro) and D-Glu evolved in detectable amounts in both wild type strains. In this respect, the amount of D-Ala was always about a magnitude higher than the amount of D-Glu. The highest amounts of D-Ala and D-Glu were found in plants after addition of D-Met and D-Phe. This holds also true for measurements in *lht1*, but compared to the corresponding wild type Col-0 and the second reference wild type C24 the smallest values after addition of a particular D-AA were always found in this mutant. In some cases of D-Glu measurement (e.g., after addition of D-Arg, D-Asp, D-Ile, D-Leu, D-Lys), the D-AA was even below detection limit (Fig. 5b). These lowered accumulations of D-Ala and D-Glu compared to wild type might be explained with the lowered import of D-AAs (Fig. 3) and the corresponding lower racemization rate.

Discussion

In the present report, the influence of D-AAs on growth and amino acid metabolism of *Arabidopsis* seedlings was characterized. As it has been shown in former analyses (Erikson et al. 2004) some D-AAs such as D-Tyr or D-Ser inhibit seedling growth at submillimolar concentrations whereas others, such as D-Asn or D-Gln have to be added to the medium in 10 mM to affect growth. A similar observation has also been made before in the case of D-Val

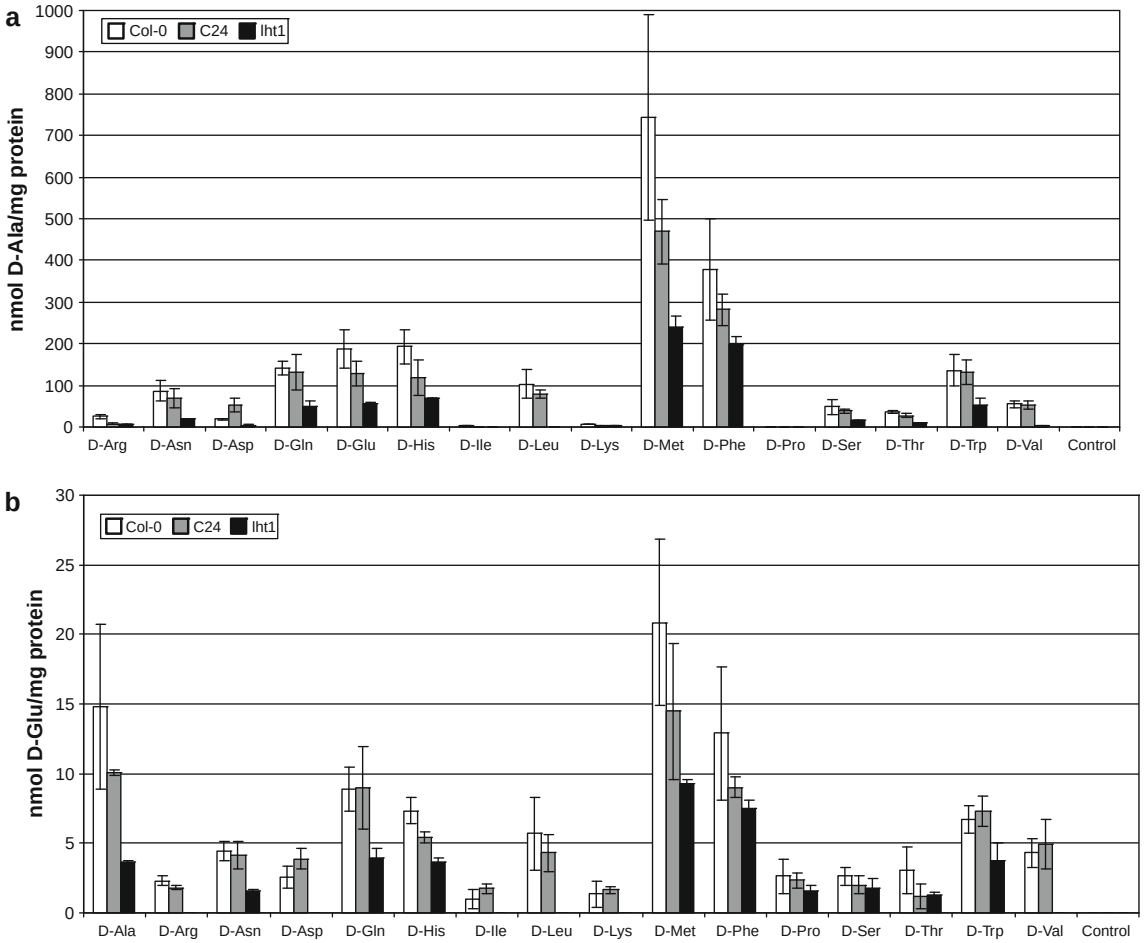

Fig. 5 Accumulation of **a** D-Ala and **b** D-Glu in plants after 20 h of growth in medium supplemented with all tested D-AAs. In the figure, the concentration of the supplemented D-AAs in the plant extracts is given in nmol of D-Ala or D-Glu per milligram protein of the protein extract. *Bars* represent the average of measurements (±SD) from three samples with four plants in each sample. The analyzed lines were the same as in Fig. 2

which leads to growth suppression from 10 mM upwards (Erikson et al. 2004). Interesting in this respect are the results of the transporter mutant *lht1*. Compared to its corresponding wild type this line shows enhanced resistance just against particular D-AAs. Furthermore, almost all exogenously offered D-AAs were also found in the mutant plants but in lower amounts than in the wild types. The mutated transporter in this line should be a major facilitator of amino acid uptake and transport in *Arabidopsis* (Forsum et al. 2008; Hirner et al. 2006; Svennerstam et al. 2007). The presented results support this observation especially because of the diminished accumulation of D-AAs in *lht1*. The residuing D-AA uptake in *lht1-1* indicates the existence of further importers for these amino acids. There are at least 53 putative amino acid transporters in the *Arabidopsis* genome (Wipf et al. 2002) which might also play a role in the D-AA import.

There are also other questions arising from these results: A decrease of D-Ala and D-Arg uptake coincides with an increase of resistance against them, but the inhibitory effect on seedling growth of other D-AAs like D-Ser or D-Trp remains the same in *lht1* although their uptake is also lowered. This observation is in conflict to the results of Forsum et al. (2008), where the overexpression of D-AA degrading enzymes led to enhanced resistance against D-Ala and D-Ser. The contradiction between reduced D-AA levels and constant concentration of growth suppression against most of these amino acids in *lht1* could be explained in different ways. One possible explanation would be that the inhibitory dose is lower than assumed and imported by other high affinity transporters. Another possibility would be the interference of most D-AAs in the rhizosphere with other essential import processes. Although there are also other explanations possible they have all in common to be highly speculative and have to be verified experimentally.

The main focus of the analyses in this study lied on the question if and how D-AAs are taken up and utilized. The

arguments for an active uptake have been debated above, but there are now also arguments in favor of utilization of the imported D-AAs by the presented results. The accumulation of the L-enantiomers of D-His, D-Phe, D-Trp and D-Met after addition to the medium can be interpreted by inhibition of degradation of the L-Enantiomers, but that would be difficult to explain for all of these amino acids with quite different metabolic contexts. It is rather easier to explain that these L-enantiomers evolved by racemization, which needs to be proven, yet. But it has to be considered that racemase activity for any of these four amino acids had just been reported for tryptophan in plants (Miura and Mills 1971; Rekoslovskaya et al. 1999). Specific racemases have not been identified for free histidine, tryptophan and methionine in any organism yet, whereas phenylalanine racemase was identified to be involved in bacterial gramicidin synthesis (Stein et al. 1995). Therefore, it is an interesting task to verify the postulated racemization processes and identify the proteins which are responsible for the putative racemization in *Arabidopsis*.

Nevertheless, the accumulation of several L-AAs after addition of their D-enantiomers shows that plants are capable to convert D-AAs and to feed the products into their pool of proteinogenic amino acids. This leaves the question open why just the accumulation of the mentioned four L-AAs was detected and not of the others. One possibility is that the velocity of depletion of the other L-AAs is too fast to observe an accumulation. This possibility has to be clarified in future experiments by feeding of radioactively labeled D-AAs and tracing the labels. Another explanation would be that just a few AAs get racemized whereas others underlie other processes like deamination to their keto acids, which have not been analyzed in the present study. The accumulation of keto acids from D-AAs had been shown in maize and ryegrass before (Aldag and Young 1970). Candidate enzymes for this process like serine racemase/dehydratase or D-AA oxidase have been identified recently in different plant genomes (Fujitani et al. 2006, 2007; Gholizadeh and Kohnehrouz 2009), but their physiological impact on amino acid metabolism is still unclear.

Another way of D-AA metabolization attracted interest after analysis of the results. Almost all D-AAs supplemented to the media were partially converted to D-Ala or D-Glu. The easiest explanation of this interconversion of D-AAs is the transfer of an amino group from a D-AA on either pyruvate or 2-oxoglutarate to form D-Ala or D-Glu, respectively. A candidate protein for this process from *Arabidopsis*, D-AA transferase, has been biochemically characterized recently (Funakoshi et al. 2008). The highest activities of this enzyme were achieved with D-Ala, D-Met and D-Phe as substrates. This corresponds with our findings, where after addition of these D-AAs also the highest accumulation rates of D-Ala and D-Glu were found. Repetition of D-AA supplementation and measurement experiments with a mutant in this single copy gene should unravel the function of this enzyme in the transamination process of D-AAs in *A. thaliana*. Regardless of the possibly responsible protein, our data imply that the transamination of D-AAs to D-Ala and D-Glu forms a central component of D-AA metabolization and degradation. In this respect, the question about the fate of D-Ala and D-Glu remains to be answered. Serine racemase as well as D-AA oxidase from plants were shown to use D-Ala as substrate (Fujitani et al. 2006; Gholizadeh and Kohnehrouz 2009) and can, therefore, be regarded as candidates for the depletion of D-Ala either to its L-enantiomer or pyruvate. Furthermore, an alanine racemase had been characterized from alfalfa and other plants (Ono et al. 2006) for the formation of L-alanine. But this protein still awaits its molecular identification.

The presented results indicate several ways for the uptake and the metabolization of D-AAs in plants. Taken together with all the other data about D-AAs in plants the question remains about the function of all the metabolic pathways and enzymes to handle D-AAs. The racemization of different D-AAs to their proteinogenic enantiomers can be interpreted in that way that they are utilized as nitrogen sources. In the debate about L-AAs as nitrogen sources it has been argued, that most tests had been performed under unphysiologically high concentrations of these compounds (Näsholm et al. 2009). The same argument also applies to physiological tests with D-AAs and their interpretation. With growth inhibition in the millimolar range these concentrations are partially orders of magnitudes higher than those naturally found in soils (Amelung and Zhang 2001; Brodowski et al. 2004). Therefore, plants just rarely encounter inhibitory concentrations of D-AAs. But these data are used as a main argument against the utilization of D-AAs from soil (Forsum et al. 2008). In our experiments, L-enantiomers accumulated after feeding of particular D-AAs. It is rather probable that this accumulation is caused by their racemization. This process would turn D-AAs into proteinogenic AAs and, therefore, be part of the plant's nitrogen pool. To verify this hypothesis on the one-hand side, the responsible racemases have to be identified and characterized physiologically (see above). On the other hand side, more detailed growth experiments with addition of D-AAs in non-inhibitory concentrations have to be made to investigate possible growth promoting effects accompanied by analyses of L- and D-AA contents of the plants.

Functions of D-AAs apart from nitrogen utilization have just rarely been reported in plants: In the case of tryptophan racemization, which had been reported in tobacco (Miura and Mills 1971) and wheat (Rekoslovskaya et al. 1999), it was assumed that D-Trp is involved in the biosynthesis of

indoleacetic acid (IAA). The sequencing of various plant genomes revealed that they contain genes of the peptidoglycan biosynthesis pathway, among them also D-Ala:D-Ala ligase. In the moss *Physcomitrella patens*, it has been shown that these genes are indispensable for chloroplast division (Machida et al. 2006). Although a part of this pathway got lost during the evolution of higher plants the D-Ala:D-Ala ligase remained in angiosperm genomes. The occurrence of D-alanyl-D-alanine in different plants (Brückner and Westhauser 2003; Robinson 1976) might be an indicator for the activity of the gene product but the functions of the gene and the dipeptide are still unknown. These examples show that the knowledge about cellular functions of D-AAs in plants is quite scarce and needs to be further investigated. Altogether actual data make the predominant usage of these amino acids as a nitrogen source for plants rather probable.

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