D-Amino acid transport, metabolism, and its link to ethylene regulation in *Arabidopsis thaliana*

Dissertation

der Mathematisch-Naturwissenschaftlichen Fakultät

der Eberhard Karls Universität Tübingen

zur Erlangung des Grades eines

Doktors der Naturwissenschaften

(Dr. rer. nat.)

vorgelegt von

Juan Sebastián Suárez Villada

aus Cali, Kolumbien

Tübingen

2019

Gedruckt mit Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät der Eberhard Karls Universität Tübingen.

Tag der mündlichen Qualifikation:	23.05.19
Dekan:	Prof. Dr. Wolfgang Rosenstiel
Berichterstatter:	Prof. Dr. Klaus Harter
Berichterstatter:	Prof. Dr. Claudia Oecking

Acknowledgements

Hereby, I want to state a few words to acknowledge the great efforts and supports that many people gave me in the last years and during the development of this thesis. I want to begin by thanking my family and to the Sänger family, and to childhood and teenage friends that have always been by my side in Colombia and Germany. This work is the fruits of all your love, patience, and support. I want to specially mention Astrid Scharfe for her lovely support and company.

From there on, people that influenced my professional, and personal life during my years of university studies, are countless. I want to thank Üner K for opening me the doors to his "about to be born" research group, and for opening the possibility for me to do a PhD. For his patiently teaching and directing from day 0 to the last day of my stay at the ZMBP as for making me feel cared, both academically and personally during all the time at Tübingen Uni. To Prof. Klaus Harter I thank his permanent support and advise, the provision of research facilities, and critical supervision of my research as well for being my doctoral supervisor. On top of that, for supporting the execution of the field trips to Colombia, and for participating in one of them.

To Dierk and Friederike Wanke, thanks for the space in the lab during the first year! It really changed my life getting out of the basement. Thanks for keeping my motivation and confidence high during so many years. Mark S, Joachim K, thanks a lot for your super positive attitude, advise and support in all analytical and technical respects. To Sabine H for her patience, and willingness to teach me many lab methods and techniques. And for taking over the task when I could not do it myself. I want to thanks Prof. Georg Felix for his support in ethylene measurements and Prof. Claudia Oecking for her interest in my research and being part of my committee and evaluation board.

I want to give special thanks to Luise B, Rebecca D, Anne M, Nina G, Natalie K, Angela F, Claudia H, Bettina S, Andy H, Michael H, Stefan F, Niklas W, Abhroop G, thanks, thanks a lot for all the information, materials, tricks and magic you showed me to survived the lab. But above all that, for making such a nice environment for having fun, during and after working, for being so open and nice to me. Last but not least, I want to thank the Deutscher Akademischer Austauschdienst for supporting my doctoral studies (DAAD 91567028). It is amazing to have had the opportunity to be supported by such an excellent scholarship.

Contents

1 Introduction **1**
1.1 Chirality of Proteinogenic Amino Acids 1
1.2 D-Amino Acids Containing Peptides and Proteins in Bacteria and Animals . 3
1.3 Soil as a Source of D-Amino Acids for Plants 4
1.4 D-Amino Acids in Plants 6

2 Aim of this thesis **11**

3 Article One **12**
3.1 D-Amino Acids in Plants: New Insights and Aspects, but also More Open Questions 12

4 Article Two **28**
4.1 D-Amino Acids Are Exuded by *Arabidopsis thaliana* Roots to the Rhizosphere 28
4.2 Supplementary Materials 41

5 Article Three **45**
5.1 AtDAT1 is a key enzyme of D-amino acid stimulated ethylene production in *Arabidopsis thaliana* 45
5.2 Supplementary Materials 87

6 Results and Discussion **99**
6.1 Transport of D-Amino Acids in *Arabidopsis thaliana* 99
6.1.1 AAPs, LHT1, and MRPs are Putative D-Amino Acids Transporters . 99
6.1.2 Passive Exudation and Active Uptake of D-Amino Acids 101
6.1.3 Fluorescent D-Ala “HADA”, a tool for D-Amino Acid detection in *Arabidopsis thaliana* 102
6.2 Metabolism of D-Amino Acids in *Arabidopsis thaliana* 103
6.2.1 Putative D-Amino Acid Metabolising Enzymes 104
6.2.2 DAT1 is a major determinant of D-Amino Acid Metabolisation in *Arabidopsis thaliana* 105
6.3 Physiological functions of D-Amino Acids in *Arabidopsis thaliana* 106

7 Outlook **109**

Bibliography **111**

List of abbreviations

A	*Arabidopsis*
AA	Amino Acid
AAPs	Amino Acid Permeases
ABC	ATP binding cassette
ACC	1-aminocyclopropane-1-carboxylic acid
ACO	ACC Oxidase
AdoMet	S-Adenosyl-methionine
Ala	Alanine
AOA	Amino-oxyacetic acid
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid or aspartate
At	*Arabidopsis thalinana*
ATP	Adenosine triphosphate
AVG	Aminoethoxy-vinylglycine
CCCP	Carbonyl Cyanide 3-Chlorophenylhydrazone
CO_2	Carbon dioxide
Col-0	Columbia-0
COOH	Carboxyl
Cys	Cysteine
D-	*Dexter*
D-AA	D-Amino Acid
D-Hyp	D-Hydroxyproline
D-Ser	D-Serine
DAOs	D-Amino Acid Oxidases
DAT1	D-Amino Acid Transferase 1
DATs	D-Amino Acid Aminotransferases
DDL	D-Ala-D-Ala ligase
DON	Dissolved Organic Nitrogen
GACC	γ-glutamyl-ACC
GFP	Green fluorescent protein
GGT	γ-glutamyl-Transferase
GLR	Glutamate Receptor
Glu	Glutamic acid
GSH	Tripeptide glutathione
H_2S	Hydrogen sulfide
HADA	7-hydroxycoumarin-3-amino-D-alanine
His	Histidine
HMW	High Molecular Weight
Hyp	Hydroxyproline
Ile	Isoleucine
JA	Jasmonic Acid
JA-ACC	Jasmonyl-ACC
kDa	kilo Dalton
KO	Knockout

L-	*Laevus*
L-AA	L-Amino Acid
Ler	Landsberg erecta
Leu	Leucine
LHT1	Lysine Histidine Transporter 1
LMW	Low Molecular Weight
Lys	Lysine
MACC	Malonyl-ACC or 1-malonylamino cyclopropane-l-carboxylic acid
Met	Methionine
mM	milli Molar
μM	micro Molar
MRP	Multidrug resistance-related protein
N	Nitrogen
N	Nitrogen
N_2	Dinitrogen
NH_2	Amine
NH_3	Ammonia
NMDA	N-Methyl-D-Aspartate
NO_3	Nitrate
O_2	Molecular oxygen
°C	Degree Celsius
OV	Orthovanadate
PCR	Polymerase chain reaction
PG	Peptidoglycan
Phe	Phenylalanine
PLP	Pyridoxalphosphate
Pro	Proline
proT2	Pro Transporter 2
ProTs	Pro Transporters
R	Side chain
SA	Salicylic Acid
SD	Standart deviation
Ser	Serine
SOM	Soil Organic Matter
SR	Serine Racemase
Trp	Tryptophan
UV	Ultraviolet
Val	Valine

Abstract

The relevance of the homochirality of proteinogenic amino acids for life is undisputed, but a growing number of biological functions could be assigned to the non-proteogenic D-enantiomers. When it comes to D-AAs in plants, information was relatively scarce for a long time. Recently, it has been shown that D-AAs are taken up by plants from soil, but could also be synthesised *de novo*, and that D-AAs cause physiological effects in plants. These were indications that D-AAs are responsible for a variety of yet poorly understood or even undiscovered functions in plants. For this reasons, I performed experiments to analyse transport, metabolism and functions of these molecules in *Arabidopsis thaliana*.

Previously, it was shown that D-AAs are taken up and utilised by plants, but how the plant reduces excessive amounts of them still remained unclear. Analyses of plant D-AA content after D-Ala and D-Glu treatment, opened the question if exudation of D-AAs into the rhizosphere takes place, and whether it plays a role in the reduction of D-AA content in plants. Exudation of D-Ala and D-Glu could be confirmed by AA analyses of growth media from seedlings treated with these D-AAs. Further tests revealed that other D-AAs are also secreted. Exudation experiments with transport inhibitors revealed that root exudation of D-AAs is rather passive and comparable to that of L-AAs, whereas uptake of D-AAs was found to be active.

It was also found that plants are able to reduce their D-AA contents by means other than exudation, and therefore metabolic processes were postulated. Analyses of the D-AA specific transaminase (AtDAT1) revealed that this enzyme is responsible for the metabolisation of the majority of tested D-AAs in *Arabidopsis thaliana*. The results of our biochemical experiments revealed that D-Met is the preferred substrate for this enzyme, with D-Ala as the major product. Furthermore, the loss of this enzyme led to a triple response and increased ethylene synthesis after D-Met treatment, indicating a functional relation between regulation of D-AA metabolism and plant development. These results show that AtDAT1 is a crucial enzyme in the D-AA metabolism in *Arabidopsis thaliana* and that these molecules have a relevant function in higher plants, contrary to what was communally accepted.

Zusammenfassung

Die Bedeutung der Homochiralität proteinogener Aminosäuren ist unbestritten, jedoch konnte auch den nicht-proteinogenen D-Enantiomeren eine wachsende Anzahl biologischer Funktionen zugeordnet werden. Über die Rolle, die D-Aminosäuren (D-AAs) in Pflanzen spielen, gibt es bislang relativ wenige Informationen. Kürzlich konnte gezeigt werden, dass Pflanzen D-AAs einerseits aus dem Boden aufnehmen, andererseits *de novo* synthetisieren können und dass D-AAs physiologische Effekte in Pflanzen bewirken. Dies weist darauf hin, dass D-AAs für eine Vielzahl von Funktionen in Pflanzen verantwortlich sein können, die bislang wenig verstandenen oder sogar noch nicht beschrieben sind. Aus diesen Gründen habe ich Experimente durchgeführt, die den Transport, den Metabolismus und die Funktion dieser Moleküle in *Arabidopsis thaliana* analysieren. In vorherigen Untersuchungen wurde gezeigt, dass D-AAs aufgenommen und verwertet werden, aber wie Pflanzen überschüssige Mengen von D-AAs reduzieren, ist bislang unklar. Konzentrationsbestimmungen von D-AAs nach Verabreichung von D-Ala und D-Glu warfen die Frage auf, ob D-AAs in die Rhizosphere exsudiert werden und ob dieser Vorgang eine Rolle für die Konzentrationsabnahme innerhalb der Pflanze spielt. Bei Keimlingen in Flüssigkultur, die mit D-Ala und D-Glu behandelt wurden, konnten diese Aminosäuren im Außenmedium nachgewiesen werden, was eine Exsudation bestätigt. Weitere Experimente zeigten, dass auch andere D-AAs ins Medium sekretiert wurden. Experimente mit Transportinhibitoren zeigten, dass die Exsudation passiv erfolgt und vergleichbar mit der Exsudation von L-AAs ist, während die Aufnahme von D-AAs einen aktiven Vorgang darstellt. Pflanzen sind nicht nur in der Lage, die Konzentration von D-AAs durch Exsudation zu reduzieren, sondern können sie auch in metabolischen Prozessen verwenden. Die Analyse der D-AA-spezifischen Transaminase (AtDAT1) ergab, dass dieses Enzym für den größten Teil des Metabolismus der D-AAs in *Arabidopsis* zuständig ist. Unsere biochemische Charakterisierung von AtDAT1 ergab, dass D-Met das bevorzugte Substrat darstellt während D-Ala das Hauptprodukt ist. Ein Verlust der Enzymfunktion führt zu einer Triple Response und verstärkter Ethylen-Synthese nach D-Met-Behandlung, was auf eine funktionelle Verbindung zwischen der Regulation des D-AA-Metabolismus und der Pflanzenentwicklung hinweist. Diese Ergebnisse zeigen, dass AtDAT1 ein zentrales Enzym des D-AA-Metabolismus in *Arabidopsis* ist und dass D-AAs, anders als bisher angenommen, eine wichtige Funktion in höheren Pflanzen ausüben.

Chapter 1

Introduction

1.1 Chirality of Proteinogenic Amino Acids

There are twenty proteinogenic amino acids (AAs). This means that out of the many AA molecules that are known, there is only a selected group of twenty L-AAs which are exclusively used by all biological systems to build up their peptides and proteins Raven et al. (2005); Taiz and Zeiger (2010). On the other hand, these twenty AAs also have non-proteinogenic D enantiomers. To understand the relevance of chirality, it is necessary to explain the meaning of it in physics, chemistry, and biology, as well as its relation to AAs. Chirality is a physical phenomenon that relates to the topological features of particular objects ranging from chemicals to proteins, and other living or non-living objects (a fan, or a screw are chiral). No matter what the object is, if it is chiral, it will include a chiral centre and will be able to occupy space in two different ways without changing any of its components. By definition, AAs are chemical organic compounds that mostly contain an asymmetric alpha carbon to which four different chemical groups are bound. These are two functional groups: Namely, an anime (-NH_2) and a carboxyl (-COOH) group. Additionally, two more groups bind to the alpha carbon a side chain (-R) that is specific for each amino acid and a hydrogen (-H) atom. Due to the alpha carbon in the amino acid, as well as the four different groups attached to it, any given amino acid (with exception of Glycine) can be synthesised as either D or L enantiomer Vollhardt and Schore (2007).

The two isomers are non-superposable. Thus they are not identical even though both consist of the same parts. In stereochemistry, those are called stereoisomers (spatial isomers), and more precisely, enantiomers or mirror images. If an enantiomer is embedded in a symmetric environment, the two isomers will have the same chemical and physical properties. However, there is one exception: Its ability to rotate plane-polarised light will be different. One isomer is able to rotate plane-polarised light to the right (in Latin denoted as *Dexter* or D-), while the other, to the left (in Latin denoted as *Laevus* or L-). These properties are

used to differentiate D- and L- enantiomeric compounds.

In a biochemical context, however, the situation changes dramatically as many biomolecules are enantiomers, and their environment is not symmetric. Therefore, in several situations the effect of the two isomers on biological systems can vary significantly Raven et al. (2005); Taiz and Zeiger (2010). A classical example is the terpenoid Carvone, which, in the L- configuration smells like mint while as D- has a spicy odor. The drug Thalidomide exemplifies a more extreme case, as its pharmaceutical effects change depending on the enantiomeric proportions of D- and L- Thalidomide in the drug Agranat et al. (2002). Thalidomide is a derivate of D- or L-glutamic acid and its solubility in water, as well as its hydrolytic cleave, differs according to its chirality. Surprisingly, pure D- or L- enantiomers have higher solubility in water than the racemate (mixture of equal amounts of D- and L- enantiomers) Eriksson et al. (2001). The L-Thalidomide has been shown to be more teratogenic than the D- form. When administrated as racemic mixture, its toxicity is reduced and it produces different pharmaceutical effects Eriksson et al. (2001).

However, it is worthwhile to mention that D-AAs can also be used *in vitro* to synthesise peptides exclusively with D-AAs. Peptides that occur naturally as “all L-AAs”like Cecropin A, from the moth *Hyalophora cecropia*; Magainin 2 amide, from the frog *Xenopus laevis* and Melittin, from the honeybee *Apis mellifera* are potent antibiotics against several gram-positive and gram-negative bacteria. When synthesised as “all D-AAs ”peptides, these antibiotics are not only equally active to the “all L-AAs”peptides, but they are significantly more resistant to enzymatic degradation Wade et al. (1990).

It is widely accepted that proteinogenic L-AAs are the basis of life. Apart of being elementary building blocks of proteins, it is well known that they are primary metabolites as well. The common usage of these AAs is also one of the fundamental reasons behind the universality of the genetic code, as every single living organism makes use of them to build and operate most of its molecular machinery in the same way. The limitation of protein-coding sequences on twenty different L-AAs is known as homochirality. It was one of the key developments in evolution to enable the compatibility between different life forms, regardless if they belong to the same species or different taxon. For instance, bacterial genes can be transcribed and translated in an eukaryotic cell because the translation machineries of both taxa use the same L-AAs Raven et al. (2005); Taiz and Zeiger (2010).

The question of why some enantiomers are dominant in biological systems is beyond the scope of this thesis. Nevertheless, it is evident that L-AAs play the predominant role in all biological systems and processes. Nonetheless, D-AAs are found in many biological systems, and they fulfil different functions there. This opens the question how D-AAs fit into the homochiral world of L-AAs.

1.2 D-Amino Acids Containing Peptides and Proteins in Bacteria and Animals

It has been proposed that inside the cell, D-AAs are products of chemical and enzymatic racemisation of L-AAs Friedman (1999) or they are synthesised by aminotransferases from other D-AAs Radkov and Moe (2014). D-AAs can be found in cells both as free D-AAs or incorporated into proteins and non-protein peptides. Nevertheless, they are not proteinogenic as this incorporations happens rarely, and natural proteins consisting exclusively of D-AAs are not known.It is different in small peptides, as D-AAs can be regular components of them.

A prominent example of such usage is the bacterial cell wall: It contains many layers of peptidoglycans (PG) which are polysaccharide chains cross-linked by oligopeptides. Constituent parts of these oligopeptides are D-Ala and D-Glu, which protect the cell due to their resistance to cleavage by conventional proteases Typas et al. (2012). Biologically active D-AAs are not exclusive for bacteria; D-(+)-octopine (a derivative of L-Arginine and D-Alanine) has been shown to act as a lactic acid analogue in muscle tissue of in invertebrates like octopus and scallop, and is also known to be produced in crown gall tumours in plants Hockachka et al. (1977); Lippincott and Lippincott (1970).

Other invertebrates are able to produce peptides containing D-AAs that act as toxins, antibiotics or hormones such as Achantin I and Fulicin from the African giant snail (*Achatina fulica*) Kamatani et al. (1989); Ohta et al. (1991). Achantin I includes a D-Phe in the second position of the peptide while Fulicin has a D-Asn in that positionCava et al. (2011). In the early eighties, demorphine, with a D-alanyl residue, was found in the opioid peptides from the skin of a frog *Phyllomedusa sauvagei* Montecucchi et al. (1981). Moreover, according to Jilek et al. (2012), a peptide with antimicrobial activity was isolated from the skin secretions of *Bombina spp.* (Fire-bellied toads) that contained a D-allo-Isoleucine (produced as a byproduct of isoleucine transamination) in the second position. It has also been possible to isolate D-AA containing peptides from mammals. For example, the venom of male Platypus (*Ornithorhynchus anatinus*) contained D-AA residues. However, its function is still unknown to date Ollivaux et al. (2014). More examples in this respect are summarised in Fujii (2002); Martínez-Rodríguez et al. (2010).

The occurrence of D-AAs is not confined to bacteria and lower animals, as it has been shown that collagen plays a particular role in the skin and articulations of mammals and fishes. Where its properties change depending on the type and content of D-AAs. In skin samples of pig and cod, D-Asp, D-Pro, and D-Hyp (D-Hydroxyproline) were detected in concentrations up to 15pmol/mg Huang et al. (2018). Various free D-AAs were found in different tissues and fluids of humans and other mammals Hamase (2007); Hamase et al. (2002). D-Asp residues were detected in α- and β- crystallines proteins of the human lenses

as in other human proteins such as phosphophoryn, osteocalcin; elastin, amyloid; elastin and, keratin Fujii et al. (2011); Ollivaux et al. (2014).

In other organs like the conjunctivae, cornea and intestine, D-Asp was also detected. However, it is not know to which protein it is associated. In all cases the D-Asp residues in proteins and peptides were associated to age related diseases Fujii et al. (2011). Free D-Asp was found in the brain tissue of newborn rats in concentrations up to 160 nmol/g, exceeding the concentration of many L-AAs. It is also present in other tissues as well as in human blood. In all the cases, the concentration of D-Asp drops drastically as the organism ages Dunlop et al. (1986). Furthermore, it has also been proven that free D-Asp plays an important role in vertebrates exocrine and endocrine glands, such as the parotid, or the Harderian gland Di Fiore et al. (2014). Anomalous levels of D-Ser and D-Asp seem also to be connected with psychological disorders and diseases of the endocrine system (for reviews see Balu and Coyle (2015); Fuchs et al. (2005); Katane and Homma (2011) and Sasabe et al. (2016). Specifically in mammals, a local increase of D-Asp concentrations facilitates the secretion of anterior pituitary hormones. At the same time, it also regulates negatively the melatonin synthesis in the pineal gland and can modulate the production of sexual steroids Di Fiore et al. (2014).

Besides D-Asp, D-Ser also has an impact on different processes in humans. Concentrations of D-Ser in the brain of mammals are relatively high, and it has been found to work as an endogenous ligand for the glycine site of the N-methyl-D-aspartate (NMDA) receptor. Therefore, it is involved in the neuron-glia interaction. Disturbances either in D-serine concentrations or in its molecular metabolism and function in the brain, induce a variety of neuropsychiatric symptoms, especially schizophrenia Hashimoto et al. (1993); Nishikawa (2005); Snyder and Kim (2000). In humans, several proteins related to certain diseases like arteriosclerosis, Parkinson and Alzheimer, contain D-AAs, in particular D-Asp and D-Ser, which are generated by racemisation of the corresponding L-AA residue in the AA chain of the protein Fujii et al. (2011) . β - Amyloid cores contain considerable amounts of D-Ser and D-Asp in their peptide. The deposition of the beta-amyloid peptide in the grey matter of the brain as senile plaques is a principal event in the neurodegenerative process, these plaques are usually found in the brain of Alzheimers disease patients Kaneko et al. (1995).

1.3 Soil as a Source of D-Amino Acids for Plants

In contrast to the knowledge about abundance and functions of D-AAs in bacteria and animals, information about these molecules in plants is relatively scarce. However, this knowledge is crucial to understand plant growth and development due to the fact that plants are permanently exposed to D-AAs in soil. Soil organic matter (SOM) contains nitrogen (N) compounds from different sources such as N_2 fixation, atmospheric deposition, animal feces, agrochemical fertiliser and decaying dead plants, microbes and animals Farrell et al. (2014).

The majority of N in soil is in the organic pool, and part of it is addressed as dissolved organic N (DON) in the soil solution. DON is composed of high molecular weight (HMW) (>1 kDa) molecules and compounds that are degraded into low molecular weight (LMW) (< 1 kDa) peptides and amino acids Farrell et al. (2011, 2014); Hill et al. (2012, 2011). LMW DON consists mainly of organic nitrogen containing oligomers and monomers, some of which can be directly taken up by soil microorganisms as well as by plants Farrell et al. (2014). This holds true independently if the plants form mycorrhizal interactions or not Raab et al. (1999).

It has been proven that in both, woodlands and managed pastures, soil DON dominated the soil N pool representing up to 70% of extractable N. In alpine and arctic soils, AAs represent up to 40% of DON Jones et al. (2002), whereas in woodland and pastures this portion of AAs is just 10-20%. Surprisingly, soil nitrate was in general the smallest N pool Prendergast-Miller et al. (2015). Miller and colleagues argue that soil microorganisms and plants are able to take up free AAs or peptides as N source and in addition, that AAs are an important source of N for plants Hill et al. (2011); Prendergast-Miller et al. (2015); Soper et al. (2011). Competition between plants and microorganisms for DON, especially for free amino acids, have been previously studied by Jones et al. (2005). In this work, it was demonstrated that glycine was taken up rapidly by microorganisms, at a concentration range of 0.1 mM to 10 mM of soil solution. However, at higher concentrations, plants were better in the capture of this AA. Furthermore, Jones et al. (2005) suggested that plant capture of soil DON may primarily occur in an organic-rich soil where concentrations of free AAs are high.

In soil and water samples, almost all D-enantiomers of proteinogenic AAs could be found in significant amounts. D-Pro, for example accounts up to 60% of Pro in the majority of soil samples. D-Lys accounts for 6 to 40% of Lys. And D-Ala together with D-Asp were found to represent between 3 to 25% of Ala and Asp pool Vranova et al. (2012). The D-AAs derived from bacterial peptidoglycan, as well as from plants, were found in significant amounts in the surface of different oceans (up to 30%) and marine aerosols (up to 20%). This was true especially for D-Asp, D-Glu, D-Ser and D-Ala Wedyan and Preston (2008). It was also reported that D-Leu and D-Ile were found exclusively in surface water samples. Another study in soil showed that D-Asp, D-Glu and D-Ala are abundant in large amounts in comparison to their corresponding L-enantiomers Pollock et al. (1977). However, these three D-AAs just represent 1.3 to 2.0% of the total amount of AA nitrogen in the analysed soils. Moreover, they found significant quantities, between 5 to 16%, of other D-AAs.

A considerable part of the D-AAs found in soil originates from bacteria in animals, as the microbiota in their guts are full of diverse bacteria species, which do just not release considerable amounts of D-AA in their host but also harbour themselves D-AAs in their cell

walls Sasabe et al. (2016). In either case, these D-AAs are later deposited from the animal as feces and dissolve in the soil. Free D-AAs from soil-borne bacteria are another source of these compounds in soil. Some gram-negative bacteria associated with environments like soil, water, and animal hosts are a source of broad-spectrum racemases (Bsr) in the rhizosphere. These enzymes are able to convert a wide variety of L-AAs into D-AAs from proteinogenic and non-proteinogenic L-AAs Espaillat et al. (2014). Consequently, the composition of D-AAs in soil is affected by the activity of these enzymes Aliashkevich et al. (2018). Thus, the amount of D-AAs in the rhizosphere can exceed 10% of the corresponding L-enantiomer Amelung et al. (2006); Brodowski et al. (2005).

It is important to highlight that the concentrations of free AAs in soil, including D-AAs, change according to the location and season. N availability fluctuates seasonally due to factors such as temperature, extracellular enzymatic activity; pH, usage of the soil; animal feces, and plant root exudation. Additionally, the demand and competition for its uptake by microorganisms and plants is very likely to change in different seasons Lipson and Näsholm (2001); Prendergast-Miller et al. (2015); Zhang et al. (2017).

1.4 D-Amino Acids in Plants

Since the seventies, it is well known that plants are able to uptake D-AAs from the soil. Evidence about this process has increased over four decades Aldag and Young (1970); Forsum et al. (2008); Svennerstam et al. (2007); Vranova et al. (2012). Furthermore, it was shown that almost all D-enantiomers of proteinogenic amino acids could be detected in *Arabidopsis thaliana* plants after their exogenous application Forsum et al. (2008); Gördes et al. (2011). But beyond that, very little is known about the mechanism involved in the uptake of D-AAs, their metabolism and the possible functions they may fulfill in plants. This is remarkable considering the fact that plant roots are permanently surrounded and challenged by D-AAs, mainly D-Ala and D-Glu, as degradation products of the peptidoglycan layer of bacterial cell walls Dworkin (2014). In general, plants respond to D-AA exposure in roots or leaves differently as the reaction is concentration dependent. It has been suggested that sub-millimolar concentrations are more realistic to be found in natural scenarios and that they would induce a physiological effects besides growth inhibition Gördes et al. (2011).

This opened the question whether active transport of D-AAs takes place, and which properties it may have. It is known that plants with mutations in the Lysine Histidine Transporter 1 (LHT1) were resistant to concentrations of 3mM D-Ala. The uptake of D-Ala was reduced by 90% in *lht1* mutants, as well as of other D-AAs but to a lower extent Svennerstam et al. (2007). This suggests that this protein is able to transport D-Ala. Also in *Arabidopsis thaliana* Pro transporters (AtProTs) have been described to facilitate the uptake of both D and L isomers of AAs. In toxicity analyses, *proT2* mutants displayed

reduced sensitivity against 8mM D-Pro Lehmann et al. (2010).

Together with LHTs, the Amino Acid Permeases (AAPs) is another group of proteins which mediate proton-dependent import of amino acids into the cell. So the fact that they transport L-AAs is well accepted and understood. LHT1, for example, was first identified by Chen and Bush (1997) and then reported to be expressed in the rhizodermis and mesophyll, enabling *Arabidopsis thaliana* to take up AAs from the roots and supply them to the leaf mesophyll via the xylem. This holds true for several AAs, as this transporter is known to have a broad-specificity and high affinity for neutral and acidic AAs Hirner et al. (2006). AAPs are involved in AA transport into the seeds and embryos and exhibit very broad specificities transporting all twenty amino acids naturally found in proteins Rentsch et al. (2007). Six AAPs in *A. thaliana* transport neutral and charged amino acids with varying specificities and affinities. AAP1-6 were shown to transport D-Ala Fischer et al. (2002). It was shown that LHT1 and AAP5 have a complementary affinity spectrum; therefore, basic amino acids are taken up by AAP5 while neutral and acidic AAs are taken up by LHT1 Svennerstam et al. (2011). They also reported that AAP1 might not be involved in AA uptake at naturally occurring concentrations. Nevertheless, AAP1 is known to transport uncharged/neutral L-AAs into roots and embryos of *Arabidopsis thaliana* Lee et al. (2007); Sanders et al. (2009); Svennerstam et al. (2011).

The uptake of D-AAs imply the necessity of their metabolism in plants. Growing evidence supports the fact that plants possess genes encoding enzymes to use D-AAs as substrates or even to produce D-AAs. Examples of such enzymes are D-Amino Acid Aminotransferases (DATs), D-Amino Acid Oxidases (DAOs) and, specific racemases for Ala, Ser, and Ile. These enzymes could participate in the metabolism of D-AAs because they confer the plant the ability to synthesize and metabolize D-AAs as well as to maintain their concentrations at required levels for any physiological activity or at least below a toxic level Aliashkevich et al. (2018); Fujitani et al. (2007, 2006); Funakoshi et al. (2008); Ono et al. (2006); Riemenschneider et al. (2005); Strauch et al. (2015). Previous works from Gördes et al. (2011) revealed that *Arabidopsis thaliana* accession Columbia-0 (Col-0) is able to convert D-AAs like D-Trp, D-Phe, D-Met and D-His partially to their respective L- enantiomers. However, the feeding of almost all tested D-AAs led mainly to the production of D-Ala and D-Glu. This did not hold true for the *Arabidopsis thaliana* accession Landsberg erecta (Ler) as it is incapable of both, the D-AA to L-AA and the D-AA to D-Ala/D-Glu conversion Gördes et al. (2013). This suggested that the responsible enzyme for the D-AA to L-AA and the D-AA to D-Ala/D-Glu conversion in Col-0 is affected in Ler. It was postulated that a single specific aminotransferase would cause these effects Vranova et al. (2012).

For a long time there was the common opinion that all D-AAs are detrimental for plant growth and therefore physiologically useless for plants. Strong growth inhibition of *Ara-*

bidopsis thaliana at concentrations of 1mM D-Ala and D-Ser was also reported. The same effect was also assigned to D-Arg and D-Ser at 0.75 mM and 0.5 mM, respectively Erikson et al. (2004); Forsum et al. (2008). But some of them, like D-Ile, D-Val, or D-Lys, can even promote plant growth at low milli-molar concentrations Aliashkevich et al. (2018); Erikson et al. (2004). In wheat, for example, D-Ala was shown to be a viable nitrogen source and its uptake was five-fold faster than the uptake of NO_3- Hill et al. (2011). As a plant-pest control, Li and Wang (2014) proved that D-Leu by alone, or in combination with copper, reduces the number of canker lesions caused by *Xanthomonas citri* in citrus trees by inhibiting the biofilm formation. Additionally, D-Ala was reported to act as a stress signal in *Landoltia punctate* duckweed, Monselise et al. (2015). Moreover, in mosses D-Ala was shown to be incorporated as a structural element into the chloroplast envelopes Hirano et al. (2016). Finally, D-Ser was shown to be involved in plant signal transduction of pollen tube growth in *Arabidopsis thaliana* and *N. benthamiana* by regulating the glutamate receptor GLR1.2. It belongs to a group of plant proteins closely related to mammalian NMDA receptors known to be regulated by D-Ser Forde and Roberts (2014); Michard et al. (2011).

Since the early sixties, it was recognised that D-AAs positively stimulate the production of ethylene in enzymatic assays with plants extracts or macerates.

Ethylene is produced in all tissues and modulates the development of many organs and processes in plants Dubois et al. (2018). It is regulated by, and it can regulate the plant response to, different biotic and abiotic stresses. Ethylene is involved in the coordination of many developmental stages of the plant. For example, its production is induced in germination, leaf abscission; flower senescence, and fruit ripening, but also by stresses like flooding, drought stress, chilling, and mechanical wounding Dubois et al. (2018); Raven et al. (2005); Taiz and Zeiger (2010). Other hormones like auxins and ethylene itself can promote ethylene biosynthesisBuchanan et al. (2015). On the other hand, inhibition of ethylene synthesis can be induced by high concentrations of CO_2, lack of O_2 and temperatures above 35°C Raven et al. (2005); Taiz and Zeiger (2010). Plants response to pathogen attack is coordinated by several hormones such as Jasmonic Acid (JA), Salicylic Acid (SA), and ethylene. In fact, it has been shown that JA and ethylene signalling operate synergistically in the expression of pathogen response genes Bari and Jones (2009).

To understand the putative role of D-AAs in ethylene production, which mainly occurs in two steps as shown in the figure 1.1: Firstly, the 1-aminocyclopropane-1-carboxylicacid (ACC) synthase uses S-Adenosyl-methionine (AdoMet) as a substrate to produce ACC. Secondly, this ACC is oxidised by the ACC Oxidase and turned into ethylene. Alternatively, ACC can be conjugated with Malonyl-CoA into malonyl-ACC (MACC) by the N-malonyltransferase as the major way to metabolise excess ACC Van de Poel and Van Der Straeten (2014).

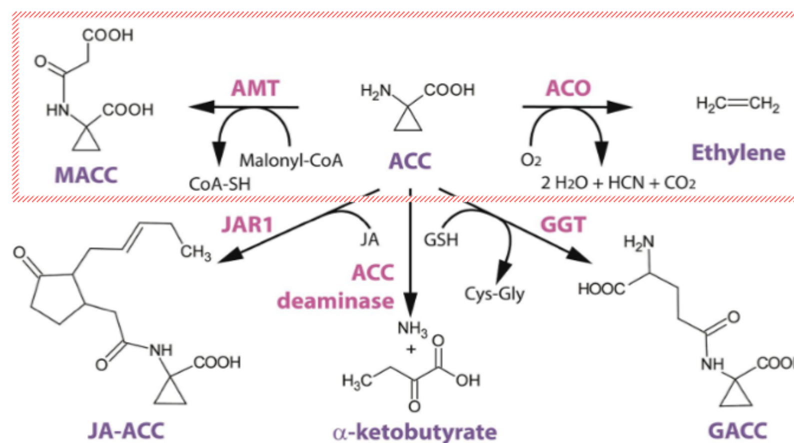


Figure 1.1: ***Structural scheme of ethylene biosynthesis and 1-aminocyclopropane-1-carboxylicacid (ACC) conjugation/metabolism. Modified from Van de Poel and Van Der Straeten (2014)***

In this case of malonylation, ACC can be outcompeted by non-polar D-AAs Kionka and Amrhein (1984). Therefore, D-AAs and ACC may compete for a common N-malonyltransferase. Accordingly, suppression of MACC formation by a D-AAs increases the level of ACC available to the ethylene-forming enzyme (ACC-Oxidase) and thus, enhance the ethylene production Kionka and Amrhein (1984). Conjugation of ACC appears to be important for hormone biosynthesis in a comparable way to conjugation of auxin and cytokinin Raven et al. (2005); Taiz and Zeiger (2010).

N-malonylation of AAs in plants is a well studied phenomenon and the malonylation of L-Trp was reported in pea, spinach, and tomato Good and Andreae (1957). Later, it was published that the same process also occurs with D-Trp Zenk and Scherf (1963). From there on, it was confirmed that N-malonylation of D-AAs is a common phenomenon of D-AA catabolism in higher plants, for example in cocklebur (*Xanthium strumarium*), mung-bean (*Vigna radiate*), and pea (*Pisum sativum*) Aldag et al. (1972); Eschrich and Hartmann (1969); Keglević et al. (1968); Ladešić et al. (1971); Rosa and Neish (1968). Additionally, Ogawa et al. (1973) confirmed the occurrence of endogenous N-malonyl-D-alanine in pea seedlings. Although the responsible N-malonyl transferase has been biochemically characterised Benichou et al. (1995), its identification is still pending. The final characterisation of this enzyme would also answer the question whether it is really responsible for D-AA stimulated ethylene production.

Chapter 2

Aim of this thesis

This thesis aims to assess the relevance of D-Amino Acids (D-AAs) in *Arabidopsis thaliana*. These compounds have been proven to have specific relevant functions in all kingdoms of life, but in plants information about these functions is scare and still under discussion. Thanks to recent findings, it is clear that plants have specific molecular mechanisms to transport and metabolise these compounds, and even more, D-AAs may have putative functions in their physiology.

Three main topics should be addressed in this thesis: Transport, metabolism, and functions of D-AAs in *A. thaliana*. For such purposes, different mutants and ecotypes of *Arabidopsis thaliana*, as well as distinct biochemical and biological approaches.

In regards to transport, the goal was to determine whether *Arabidopsis thaliana* is able to take up or import free D-AAs from the rhizosphere. In addition, I wanted to investigate whether active export from the roots to the rhizosphere or translocation is taking place. With that aim, mutant lines of *Arabidopsis thaliana* affected in amino acid transport like *lht1*, *aap5*, *aap1*, and *mrp5* were used to analyze the flux of D-AAs in the root-rhizosphere interphase. In order to test the hypothesis whether *Arabidopsis thaliana* is able to metabolize D-AAs via enzymatic transamination, the difference between the *Arabidopsis* accessions Col-0 and Led to metabolise these compounds was used as a starting point. Additionally, genetical and biochemical analyses of the D-Amino Acid Transferase 1 (DAT1) in different ecotypes and in the knock out mutants of the encoding gene should be carried out.

Finally, this thesis also addresses the physiological functions of D-AAs in *Arabidopsis thaliana* as recent publications suggest possible functions of D-AAs in plants, such as nitrogen utilisation, stress adaptation or chloroplast division. The aim was to find out if the lack of DAT1 is sufficient to causes phenotypic changes in *Arabidopsis thaliana* plants.

Chapter 3

Article One

3.1 D-Amino Acids in Plants: New Insights and Aspects, but also More Open Questions

Status: Published

Position of candidate in list of authors: Co-author.

Scientific ideas by the candidate: 50%

Data generation by the candidate: 75%

Analysis and Interpretation by the candidate: 50%

Paper writing done by the candidate: 30%

Chapter 7

D-Amino Acids in Plants: New Insights and Aspects, but also More Open Questions

Üner Kolukisaoglu and Juan Suarez

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.68539

Abstract

The relevance of the homochirality of proteinogenic amino acids for life is undisputed, but also to their D-enantiomers a growing number of biological functions could be assigned. When it comes to D-amino acids in plants, information was relatively scarce for a long time. Nowadays, also in this field, knowledge is growing which will be presented and discussed in this review. In this respect, it was shown that D-amino acids are taken up by plants from soil but could also be synthesized de novo. Investigations of plant D-amino acid metabolism as well as other studies revealed a central function of D-Ala in plants, which await further elucidation. Also other D-amino acids are shown to cause physiological effects in plants, ranging from nitrogen utilization over stress adaptation to chloroplast division, and indicate that D-amino acids are responsible for a variety of yet poorly understood or even undiscovered functions in plants.

Keywords: D-amino acids in plants, D-amino acid biochemistry, functions of D-amino acids in plants and bacteria, D-alanine

1. Introduction

L-amino acids (L-AAs) are the basis of life on our planet (and maybe also on other animate ones), mainly due to their property to be the building blocks of all proteins. These proteinogenic amino acids are also one of the fundamentals of the universality of the genetic code. The limitation of protein coding sequences on 20 different L-AAs was one of the key developments in evolution to ensure the compatibility between different life forms, regardless if they belong to the same species or if their genetic material is exchanged between a bacterial pathogen and its plant host, as it is the case in the *Agrobacterium*-plant relationship.

INTECH
open science | open minds

© 2017 The Author(s). Licensee InTech. This chapter is distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/3.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. CC BY

In the course of limitation to 20 proteinogenic amino acids, also the convention of exclusive usage of L-AAs (homochirality) in the primary structure of proteins evolved to ensure the intended structure and functionality of a protein. But since the very beginning of evolution also the enantiomers of L-AAs, the D-amino acids (D-AAs) were existent. These D-AAs are mainly products of abiotic and enzymatic racemization of L-AAs [1] or synthesized by aminotransferases from other D-AAs [2]. One possibility of organisms during evolution to handle D-AAs would have been to develop mechanisms for their elimination. But instead almost all organismal classes in the tree of life learned to live with substantial amounts of D-AAs and even made use of them. One prominent example of such usage is the bacterial cell wall. It contains many layers of peptidoglycan, polysaccharide chains cross linked by oligopeptides. Parts of these oligopeptides are D-AAs, especially D-Ala and D-Glu, which protect the cell due to their resistance to cleavage by conventional proteases [3].

The decay of the bacterial cell wall is also one of the major sources of D-AAs in soil [4] and the reason why plants are specifically surrounded and challenged by D-AAs. In soil samples, almost all D-enantiomers of proteinogenic amino acids could be found in significant amounts [4–7]. For a long time, it was assumed that D-AAs are just inhibitory for plant growth and therefore plants evolved mechanisms to avoid and eliminate them. But recent studies have shown that plants are instead able to import D-AAs and metabolize them. They even synthesize D-AA themselves for physiological reasons, which raised the question about the beneficial effects of D-AAs for plants. In this review, we want to summarize the current knowledge about these processes and highlight different aspects and questions of future research with a focus on *Arabidopsis thaliana* as a model plant to investigate D-AAs in plants.

2. How do D-AAs get into the plant?

It is a widely accepted fact that plants harbour free D-AAs as they could be identified in different plant species and tissues [8–12]. In this regard, the question arose, if all these amino acids are synthesized by plants themselves or also taken up from the soil. By detecting various D-AAs in seedlings of runner and soy beans, garden and water cress, as well as alfalfa, raised on amino acid free media in Ref. [10], first indirect indications were given that these plants are able to synthesize D-AAs de novo. This hypothesis was supported by the discovery, identification and characterization of alanine, serine and isoleucine racemases from different plant species [13–16]. The toxicity of D-AAs on Arabidopsis [12, 17] and the toxic effect of D-Ser on other species [18] were first hints for a general D-AA uptake mechanism in plants. Furthermore, it was shown that almost all D-enantiomers of proteinogenic amino acids could be detected in Arabidopsis plants after their exogenous application [12, 19]. The direct uptake of D-Ala and its utilization could be demonstrated for the first time in wheat [20].

At that point, it was interesting which transporters are involved in the uptake of D-AAs and which properties they have. One of the first hints in this respect was given by the works of Ref. [21]. In an initial screen, they germinated Arabidopsis mutants on 3 mM D-Ala, a toxic D-Ala concentration for wild-type plants and found plants to survive with mutations in the *LYSINE HISTIDINE TRANSPORTER 1 (LHT1)* gene. Furthermore, the uptake for D-Ala in

these plants was reduced by more than 90% in these mutants. Also the uptake of many other D-AAs was found to be reduced in *lht1* mutants [12]. This was the first evidence that a broad range specificity L-AA transporter in plants was also able to take up D-AAs from soil. A second example for plant D-AA transporters are the proline transporters of *A. thaliana* (AtProTs); they facilitate the uptake of L- and D-Pro, and mutants of *AtProT2* show reduced sensitivity against D-Pro [22].

These reports implied that transporters involved in the uptake and transport of L-AAs could also be responsible for the same processes of D-AAs. That D-AA transporting proteins are most probably not restricted to the LHT, and ProT families were given by experiments of our group: in toxicity tests, performed as described before [12], an Arabidopsis mutant of *LHT1* was confirmed to be less sensitive against D-Ala than the corresponding wild type (**Figure 1A**). A mutant of *AAP1*, belonging to the *Amino Acid Permease* family and shown to be responsible for root uptake of uncharged L-AAs [23], revealed a higher resistance against D-Met and D-Phe than Col-0 (**Figure 1B** and **C**). This result implies that AAP1 is involved in the import of D-AAs, specifically of D-Met and D-Phe.

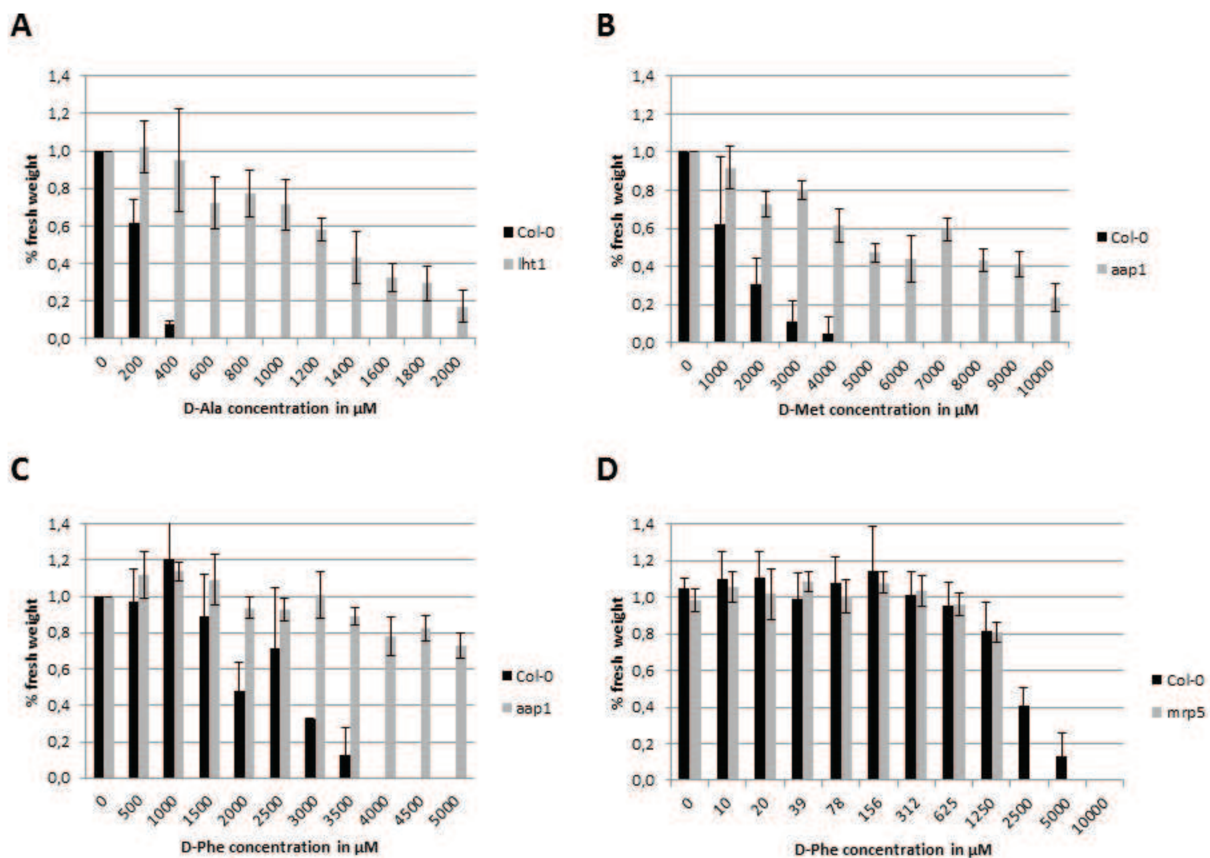


Figure 1. Seed growth inhibition of D-AAs on different transporter mutants compared to their corresponding background accession Col-0.

For these diagrams, different mutants and their corresponding background line Col-0 were germinated for 14 days in ½ MS + 1% sucrose including different D-AAs. Afterward the fresh

weight was recorded. For each measurement, three times eight seedlings were measured. In (A), *lht1* was treated with D-Ala, in (B) and (C), *aap1* was treated with D-Met and D-Phe, respectively, and in (D), *mrp5* was treated with D-Phe. Mutant values are always represented by grey blocks, control values in black blocks. All values are calculated and given in relation to the untreated control seedlings. Error bars indicate standard deviation.

But the interpretation of toxicity experiments using transporter mutants should be handled with care as another example shows: in a series of toxicity tests with different D-AAs, an Arabidopsis mutant of *AtMRP5* showed less resistance against D-Phe compared to the corresponding control (**Figure 1D**), instead of increased resistance like in the case of the tested *AAP1* mutant (**Figure 1C**). *AtMRP5* belongs to a gene family of 14 ABC transporters in the Arabidopsis genome [24] and found to transport inositol phosphate for phytate storage [25]. A functionality as amino acid transporter has not been reported for this protein, yet. Surprisingly, at *mrp5* mutant allele showed also drastically reduced root exudation of almost all L-AAs [26]. It is tempting to speculate whether the reduced D-Phe resistance of the *mrp5* mutant in our experiments may be a consequence of reduced exudation of this amino acid, which may lead to accumulation of it to toxic levels.

Altogether the presented studies in this chapter indicate that plants seem to take up D-AAs actively from their rhizosphere. As also shown above candidate transporter proteins for this uptake are found among L-AA transporting proteins like LHT1, ProT2 and AAP1. There is a certain possibility that these three transporters are not the only ones within their families to transport D-AAs. In the Arabidopsis genome, there are 10 LHTs, 3 ProTs and 8 AAPs encoded [22, 27]. This means that at least among the members of these three transporter families, further D-AA transport proteins may be found. Taking into account that members of other amino acid transporter families may also be able to do so, raises the number of candidates in Arabidopsis up to 63. Even more could be found in other plant species, as there are for instance 189 putative amino acid transporter genes encoded in the soybean genome [28].

3. What happens to D-AAs in the plant?

The fact that plants take up D-AAs from their surrounding rhizosphere leads to the question what happens to them in consequence in the plant. An approach to answer this question was given by our group by feeding Arabidopsis mutants and accessions with different D-AAs to measure the D- and L-AA contents in these plants afterwards [12, 29]. These analyses revealed two major metabolic processes which could be observed; one of them was the conversion of particular D-AAs like D-His, D-Met, D-Phe and D-Trp to their L-enantiomers. In this respect, the increase of these L-AAs was about 2–50 times compared to the untreated control plants, depending on the applied D-AA. The other one was the increase of D-Glu and D-Ala contents after treatment with any given D-AA. In this regard, D-Ala was the major compound to be found after D-AA application with concentrations more than 20 times higher than the ones of D-Glu. These observations led to speculations about the metabolic processes responsible for these effects. To explain the outcome of our amino acid profiling three different possibilities of enzymatic

http://dx.doi.org/10.5772/intechopen.68539

reaction have been discussed: racemization, deamination and transamination [4]. Recent studies of our group revealed that as well the D- to L-AA conversion as also the occurrence of D-Glu and D-Ala can be explained by the activity of a single D-AA specific transaminase in the Arabidopsis genome (Suarez et al., unpublished results).

All these studies shifted our focus towards the evolution and metabolic fate of D-Ala in plants. D-Ala appears to be the major product of D-AA metabolism in Arabidopsis, but, at the same time, it is one of the most toxic D-AAs for this species when applied exogenously [12, 17]. This raises the question how plants process D-Ala specifically and why it is the preferred product of D-AA metabolization. Several possibilities for this process are summarized in **Figure 2**.

A common feature of all possible pathways in **Figure 2** is that none of them have been characterized sufficiently to date in plants, especially in Arabidopsis. But there are a series of reports and evidences arguing for the scheme in this figure, which will be discussed in this section.

The ligation of D-Ala to its dipeptide D-Ala-D-Ala is among the best characterized ways to metabolize D-Ala (**Figure 2**). D-Ala-D-Ala could be detected in different grasses and tobacco long before [30–32], indicating the existence of a D-Ala ligating enzyme. Recently, this enzyme, D-Ala-D-Ala ligase (DDL), could be characterized physiologically for the first time from a plant source, PpDDL1 from the moss *Physcomitrella patens* [33]. As it can be seen in **Table 1** also in the Arabidopsis genome, a DDL encoding gene could be found, an orthologue of PpDDL1, which has not been characterized biochemically, yet. The situation is similar for a putative D-amino acid oxidase (DAO) from Arabidopsis: Its homologue from maize has been biochemically characterized and shown to oxidize preferably D-Ala [34], but its Arabidopsis homologue has not been characterized biochemically or physiologically, yet.

When it comes to the alanine racemase in plants (**Figure 2**), knowledge is rather scarce; Alanine racemase enzyme activity and its corresponding enzyme activity could be isolated and measured in *Chlamydomonasreinhardtii* and alfalfa [13, 35], but identification of the

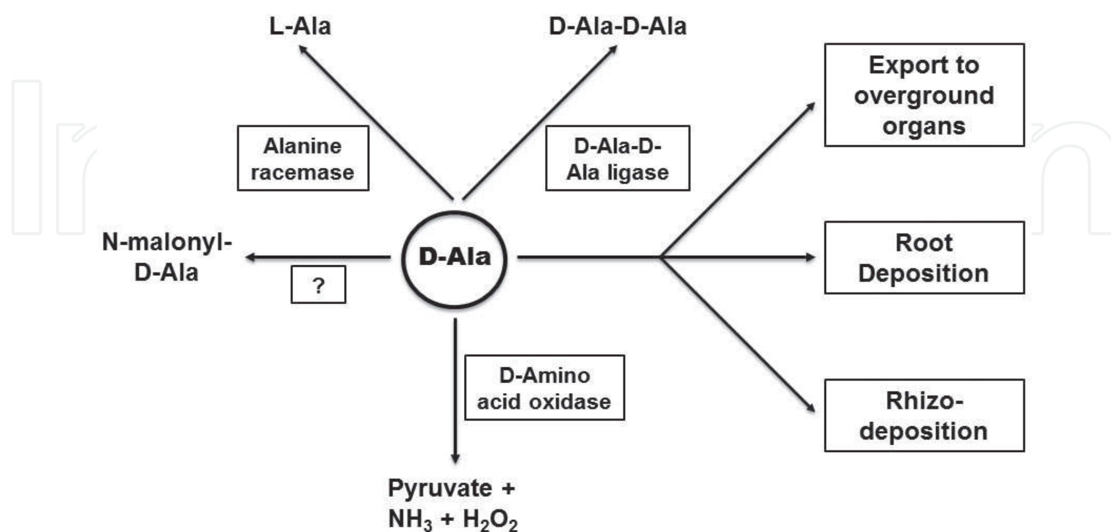


Figure 2. D-Ala as central product of D-AA metabolism and possible metabolic fates of it in plants.

corresponding gene is still pending. In the Arabidopsis genome, two genes with homologies to alanine racemases are annotated (**Table 1**), but characterization is still pending. Even less is known about malonylation of D-Ala, which is given as the fourth major enzymatic way to metabolize D-Ala in **Figure 2**: In pea seedlings, N-malonyl-D-Ala had been detected [36, 37]. Additionally, in mung bean seedlings, D-Ala malonylating activity could be shown [38], but the corresponding enzyme still awaits identification.

Apart from the enzymatic metabolization of D-Ala, other ways of its deposition have to be taken into account as depicted in **Figure 2**: The spatial distribution of D-Ala within the plant, but also of D-AAs in general, would be of interest in this respect. Then, the question could be answered if D-Ala is deposited in the root or if it is transported to other organs, in order to dilute its toxicity. Another possibility would be rhizodeposition, the exudation of metabolites from the root into soil. Rhizodeposition of L-AAs has been shown for plants several times [26, 39, 40]. This process has a strong impact on the microbial community in the rhizosphere, but reports of rhizodeposition of D-AAs are still missing.

Another look into **Table 1** reveals further D-AA processing enzymes in the Arabidopsis genome apart from either synthesizing or metabolizing D-Ala. First of all, there are four D-AA

Function	Name	AGI code	Localization	References
D-amino acid transaminase	AtDAAT1	At5g57850	chloroplast	[41]
		At3g05190	(unknown)	
		At3g54970	(unknown)	
		At5g27410	(unknown)	
Alanine racemase		At1g11930	(chloroplast)	
		At4g26860	(chloroplast)	
D-amino acid racemase	AtDAAR1	At4g02850	(unknown)	[16]
	AtDAAR2	At4g02860	(cytosol)	[16]
Serine racemase	AtSR1	At4g11640	(unknown)	[14]
Asp-Glu racemase		At1g15410	(chloroplast)	
D-aminoacyl-tRNAdeacylase	AtGEK1	At2g03800	cytosol, nucleus	[55]
D-Tyr-tRNAdeacylase		At4g18460	(unknown)	
D-amino acid oxidase		At5g67290	(chloroplast)	
D-Cysdesulfhydrase		At1g48420	mitochondria	[42]
D-Ala-D-Ala ligase		At3g08840	(chloroplast)	

Localization refers to the experimentally determined subcellular localization; localization predictions on the basis of peptide sequencing data by the Plant Proteome Database (PPD; http://ppdb.tc.cornell.edu) and unknown localizations are given in parentheses.

Table 1. Putative D-AA metabolizing genes in the Arabidopsis genome.

specific transaminases, from which just one has been shown to produce D-Ala and preferably D-Glu using various D-AAs as substrates with different affinities [41]. Beside the already mentioned alanine racemase, also three other racemases with specificities for other amino acids can be found in the Arabidopsis genome: First, there is a putative Asp-Glu racemase encoded in the Arabidopsis genome, but currently, there are no reports available about it. The second one is the serine racemase AtSR1, which catalyses the racemization of serine, but also to lesser extent alanine, arginine and glutamine. Beside its racemase activity, it acts also as a dehydratase on D- and L-serine [13]. The third one are the so-called D-amino acid racemases AtDAAR1 and AtDAAR2, which are indeed specific for Ile with D-*allo*-Ile as a product. Leu and Val were just racemized with 1 and 5% relative activity, respectively [16]. The D-Cys desulfhydrase from Arabidopsis is another example for a D-AA metabolizing enzyme with a specificity apart from D-Ala; this specificity to catabolize D-Cys to pyruvate, NH_3 and H_2S has been shown previously [42], but the physiological function of this enzyme and especially of D-Cys still remains unclear and will be discussed in the next chapter. Altogether the collection of D-AA processing enzymes in **Table 1** is a reminder that D-Ala seems to be central product of D-AA metabolism, but that there are far more putative enzyme encoding genes annotated to produce and process also other D-AAs.

4. What are the effects and functions of D-AAs in the plant?

The abundance and fate of D-AAs in plants are indicators that these compounds are actively processed and therefore play a role in the physiology of plants. This leads to the question: Which role(s) are these? In the last years, three different scenarios about the effects of D-AAs on plants were discussed. The first one was that D-AAs have either no effect on plants or even inhibit growth and therefore have to be considered as toxins for plants [19]. In contrast, it could be shown before that at least D-Ile and D-Val promoted seedling growth [17], and that for different D-AAs even the highest tested concentration did not cause growth inhibition [12]. Together with the de novo synthesis of various D-AAs in plants described above a general toxic function of all D-AAs is rather unlikely and depends on dosage, which also applies to many L-AAs.

There are also other arguments speaking against this scenario like the utilization of D-AAs as possible nitrogen source [4, 40], which is the second major postulated function of D-AAs in plants. In this respect, it could be shown that wheat plants are able to assimilate D-Ala as well as D-trialanine, which they took up from the soil [20]. This was the first evidence of direct utilization of D-AAs as a nitrogen source. Additionally, it revealed that plants are able to utilize not just free forms of D-AAs but also as oligomers, as also found as a degradation product of the bacterial cell wall. Nevertheless, more plant species and other D-AAs have to be analysed in this respect to confirm the general utilization of D-AAs as nitrogen sources for plants.

The third major complex of functions of D-AAs in plants is the ones, which have been either just recently discovered, and need to be further analysed and characterized, or which have not been discovered at all. Among these novel functions is, for instance, D-Ala as a stress signal: It has been reported that duckweed seedlings accumulate D-Ala after UV light exposure [43], but the confirmation of this finding by other groups or in other

species is still pending. Another, more prominent, example of a novel physiological function of a D-AA in plants is the impact of D-Ser on pollen tube growth in Arabidopsis [44]; In that report, the authors provided evidence that D-Ser affects pollen tube growth via its agonistic action on the glutamate receptor AtGLR1.2. Furthermore, it was shown that the loss of the serine racemase AtSR1 leads to aberrant pollen tube growth. AtGLR1.2 belongs to a protein family of 20 members in Arabidopsis with highest homologies towards the mammalian ionotropic glutamate receptors (GLRs), also known as N-methyl-D-Aspartate (NMDA) receptors [45]. In humans and other mammalians, these receptors, involved in neurotransmission, have been shown to be activated by L-Glu and D-Ser synergistically [46]. The homologous action of D-Ser on GLRs in animals and plants together with the relatively large number of GLRs in the Arabidopsis genome implies further effects of D-Ser on physiological functions in plants, especially on pathogen response, which may be regulated by GLRs, too [45].

Another type of novel functions of D-AAs was unravelled by the analyses of the structure of the chloroplast membrane of mosses [33]. The authors provided evidence that the membranes of chloroplasts from the moss *P. patens* contain the dipeptide D-Ala-D-Ala and therefore possess a major structural component of peptidoglycan, the building block of bacterial cell walls [3]. Another indication of the structural similarity of bacterial cell wall and plastidial envelopes in mosses was given by genetical experiments. Loss-of-function mutants of the Physcomitrella D-Ala-D-Ala ligase, *PpDDL1*, were not able for chloroplast division and therefore showed megachloroplasts in their protonema cells [33]. All these findings fit to the observation made before in the Physcomitrella genome, which harboured all gene homologues from bacteria to synthesize peptidoglycan including *PpDDL1* [47]. The structural similarity between bacterial cell walls and plastidial envelopes seems to be limited to cryptogamic plants, because loss-of-function mutants of *AtDDL1* did not show the megachloroplast phenotype observed in mosses [33]. This observation seems to be in concordance to the situation in the Arabidopsis genome which harbours just four homologues of the ten mentioned genes needed for peptidoglycan synthesis [47]. It is interesting in this respect that homologues of these four genes were found in all higher plant genomes [48].

Nevertheless, D-Ala seems to play a role in chloroplasts of higher plants as well. Many proteins directly involved in D-Ala metabolism in Arabidopsis were either found in the chloroplast or were predicted to be localized there (**Table 1**). Furthermore, we were able to synthesize a fluorescent D-Ala analogue, HADA (7-hydroxycoumarin-3-amino-D-alanine), according to a previously published protocol [49], and fed it to Arabidopsis seedlings. In these experiments, we could trace the HADA fluorescence evenly distributed in the chloroplasts (**Figure 3**). This targeting of the D-Ala analogue to the chloroplasts indicated a central metabolization of this compound in this compartment. The even distribution of it, in contrast to the accumulation, found in moss chloroplast envelopes [33] points to a different function of D-Ala in higher plant chloroplasts, which still awaits to be unravelled.

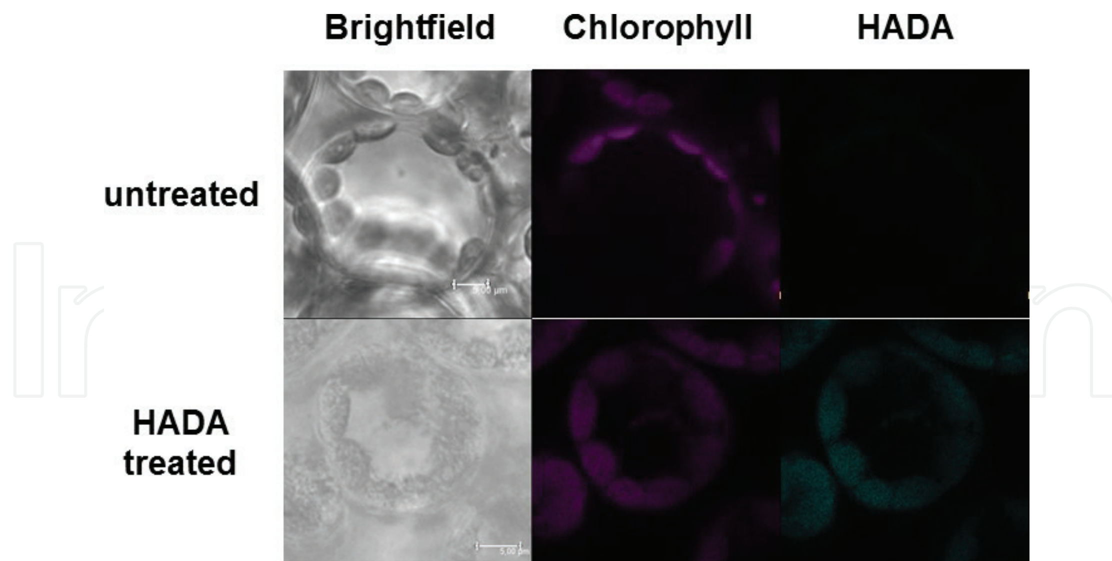


Figure 3. Fluorescent D-Ala analogue HADA accumulates in chloroplasts of Arabidopsis leaves.

Fourteen days old *Col-0* seedlings grown in liquid culture were incubated overnight in 0.1 mM HADA and then analysed microscopically. The pictures in the first column show bright field images of sponge parenchyma cells. The chlorophyll in the chloroplasts was detected by its autofluorescence (Chlorophyll, second column), and fluorescence of HADA was recorded in the DAPI channel (HADA, third column) with a laser-scanning microscope. The upper row shows cells without HADA treatment (untreated) as control, the lower one with HADA treatment (HADA treated). The size bars indicate 5 μm.

Among D-AAs with novel functions in plants, there are D-AAs to be known to affect specific proteins, but not how they cause the associated physiological reactions. One example for such a relationship is the one between D-Cys and drought resistance. As described above, a desulfhydrase specific for D-Cys could be characterized from Arabidopsis, which also produces H_2S [42]. In further experiments, it turned out that increased H_2S production leads to enhanced drought resistance [50], which can be partially assigned to increased D-Cysdesulfhydrase activity [51]. This effect seems to be related to ethylene induced stomatal aperture. Furthermore, H_2S production leads to cross adaptation of plants to several other stress factors [52]. But nevertheless, the source of D-Cys, its significance in stress signalling and adaptation and its detailed way of action still need to be elucidated.

Another example for an enigmatic relation between a D-AA and physiological response was described previously: In this case, an Arabidopsis mutant with hypersensitivity to ethanol, *gek1*, was isolated [53], where the respective mutation could be assigned to a D-aminoacyl-tRNAdeacylase (*AtGEK1*) [54]. Later, it was found that this gene encodes an active enzyme with broad substrate specificity [55]. But its overexpression led neither in *Escherichia coli* nor in yeast to an increase of ethanol tolerance [54]. Therefore, a functional explanation how the loss of *AtGEK1*, and therefore the inability to repair accidental loading of tRNAs with D-AAs, causes ethanol hypersensitivity in plants is still missing.

5. Conclusions

As discussed largely in this text, knowledge gathered in the last decade implies that D-AAs are involved in more plant physiological processes than assumed before. Furthermore, the view of D-AAs as generally toxic molecules needs to be changed to a view of them as physiologically active compounds, which can cause detrimental effects by over dosage. Therefore, the investigation of their uptake and elimination, their metabolic pathways, and their physiological functions in plants will gain more interest and significance in the future.

To understand the transport processes for uptake, distribution (intracellular, intercellular and also at long distances) as well as of possible excretion of D-AAs will be one of the major fields to be investigated. Although there are candidate transport proteins given to be analysed with the classical L-AA transporters as described above, also other, yet unknown, proteins may contribute to D-AA transport processes, as the D-Phe toxicity on *mrp5* mutants demonstrates (**Figure 1**). In this regard, one of the first questions to be solved would be the proof of active uptake of D-AAs by candidate transporters instead of indirect evidences.

When it comes to the metabolization of D-AAs, the formation of D-Ala appeared to be central, which puts also this molecule into the centre of future investigations. As it has been shown in the preceding chapter, D-Ala seems to accumulate in the chloroplast. In this respect, D-Ala may play a double role. On the one hand, it is an intermediate metabolite, which needs to be further metabolized due its toxicity in excess concentration. In this regard, the different putative metabolic pathways await elucidation. On the other hand, D-Ala is a physiologically active compound as it has been shown as a building block of moss chloroplast envelopes. In this context, the function of D-Ala in chloroplasts of higher plant and the concentration of different D-AA-related enzymes in this compartment will be of specific interest. Furthermore, it would be interesting if also other D-AAs take this way over chloroplasts and what functions they fulfil there.

Finally, which physiological role(s) the different D-AAs play in plants are the major questions to be solved. One of these questions will be, to which extent and under which circumstances D-AAs from the rhizosphere are utilized as nitrogen sources. As it was mentioned above, the accumulation of D-Ala and of D-AA-related enzymes in the chloroplasts may point to their involvement in plastid biogenesis, assembly and maintenance. The unresolved functions of D-Cys in stress resistance and the unclear involvement of AtGEK1 in ethanol resistance indicate that there is a high probability that there are still many D-AA-related functions and processes in plants waiting to be discovered.

Acknowledgements

We are grateful to Prof. Klaus Harter (Centre for Plant Molecular Biology, University of Tübingen, Germany) for the provision of research facilities, where the majority of our studies were performed. Studies of Juan Suarez on the effects of D-AAs on Arabidopsis were supported by the DeutscherAkademischerAustauschdienst (DAAD 91567028). Additionally, we would like to thank to Laura Menius for her work on the toxicity of D-AAs on the *mrp5* mutant.

Author details

Üner Kolukisaoglu* and Juan Suarez

*Address all correspondence to: uener.kolukisaoglu@zmbp.uni-tuebingen.de

Department of Plant Physiology, Center for Plant Molecular Biology (ZMBP), Eberhard Karls University of Tübingen, Tübingen, Germany

References

[1] Friedman M. Chemistry, nutrition, and microbiology of D-amino acids. Journal of Agricultural and Food Chemistry. 1999;**47**:3457–3479. DOI: 10.1021/jf990080u

[2] Radkov AD, Moe LA. Bacterial synthesis of D-amino acids. Applied Microbiology and Biotechnology. 2014;**98**:5363–5374. DOI: 10.1007/s00253-014-5726-3

[3] Typas A, Banzhaf M, Gross CA, Vollmer W. From the regulation of peptidoglycan synthesis to bacterial growth and morphology. Nature Reviews Microbiology. 2011;**10**:123–136. DOI: 10.1038/nrmicro2677

[4] Vranova V, Zahradnickova H, Janous D, Skene KR, Matharu KR, Rejsek K, Formanek P. The significance of D-amino acids in soil, fate and utilization by microbes and plants: Review and identification of knowledge gaps. Plant and Soil. 2012;**354**:21–39. DOI: 10.1007/s11104-011-1059-5

[5] Pollock GE, Cheng CN, Cronin SE. Determination of the D and L isomers of some protein amino acids present in soils. Analytical Chemistry. 1977;**49**:2–7. DOI: 10.1021/ac50009a008

[6] Amelung W, Zhang X. Determination of amino acid enantiomers in soil. Soil Biology and Biochemistry. 2001;**33**:553–562. DOI: 10.1016/S0038-0717(00)00195-4

[7] Amelung W, Zhang X, Flach KW. Amino acids in grassland soils: Climatic effects on concentrations and chirality. Geoderma. 2006;**130**:207–217. DOI: 10.1016/j.geoderma.2005.01.017

[8] Robinson T. D-amino acids in higher plants. Life Sciences. 1976;**19**:1097–1102. DOI: 10.1016/0024-3205(76)90244-7

[9] Brückner H, Westhauser T. Chromatographic determination of D-amino acids as native constituents of vegetables and fruits. Chromatographia. 1994;**39**:419–426. DOI: 10.1007/BF02278756

[10] Brückner H, Westhauser T. Chromatographic determination of L- and D-amino acids in plants. Amino Acids. 2003;**24**:43–55. DOI: 10.1007/s00726-002-0322-8

[11] Herrero M, Ibáñez E, Martín-Alvarez PJ, Cifuentes A. Analysis of chiral amino acids in conventional and transgenic maize. Analytical Chemistry. 2007;**79**:5071–5077. DOI: 10.1021/ac070454f

[12] Gördes D, Kolukisaoglu Ü, Thurow K. Uptake and Conversion of D-Amino Acids in *Arabidopsis thaliana*. Amino Acids. 2011;**40**:553–563. DOI: 10.1007/s00726-010-0674-4

[13] Ono K, Yanagida K, Oikawa T, Ogawa T, Soda K. Alanine racemase of alfalfa seedlings (*Medicagosativa* L.): First evidence for the presence of an amino acid racemase in plants. Phytochemistry. 2007;**67**:856–860. DOI: 10.1016/j.phytochem.2006.02.017

[14] Fujitani Y, Nakajima N, Ishihara K, Oikawa T, Ito K, Sugimoto M. Molecular and biochemical characterization of a serine racemase from *Arabidopsis thaliana*. Phytochemistry. 2006;**67**:668–674. DOI: 10.1016/j.phytochem.2006.01.003

[15] Fujitani Y, Horiuchi T, Ito K, Sugimoto M. Serine racemases from barley, *Hordeum vulgare* L., and other plant species represent a distinct eukaryotic group: Gene cloning and recombinant protein characterization. Phytochemistry. 2007;**68**:1530–1536. DOI: 10.1016/j.phytochem.2007.03.040

[16] Strauch RC, Svedin E, Dilkes B, Chapple C, Li X. Discovery of a novel amino acid racemase through exploration of natural variation in *Arabidopsis thaliana*. Proceedings of the National Academy of Sciences U S A. 2015;**112**:11726–11731. DOI: 10.1073/pnas.1503272112

[17] Erikson O, Hertzberg M, Näsholm T. A conditional marker gene allowing both positive and negative selection in plants. Nature Biotechnology. 2004;**22**:455–458. DOI: 10.1038/nbt946

[18] Erikson O, Hertzberg M, Näsholm T. The *dsdA* gene from *Escherichia coli* provides a novel selectable marker for plant transformation. Plant Molecular Biology. 2005;**57**:425–433. DOI: 10.1007/s11103-004-7902-9

[19] Forsum O, Svennerstam H, Ganeteg U, Näsholm T. Capacities and constraints of amino acid utilization in Arabidopsis. New Phytologist. 2008;**179**:1058–1069. DOI: 10.1111/j.1469-8137.2008.02546.x

[20] Hill PW, Quilliam RS, DeLuca TH, Farrar J, Farrell M, Roberts P, Newsham KK, Hopkins DW, Bardgett RD, Jones DL. Acquisition and assimilation of nitrogen as peptide-bound and D-enantiomers of amino acids by wheat.PLoS One. 2011;**6**:e1922. DOI: 10.1371/journal.pone.0019220

[21] Svennerstam H, Ganeteg U, Bellini C, Näsholm T. Comprehensive screening of Arabidopsis mutants suggests the lysine histidine transporter 1 to be involved in plant uptake of amino acids. Plant Physiology. 2007;**143**:1853–1860. DOI: 10.1105/tpc.106.041012

[22] Lehmann S, Gumy C, Blatter E, Boeffel S, Fricke W, Rentsch D. In planta function of compatible solute transporters of the AtProT family. Journal of Experimental Botany. 2011;**62**:787–796. DOI: 10.1093/jxb/erq320

[23] Lee YH, Foster J, Chen J, Voll LM, Weber AP, Tegeder M. AAP1 transports uncharged amino acids into roots of Arabidopsis. The Plant Journal. 2007;**50**:305–319. DOI: 10.1111/j.1365-313X.2007.03045.x

[24] Verrier PJ, Bird D, Burla B, Dassa E, Forestier C, Geisler M, Klein M, Kolukisaoglu U, Lee Y, Martinoia E, Murphy A, Rea PA, Samuels L, Schulz B, Spalding EJ, Yazaki K, Theodoulou FL. Plant ABC proteins – A unified nomenclature and updated inventory. Trends in Plant Sciences. 2008;**13**:151–159. DOI: 10.1016/j.tplants.2008.02.001

[25] Nagy R, Grob H, Weder B, Green P, Klein M, Frelet-Barrand A, Schjoerring JK, Brearley C, Martinoia E. The Arabidopsis ATP-binding cassette protein AtMRP5/AtABCC5 is a high affinity inositol hexakisphosphate transporter involved in guard cell signaling and phytate storage. The Journal of Biological Chemistry. 2009;**284**:33614–33622. DOI: 10.1074/jbc.M109.030247

[26] Zhou D, Huang XF, Chaparro JM, Badri DV, Manter DK, Vivanco JM, Guo J. Root and bacterial secretions regulate the interaction between plants and PGPR leading to distinct plant growth promotion effects. Plant and Soil. 2016;**401**:259. DOI: 10.1007/s11104-015-2743-7

[27] Tegeder M, Ward JM. Molecular Evolution of Plant AAP and LHT Amino Acid Transporters. Frontiers in Plant Science. 2012;**13**:21. DOI: 10.3389/fpls.2012.00021

[28] Cheng L, Yuan HY, Ren R, Zhao SQ, Han YP, Zhou QY, Ke DX, Wang YX, Wang L. Genome-wide identification, classification, and expression analysis of *Amino Acid Transporter* gene family in *Glycine Max*. Frontiers in Plant Science. 2016;**20**;7:515. DOI: 10.3389/fpls.2016.00515

[29] Gördes D, Koch G, Thurow K, Kolukisaoglu U. Analyses of Arabidopsis ecotypes reveal metabolic diversity to convert D-amino acids. Springerplus. 2013;**2**:559. DOI: 10.1186/2193-1801-2-559

[30] Noma M, Noguchi M, Tamaki E. Isolation and characterization of D-alanyl-D-alanine from tobacco leaves. Agricultural and Biological Chemistry. 1973;**37**:2439. DOI: 10.1271/bbb1961.37.2439

[31] Frahn JL, Illman RJ. The occurrence of D-alanine and D-alanyl-D-alanine in *Phalaristube-rosa*. Phytochemistry. 1975;**14**:1464–1465

[32] Manabe H. Occurrence of D-Alanyl-D-alanine in *Oryzaaustraliensis*. Agricultural and Biological Chemistry. 1985;**49**:1203–1204. DOI: 10.1080/00021369.1985.10866877

[33] Hirano T, Tanidokoro K, Shimizu Y, Kawarabayasi Y, Ohshima T, Sato M, Tadano S, Ishikawa H, Takio S, Takechi K, Takano H. Moss chloroplasts are surrounded by a peptidoglycan wall containing D-amino acids. Plant Cell. 2016;**28**:1521–1532. DOI: 10.1105/tpc.16.00104

[34] Gholizadeh A, Kohnehrouz BB. Molecular cloning and expression in *Escherichia coli* of an active fused *Zea mays L*. D-amino acid oxidase. Biochemistry (Moscow). 2009;**74**:137–144. DOI: 10.1134/S0006297909020035

[35] Nishimura K, Tomoda Y, Nakamoto Y, Kawada T, Ishii Y, Nagata Y. Alanine racemase from the green alga *Chlamydomonasreinhardtii*. Amino Acids.2007;**32**:59–62. DOI:10.1007/s00726-006-0352-8

[36] Fukuda M, Tokumura A, Ogawa T. D-Alanine in germinating *Pisumsativum* seedlings. Phytochemistry. 1973;**12**:2893–2595. DOI: 10.1016/0031-9422(73)85061-7

[37] Ogawa T, Fukuda M, Sasaoka K. Occurrence of N-malonyl-D-alanine in pea seedlings. Biochimica et Biophysica Acta. 1973;**297**:60–69. DOI: 10.1016/0304-4165(73)90049-4

[38] Kionka C, Amrhein N. The enzymatic malonylation of 1-aminocyclopropane-1-carboxylic acid in homogenates of mung-bean hypocotyls. Planta. 1984;**162**:226–35. DOI: 10.1007/BF00397444

[39] Chaparro JM, Badri DV, Bakker MG, Sugiyama A, Manter DK, Vivanco JM. Root exudation of phytochemicals in arabidopsis follows specific patterns that are developmentally programmed and correlate with soil microbial functions. PLoS One. 2013;**8**:e55731. DOI:10.1371/journal.pone.0055731

[40] Moe LA. Amino acids in the rhizosphere: From plants to microbes. American Journal of Botany. 2013;**100**:1692–1705. DOI: 10.3732/ajb.1300033

[41] Funakoshi M, Sekine M, Katane M, Furuchi T, Yohda M, Yoshikawa T, Homma H. Cloning and functional characterization of *Arabidopsis thaliana* D-amino acid aminotransferase – D-aspartate behavior during germination. The FEBS Journal. 2008;**275**:1188–1200. DOI: 10.1111/j.1742-4658.2008.06279.x

[42] Riemenschneider A, Wegele R, Schmidt A, Papenbrock J. Isolation and characterization of a D-cysteine desulfhydrase protein from *Arabidopsis thaliana*. The FEBS Journal. 2005;**272**:1291–1304. DOI: 10.1111/j.1742-4658.2005.04567.x

[43] Monselise EB, Levkovitz A, Kost D. Ultraviolet radiation induces stress in etiolated *Landoltiapunctata*, as evidenced by the presence of alanine, a universal stress signal: a ^{15}N NMR study. Plant Biology (Stuttg). 2015;**17**(Suppl 1):101–107. DOI: 10.1111/plb.12198

[44] Michard E, Lima PT, Borges F, Silva AC, Portes MT, Carvalho JE, Gilliham M, Liu LH, Obermeyer G, Feijó JA. Glutamate receptor-like genes form Ca^{2+} channels in pollen tubes and are regulated by pistil D-serine. Science. 2011;**332**:434–437. DOI: 10.1126/science.1201101

[45] Forde BG, Roberts MR. Glutamate receptor-like channels in plants: A role as amino acid sensors in plant defence? F1000Prime Rep. 2014;**6**:37. DOI: 10.12703/P6-37

[46] Wolosker H. NMDA receptor regulation by D-serine: New findings and perspectives. Molecular Neurobiology. 2007;**36**:152–164. DOI: 10.1007/s12035-007-0038-6

[47] Machida M, Takechi K, Sato H, Chung SJ, Kuroiwa H, Takio S, Seki M, Shinozaki K, Fujita T, Hasebe M, Takano H. Genes for the peptidoglycan synthesis pathway are essential for chloroplast division in moss. Proceedings of the National Academy of Sciences U S A. 2006;**103**:6753–6758. DOI: 10.1073/pnas.0510693103

[48] van Baren MJ, Bachy C, Reistetter EN, Purvine SO, Grimwood J, Sudek S, Yu H, Poirier C, Deerinck TJ, Kuo A, Grigoriev IV, Wong CH, Smith RD, Callister SJ, Wei CL, Schmutz J, Worden AZ. Evidence-based green algal genomics reveals marine diversity

and ancestral characteristics of land plants. BMC Genomics. 2016;**17**:267. DOI: 10.1186/s12864-016-2585-6

[49] Kuru E, Tekkam S, Hall E, Brun YV, Van Nieuwenhze MS. Synthesis of fluorescent D-amino acids and their use for probing peptidoglycan synthesis and bacterial growth in situ. Nature Protocols. 2015;**10**:33–52. DOI: 10.1038/nprot.2014.197

[50] Jin Z, Shen J, Qiao Z, Yang G, Wang R, Pei Y. Hydrogen sulfide improves drought resistance in *Arabidopsis thaliana*. Biochemical and Biophysical Research Communications. 2011;**414**:481–486. DOI: 10.1016/j.bbrc.2011.09.090

[51] Hou Z, Wang L, Liu J, Hou L, Liu X. Hydrogen sulfide regulates ethylene-induced stomatal closure in *Arabidopsis thaliana*. Journal of Integrative Plant Biology. 2013;**55**:277–289. DOI: 10.1111/jipb.12004

[52] Li ZG, Min X, Zhou ZH. Hydrogen Sulfide: A Signal Molecule in Plant Cross-Adaptation. Frontiers in Plant Science. 2016;**7**:1621. DOI: 10.3389/fpls.2016.01621

[53] Hirayama T, Fujishige N, Kunii T, Nishimura N, Iuchi S, Shinozaki K. A novel ethanol-hypersensitive mutant of Arabidopsis. Plant and Cell Physiology. 2004;**45**:703–711. DOI: 10.14841/jspp.2008.0.0890.0

[54] Fujishige N, Nishimura N, Iuchi S, Kunii T, Shinozaki K, Hirayama T. A novel Arabidopsis gene required for ethanol tolerance is conserved among plants and archaea. Plant and Cell Physiology. 2004;**45**:659–666. DOI: 10.1093/pcp/pch086

[55] Wydau S, Ferri-Fioni ML, Blanquet S, Plateau P. GEK1, a gene product of *Arabidopsis thaliana* involved in ethanol tolerance, is a D-aminoacyl-tRNAdeacylase. Nucleic Acids Research. 2007;**35**:930–938. DOI: 10.1093/nar/gkl1145

IntechOpen

Chapter 4

Article Two

4.1 D-Amino Acids Are Exuded by *Arabidopsis thaliana* Roots to the Rhizosphere

Status: Published

Position of candidate in list of authors: Co-author.

Scientific ideas by the candidate: 30%

Data generation by the candidate: 30%

Analysis and Interpretation by the candidate: 20%

Paper writing done by the candidate: 20%

International Journal of
Molecular Sciences

MDPI

Article

D-Amino Acids Are Exuded by *Arabidopsis thaliana* Roots to the Rhizosphere

Claudia Hener [1], Sabine Hummel [1], Juan Suarez [1], Mark Stahl [1,*] and Üner Kolukisaoglu [1,*]

Center for Plant Molecular Biology (ZMBP), University of Tübingen, Auf der Morgenstelle 32, 72076 Tübingen, Germany; claudia.hener@zmbp.uni-tuebingen.de (C.H.); sabine.hummel@zmbp.uni-tuebingen.de (S.H.); juan.suarez@zmbp.uni-tuebingen.de (J.S.)

* Correspondence: mark.stahl@zmbp.uni-tuebingen.de (M.S.); uener.kolukisaoglu@zmbp.uni-tuebingen.de (Ü.K.); Tel.: +49-7071-29-73095 (Ü.K.)

Received: 8 March 2018; Accepted: 5 April 2018; Published: 7 April 2018

Abstract: Proteinogenic L-amino acids (L-AAs) are essential in all kingdoms as building blocks of proteins. Their D-enantiomers are also known to fulfill important functions in microbes, fungi, and animals, but information about these molecules in plants is still sparse. Previously, it was shown that D-amino acids (D-AAs) are taken up and utilized by plants, but their ways to reduce excessive amounts of them still remained unclear. Analyses of plant D-AA content after D-Ala and D-Glu feeding opened the question if exudation of D-AAs into the rhizosphere takes place and plays a role in the reduction of D-AA content in plants. The exudation of D-Ala and D-Glu could be confirmed by amino acid analyses of growth media from plants treated with these D-AAs. Further tests revealed that other D-AAs were also secreted. Nevertheless, treatments with D-Ala and D-Glu showed that plants are still able to reduce their contents within the plant without exudation. Further exudation experiments with transport inhibitors revealed that D-AA root exudation is rather passive and comparable to the secretion of L-AAs. Altogether, these observations argued against a dominant role of exudation in the regulation of plant D-AA content, but may influence the composition of the rhizosphere.

Keywords: D-amino acids; chiral LC-MS; root exudation; plant-rhizosphere interactions; molecular transport

1. Introduction

The proteinogenic L-amino acids (L-AAs) are, according to textbook knowledge, ubiquitously found in all living organisms. Many of their functions are essential, especially as primary metabolites and building blocks of proteins. Their enantiomers, the D-amino acids (D-AAs), are also widely distributed in nature, but their functions are still cryptic in many cases. The most prominent example of D-AA utilization is found in bacteria which incorporate D-Ala and D-Glu into their cell wall as structural elements and to protect it from proteases [1]. However, D-AAs are also widespread in eukaryotes: in bound forms within bioactive peptides from crustaceans to vertebrates or within long living proteins from humans (for a review, see [2]). Furthermore, D-AAs also fulfill physiological functions in their free form. A prominent example for such a case is given by the *N*-methyl-D-aspartate (NMDA) receptor in mammals, which binds D-Ser as a co-agonist. In humans, it was found that reduced levels of D-Ser and resulting hypofunction of NMDA receptors leads to schizophrenia [3].

These examples show that the availability of particular D-AAs can be essential for many organisms. In many cases, these organisms are able to produce them de novo, such as in the case of bacteria which possess various types of amino acid racemases. These enzymes, which catalyze the interconversion of L- and D-AAs, are the major drivers of D-AA production in bacteria, but also in animals (for overviews,

see [4–6]). Another major pool of D-AAs, especially for higher eukaryotes, is their uptake either by nutrition, in animals, or by root uptake, in plants [7,8]. All these organisms are not just dependent on possessing sufficient amounts of particular D-AAs, but also on regulated ways to metabolize them for the prevention of toxic effects by overdosage of particular D-AAs, observable in mammals [7] or plants [9]. Therefore, a major way to catabolize D-AAs is deamination, mostly in an oxidative way by D-AA oxidases (for reviews, see [10–12]).

When it comes to D-AA production, uptake, function, and metabolism in plants, there is a remarkable lack of knowledge in these fields compared to other organismal groups. For a long time, D-AAs were regarded as physiologically useless for plants due to their toxicity and low metabolization capacity [13]. However, the physiological value of D-AAs for plants has to be reviewed in the light of recent findings: it has been shown before that not all D-AAs are detrimental to plant growth; even some L-AAs show more inhibitory capacity than their D-enantiomers [8,9]. Furthermore, it has been shown that wheat plants are able to utilize D-Ala as a nitrogen source [14]. However, the functions of D-AAs in plants are not confined to nitrogen delivery. Previously, it has been shown that D-Ser is involved in pollen tube growth in *Arabidopsis* [15]. D-Ala acts as a stress signal in duckweed [16], and it is incorporated into moss chloroplast envelopes as a structural element [17]. Together with the mentioned role of D-Ala as a nitrogen source, D-AAs seem to fulfill a broad range of physiological functions in plants, and many of them remain yet to be unraveled.

In regard to the different functions of D-AAs in plants, their metabolism has come into the focus of plant physiologists. Plant roots are surrounded by D-AAs in their rhizosphere, which are mainly from bacterial origin and are also utilized by bacteria [18,19]. Therefore, it is not astonishing that plants are also able to take up a large variety of D-AAs [20]. With the amino acid transporters LHT1 and ProT2, there are at least two candidates for which D-AA import could be identified [21,22]. Additionally, the ability of plants to synthesize D-AAs de novo has been reported before [23], and with the serine racemase from *Arabidopsis*, also the first D-AA-synthesizing enzyme could be identified in plants [24]. In contrast to the uptake and synthesis of D-AAs, the situation is less clear with respect to the regulation of D-AA content in plants. It has been observed before that exogenously applied D-AAs are partially converted to their L-enantiomers, but all of them are transformed into D-Ala and D-Glu in *Arabidopsis* [8,20]. Recently, it could be shown that a D-amino acid specific transaminase, AtDAT1, is responsible for these processes [25]. However, the question remained about the further fate of D-Ala and D-Glu as major products of this enzyme reaction in plants.

This question takes the center stage of the present study. As one possibility for reducing the D-Ala and D-Glu contents in plants, rhizodeposition has been suggested [26]. In this study, it is shown that exogenously applied D-Ala and D-Glu is significantly reduced in *Arabidopsis* seedlings within 24 h. Furthermore, exudation of these and other D-AAs could be observed. Experiments with uncoupling agents such as CCCP and orthovanadate indicated that the exudation of D- and L-AAs may be a passive mechanism. Although root exudation of D-AAs does not contribute significantly to the reduction of its content in plants, the question about its functions remains and will be discussed.

2. Results

2.1. D-Ala and D-Glu Are Degraded Rapidly in Seedlings

In the beginning of our studies was the question about the fate of the major intermediates of D-AA conversion: D-Ala and D-Glu [8,20]. Especially the reduction of the D-Ala content was of interest, due to its relatively high toxicity [8,9]. To analyze the capacity of *Arabidopsis thaliana* seedlings to reduce their D-Ala and D-Glu contents, they were germinated first for 14 d in a liquid medium in 96-well microtiter plates. Then, 1 mM of either D-Ala or D-Glu were applied to the media. After 24 h, seedlings were washed and transferred to fresh media, and seedlings were sampled for another 24 h to analyze their different AA contents. As can be seen in Figure 1, the contents of both D-Ala and D-Glu decreased in the seedlings in this time without reaching the levels of untreated control plants.

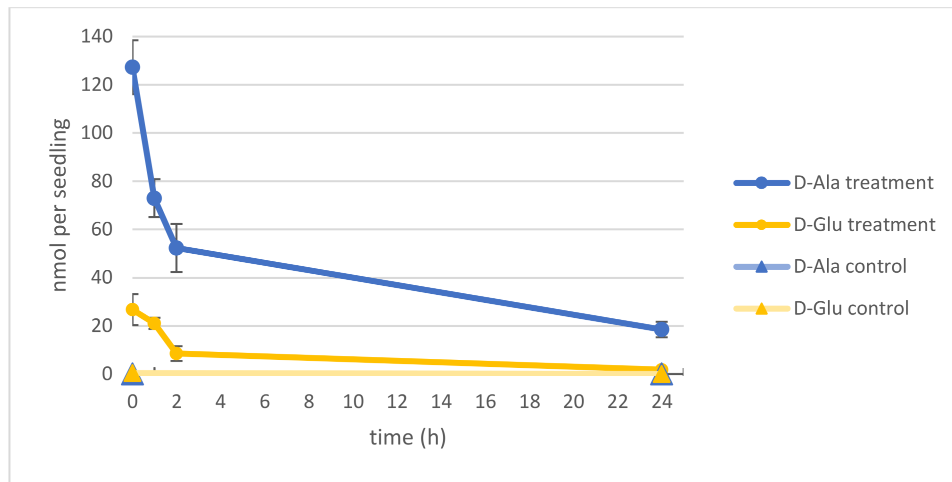
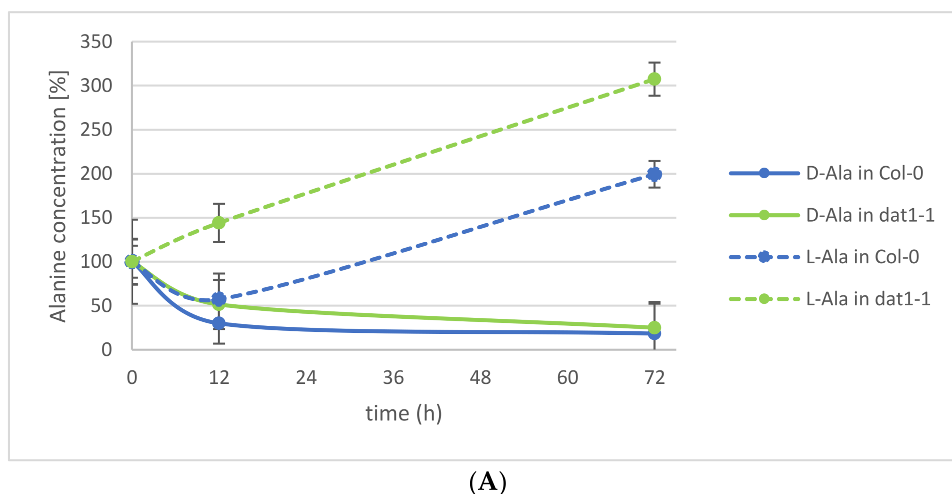


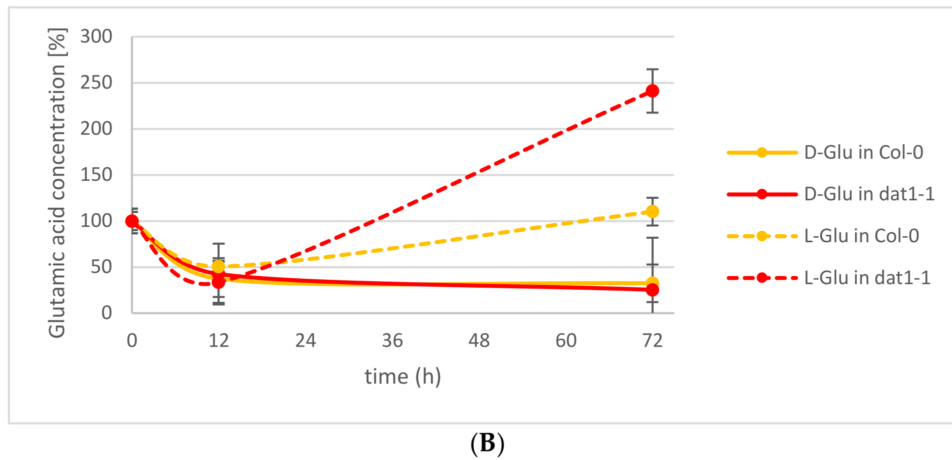
Figure 1. Decrease of D-Ala and D-Glu levels in *Arabidopsis* seedlings within 24 h. Dark blue lines represent the D-Ala and dark yellow lines the D-Glu contents of seedlings, respectively. Measurements from D-amino acid (AA)-treated plants are marked with circles; untreated control plants with lighter colors and triangles. The measurements started directly at transfer to fresh media up to 24 h later. Error bars: ± SD.

This observation led to the question of which processes may contribute to this D-AA reduction in the plants. Various enzymatic and nonenzymatic processes have been suggested elsewhere [26]. Among the putative enzymatic metabolizations of D-AAs, we tested the impact of D-AA-specific transamination. Recently, the responsible enzyme for the almost-complete D-AA transamination activity in *Arabidopsis* could be identified as AtDAT1 [25], an enzyme which had been characterized as a D-Asp transaminase before [27]. The loss of this enzyme leads to the inability of the plants to convert any D-AA into D-Ala and D-Glu, as it has been observed for the *Arabidopsis* accession Landsberg erecta (Ler) [20,25]. To analyze the portion of transamination in the reduction of D-Ala and D-Glu in seedlings, *Arabidopsis* Columbia-0 (Col-0) wild-type and a dat1 mutant line (*dat1-1*) were treated with both D-AAs as described above, and their Ala and Glu contents were determined for 72 h. These analyses revealed that the reduction of D-Ala and D-Glu is not different between both tested lines over the observed time. Instead, the content of the corresponding L-enantiomer of the applied D-AA increases significantly (Figure 2A,B). This implies the involvement of racemases in the reduction of D-Ala and D-Glu levels.



(A)

Figure 2. *Cont.*



(**B**)

Figure 2. Relative alanine and glutamic acid concentrations in Col-0 wild-type and dat1 mutant line (*dat1-1*) seedlings within 72 h. Seedlings were exogenously applied with (**A**) D-Ala and (**B**) D-Glu, and their D- and L-Ala or D- and L-Glu contents were determined, respectively. Solid lines mark the D-enantiomer; dotted lines the L-enantiomer. In (**A**), blue lines represent Ala from Col-0; green ones Ala from *dat1-1*. In (**B**), yellow lines represent Glu from Col-0; red ones Glu from *dat1-1*. Error bars: ± SD.

2.2. D-AAs Are Exuded by Roots into the Medium

Another possible mechanism for D-AA reduction to be tested was rhizodeposition [26]. It is a well-established fact that plants release proteinogenic AAs into their rhizosphere [28], but the root exudation of D-AAs has not been reported before. Therefore, *Arabidopsis* seedlings were fed with D-Ala and D-Glu, washed, and then transferred to fresh media. These media were then analyzed after 12 and 72 h. The results of these experiments are presented in Figure 3. In both cases, there is a comparable release of both D-AAs to the medium, which is significantly higher than in the untreated control samples. It is noteworthy that the D-Ala concentration in the medium decreases significantly after 72 h, whereas D-Glu stays almost constant.

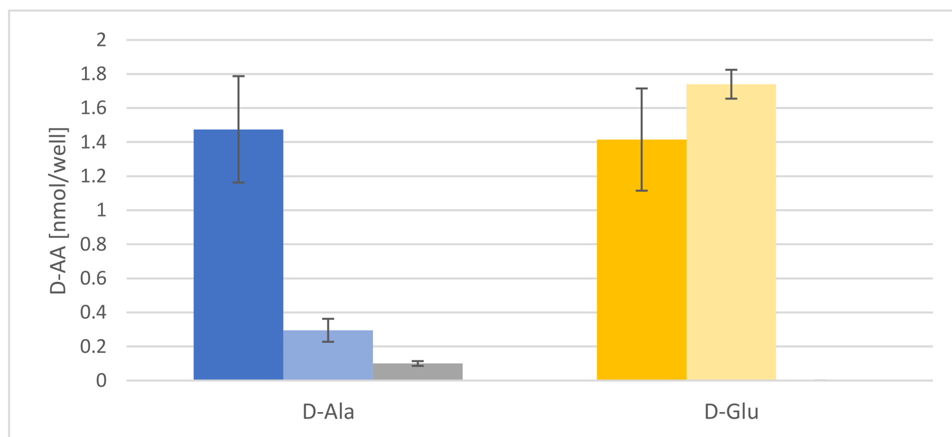
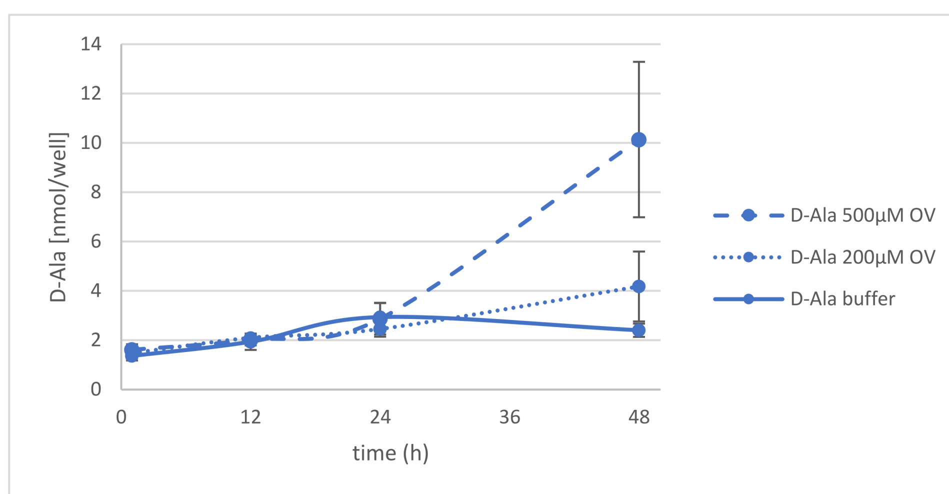


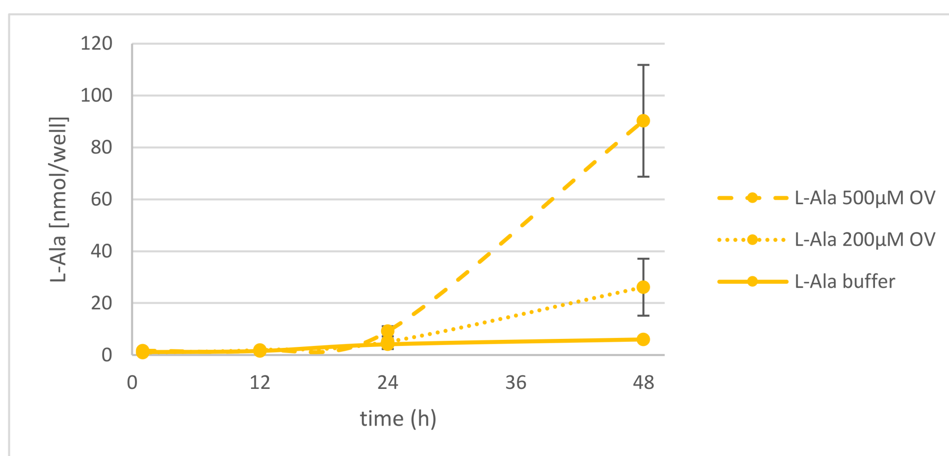
Figure 3. Contents of D-Ala and D-Glu in media released by *Arabidopsis* seedlings treated with both D-AAs. Dark blue and light blue bars represent D-Ala content per well 12 and 72 h after transfer from the D-Ala application medium to fresh medium, respectively. The grey bar represents the 72 h values for media from control plants without D-Ala treatment. Dark-yellow and light-yellow bars represent D-Glu concentration per well 12 and 72 h after transfer from the D-Glu application medium to fresh medium, respectively. D-Glu was not detected in the medium of untreated plants. Error bars: ± SD.

2.3. The Energetization of D*-Ala Exudation*

The observation of D-AA exudation into the rhizosphere led to the questions of if this process is comparable to the exudation of L-AAs and how it is energized. It has been shown before that L-AA exudation is related to ATP hydrolysis by ATP binding cassette (ABC) transporters [29,30]. To find out if this also holds true for D-AA exudation, seedlings were treated with D-Ala and afterwards transferred to fresh media with Na-orthovanadate (OV) or carbonyl cyanide 3-chlorophenylhydrazone (CCCP). The analysis of the AA composition in the medium after treatment with 200 and 500 µM OV, an inhibitor of ATP hydrolysis, revealed an increase of D-Ala exudation over time compared to the untreated control plants (Figure 4A). In the same time, the D-Ala content within treated and untreated plants decreased comparably (Figure S1A). The release of the corresponding L-enantiomer into the medium also increased upon OV application even to a greater extent (Figure 4B). In contrast to D-Ala, the L-Ala concentration in the plants rose with increasing OV concentration (Figure S1B).



(**A**)



(**B**)

Figure 4. Release of D- and L-Ala into the medium under Na-orthovanadate (OV) treatment. Seedlings were treated with D-Ala and then transferred to fresh medium without OV (solid line), with 200 µM OV (dotted line), and with 500 µM OV (dashed line). Then, the D-Ala (**A**) and L-Ala (**B**) contents in the media were analyzed from 1 to 48 h after transfer. Error bars: ± SD.

Application of another ABC transporter blocking agent, CCCP, which causes the dissipation of proton gradients, also led to an increase of D-Ala and L-Ala exudation (Figure 5A). As observed before

with OV application, the D-Ala content in the plants decreased too, whereas the L-Ala remained almost constant (Figure S2A). Additionally, it has been shown before that application of D-Ala leads to the accumulation of D-Glu in the plant [20]. Thus, the exudation of D- and L-Glu could also be analyzed without external application. There was an increased exudation of D-Glu observable, as well as of its L-enantiomer, after the application of D-Ala (Figure 5B). There were no significant changes observed of the Glu levels in treated and untreated plants (Figure S2B).

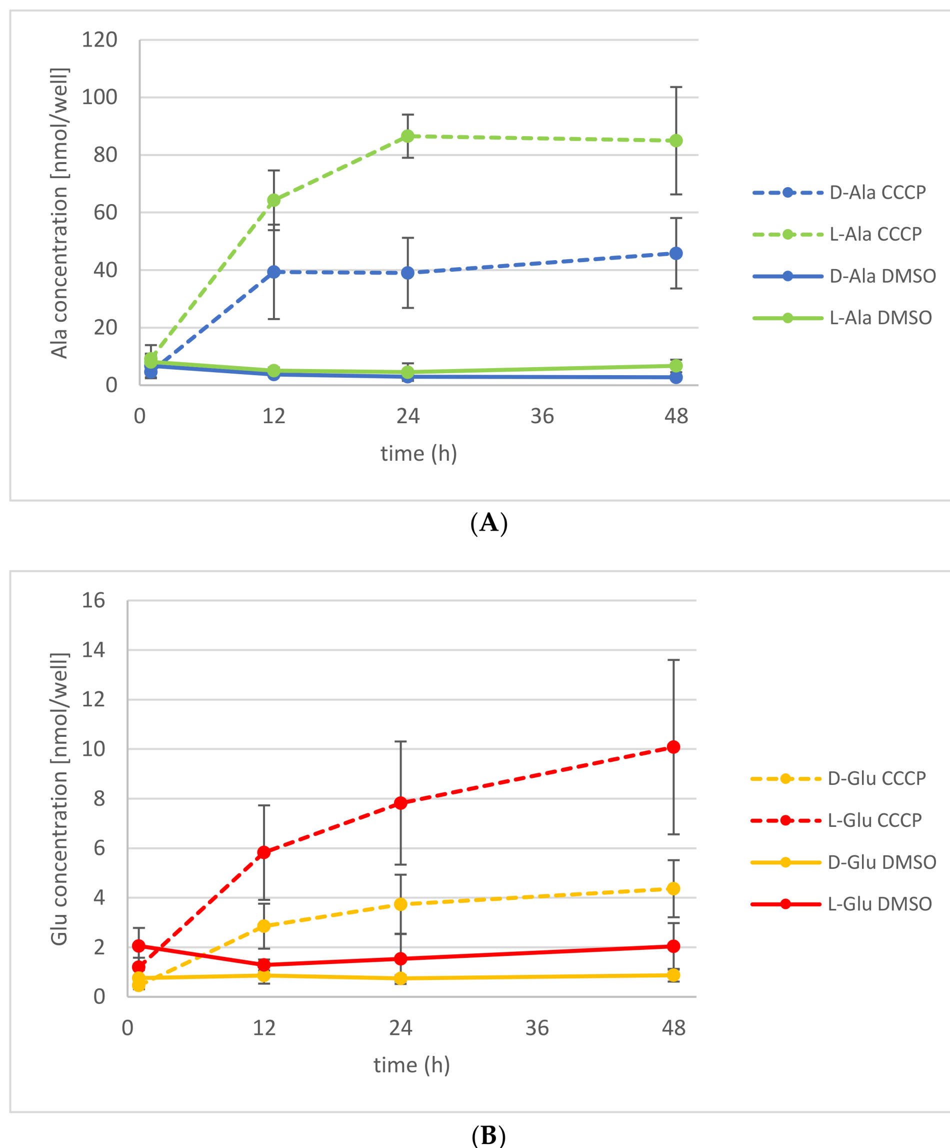


Figure 5. Release of D-/L-Ala and D-/L-Glu into the medium under CCCP treatment. Seedlings were treated with D-Ala and then transferred to fresh medium with 50 µM CCCP (dashed line) or with the solvent of CCCP, dimethyl sulfoxide (DMSO; solid line). (**A**) The D-Ala (blue lines) and L-Ala (green lines) contents in the media were analyzed from 1 to 48 h after transfer. (**B**) In the same media, the D-Glu (yellow lines) and L-Glu (red lines) contents were also determined. Error bars: ± SD.

2.4. Root Exudation of Other D-AAs

After initial characterization of D-Ala and D-Glu exudation, the question arose if also other D-AAs are secreted in this way. Therefore, five additional D-AAs (D-Asp, D-Leu, D-Lys, D-Phe, and D-Pro) were chosen for seedling treatment and AA release measurement into the medium, as performed before (see Section 2.2, Figure 3). The results of these experiments are summarized in Table 1.

Table 1. D- and L-AA contents of media (in nmol/well) 12 and 72 h after 24 h treatment in different D-AAs and transfer to fresh media.

		D-AA Treated Plants				Control Plants			
		D		L		D		L	
		mean	(±SD)	mean	(±SD)	mean	(±SD)	mean	(±SD)
12 h	Aspartate	1.38	(±0.30)	2.15	(±0.35)	0.01	(±0.01)	1.85	(±0.39)
	Leucine	0.32	(±0.03)	0.28	(±0.21)	0.01	(±0.01)	0.14	(±0.13)
	Lysine	1.46	(±1.03)	0.03	(±0.02)	0.00	(±0.00)	0.00	(±0.00)
	Phenylalanine	0.46	(±0.10)	0.00	(±0.00)	0.00	(±0.00)	0.00	(±0.00)
	Proline	2.44	(±0.62)	0.18	(±0.02)	0.76	(±0.16)	0.29	(±0.06)
72 h	Aspartate	0.77	(±0.08)	5.54	(±1.51)	0.03	(±0.01)	5.90	(±0.53)
	Leucine	0.12	(±0.09)	0.00	(±0.00)	0.00	(±0.00)	0.00	(±0.00)
	Lysine	0.20	(±0.16)	0.00	(±0.00)	0.00	(±0.00)	0.00	(±0.00)
	Phenylalanine	0.06	(±0.06)	0.00	(±0.00)	0.00	(±0.00)	0.00	(±0.00)
	Proline	2.48	(±0.27)	0.01	(±0.01)	1.27	(±0.09)	0.09	(±0.06)

There are some tendencies about AA exudation in this data set attracting interest: First of all, exudation of all additionally tested D-AAs could be detected. The exudation levels for D-Asp and D-Leu were similar to that of their corresponding L-enantiomers, in the beginning. Additionally, the levels of some D-AAs in the medium of D-AA-treated plants decreased over time (D-Asp, D-Leu, D-Lys, and D-Phe), whereas D-Pro levels in the medium stayed constant over time. Both exudation patterns (decreasing and constant ones) were also observed for D-Ala and D-Glu, respectively (Figures 3–5).

3. Discussion

In the beginning of this study, there was the question about the fate of D-Ala and D-Glu in plants, being the major conversion products of D-AA metabolism. The presented analyses revealed that exogenously applied D-Ala and D-Glu were reduced in the plants to less than one-fifth within the first 24 h (Figure 1), and further reduction of the remainder needed more than 72 h (Figure 2A,B). Although these results do not answer the initial question finally, they point to different mechanisms involved in D-Ala and D-Glu reduction in plants. The decrease of D-Ala and D-Glu contents and the simultaneous increase of their respective L-enantiomers after D-AA feeding (Figure 2A,B) imply an enzymatic interconversion of D- to L-AA. The most obvious reaction to explain this interconversion would be racemization. Although biochemical evidences for a plant alanine racemase have been found previously in *Chlamydomonas* [31] and *Medicago* [32], the identification of its encoding gene in plants is still pending. Additionally, an enzyme in plants with glutamate racemase activity has not yet been reported.

However, there are also indirect ways to degrade D-Ala and D-Glu or to form L-Ala and L-Glu from them. The easiest one in this respect would be the deamination of the D-AAs by an oxidase, lyase, or dehydrogenase to NH_3 and its corresponding keto acids. The subsequent transamination of the keto acid by an L-AA transaminase would then result in the formation of the corresponding L-AAs. However, the reports about D-AA-deaminating enzymes in plants, especially D-AA oxidases, are scarce and contradictory. Although it has been stated before that plants possess low capacity to metabolize D-AAs and lack D-AA oxidases [13], there is also a report biochemically characterizing a putative D-AA oxidase from corn [33]. Furthermore, the *Arabidopsis* genome harbors at least one putative D-AA oxidase gene [26].

Another possibility of D-Ala and D-Glu reduction in our experiments is D-AA-specific transamination, as implied in previous studies. Previously, it was found that almost all D-AAs are converted into D-Ala and D-Glu; also both D-AAs into each other [8]. In a follow-up report [20], the *Arabidopsis* accession *Ler* was found to be unable to perform this conversion. Later, it was shown that a defective DAT1 protein in this accession is responsible for this effect, which leads to the loss of this major determinant in plant D-AA metabolism [25]. In Figure 2A,B, it can be seen that reduction of the tested D-AAs does not

differ between the wild-type and the *dat1* mutant. This shows, at least, that plants possess alternatives to DAT1-catalyzed transamination with comparable capacity to metabolize D-AAs, which await to be unraveled.

Another principal way of reducing the contents of D-Ala and D-Glu in plants would have been exudation, which was another major focus of this study. The presented data revealed that D-Ala and D-Glu, as well as all other tested D-AAs, were secreted to the rhizosphere in amounts of <10 nmol per seedling (Table 1, Figure 5A,B). Despite the fact that the levels of D-Ala and D-Glu within the plants were about an order of magnitude higher, the D-AA levels in the medium did not increase over time significantly (Figure 4 and Figure S1, Figure 5 and Figure S2). Therefore, the exudation of D-AAs does not seem to contribute crucially to the reduction of these compounds in plants. Instead, the D-Ala and D-Glu levels remain constant in the media over time, but increase drastically after inhibition of active transport (Figures 4 and 5).

This leads to the question of how this observation can be explained. It is a long-lasting matter of debate whether root secretion is a passive or actively energized process (for summaries, see [28,34]). In respect to the exudation of L-AAs, there are two publications reporting aberrant L-AA profiles in ATP-binding cassette (ABC) transporter mutant lines: In one case, the knockout (KO) of AtMRP2, belonging to the multidrug resistance-related protein (MRP) subclass of ABC transporters, leads to a significant increase of L-Pro, L-Tyr, L-Phe, and L-Ala in root exudate [28]. In the other case, the loss of AtMRP5 causes the opposite effect, where all analyzed L-AAs are decreased in the exudate [30]. In our inhibitor experiments in the present report, the secretion of D-Ala and D-Glu was increased by OV, a general ABC transport inhibitor [35], and CCCP, a potent protonophore and inhibitor of metabolically active processes [36]. Furthermore, the content of the corresponding L-enantiomers was also higher in exudates of inhibitor-treated seedlings (Figures 4 and 5).

These observations point to two things: First, the exudations of D- and L-AAs, as far as it concerns Ala and Glu, are similarly regulated processes. This is interesting insofar that knowledge from previous studies about L-AA exudation [36] may also be transferred to root secretion of their D-forms. However, this has to be confirmed in future studies. Furthermore, it would be worthwhile for the future to analyze the chirality of AAs in exudates, as the presented results showed that L- and D-forms are both secreted (Figure 5 and Figure S2). Second, there is at least one passive efflux process, which contributes to AA exudation to a certain extent. If uptake and exudation are exclusively active processes, one would expect either a block of exudation after OV and CCCP treatment or at least a decrease by increasing or prolonged inhibitor treatment. However, the opposite takes place (Figures 4 and 5). For the moment, the best explanation for this scenario would be that a crucial portion of exudation runs via passive efflux, whereas a significant part of the uptake, especially the reuptake of secreted AAs, is ATP-dependent. This scenario is supported by several findings: The uptake of D-Ala in *Arabidopsis* is primarily achieved by the AA transporter LHT1 [8,21], which is CCCP-sensitive [37]. Reuptake of D-Ala and other D-AAs has been observed before [25]. Also, AA root secretion was assumed before not to be energized [28,34,38].

Although the suggested scenario is a working hypothesis and needs to be thoroughly confirmed, it can be concluded that D-AAs are part of the composition of plant root exudates. This finding leaves and opens several questions. For instance, it is still open if just the tested D-AAs, or even all of them, can be secreted, as the results in Table 1 imply. In this respect, it would also be interesting as to what extent the D-AA concentration in the plant influences the exudation process and rate. Together with the transport aspects of the D-AAs, the responsible transporters need to be identified. Several L-AA transporter families in plants have been identified and characterized in the past (for a latest review, see [39]). For some of their members, even the transport of D-AAs was shown: besides the role of AtLHT1 in D-Ala uptake (see above), AtProT1 and AtProT2 have been shown to transport D-Pro [22], and AtAAP1 seems to be involved in D-Met uptake [26]. These examples show that the known amino acid transporters are also able to facilitate D-AA transport and maybe also contribute to their root exudation.

All the questions mentioned before lead to the physiological role of D-AA root exudation. Generally, root exudates are composed of a large variety of chemical compounds, and have been once classified either as mediating plant–plant interactions or playing a role in plant–microbe interactions [40]. In respect to the latter interactions, the exudates may fulfill a rhizosphere-forming function by defending the plant from pathogenic microbes. A possible role of the exudation of D-AAs may be also the attraction of specific microorganisms [28,34]. It has been shown before that the amino acid content of root exudates influences the soil either towards production of antibacterial or antifungal volatiles [41]. Additionally, D-AAs are also generally utilized by bacteria and are able to grow on them as a sole source of carbon and nitrogen [19,42,43]. These properties make D-AAs a potential attractant for bacteria. When it comes to the impact of D-AAs on plant-plant interactions, a possible role of them may be neighbor recognition. The influence of root exudates on kin recognition has been discussed before [44]. Furthermore, nonproteinogenic amino acids, such as meta-tyrosine, produced by plants can act as herbicides and inhibit the growth and settling of other species [45]. Future studies will show if D-AA exudation has an impact on rhizosphere composition, but also on the growth and composition of plant populations.

4. Materials and Methods

4.1. Chemicals

MS media components were purchased from Duchefa (Haarlem, The Netherlands). To apply, determine, and quantify amino acids in plant extracts and media, standard materials were purchased from Sigma-Aldrich (Steinheim, Germany) or in LC/MS grade from Roth (Karlsruhe, Germany).

4.2. Plant Material and Growth Conditions

Arabidopsis seeds of the *dat1-1* mutant and its corresponding wild-type Col-0 were ordered from the Nottingham *Arabidopsis* Stock Centre (University of Nottingham, UK) and genetically characterized as described elsewhere [23]. All seedlings were germinated for 14 d under long-day conditions in microtiter plates as described before [18]. For D-AA uptake and metabolization analyses, D-AAs were added to the media to a final concentration of 1 mM for 24 h. Subsequent transfer into fresh media was done after two washing steps in MilliQ water and brief drying of the seedlings on tissues to remove excess water. Growth conditions were the same as given above.

4.3. Extraction of Amino Acids from Seedlings and Media and Their Derivatization

Amino acid extraction from seedlings and derivatization of AAs in solution were performed as described elsewhere [8]. To derivatize AAs in media, the buffer conditions were adjusted to 0.1 M Tris/HCl, pH 8, such as in plant extracts, by adding 1 M Tris/HCl. The incubation time of derivatization was elongated to 3 h and the derivatized liquid volume was adjusted with acetonitrile instead of methanol for all derivatizations.

4.4. LC/MS Determination of D- and L-AAs

In the course of the studies, the following amino acids were measured: D/L-alanine, D/L-aspartate, D/L-glutamate, D/L-leucine, D/L-lysine, D/L-phenylalanine, and D/L-proline. An Acquity–SynaptG2 LC/MS system from Waters (Manchester, UK) was used for quantification, and operated in positive electrospray ionization mode. The mass spectrometer was operated at a capillary voltage of 3000 V and a resolution of 20,000. Separation of the abovementioned amino acids was carried out on a RP Acquity HSST3 1 × 150 mm, 1.8 µm column with a flow rate of 50 µL/min and a 22 min gradient from 70% water to 99% acetonitrile (both with 0.1% formic acid). For quantification, 3 µL of sample were injected with a 6-point calibration from 0.125 µM to 1250 µM.

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/1422-0067/19/4/1109/s1.

Acknowledgments: We are indebted to Klaus Harter (Center for Plant Molecular Biology, University of Tübingen, Germany) for the provision of research facilities and the opportunity to perform our studies. Investigations of Juan Suarez were supported by the Deutscher Akademischer Austauschdienst (DAAD 91567028). We acknowledge support by Deutsche Forschungsgemeinschaft and Open Access Publishing Fund of Tübingen University.

Author Contributions: Claudia Hener, Juan Suarez, Mark Stahl, and Üner Kolukisaoglu conceived and designed the experiments; Claudia Hener and Sabine Hummel performed the experiments; Claudia Hener, Üner Kolukisaoglu, and Mark Stahl analyzed the data; Juan Suarez and Sabine Hummel contributed reagents/materials/analysis tools; Mark Stahl and Üner Kolukisaoglu wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Typas, A.; Banzhaf, M.; Gross, C.A.; Vollmer, W. From the regulation of peptidoglycan synthesis to bacterial growth and morphology. *Nat. Rev. Microbiol.* **2011**, *10*, 123–136. [CrossRef] [PubMed]
2. Ollivaux, C.; Soyez, D.; Toullec, J.Y. Biogenesis of D-amino acid containing peptides/proteins: Where, when and how? *J. Pept. Sci.* **2014**, *20*, 595–612. [CrossRef] [PubMed]
3. Balu, D.T.; Coyle, J.T. The NMDA receptor 'glycine modulatory site' in schizophrenia: D-serine, glycine, and beyond. *Curr. Opin. Pharmacol.* **2015**, *20*, 109–115. [CrossRef] [PubMed]
4. Hernandez, S.B.; Cava, F. Environmental roles of microbial amino acid racemases. *Environ. Microbiol.* **2016**, *18*, 1673–1685. [CrossRef] [PubMed]
5. Sacchi, S. D-Serine metabolism: New insights into the modulation of D-amino acid oxidase activity. *Biochem. Soc. Trans.* **2013**, *41*, 1551–1556. [CrossRef] [PubMed]
6. Di Fiore, M.M.; Santillo, A.; Chieffi Baccari, G. Current knowledge of D-aspartate in glandular tissues. *Amino Acids* **2014**, *46*, 1805–1818. [CrossRef] [PubMed]
7. Friedman, M. Origin, microbiology, nutrition, and pharmacology of D-amino acids. *Chem. Biodivers.* **2010**, *7*, 1491–1530. [CrossRef] [PubMed]
8. Gordes, D.; Kolukisaoglu, U.; Thurow, K. Uptake and conversion of D-amino acids in *Arabidopsis* thaliana. *Amino Acids* **2011**, *40*, 553–563. [CrossRef] [PubMed]
9. Erikson, O.; Hertzberg, M.; Nasholm, T. A conditional marker gene allowing both positive and negative selection in plants. *Nat. Biotechnol.* **2004**, *22*, 455–458. [CrossRef] [PubMed]
10. Takahashi, S.; Abe, K.; Kera, Y. Bacterial d-amino acid oxidases: Recent findings and future perspectives. *Bioengineered* **2015**, *6*, 237–241. [CrossRef] [PubMed]
11. Molla, G. Competitive Inhibitors Unveil Structure/Function Relationships in Human D-Amino Acid Oxidase. *Front. Mol. Biosci.* **2017**, *4*, 80. [CrossRef] [PubMed]
12. Genchi, G. An overview on D-amino acids. *Amino Acids* **2017**, *9*, 1521–1533. [CrossRef] [PubMed]
13. Nasholm, T.; Kielland, K.; Ganeteg, U. Uptake of organic nitrogen by plants. *New Phytol.* **2009**, *182*, 31–48. [CrossRef] [PubMed]
14. Hill, P.W.; Quilliam, R.S.; DeLuca, T.H.; Farrar, J.; Farrell, M.; Roberts, P.; Newsham, K.K.; Hopkins, D.W.; Bardgett, R.D.; Jones, D.L. Acquisition and assimilation of nitrogen as peptide-bound and D-enantiomers of amino acids by wheat. *PLoS ONE* **2011**, *6*, e19220. [CrossRef] [PubMed]
15. Michard, E.; Lima, P.T.; Borges, F.; Silva, A.C.; Portes, M.T.; Carvalho, J.E.; Gilliham, M.; Liu, L.H.; Obermeyer, G.; Feijo, J.A. Glutamate receptor-like genes form Ca^{2+} channels in pollen tubes and are regulated by pistil D-serine. *Science* **2011**, *332*, 434–437. [CrossRef] [PubMed]
16. Monselise, E.B.; Levkovitz, A.; Kost, D. Ultraviolet radiation induces stress in etiolated *Landoltia punctata*, as evidenced by the presence of alanine, a universal stress signal: A ^{15}N NMR study. *Plant. Biol. (Stuttg.)* **2015**, *17* (Suppl. 1), 101–107. [CrossRef] [PubMed]
17. Hirano, T.; Tanidokoro, K.; Shimizu, Y.; Kawarabayasi, Y.; Ohshima, T.; Sato, M.; Tadano, S.; Ishikawa, H.; Takio, S.; Takechi, K.; et al. Moss Chloroplasts Are Surrounded by a Peptidoglycan Wall Containing D-Amino Acids. *Plant Cell* **2016**, *28*, 1521–1532. [CrossRef] [PubMed]

18. Vranova, V.; Zahradnickova, H.; Janous, D.; Skene, K.R.; Matharu, A.S.; Rejsek, K.; Formanek, P. The significance of D-amino acids in soil, fate and utilization by microbes and plants: Review and identification of knowledge gaps. *Plant Soil* **2012**, *354*, 21–39. [CrossRef]
19. Radkov, A.D.; McNeill, K.; Uda, K.; Moe, L.A. D-Amino acid catabolism is common among soil-dwelling bacteria. *Microbes Environ.* **2016**, *31*, 165–168. [CrossRef] [PubMed]
20. Gordes, D.; Koch, G.; Thurow, K.; Kolukisaoglu, U. Analyses of *Arabidopsis* ecotypes reveal metabolic diversity to convert D-amino acids. *Springerplus* **2013**, *2*, 559. [CrossRef] [PubMed]
21. Svennerstam, H.; Ganeteg, U.; Bellini, C.; Nasholm, T. Comprehensive screening of *Arabidopsis* mutants suggests the lysine histidine transporter 1 to be involved in plant uptake of amino acids. *Plant Physiol.* **2007**, *143*, 1853–1860. [CrossRef] [PubMed]
22. Lehmann, S.; Gumy, C.; Blatter, E.; Boeffel, S.; Fricke, W.; Rentsch, D. In planta function of compatible solute transporters of the AtProT family. *J. Exp. Bot.* **2011**, *62*, 787–796. [CrossRef] [PubMed]
23. Bruckner, H.; Westhauser, T. Chromatographic determination of L- and D-amino acids in plants. *Amino Acids* **2003**, *24*, 43–55. [PubMed]
24. Fujitani, Y.; Nakajima, N.; Ishihara, K.; Oikawa, T.; Ito, K.; Sugimoto, M. Molecular and biochemical characterization of a serine racemase from *Arabidopsis* thaliana. *Phytochemistry* **2006**, *67*, 668–674. [CrossRef] [PubMed]
25. Suarez, J.; Hummel, S.; Hener, C.; Stahl, M.; Kolukisaoglu, Ü. AtDAT1 plays a major role in D-amino acid metabolism of *Arabidopsis thaliana*. *New Phytol.* **2018**. under review.
26. Kolukisaoglu, Ü.; Suarez, J. D-Amino Acids in Plants: New Insights and Aspects, but also More Open Questions. In *Amino Acid-New Insights and Roles in Plant and Animal*; InTech: London, UK, 2017.
27. Funakoshi, M.; Sekine, M.; Katane, M.; Furuchi, T.; Yohda, M.; Yoshikawa, T.; Homma, H. Cloning and functional characterization of *Arabidopsis* thaliana D-amino acid aminotransferase—D-aspartate behavior during germination. *FEBS J.* **2008**, *275*, 1188–1200. [CrossRef] [PubMed]
28. Badri, D.V.; Vivanco, J.M. Regulation and function of root exudates. *Plant Cell Environ.* **2009**, *32*, 666–681. [CrossRef] [PubMed]
29. Badri, D.V.; Quintana, N.; El Kassis, E.G.; Kim, H.K.; Choi, Y.H.; Sugiyama, A.; Verpoorte, R.; Martinoia, E.; Manter, D.K.; Vivanco, J.M. An ABC transporter mutation alters root exudation of phytochemicals that provoke an overhaul of natural soil microbiota. *Plant Physiol.* **2009**, *151*, 2006–2017. [CrossRef] [PubMed]
30. Zhou, D.; Huang, X.-F.; Chaparro, J.M.; Badri, D.V.; Manter, D.K.; Vivanco, J.M.; Guo, J. Root and bacterial secretions regulate the interaction between plants and PGPR leading to distinct plant growth promotion effects. *Plant Soil* **2016**, *401*, 259–272. [CrossRef]
31. Nishimura, K.; Tomoda, Y.; Nakamoto, Y.; Kawada, T.; Ishii, Y.; Nagata, Y. Alanine racemase from the green alga Chlamydomonas reinhardtii. *Amino Acids* **2007**, *32*, 59–62. [CrossRef] [PubMed]
32. Ono, K.; Yanagida, K.; Oikawa, T.; Ogawa, T.; Soda, K. Alanine racemase of alfalfa seedlings (*Medicago sativa* L.): First evidence for the presence of an amino acid racemase in plants. *Phytochemistry* **2006**, *67*, 856–860. [CrossRef] [PubMed]
33. Gholizadeh, A.; Kohnehrouz, B. Molecular cloning and expression in *Escherichia coli* of an active fused *Zea mays* L. D-amino acid oxidase. *Biochemistry (Moscow)* **2009**, *74*, 137–144. [CrossRef]
34. Baetz, U.; Martinoia, E. Root exudates: The hidden part of plant defense. *Trends Plant Sci.* **2014**, *19*, 90–98. [CrossRef] [PubMed]
35. Sugiyama, A.; Shitan, N.; Yazaki, K. Involvement of a soybean ATP-binding cassette-type transporter in the secretion of genistein, a signal flavonoid in legume-Rhizobium symbiosis. *Plant Physiol.* **2007**, *144*, 2000–2008. [CrossRef] [PubMed]
36. Lesuffleur, F.; Cliquet, J.B. Characterisation of root amino acid exudation in white clover (*Trifolium repens* L.). *Plant Soil* **2010**, *333*, 191–201. [CrossRef]
37. Hirner, A.; Ladwig, F.; Stransky, H.; Okumoto, S.; Keinath, M.; Harms, A.; Frommer, W.B.; Koch, W. *Arabidopsis* LHT1 is a high-affinity transporter for cellular amino acid uptake in both root epidermis and leaf mesophyll. *Plant Cell* **2006**, *18*, 1931–1946. [CrossRef] [PubMed]
38. Okumoto, S.; Pilot, G. Amino Acid Export in Plants: A Missing Link in Nitrogen Cycling. *Mol. Plant* **2011**, *4*, 453–463. [CrossRef] [PubMed]
39. Dinkeloo, K.; Boyd, S.; Pilot, G. Update on amino acid transporter functions and on possible amino acid sensing mechanisms in plants. *Semin. Cell Dev. Biol.* **2018**, *74*, 105–113. [CrossRef] [PubMed]

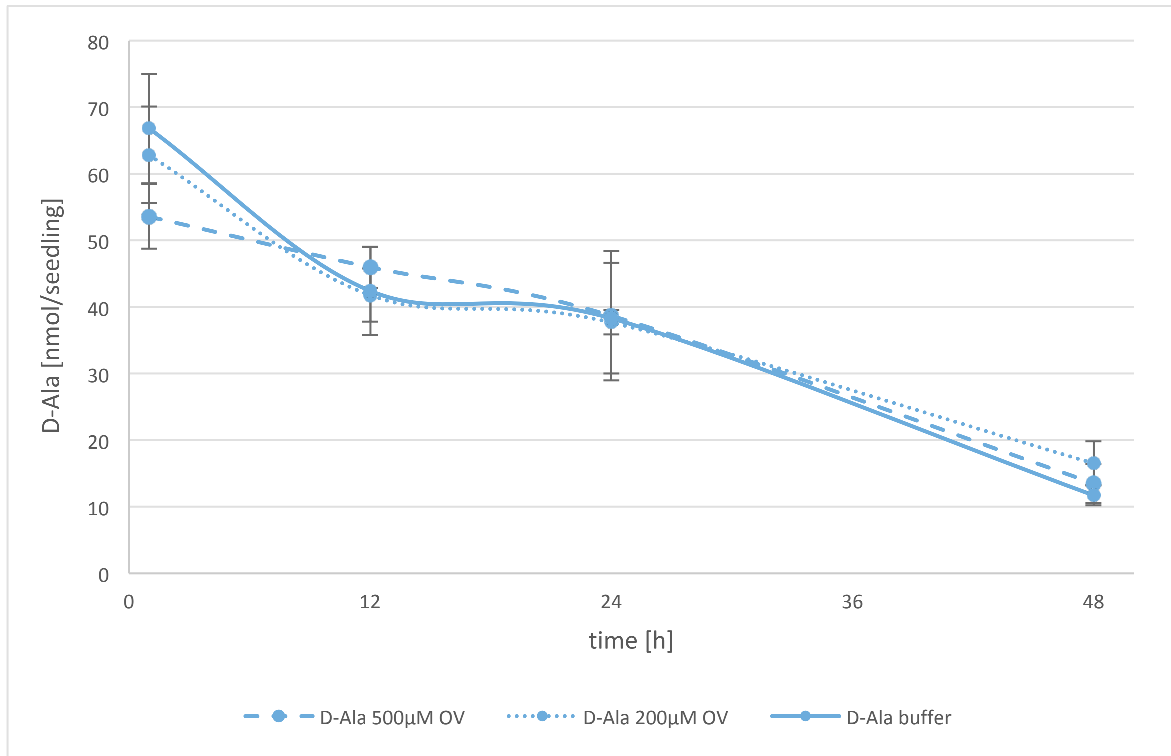
40. Bais, H.P.; Weir, T.L.; Perry, L.G.; Gilroy, S.; Vivanco, J.M. The role of root exudates in rhizosphere interations with plants and other organisms. *Annu. Rev. Plant Biol.* **2006**, *57*, 233–266. [CrossRef] [PubMed]
41. Mendes, R.; Garbeva, P.; Raaijmakers, J.M. The rhizosphere microbiome: Significance of plant beneficial, plant pathogenic, and human pathogenic microorganisms. *FEMS Microbiol. Rev.* **2013**, *37*, 634–663. [CrossRef] [PubMed]
42. Zhang, G.S.; Sun, H.J. Racemization in Reverse: Evidence that D-Amino Acid Toxicity on Earth Is Controlled by Bacteria with Racemases. *PLoS ONE* **2014**, *9*, e92101. [CrossRef] [PubMed]
43. Zhang, Z.L.; Zheng, Q.; Jiao, N.Z. Microbial D-amino acids and marine carbon storage. *Sci. China Earth Sci.* **2016**, *59*, 17–24. [CrossRef]
44. Semchenko, M.; Saar, S.; Lepik, A. Plant root exudates mediate neighbour recognition and trigger complex behavioural changes. *New Phytol.* **2014**, *204*, 631–637. [CrossRef] [PubMed]
45. Bertin, C.; Weston, L.A.; Huang, T.; Jander, G.; Owens, T.; Meinwald, J.; Schroeder, F.C. Grass roots chemistry: Meta-tyrosine, an herbicidal nonprotein amino acid. *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 16964–16969. [CrossRef] [PubMed]

© 2018 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

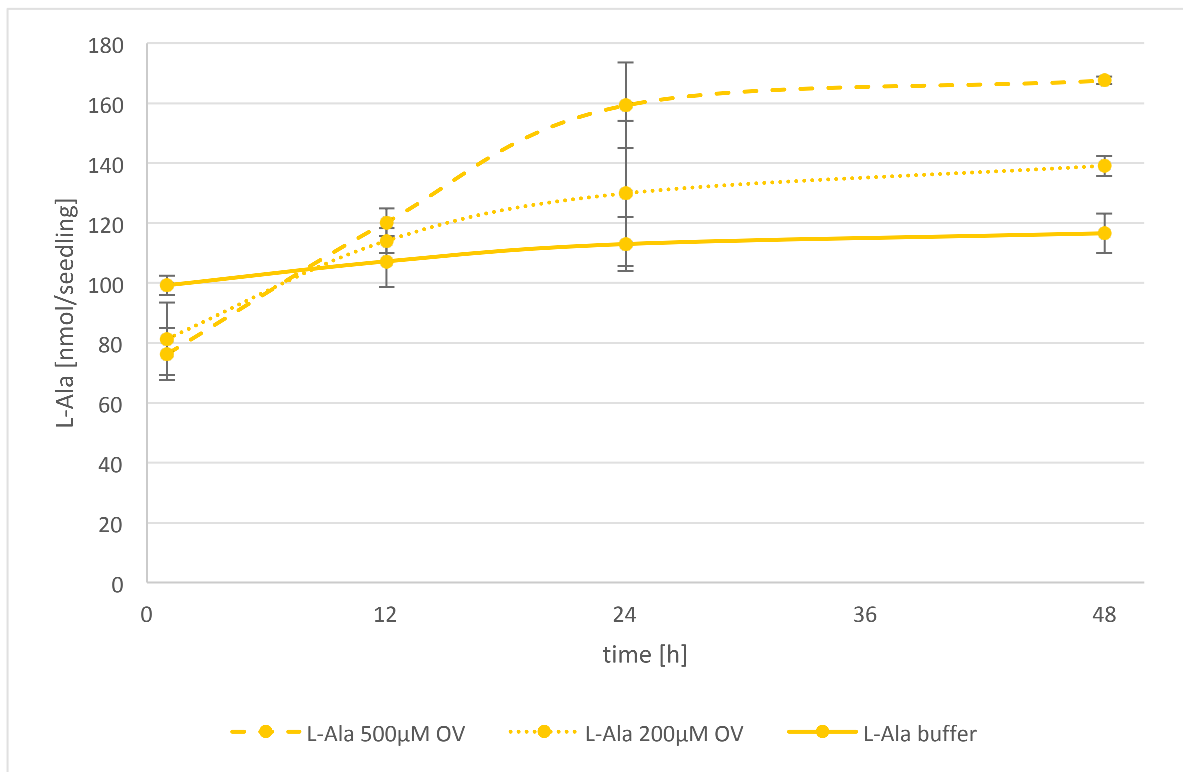
4.2 Supplementary Materials: D-Amino Acids are Exudated by *Arabidopsis thaliana* Roots to the Rhizosphere

Supplementary Materials: D-Amino Acids are Exudated by *Arabidopsis Thaliana* Roots to the Rhizosphere

Claudia Hener, Sabine Hummel, Juan Suarez, Mark Stahl and Üner Kolukisaoglu

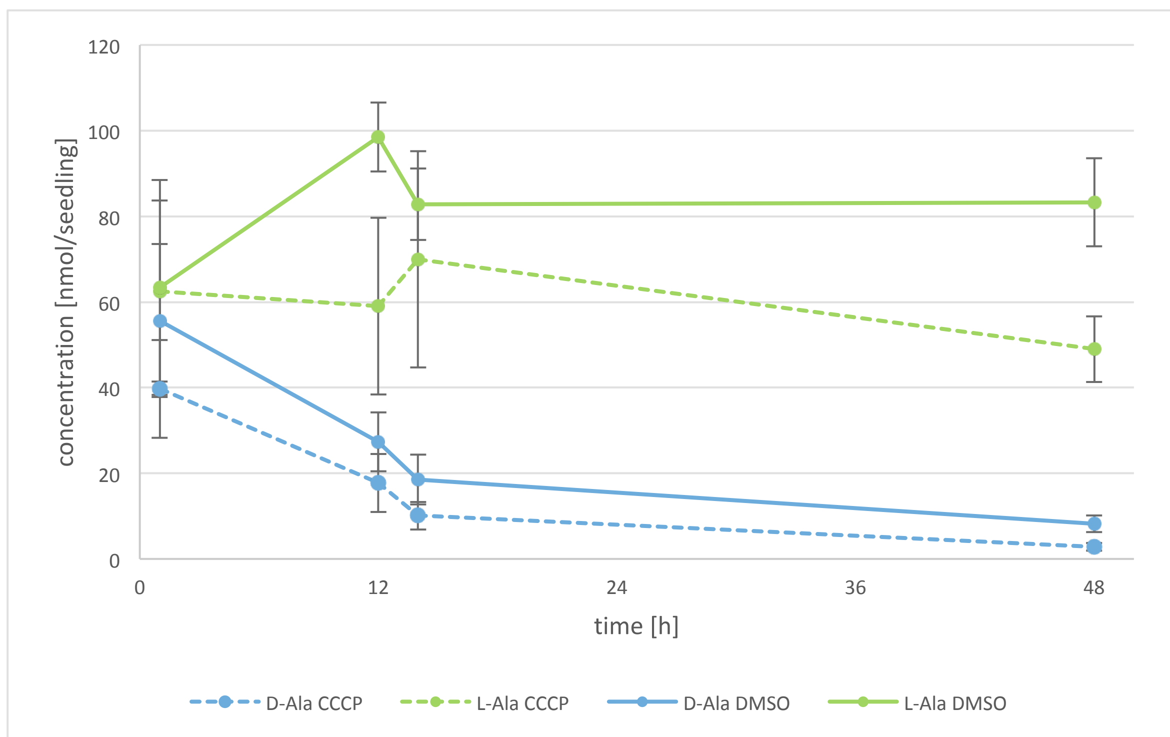


(**A**)

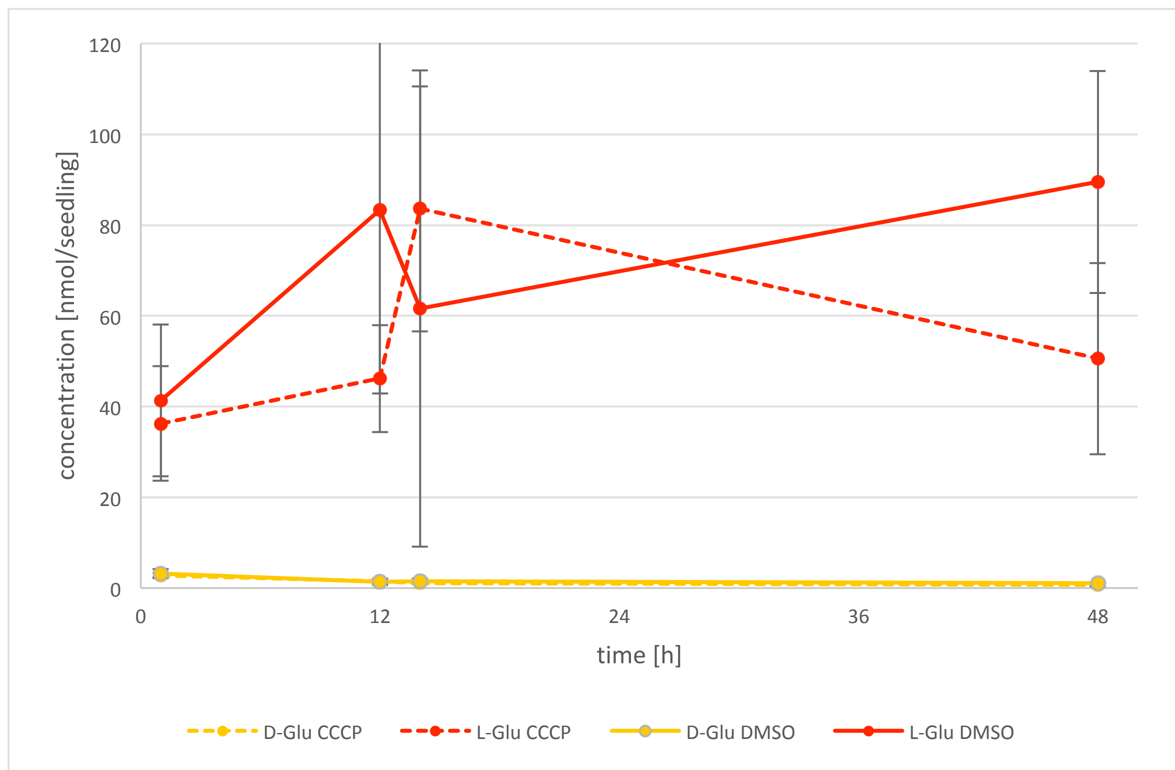


(**B**)

Figure S1. Contents of D- and L-Ala in seedlings under OV treatment. Seedlings were treated with D-Ala and then transferred to fresh medium without (solid line), with 200 µM OV (dotted line) and 500 µM OV (dashed line). Then the D-Ala (A) and L-Ala (B) contents in the seedlings were analyzed from 1-48 h after transfer. Error bars: ±SD.



(**A**)



(**B**)

Figure S2. Contents of D-/L-Ala and D-/L-Glu in seedlings under CCCP treatment. Seedlings were treated with D-Ala and then transferred to fresh medium with DMSO (solid line) or with 10 µM CCCP (dotted line). (A) The D-Ala (blue lines) and L-Ala (green lines) contents in the seedlings were analyzed from 1-48 h after transfer. (B) In the same media also the D-Glu (yellow lines) and L-Glu (red lines) contents of the seedlings were determined. Error bars: ±SD.

Chapter 5

Article Three

5.1 AtDAT1 is a key enzyme of D-amino acid stimulated ethylene production in *Arabidopsis thaliana*

Status: Submitted

Position of candidate in list of authors: First author.

Scientific ideas by the candidate: 60%

Data generation by the candidate: 40%

Analysis and Interpretation by the candidate: 40%

Paper writing done by the candidate: 20%

the plant journal

AtDAT1 is a key enzyme of D-amino acid stimulated ethylene production in Arabidopsis thaliana

Journal:	*The Plant Journal*
Manuscript ID	TPJ-00044-2019
Manuscript Type:	Original Article
Key Words:	D-amino acids in plants, D-amino acid-stimulated ethylene production, D-amino acid specific transaminase, D-methionine, 1-aminocyclopropane, ethylene, amino acid malonylation

SCHOLARONE™
Manuscripts

SUBMITTED MANUSCRIPT

AtDAT1 is a key enzyme of D-amino acid stimulated ethylene production in *Arabidopsis thaliana*

Juan Suarez*, Claudia Hener*, Vivien-Alisa Lehnhardt, Sabine Hummel, Mark Stahl, Üner Kolukisaoglu

Center for Plant Molecular Biology (ZMBP), University of Tübingen, Auf der Morgenstelle 32, 72076 Tübingen, Germany

* authors contributed equally to the manuscript

Corresponding author: Üner Kolukisaoglu, Center for Plant Molecular Biology (ZMBP), University of Tübingen, Auf der Morgenstelle 32, 72076 Tübingen, Germany

email: uener.kolukisaoglu@zmbp.uni-tuebingen.de

Tel.: +49 7071 29-73095

CONFIDENTIAL

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

18 **Summary**

19 D-enantiomers of proteinogenic amino acids (D-AAs) are found ubiquitously, but the
20 knowledge about their metabolism and functions in plants is scarce. A long forgotten
21 phenomenon in this regard is the D-AA-stimulated ethylene production in plants. As a
22 starting point to investigate this effect the *Arabidopsis* accession *Landsberg erecta*
23 (*Ler*) got into focus as it was found defective in metabolizing D-AAs. Combining
24 genetics and molecular biology of T-DNA lines and natural variants together with
25 biochemical and physiological approaches we could identify AtDAT1 as a major D-AA
26 transaminase in *Arabidopsis*. *atdat1* loss-of-function mutants and *Arabidopsis*
27 accessions with defective *AtDAT1* alleles were not able to produce D-Ala, D-Glu and
28 L-Met in response to D-Met anymore. This result corroborates the biochemical
29 characterization of AtDAT1, which showed highest activity using D-Met as substrate.
30 Germination of seedlings in light and dark led to enhanced growth inhibition of *atdat1*
31 mutants on D-Met. Ethylene measurements revealed an enhanced D-AA stimulated
32 ethylene production in these mutants. This effect could be partially assigned to the
33 deregulation of the ethylene precursor 1-aminocyclopropane (ACC) content due to the
34 malonylation of D-Met instead of ACC resulting in ethylene increase. With AtDAT1, the
35 first central regulator of plant D-AA metabolism was characterized biochemically and
36 physiologically. The specific effects of D-Met on ethylene production and plant
37 development and the impact of AtDAT1 on these effects raise questions about further
38 physiological functions of D-AAs for plants.

CONFIDENTIAL

39

40 **Significance Statement**

41 The metabolism and functions of D-amino acids in plants is still cryptic, but analyses
42 of the D-amino acid specific transaminase AtDAT1 revealed that this enzyme is
43 responsible for the metabolization of the majority of these non-canonical amino acids.
44 Furthermore, the loss of this gene leads to increased triple response and ethylene
45 synthesis after D-methionine application in *dat1* mutants, indicating functional relations
46 between regulation of D-amino acid metabolism and plant development.

47

48 **Keywords**

D-amino acids in plants, D-amino acid-stimulated ethylene production, D-amino acid specific transaminase, D-methionine, 1-aminocyclopropane, ethylene, amino acid malonylation

Introduction

It is widely accepted that L-amino acids (L-AAs), especially the proteinogenic ones, are essential in all kingdoms of life, both as primary metabolites as well as elementary building blocks of proteins. In contrast, the metabolism and functions of the D-forms of proteinogenic amino acids (D-AAs) is far less clear and defined. Major reasons for this discrepancy in our knowledge are the large diversity and different functions of D-AAs in organisms. For instance, bioactive peptides like octopine from octopus and scallop, antibiotics from bacteria and opioids from frogs were among the first substances reported to contain D-AAs. (Fujii, 2002, Martínez-Rodríguez *et al.*, 2010, Ollivaux *et al.*, 2014) In humans, several proteins related to diseases like arteriosclerosis, Alzheimer or Parkinson contain D-AAs, especially D-Asp, which are generated by racemization of the corresponding L-AA (Fujii *et al.*, 2011). Various free D-AAs were detected in different tissues and fluids of humans and other mammals (Hamase *et al.*, 2002, Hamase, 2007). The most prominent example in this respect is the impact of D-Asp and D-Ser on the functions of the N-methyl-D-aspartate (NMDA) receptor in mammals: Aberrant levels of these D-AAs seem also to be connected with psychological disorders and diseases of the endocrine system (for reviews see Fuchs *et al.* (2005), D'aniello (2007), Katane and Homma (2011), Balu and Coyle (2015)).

Far less is known about the metabolism and functions of D-AAs in plants. This is astonishing against the background that plant roots are surrounded by D-AAs, namely D-Ala and D-Glu, as degradation products of the peptidoglycan layer of bacterial cell walls (Dworkin, 2014). Thus, the amount of D-AAs in the rhizosphere can be more than 10% of the corresponding L-enantiomer (Brodowski *et al.*, 2005, Amelung *et al.*, 2006). This led to the question if D-AAs are actively utilized by plants. For a long time, D-AAs were considered as toxins due to the fact, that some of them inhibit seedling growth in submillimolar concentrations (Erikson *et al.*, 2004, Forsum *et al.*, 2008). But several reports suggest that D-AAs take up a similarly crucial position in plants as in microbes and animals. For instance, the D-Ala amount in duckweed was demonstrated to be increased during UV stress (Monselise *et al.*, 2015) and D-Ser is involved in pollen

SUBMITTED MANUSCRIPT

tube growth in Arabidopsis by regulating the glutamate receptor GLR1.2, which belongs to a group of plant proteins closely related to mammalian NMDA receptors (Michard *et al.*, 2011, Forde and Roberts, 2014). In mosses D-Ala and D-Glu were detected in the plastidial envelope, which resembles to bacterial peptidoglycan (Hirano *et al.*, 2016). This finding and others led to the conclusion that peptidoglycan, containing D-Ala and D-Glu, is an integral part of the plastidial envelope not only in cryptophytes (for a review see Chen *et al.* (2018)).

The number of enzymes predicted to be specific for processing D-AAs annotated in plant genomes imply much more functions for these amino acids than currently known (Naranjo-Ortíz *et al.*, 2016). However, it also raises the question about its metabolism in plants, especially how the abundance of different D-AAs is regulated. On the one hand their contents have to be maintained at required levels to ensure their activity. On the other hand the intracellular concentrations must be limited below toxic levels. This restriction is of specific importance due to the fact that the rhizosphere is the natural major source of D-AAs for plants (Vranova *et al.*, 2012) and that D-AAs are taken up by roots in considerable amounts compared to L-AAs (Hill *et al.*, 2011, Gordes *et al.*, 2013). In this respect, the question arises which processes facilitate the catabolization of D-AAs in plants.

In the course of these previous studies D-Met got into our focus. This D-AA caused the greatest conversion rates in almost all tested accessions of *Arabidopsis thaliana* except in *Ler* (Gordes *et al.*, 2013). This attracted our interest insofar as methionine represents a relatively small portion of soil amino acids (Vranova *et al.*, 2012). But it had been detected in soil (Amelung and Zhang, 2001), and there have also been several bacterial species isolated from soil which are specialized to the utilization of D-Met as sole carbon and nitrogen source (Radkov *et al.*, 2016). Furthermore, it is produced by different bacteria, incorporated into their cell wall and even released to their environment in order to disassemble biofilms (for a review see Cava *et al.* (2011). Nevertheless, D-Met has not been reported yet to be produced by plants.

More than 30 years ago it was reported that feeding D-Met to seedlings of cocklebur, pumpkin, sunflower, mung bean, water melon, and pea leads to increased ethylene production (Satoh and Esashi, 1980, Liu *et al.*, 1983, Kionka and Amrhein, 1984). This phenomenon was coined as “D-amino-acid-stimulated ethylene production” (Satoh and Esashi, 1980) due to the increase of ethylene content also induced by some other

SUBMITTED MANUSCRIPT

CONFIDENTIAL

D-AAs. The authors tried to explain the effect by competitive malonylation of D-Met and 1-aminocyclopropane (ACC), the precursor of ethylene. According to this hypothesis D-Met would compete with ACC for the same putative malonyl transferase (Liu *et al.*, 1983, Ling-Yuan *et al.*, 1985, Benichou *et al.*, 1995, Wu *et al.*, 1995), which would lead to an increase of ACC level and subsequently the ethylene production would rise (S F Yang and Hoffman, 1984). However, this hypothesis could not be verified because the corresponding malonyl transferase has not been identified to date. Nevertheless, the question about the mechanism and function of this phenomenon remains.

Our starting point to address this question were previous works from Gördes *et al.* (2011), which revealed that *Arabidopsis* plants convert particular D-AAs like D-Met, D-Trp, D-Phe and D-His partially to their respective L-enantiomers. Additionally, the feeding of almost all tested D-AAs led mainly to the formation of D-Ala and D-Glu. By contrast, the *Arabidopsis* accession *Landsberg erecta* (*Ler*) is incapable of both, the D-AA to L-AA and the D-AA to D-Ala/D-Glu conversion (Gordes *et al.*, 2013). These observations point to a central metabolic step, in which D-AAs, with a high preference to D-Met, are converted to D-Ala and D-Glu by a D-AA specific transaminase (Vranova *et al.*, 2012, Gordes *et al.*, 2013).

In this paper we describe the identification and characterization of *Arabidopsis* loss-of-function mutant alleles in the *Columbia-0* (*Col-0*) accession for a previously characterized D-AA specific transaminase called D-AAT (Funakoshi *et al.*, 2008), which we named AtDAT1. The mutants of this gene showed almost identical defects as *Ler* to metabolize D-AAs, with D-Met as strongest effector. Indeed, we could show that the affected gene in the *Ler* accession is an almost non-functional *AtDAT1* allele. Biochemical analyses revealed that this enzyme prefers D-Met as amino donor and pyruvate over 2-oxoglutarate as amino acceptor, in contrast to the findings of Funakoshi *et al.* (2008). The preferential production of D-Ala offers also an explanation why it is found as a predominant conversion product of D-AAs. Here we report data that D-Met causes growth inhibition in Arabidopsis seedlings due to a deregulated metabolism of the ethylene precursor ACC, which is mainly regulated by AtDAT1. Based on these findings *atdat1* mutants offered a tool to investigate the influence of D-AAs on plant growth. Furthermore, these findings point to functions of D-Met in central plant processes beyond unspecific growth inhibition.

SUBMITTED MANUSCRIPT

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

148

149 **Results**

150 AtDAT1 as a candidate gene for D-AA metabolization

151 Initially, we observed the almost absence of both D- to L-AA and D-AA to D-Ala/D-Glu
152 conversion in *Ler* in comparison to other ecotypes (Gördes *et al.*, 2013). According to
153 the transamination hypothesis, the mutation of at least one D-AA specific transaminase
154 could be responsible for this metabolic phenotype. With AtDAAT1, one candidate
155 protein had been previously identified biochemically as such an enzyme (Funakoshi *et*
156 *al.*, 2008). In order to investigate its role *in planta* we wanted to analyze T-DNA
157 insertion lines of the corresponding gene (At5g57850) regarding their D-AA
158 metabolization capability.

159 Homozygous plants for such insertion lines, SALK_011686 and SALK_111981 (Figure
160 1a), were isolated and propagated for further analyses (see Table S1 for primer
161 sequences). RT-PCR analysis of *AtDAT1* expression displayed no transcripts in the
162 *dat1-1* and *dat1-2* mutant compared to the respective *Col-0* wild type (Figure 1b).
163 However, the *AtDAT1* transcript level in the *Ler* accession was similar to the wild type
164 *Col-0*. As shown before (Gördes *et al.*, 2011; 2013) feeding with D-Met caused the
165 highest accumulation of D-Ala, D-Glu and their respective L-enantiomers in
166 *Arabidopsis* seedlings. Therefore, mutant and corresponding wild type (*Col-0*)
167 seedlings derived from the insertion lines were grown for 14 days on liquid MS medium
168 in light, then supplemented with D-Met and subsequently analyzed for their AA content.
169 In sharp contrast to *Col-0*, both insertion mutants of At5g57850 (afterwards designated
170 as *AtDAT1*, the mutants SALK_011686 and SALK_111981 as *dat1-1* and *dat1-2,*
171 respectively; Figure 1a), were neither able to produce D-Ala, D-Glu nor additional L-
172 Met after application of D-Met. This AA profile was very similar to that found in
173 seedlings of the *Ler* accession (Figure 1c).

174 Further *in silico* analyses of public transcriptomic data (Lempe *et al.*, 2005) revealed
175 that the accession *M7323S* displayed a strongly reduced *AtDAT1* transcript level,
176 which could be confirmed by RT-PCR (Fig. 1b). When this accession was grown on D-
177 Met supplemented medium defects in AA metabolism were observed (Figure S1),
178 comparable to the ones in *Ler* and the *dat1* mutants. Sequencing of the genomic locus
179 as well as the cDNA of *AtDAT1* from *M7323S* revealed that this gene contains a T→A
180 mutation at genomic position +1259. This leads to a nonsense mutation on cDNA level

SUBMITTED MANUSCRIPT

CONFIDENTIAL

at the third position of a cysteine codon (TGT) to a stop codon (TGA) at position 248 of the AA sequence (C248STOP) (Fig. 1a). In contrast, sequencing the genomic locus and the cDNA of *AtDAT1* from *Ler* just revealed two mutations leading to AA exchanges of the peptide sequence (A77T and T303S) (Fig. 1a).

To examine whether these mutations in the *AtDAT1 Ler* allele are responsible for the aberrant metabolic phenotype in the *Ler* accession, we performed different genetic approaches. Firstly, ubiquitin promoter-driven expression of the *AtDAT1 Col-0* allele in transgenic *Ler* plants led to the reconstitution of the D-Met metabolism in *Ler* and its complementation of the *dat1-2* mutant (Figure 2a). Secondly, the AA analyses of F1 seedlings derived from crosses between *Col-0* and *Ler* and between *Col-0* and *dat1-2* displayed no D-Met metabolization defect, irrespective of the maternal origin. In contrast, the offspring of *Ler* x *dat1-2* was not able to metabolize D-Met (Fig. 2b). These data unequivocally prove the loss of *AtDAT1* function in the *Ler* accession and *dat1-2* T-DNA insertion mutant.

To answer the remaining question which process leads to the functional defect of AtDAT1 in *Ler* the expression of this gene was analyzed. By analysis of pDAT1::GUS plants it can be seen that this gene is expressed in seedlings and adult plants with a lack of GUS staining in late floral stages and seeds (Figure S2a). Comparing the activity of the *AtDAT* promoters derived from the *Col-0* and *Ler* alleles no apparent difference could be observed in seedlings either in the absence or in the presence of L-Met or D-Met in the growth media (Figure S2b). This finding was supported by RT-PCR expression analysis of the *AtDAT1* allele in *Ler*, which was also not altered significantly in comparison to *Col-0*, in contrast to the *dat1-1* and *dat1-2* mutants displaying no detectable transcript (Fig. 1b).

Subcellular mis-localization would have been another reason for affected AtDAT1 function in *Ler*. Therefore, GFP-tagged AtDAT1 protein variants derived from cDNA of both ecotypes expressed under the control of the ubiquitin 10 promoter were transiently transformed into tobacco leaves (Figure S3). Both were shown to be localized in plastids. Therefore, a possible mis-expression or mis-localization caused by the mutation in the *Ler* allele of *AtDAT1* is not the reason of its aberrant D-Met metabolization.

A missense mutation of the *AtDAT1 Ler* allele leads to an almost complete loss of the enzymatic activity

SUBMITTED MANUSCRIPT

To clarify why the enzyme encoded by the *Ler AtDAT1* allele is not able to transaminate D-AAs, the *Ler* and, as a control, the *Col-0* versions of AtDAT1 were expressed with an N-terminal GST-tag in *E. coli*. After purification by affinity chromatography (for results of purification see Figure S4) their enzymatic capabilities were tested according to Funakoshi *et al.* (2008).

To seek out the optimal substrate D-AA and experimental conditions, we initially tested the *Col-0* version of GST-AtDAT1 for its capability to transaminate 2-oxoglutarate (2-OG), as it had been done by Funakoshi *et al.* (2008), or pyruvate using 16 different D-AAs as amino group donors. When 2-OG was used as amino group acceptor, a significant transaminase reaction was only observed for the donors D-Met, D-Trp and D-Ala (Table S2), whereas with pyruvate as acceptor almost all applied D-AAs (with the exception of D-Pro) led to the formation of the reaction product D-Ala (Figure 3). Furthermore, we measured an over 100 times higher activity for the enzymatic reaction with pyruvate as acceptor than with 2-OG, irrespective of the D-AA used as amino group donor (Table S2). The comparison of the $AtDAT1_{(Col\text{-}0)}$ activities using different D-AAs and pyruvate as substrates revealed that D-Met was the best tested amino group donor (Figure 3). Using these two compounds as substrates we determined the K_M and V_{max} of $AtDAT1_{(Col\text{-}0)}$ to be 17.4 mM and 0.07 nkat, respectively.

To investigate the enzymatic activity of $AtDAT1_{(Ler)}$ assays were performed with two substrate combinations: Firstly, with D-Met and pyruvate as amino group donor and acceptor, respectively, as it was the best substrate combination for $AtDAT1_{(Col\text{-}0)}$ and, secondly, with D-Ala and 2-OG to evaluate previously published data (Funakoshi *et al.*, 2008). As shown in Figures 4a and 4b for both substrate combinations the activity of $AtDAT1_{(Ler)}$ dropped to 0-5% compared to $AtDAT1_{(Col\text{-}0)}$.

At that point the question arose if just one of the missense mutations (A77T and T303S) was sufficient to cause this activity loss in $AtDAT1_{(Ler)}$. The alignment of DAT1 amino acid sequences from different algal and plant species revealed that the alanine at position 77 seems to be more conserved than the threonine at position 303 (Figure S5). To analyze the impact of the mutations of $AtDAT1_{(Ler)}$, $AtDAT1_{(Col\text{-}0)}$ derived isoforms harboring single amino acid exchanges were also expressed as N-terminal GST fusions in *E. coli*, affinity-purified and tested for their activity. Whereas the enzyme isoform with the Thr 303 to Ser single amino acid exchange $AtDAT1_{(303S)}$ showed an activity comparable to $AtDAT1_{(Col\text{-}0)}$ (Figures 4a and 4b), the mutation of Ala 77 to Thr

SUBMITTED MANUSCRIPT

led to a strong decrease in the production of D-Glu (Figure 4a) and D-Ala (Figure 4b) with 2-oxoglutarate or pyruvate as amino acceptors, respectively. The enzymatic defect of $AtDAT1_{(A77T)}$ was quantitatively similar to that of $AtDAT1_{(Ler)}$. From these data we concluded that solely the amino acid exchange A77T is responsible for the activity loss of AtDAT1 in *Ler*. But the data also revealed that the *Ler* variant is not completely inactive with about 5% remaining activity in comparison to *Col-0* (Figure 4b).

The loss of AtDAT1 leads to decreased seedling growth in response to D-Met

After identification of AtDAT1 as a central enzyme for metabolization of D-AAs, the question arose, to which physiological effects in *Arabidopsis* growth and development the loss of *AtDAT1* gene function would lead. Under greenhouse conditions in soil growth behavior of *dat1-1* and *dat1-2* mutant plants could not be distinguished from corresponding wild type (Figure S6).

An obvious question in this respect was how these mutant lines would grow in presence of D-Met. In advance several other D-AAs were tested, but none of them led to such drastic differences in seedling growth of *dat1* mutants compared to *Col-0*. Germination on D-Met in concentrations of 500 µM affected *dat1-1* and *dat1-2* seedling growth significantly compared to the corresponding wild type, whereas *Ler* took an intermediate position (Figure 5a). Testing this growth behavior in the dark revealed an even more pronounced growth difference between *dat1* mutants and *Col-0* (Figure 5b). All these growth differences were specific for D-Met, whereas the addition of the same concentrations of L-Met did not lead to these differential effects (Figure 5a and 5b). Altogether, D-Met inhibited seedling growth specifically in AtDAT1 affected lines.

atdat1 mutants display enhanced D-AA stimulated ethylene production

Especially the growth effects of *dat1-1* and *dat1-2* in the dark (Figure 5b) indicate a triple response caused by the gaseous plant hormone ethylene. This gets even clearer with a look on the hypocotyl length of the four dark grown compared lines (Figure 5c): There was a highly significant decrease of *dat1-1* and *dat1-2* hypocotyl length of about one eighth compared to Col-0 grown on 500 µM D-Met. Increasing L-Met concentrations also led to shorter hypocotyls, but to similar extent in mutants and wild type plants (Figure S7). Furthermore, the growth inhibition was by far not as strong as with D-Met.

SUBMITTED MANUSCRIPT

1
2
3 279 To elucidate if this observation was really caused by ethylene we measured its
4
5 280 production in *dat1* mutants and wild type plants grown in the dark. As it can be seen in
6
7 281 Figure 6a the addition of 500 µM D-Met was sufficient to induce a significant increase
8
9 282 of up to threefold of ethylene production in light grown *dat1* mutants compared to *Col-0*.
10 283 Even stronger changes in ethylene production could be observed for both *dat1* mutant
11
12 284 lines grown in the presence of D-Met (Figure 6b) also in the dark.
13
14 285 As mentioned above the increase of ethylene production by D-AAs was attributed to
15
16 286 competitive malonylation of D-AAs instead of ACC, which should lead to ACC oxidation
17
18 287 resulting in higher ethylene concentration (S F Yang and Hoffman, 1984). To verify this
19
20 288 assumption, we measured the contents of malonyl-methionine and malonyl-ACC in D-
21 289 Met treated seedlings. In these measurements we could detect a significant increase
22
23 290 of malonyl-methionine in Col-0 and *dat1* seedlings upon D-Met treatment (Figure 7a
24
25 291 and 7b). This accumulation of up to 5 fold compared to the corresponding wild type
26 292 was clearly detectable in both mutants, irrespective of the light regime. Since the
27
28 293 amount of malonyl-ACC in these experiments was below our detection limit, we added
29
30 294 10 µM ACC to the media and measured once more malonyl-ACC in *dat1-1* seedlings.
31 295 In this case we could detect large amounts of malonyl-ACC which decreased
32
33 296 drastically upon D-Met addition both in Col-0 and the mutant (Figure 7c). The decrease
34
35 297 was higher in *dat1-1* than in Col-0, but this difference was not significant. D-Met
36
37 298 induced malonyl-ACC reduction was not due to production of malonyl-methionine
38 299 caused by ACC, which was comparable with and without ACC addition (Table S3).
39
40
41 300
42
43 301 **Discussion**
44
45 302 For several decades the detrimental, but partially also beneficial, effects of D-AAs on
46
47 303 plants have been investigated (Valdovinos and Muir, 1965, Aldag and Young, 1970,
48
49 304 Erikson *et al.*, 2004, Erikson *et al.*, 2005, Gordes *et al.*, 2011, Hill *et al.*, 2011).
50 305 Especially in recent years there was growing evidence that almost all D-enantiomers
51
52 306 of proteinogenic L-AAs are taken up by plants (Aldag and Young, 1970, Forsum *et al.*,
53
54 307 2008, Gordes *et al.*, 2011, Hill *et al.*, 2011). They are partially synthesized *de novo*
55
56 308 (Bruckner and Westhauser, 2003, Strauch *et al.*, 2015) and also metabolized in
57 309 significant amounts (Aldag & Young, 1970; Gördes *et al.*, 2011). With the evidence
58
59 310 provided here, the long standing question was addressed how D-AAs are metabolized
60 311 in plants.

SUBMITTED MANUSCRIPT

CONFIDENTIAL

In the light of the observations of Gördes *et al.* (2011), three possible mechanisms for this process had been suggested: racemization, deamination and transamination of D-AAs (Vranova *et al.*, 2012; Gördes *et al.*, 2013). Our data indicate that transamination by AtDAT1 is responsible for major steps of D-AA turnover in *Arabidopsis*, which is reflected by its broad range of D-AA specificity (Figure 3). Furthermore, we showed that the major product of this enzymatic reaction is D-Ala with D-Met as the favored amino group donor. This finding led us to the analysis of D-Met induced ethylene production and to the characterization of AtDAT1 as a regulatory enzyme in this process in Arabidopsis.

An open question could be answered by the characterization of AtDAT1: D-Ala was known as a primary product of D-AA metabolization. This effect is caused by the property of AtDAT1 to prefer pyruvate over 2-OG (Table S2). In comparison to the work of Funakoshi *et al.* (2008), who used 2-OG as amino group acceptor for the characterization of AtDAT1, our results revealed a higher V_{max} with pyruvate as substrate. Most interestingly, the different enzymatic activities with pyruvate and 2-OG as amino acceptors with ratios of 100:1 and more (Table S2) were in a comparable range as the D-Ala/D-Glu ratios found in plants after D-AA application (Gördes *et al.*, 2011). This preferential accumulation of D-Ala compared to D-Glu in D-AA treated plants is in accordance to the presented preference for pyruvate as substrate of AtDAT1.

Nevertheless, further degradation of D-Ala in plants is still unresolved. Due to its toxicity on Arabidopsis in submillimolar concentration (Erikson *et al.*, 2004; Gördes *et al.*, 2011), accumulation of D-Ala to higher concentrations in plants is not likely, but there are four possible ways of D-Ala to be further processed. Beside malonylation (Fukuda *et al.*, 1973, Ogawa *et al.*, 1973), oxidation (Gholizadeh and Kohnehrouz, 2009), and racemization (Ono *et al.*, 2006, Nishimura *et al.*, 2007) the dimerization to its dipeptide D-Ala-D-Ala is of specific interest. It has been reported recently that mosses are able to integrate D-Ala as the dipeptide D-Ala-D-Ala into the envelope of their chloroplasts as found in peptidoglycans of bacterial cell walls (Typas *et al.*, 2012). The loss of the enzyme for this D-Ala ligation, D-Ala-D-Ala ligase (DDL), causes defects in chloroplast division in mosses, whereas loss-of-function alleles of the single ortholog in *Arabidopsis* (*AtDDL1*) did not show such an aberrant phenotype (Hirano *et al.*, 2016). The occurrence of the dipeptide D-Ala-D-Ala in leaf tissue of different

SUBMITTED MANUSCRIPT

angiosperms was shown long ago (Noma *et al.*, 1973, Frahn and Illman, 1975, Manabe, 1985, Manabe, 1992) and also confirms the existence of active DDL in higher plants. But other functions for D-Ala-D-Ala in plants apart from its integration into peptidoglycan-like structures in mosses await elucidation.

In this regard it is noteworthy that AtDAT1-GFP was exclusively found in chloroplasts (Figure S3). This means that this step of D-AA metabolism in plants is also confined to this compartment and would require transport of D-AAs thereto. This assumption was supported by Gisby *et al.* (2012) who provided genetic evidence for plastidial import of D-Ala and D-Val in transgenic tobacco plants. A summary of potentially D-AA metabolizing enzymes in the Arabidopsis genome revealed their putative subcellular localization mostly in plastids (Kolukisaoglu and Suarez, 2017). The question why D-AA related processes seem to be focused to this compartment also awaits to be answered.

Another open question is the role of AtDAT1 in D-Met stimulated ethylene production. As it could be shown in this report this phenomenon is tightly connected to AtDAT1. The loss of this protein leads to significant increase of ethylene after D-Met application (Figure 6), resulting primarily in shortening of the hypocotyl of *dat1* mutants irrespective of the light regime (Figure 5). This treatment led also to increasing production of malonyl-methionine especially in the mutants, and the content of malonyl-ACC developed reciprocally (Figure 7). Especially, the decreasing capability of ACC degradation by malonylation implies that this results in the specific increase in ethylene production and enhanced triple response. But this conclusion needs further confirmation, because ACC malonylation does not differ significantly between M-Met treated Col-0 and *dat1* mutants. In this regard it must not be forgotten that small molecules like L-Met, D-Ala or D-Glu, evolve in response to D-Met in Col-0, but not in *dat1* mutants, and may also cause the observed ethylene production. At least for D-Ala it has been shown to inhibit the ACC oxidase (Brunhuber *et al.*, 2000, Charng *et al.*, 2001).

Undoubtedly, AtDAT1 is a key enzyme of D-Met stimulated ethylene production. But the question remains if D-Met is the natural substrate of this enzyme, which also needs future investigations. Assuming D-Met as the natural substrate leads to the problem that this D-AA has never been reported in plants until to date. In contrast, it has been shown previously that D-Met is released by bacterial biofilms (Kolodkin-Gal *et al.*, 2010,

SUBMITTED MANUSCRIPT
CONFIDENTIAL

Vlamakis *et al.*, 2013) and that different rhizosphere colonizing bacterial species are able to utilize D-Met as sole carbon and nitrogen source (Radkov *et al.*, 2016). Biofilm formation on root surfaces as a bacterial pathogen protection strategy has been reported before (for a review see Vlamakis *et al.* (2013)). Possibly, AtDAT1 is part of bacterial biofilm recognition and therefore may be involved in plant-bacterial interaction.

This possibility would also offer an explanation why AtDAT1 gets lost in particular Arabidopsis accessions as it has been shown for *Ler* and *M7323S* in this report. In a habitat without D-Met releasing bacteria in the rhizosphere, a recognition system for this compound would be also dispensable for the plant. But this relation needs to be proven first. It is noteworthy in this respect that DAT1 encoding genes seem to be found in almost all sequenced plant genomes (for a selection see Figure S5), and that ethylene production in other plant species than Arabidopsis is also induced by additional D-AAs like D-Leu, D-Thr, D-Val or D-Phe (Satoh and Esashi, 1980, Satoh and Esashi, 1982, Liu *et al.*, 1983). It would be interesting in this regard if DAT1 proteins from different species have also differing substrate specificities and therefore contribute to the adaptation of plants to changing microbial environments.

Experimental procedures

Plant Material and Growth Conditions

All *Arabidopsis* ecotypes as well as T-DNA insertion lines analyzed in this study were either provided by the Nottingham Arabidopsis Stock Centre (University of Nottingham, UK) or the Arabidopsis Biological Resource Center (University of Ohio, Columbus, OH). All T-DNA insertion lines were selected and verified by PCR to harbor the correct insertion homozygously.

Seedlings for amino acid extraction and profiling were germinated in microtiter plates as described before (Gördes *et al.*, 2013). For phenotypic analysis of seedlings and subsequent measurement of malonylated methionine and ACC in their extracts plants were either germinated for six days in darkness or at 16 h light/8 h darkness (all at 22°C). As solid growth media ½ x MS salts with 1% sucrose and 1% phytoagar, including conditional further additions (e.g. D-AAs, ACC) were applied. For all analyses of adult plants these were grown in the greenhouse in soil.

SUBMITTED MANUSCRIPT

CONFIDENTIAL

PCR Genotyping and RT-PCR analysis of *Arabidopsis* Lines and Accessions

Plant DNA for PCR analysis was extracted from seedlings or leaves of adult plants according to Edwards *et al.* (1991). To determine zygosity of T-DNA insertion lines either a gene specific primer and a border primer or two gene specific primers flanking the insertion (for primer combinations and sequences see Supplemental Table S1) were used in a PCR reaction with Taq polymerase from New England Biolabs (Frankfurt am Main, Germany) according to manufacturer´s protocol. To determine the AtDAT1 sequence in different *Arabidopsis* ecotypes the complete coding sequences were amplified from genomic DNA and cDNA as described above and the PCR amplificates were sequenced directly by GATC (Konstanz, Germany). For cDNA synthesis RNA of 14 days old seedlings germinated in liquid media was extracted with the RNeasy Mini Kit from Qiagen (Düsseldorf, Germany) and cDNA was synthesized with RevertAid H Minus Reverse Transcriptase from Thermo Fisher Scientific (Karlsruhe, Germany), both according to manufacturers´ protocols. This cDNA was also used for cloning purposes (see below) and RT-PCR analysis.

Cloning of AtDAT1 Variants for Recombinant Expression

For cloning AtDAT1 from cDNA of *Arabidopsis* accessions Col-0 and Ler, the complete coding sequence was amplified with KOD DNA Polymerase from Merck Millipore (Schwalbach am Taunus, Germany) with the primer combination DAT1-Start/DAT1-A1 (Supplemental Table S1). Amplificates were cloned into pENTR/D-TOPO according to manufacturer´s protocol (Thermo Fisher Scientific, Karlsruhe, Germany), leading to the constructs pENTR-AtDAT1$_{(Col\text{-}0)}$ and pENTR-AtDAT1$_{(Ler)}$. To create AtDAT1 coding sequences with the single point mutations A77T and T303S the previously described clones were cleaved with *Pst I* and *Not I*, creating a 0.5 kb fragment. This was then ligated from pENTR-AtDAT1$_{(Col\text{-}0)}$ to pENTR-AtDAT1$_{(Ler)}$ and vice versa, resulting in the constructs pENTR-AtDAT1$_{(A77T)}$ and pENTR-AtDAT1$_{(T303S)}$. After sequence verification of the constructs they were all used for LR reaction using the kit form Invitrogen (Karlsruhe, Germany) according to manufacturer's protocol into pGEX-2TM-GW (kindly received from Bekir Ülker) for expression in *E. coli* with N-terminal GST tag and C-terminal His tag. Additionally, the pENTR-AtDAT1$_{(Col\text{-}0)}$ and pENTR-AtDAT1$_{(Ler)}$ were used for Gateway-based cloning into pUB-DEST-GFP for expression in plants with C-

SUBMITTED MANUSCRIPT

1 2 3 443 terminal GFP tag. pENTR-AtDAT1$_{(Col-0)}$ was used for Gateway-based cloning into pUB-
4 5 444 DEST (Grefen *et al.*, 2010) for complementing AtDAT1 defective plants.
6 7 445
8 9 10 446 <u>Arabidopsis Transformation and tobacco leaf infiltration</u>

11 12 447 All plant transformation vectors were transformed into *Agrobacterium tumefaciens* cv.
13 14 448 pMP90-RK GV3101. Plant transformation was performed by floral dipping (Clough and
15 16 449 Bent, 1998). For selection of transformants, seeds were either germinated on 1/2 MS-
17 18 450 Agar with 1% sucrose containing hygromycin or germinated on soil and sprayed with
19 451 2% BASTA from AgrEvo (Düsseldorf, Germany) depending on the used vector.

20 21 452 For tobacco leaf infiltration transformed Agrobacterium containing pUB10-GFP::DAT1
22 23 453 was mixed with a strain of transformed Agrobacterium for expression of the mCherry
24 25 454 plastid marker (CD3-999 pt-rk; Nelson *et al.*, 2007) and P19 *Agrobacterium*
26 27 455 *tumefaciens* cells into infiltration media (10 mM MES-KOH [pH 5.7], 10 mM] $MgCl_{2,}$ 0.2
28 456 mM Acetosyringone). Using a syringe 1mL of infiltration media with the mix of the 3
29 30 457 type of cells was infiltrated in the abaxial side of *Nicotiana benthamiana* leaves. Plants
31 32 458 were then watered and kept on the lab bench for 2 d. Afterwards, single leaf discs were
33 459 excised for confocal fluorescence microscopy.

CONFIDENTIAL

34 35 460
36 37 38 461 <u>Fluorescence Microscopy</u>

39 40 462 Imaging was performed using a Leica laser scanning microscope SP8 with the
41 42 463 corresponding software LCS or LASAF X (Leica Microsystems, Wetzlar, Germany).
43 44 464 For excitation of GFP-fusion proteins the Argon laser was used at 488 nm and the
45 46 465 detection range was from 500 to 550 nm. For m-RFP excitation was set to 561 nm and
47 466 detection was from 600 to 650 nm. All autofluorescence of chloroplasts was detected
48 49 467 in the range from 670 nm to 725 nm.

50 51 468
52 53 469 <u>Promoter::GUS Transgenic Analysis</u>

54 55 470 The promoter region from -677 to +11 of the genomic locus of AtDAT1 from *Col-0* and
56 57 471 *Ler* were amplified by PCR with the primer pair ProDAT1-SGW/ProDAT1-AGW (for
58 59 472 sequences see Supplemental Table S1). The respective fragment was cloned into
60

pENTR/D-TOPO and then into pMDC163 (Curtis and Grossniklaus, 2003), to be transformed into *Arabidopsis* by Agrobacterium-mediated gene transfer.

Histochemical staining of GUS activity was analyzed in plants of the T2-generation that had been germinated on liquid media. For GUS staining seedlings and adult plants were washed in sodium phosphate buffer and afterwards incubated overnight at 37°C in this buffer containing 1 mM X-Gluc (5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid) and 0.5 mM $K_3Fe(CN)_6$. Afterwards chlorophyll was removed for documentation by several washings with hot ethanol.

Recombinant Expression of AtDAT1 Variants in *E. coli*

E. coli strain BL21(DE3) RIL was transformed with cDNA of AtDAT1 variants in pGEX-2TM-GW (see above) and grown in LB medium with appropriate antibiotics until they reached an OD_{600} of 0.5. Then expression was induced by addition to a final concentration of 0.1 mM isopropyl-β-D-galactoside (IPTG) and the culture was grown for 20 h at 18°C. Afterwards cells were pelleted by centrifugation and washed once with TE buffer including 100 mM NaCl. After further centrifugation cells were resuspended in 20 mM Tris, pH 8, with Protease Inhibitor Cocktail from Biotool (Oberasbach, Germany). This suspension was sonicated and afterwards centrifuged with 18,000 x g to clear the crude extract from cell debris.

The recombinant His-tagged AtDAT1 protein variants from this crude extract were purified with Protino Ni-NTA agarose from Macherey-Nagel, (Weilmünster Germany) according to manufacturer´s protocol. Therefore, the column was equilibrated and loaded with 10 mM imidazole, washed with 20 mM imidazole, and elution of His-tagged proteins was achieved with 250 mM imidazole. Imidazole was removed by dialysis with Float-A-Lyzer Dialysis Device from Roth (Karlsruhe, Germany) in 10 mM potassium phosphate, pH 8. Protein content was determined with the Bio-Rad Protein Assay (Bio-Rad, München, Germany) according to manufacturer´s protocol. Specific detection of His tagged proteins on a western blot was achieved with a monoclonal His Tag antibody conjugated to alkaline phosphatase (antikoerper-online.de, Aachen, Germany).

Enzyme assays to determine D-AA specific aminotransferase activity

SUBMITTED MANUSCRIPT

CONFIDENTIAL

The standard reaction mixture with 2-OG as amino group acceptor contained D-Ala (10 mM), 2-OG (50 mM) and pyridoxalphosphate (PLP; 50 µM) in potassium phosphate buffer (100 mM, pH 8). For assays with pyruvate as amino group acceptor D-Ala and 2-OG were replaced by D-Met (10 mM) and pyruvate (50 mM), respectively. To determine substrate specificity, the tested D-AAs were all applied in 10 mM concentration. All assay reactions in triplicates were started by addition of 3-8 µg of purified protein, incubated at 37°C, and samples were taken at different time points up to 90 min. Each sample was derivatized and the amino acids measured as described below.

For the determination of K_M and V_{max} values different D-Met concentrations (0.1, 0.5, 1.0, 2.0, 5.0, 10.0, 20.0 and 50.0 mM D-Met) have been incubated with the enzyme AtDAT1 and pyruvate as cosubstrate (50 mM). Produced D-Alanine was analyzed after 0, 5 and 10 min. With the means of three biological replicates for any D-Met concentration and time point the slope of the time course was calculated and normalized to the protein amount used. To determine K_M and V_{max} values a linearization according to Hofstee (1959) was used.

Amino acid extraction and determination from plant material

Amino acid extraction and derivatization was performed as described before (Gördes *et al.*, 2011). The incubation time of derivatization was elongated to 3 h and the derivatized liquid volume was adjusted with acetonitrile instead of methanol.

Almost all experiments were focused on the measurement of D/L-Alanine, D/L-Glutamate and D/L-Methionine. To determine and quantify these amino acids in plant extracts and enzyme assays standard materials were purchased from Sigma-Aldrich (Steinheim, Germany). Other chemicals were obtained in LC/MS grade from Roth (Karlsruhe, Germany). An Acquity–SynaptG2 UPLC-MS system from Waters (Manchester, England) was used for quantification, operated in positive electrospray ionization mode. The mass spectrometer was operated at a capillary voltage of 3000 V and a resolution of 20000. Separation of the above mentioned amino acids was carried out on a RP Acquity HSS T3, 1 x 150 mm, 1,8 µm column with a flow rate of 50 µl/min and a 22 min stepwise gradient from 99 % water to 99 % methanol (both with 0,1 % formic acid). For quantification, 3 µl of sample were injected and a 5-point calibration from 0,125 µM to 1250 µM was used.

SUBMITTED MANUSCRIPT

CONFIDENTIAL

The quantification of malonyl-methionine ([M+H+] 218,022) and malonyl-ACC ([M+H+] 188,050) was performed relatively using the same LC/MS system described above. However, the stationary phase was changed into RP Acquity HSS T3, 2,1 x 100 mm, 1,8 µm column, and the mobile phase was a 15 min gradient from 99 % water to 99 % methanol with a flow of 0,2 ml/min. The detection of the malonylated compounds was carried out via mass identification in the positive ionization mode after injection of 7 µL underivatized pure plant extract.

Analysis of ethylene

For assaying ethylene production, Arabidopsis seedlings were grown in glass vials (18 ml) containing 3 ml solid medium (30 seedlings per vial) for six days. The vials were closed with rubber septa and opened ones before measuring. After 30-90 min of further incubation ethylene accumulating in the free air space was measured by gas chromatography using a gas chromatograph equipped with a flame-ionization detector (Felix *et al.*, 1991).

Statistical evaluation

Data was analyzed with IBM SPSS Statistics 24. Significances were analyzed using an independent two sample Student´s t-test. For testing the homogeneity of variances a levene test was applied.

Acknowledgements

We would like to acknowledge Prof. Klaus Harter (Center for Plant Molecular Biology, University of Tübingen, Germany) for the provision of research facilities and critically reading the manuscript. Efforts of J.S. were supported by the Deutscher Akademischer Austauschdienst (DAAD 91567028). Furthermore, we would like to thank to Nina Glöckner and Friederike Wanke for their excellent support in confocal fluorescence microscopy and to Georg Felix for his support in ethylene measurements.

Supporting Information

Figure S1: D-Met metabolization in *M7323S* and *Col-0*

SUBMITTED MANUSCRIPT

CONFIDENTIAL

1
2
3 569 **Figure S2:** Expression patterns of AtDAT1
4
5
6 570 **Figure S3**: AtDAT1 from Col-0 and Ler localize both in plastids
7
8 571 **Figure S4:** Western blot of the purified AtDAT1 variants
9
10 572 **Figure S5:** Alignment of DAT1 protein sequences from different plants and algae
11
12 573 **Figure S6:** Loss of AtDAT1 does not lead to apparent growth defects in adult plants
13
14 574 **Figure S7**: Seedling growth is not differentially suppressed by L-Met in *AtDAT1*
15
16 575 knock out-lines
17
18 576 **Table S1:** Primers used in this study
19
20
21 577 **Table S2**: Comparison of the transaminase activity of AtDAT1 with different amino
22 578 donors and acceptors
23
24
25 579 **Table S3**: Relative malonyl-methionine contents in seedlings grown with or without
26 580 addition of 10µM ACC
27
28
29 581
30
31 582 **References**
32

CONFIDENTIAL

33 583 **Aldag, R. and Young, J.** (1970) D-Amino Acids in Soils. I. Uptake and Metabolism by Seedling Maize
34 584 and Ryegrass 1. *Agronomy Journal*, **62**, 184-189.
35 585 **Amelung, W. and Zhang, X.** (2001) Determination of amino acid enantiomers in soils. *Soil Biology and*
36 586 *Biochemistry*, **33**, 553-562.
37 587 **Amelung, W., Zhang, X. and Flach, K.** (2006) Amino acids in grassland soils: climatic effects on
38 588 concentrations and chirality. *Geoderma*, **130**, 207-217.
39
40 589 **Balu, D.T. and Coyle, J.T.** (2015) The NMDA receptor 'glycine modulatory site' in schizophrenia: D-
41 590 serine, glycine, and beyond. *Curr Opin Pharmacol*, **20**, 109-115.
42 591 **Benichou, M., Martinez-Reina, G., Romojaro, F., Pech, J.C. and Latché, A.** (1995) Partial purification
43 592 and properties of a 36-kDa 1-aminocyclopropane-1-carboxylate N-malonyltransferase from
44 593 mung bean. *Physiologia Plantarum*, **94**, 629-634.
45 594 **Brodowski, S., Amelung, W., Lobe, I. and Du Preez, C.C.** (2005) Losses and biogeochemical cycling of
46 595 soil organic nitrogen with prolonged arable cropping in the South African Highveld—evidence
47 596 from D-and L-amino acids. *Biogeochemistry*, **71**, 17-42.
48 597 **Bruckner, H. and Westhauser, T.** (2003) Chromatographic determination of L- and D-amino acids in
49 598 plants. *Amino Acids*, **24**, 43-55.
50
51 599 **Brunhuber, N.M., Mort, J.L., Christoffersen, R.E. and Reich, N.O.** (2000) Steady-state kinetic
52 600 mechanism of recombinant avocado ACC oxidase: initial velocity and inhibitor studies.
53 601 *Biochemistry*, **39**, 10730-10738.
54 602 **Cava, F., Lam, H., De Pedro, M.A. and Waldor, M.K.** (2011) Emerging knowledge of regulatory roles
55 603 of D-amino acids in bacteria. *Cellular and Molecular Life Sciences*, **68**, 817-831.
56 604 **Charng, Y.-y., Chou, S.-J., Jiaang, W.-T., Chen, S.-T. and Yang, S.F.** (2001) The catalytic mechanism of
57 605 1-aminocyclopropane-1-carboxylic acid oxidase. *Archives of biochemistry and biophysics*,
58
59 606 **385**, 179-185.
60 607 **Chen, C., MacCready, J.S., Ducat, D.C. and Osteryoung, K.W.** (2018) The molecular machinery of
608 chloroplast division. *Plant physiology*, **176**, 138-151.

1
2
3 609 **Clough, S.J. and Bent, A.F.** (1998) Floral dip: a simplified method for Agrobacterium-mediated
4 610 transformation of Arabidopsis thaliana. *The plant journal*, **16**, 735-743.
5 611 **Curtis, M.D. and Grossniklaus, U.** (2003) A gateway cloning vector set for high-throughput functional
6 612 analysis of genes in planta. *Plant physiology*, **133**, 462-469.
7 613 **D'aniello, A.** (2007) D-Aspartic acid: an endogenous amino acid with an important neuroendocrine
8 614 role. *Brain research reviews*, **53**, 215-234.
9 615 **Dworkin, J.** (2014) The medium is the message: interspecies and interkingdom signaling by
10 616 peptidoglycan and related bacterial glycans. *Annual review of microbiology*, **68**, 137-154.
11 617 **Edwards, K., Johnstone, C. and Thompson, C.** (1991) A simple and rapid method for the preparation
12 618 of plant genomic DNA for PCR analysis. *Nucleic acids research*, **19**, 1349.
13 619 **Erikson, O., Hertzberg, M. and Nasholm, T.** (2004) A conditional marker gene allowing both positive
14 620 and negative selection in plants. *Nat Biotechnol*, **22**, 455-458.
15 621 **Erikson, O., Hertzberg, M. and Näsholm, T.** (2005) The dsdA gene from Escherichia coli provides a
16 622 novel selectable marker for plant transformation. *Plant molecular biology*, **57**, 425-433.
17 623 **Felix, G., Grosskopf, D.G., Regenass, M., Basse, C.W. and Boller, T.** (1991) Elicitor-induced ethylene
18 624 biosynthesis in tomato cells: characterization and use as a bioassay for elicitor action. *Plant*
19 625 *Physiology*, **97**, 19-25.
20 626 **Forde, B.G. and Roberts, M.R.** (2014) Glutamate receptor-like channels in plants: a role as amino
21 627 acid sensors in plant defence? *F1000prime reports*, **6**.
22 628 **Forsum, O., Svennerstam, H., Ganeteg, U. and Näsholm, T.** (2008) Capacities and constraints of
23 629 amino acid utilization in Arabidopsis. *New Phytologist*, **179**, 1058-1069.
24 630 **Frahn, J.L. and Illman, R.J.** (1975) The occurrence of D-alanine and D-alanyl-D-alanine in Phalaris
25 631 tuberosa. *Phytochemistry (UK)*.
26 632 **Fuchs, S.A., Berger, R., Klomp, L.W. and de Koning, T.J.** (2005) D-amino acids in the central nervous
27 633 system in health and disease. *Molecular genetics and metabolism*, **85**, 168-180.
28 634 **Fujii, N.** (2002) D-amino acids in living higher organisms. *Origins of Life and Evolution of the*
29 635 *Biosphere*, **32**, 103-127.
30 636 **Fujii, N., Kaji, Y. and Fujii, N.** (2011) d-Amino acids in aged proteins: analysis and biological relevance.
31 637 *Journal of Chromatography B*, **879**, 3141-3147.
32 638 **Fukuda, M., Tokumura, A., Ogawa, T. and Sasaoka, K.** (1973) D-alanine in germinating Pisum
33 639 sativum seedlings. *Phytochemistry*, **12**, 2593-2595.
34 640 **Funakoshi, M., Sekine, M., Katane, M., Furuchi, T., Yohda, M., Yoshikawa, T. and Homma, H.** (2008)
35 641 Cloning and functional characterization of Arabidopsis thaliana D-amino acid
36 642 aminotransferase--D-aspartate behavior during germination. *FEBS J*, **275**, 1188-1200.
37 643 **Gholizadeh, A. and Kohnehrouz, B.** (2009) Molecular cloning and expression in Escherichia coli of an
38 644 active fused Zea mays L. D-amino acid oxidase. *Biochemistry (Moscow)*, **74**, 137-144.
39 645 **Gisby, M.F., Mudd, E.A. and Day, A.** (2012) Growth of transplastomic cells expressing D-amino acid
40 646 oxidase in chloroplasts is tolerant to D-alanine and inhibited by D-valine. *Plant physiology*,
41 647 pp. 112.204107.
42 648 **Gordes, D., Koch, G., Thurow, K. and Kolukisaoglu, U.** (2013) Analyses of Arabidopsis ecotypes reveal
43 649 metabolic diversity to convert D-amino acids. *Springerplus*, **2**, 559.
44 650 **Gordes, D., Kolukisaoglu, U. and Thurow, K.** (2011) Uptake and conversion of D-amino acids in
45 651 Arabidopsis thaliana. *Amino Acids*, **40**, 553-563.
46 652 **Grefen, C., Donald, N., Hashimoto, K., Kudla, J., Schumacher, K. and Blatt, M.R.** (2010) A
47 653 ubiquitin-10 promoter-based vector set for fluorescent protein tagging facilitates temporal
48 654 stability and native protein distribution in transient and stable expression studies. *The Plant*
49 655 *Journal*, **64**, 355-365.
50 656 **Hamase, K.** (2007) Sensitive two-dimensional determination of small amounts of D-amino acids in
51 657 mammals and the study on their functions. *Chemical and pharmaceutical bulletin*, **55**, 503-
52 658 510.
53 659 **Hamase, K., Morikawa, A. and Zaitsu, K.** (2002) D-Amino acids in mammals and their diagnostic
54 660 value. *Journal of chromatography B*, **781**, 73-91.
55
56
57
58
59
60

CONFIDENTIAL

1
2
3 661 **Hill, P.W., Quilliam, R.S., DeLuca, T.H., Farrar, J., Farrell, M., Roberts, P., Newsham, K.K., Hopkins,**
4 662 **D.W., Bardgett, R.D. and Jones, D.L.** (2011) Acquisition and assimilation of nitrogen as
5 663 peptide-bound and D-enantiomers of amino acids by wheat. *PLoS One*, **6**, e19220.
6 664 **Hirano, T., Tanidokoro, K., Shimizu, Y., Kawarabayasi, Y., Ohshima, T., Sato, M., Tadano, S.,**
7 665 **Ishikawa, H., Takio, S., Takechi, K. and Takano, H.** (2016) Moss Chloroplasts Are Surrounded
8 666 by a Peptidoglycan Wall Containing D-Amino Acids. *Plant Cell*, **28**, 1521-1532.
9 667 **Hofstee, B.** (1959) Non-inverted versus inverted plots in enzyme kinetics. *Nature*, **184**, 1296.
10 668 **Katane, M. and Homma, H.** (2011) d-Aspartate—an important bioactive substance in mammals: a
11 669 review from an analytical and biological point of view. *Journal of Chromatography B*, **879**,
12 670 3108-3121.
13 671 **Kionka, C. and Amrhein, N.** (1984) The enzymatic malonylation of 1-aminocyclopropane-1-carboxylic
14 672 acid in homogenates of mung-bean hypocotyls. *Planta*, **162**, 226-235.
15 673 **Kolodkin-Gal, I., Romero, D., Cao, S., Clardy, J., Kolter, R. and Losick, R.** (2010) D-amino acids trigger
16 674 biofilm disassembly. *Science*, **328**, 627-629.
17 675 **Kolukisaoglu, Ü. and Suarez, J.** (2017) D-Amino Acids in Plants: New Insights and Aspects, but also
18 676 More Open Questions. In *Amino Acid-New Insights and Roles in Plant and Animal*: InTech.
19 677 **Lempe, J., Balasubramanian, S., Sureshkumar, S., Singh, A., Schmid, M. and Weigel, D.** (2005)
20 678 Diversity of flowering responses in wild Arabidopsis thaliana strains. *PLoS genetics*, **1**, e6.
21 679 **Ling-Yuan, S., Yu, L. and Shang, F.Y.** (1985) Relationship between 1-aminocyclopropanecarboxylate
22 680 malonyltransferase and D-amino acid malonyltransferase. *Phytochemistry*, **24**, 1141-1145.
23 681 **Liu, Y., Hoffman, N.E. and Yang, S.F.** (1983) Relationship between the malonylation of 1-
24 682 aminocyclopropane-1-carboxylic acid and D-amino acids in mung-bean hypocotyls. *Planta*,
25 683 **158**, 437-441.
26 684 **Manabe, H.** (1985) Occurrence of D-alanyl-D-alanine in Oryza australiensis. *Agricultural and*
27 685 *Biological Chemistry*, **49**, 1203-1204.
28 686 **Manabe, H.** (1992) Formation of dipeptides containing D-alanine in wild rice plants. *Phytochemistry*,
29 687 **31**, 527-529.
30 688 **Martínez-Rodríguez, S., Martínez-Gómez, A.I., Rodríguez-Vico, F., Clemente-Jiménez, J.M. and Las**
31 689 **Heras-Vázquez, F.J.** (2010) Natural occurrence and industrial applications of D-amino acids:
32 690 an overview. *Chemistry & biodiversity*, **7**, 1531-1548.
33 691 **Michard, E., Lima, P.T., Borges, F., Silva, A.C., Portes, M.T., Carvalho, J.E., Gilliham, M., Liu, L.H.,**
34 692 **Obermeyer, G. and Feijo, J.A.** (2011) Glutamate receptor-like genes form Ca2+ channels in
35 693 pollen tubes and are regulated by pistil D-serine. *Science*, **332**, 434-437.
36 694 **Monselise, E.I., Levkovitz, A. and Kost, D.** (2015) Ultraviolet radiation induces stress in etiolated L
37 695 andoltia punctata, as evidenced by the presence of alanine, a universal stress signal: a 15 N
38 696 NMR study. *Plant Biology*, **17**, 101-107.
39 697 **Naranjo-Ortíz, M.A., Brock, M., Brunke, S., Hube, B., Marcet-Houben, M. and Gabaldón, T.** (2016)
40 698 Widespread inter-and intra-domain horizontal gene transfer of d-amino acid metabolism
41 699 enzymes in eukaryotes. *Frontiers in microbiology*, **7**, 2001.
42 700 **Nelson, B.K., Cai, X. and Nebenführ, A.** (2007) A multicolored set of in vivo organelle markers for
43 701 co-localization studies in Arabidopsis and other plants. *The Plant Journal*, **51**, 1126-1136.
44 702 **Nishimura, K., Tomoda, Y., Nakamoto, Y., Kawada, T., Ishii, Y. and Nagata, Y.** (2007) Alanine
45 703 racemase from the green alga Chlamydomonas reinhardtii. *Amino Acids*, **32**, 59-62.
46 704 **Noma, M., Noguchi, M. and Tamaki, E.** (1973) Isolation and characterization of D-alanyl-D-alanine
47 705 from tobacco leaves. *Agricultural and Biological Chemistry*, **37**, 2439-2439.
48 706 **Ogawa, T., Fukuda, M. and Sasaoka, K.** (1973) Occurrence of N-malonyl-D-alanine in pea seedlings.
49 707 *Biochimica et biophysica acta*, **297**, 60.
50 708 **Ollivaux, C., Soyez, D. and Toullec, J.Y.** (2014) Biogenesis of D-amino acid containing
51 709 peptides/proteins: where, when and how? *J Pept Sci*, **20**, 595-612.
52 710 **Ono, K., Yanagida, K., Oikawa, T., Ogawa, T. and Soda, K.** (2006) Alanine racemase of alfalfa
53 711 seedlings (Medicago sativa L.): first evidence for the presence of an amino acid racemase in
54 712 plants. *Phytochemistry*, **67**, 856-860.
55
56
57
58
59
60

CONFIDENTIAL

Radkov, A.D., McNeill, K., Uda, K. and Moe, L.A. (2016) D-Amino acid catabolism is common among soil-dwelling bacteria. *Microbes and environments*, **31**, 165-168.

S F Yang, a. and Hoffman, N.E. (1984) Ethylene Biosynthesis and its Regulation in Higher Plants. *Annual Review of Plant Physiology*, **35**, 155-189.

Satoh, S. and Esashi, Y. (1980) D-Amino-acid-stimulated ethylene production in seed tissues. *Planta*, **149**, 64-68.

Satoh, S. and Esashi, Y. (1982) Effects of α-aminoisobutyric acid and D-and L-amino acids on ethylene production and content of 1-aminocyclopropane-1-carboxylic acid in cotyledonary segments of cocklebur seeds. *Physiologia Plantarum*, **54**, 147-152.

Strauch, R.C., Svedin, E., Dilkes, B., Chapple, C. and Li, X. (2015) Discovery of a novel amino acid racemase through exploration of natural variation in Arabidopsis thaliana. *Proceedings of the National Academy of Sciences*, **112**, 11726-11731.

Typas, A., Banzhaf, M., Gross, C.A. and Vollmer, W. (2012) From the regulation of peptidoglycan synthesis to bacterial growth and morphology. *Nature Reviews Microbiology*, **10**, 123.

Valdovinos, J.G. and Muir, R.M. (1965) Effects of D and L amino acids on foliar abscission. *Plant physiology*, **40**, 335.

Vlamakis, H., Chai, Y., Beauregard, P., Losick, R. and Kolter, R. (2013) Sticking together: building a biofilm the Bacillus subtilis way. *Nature Reviews Microbiology*, **11**, 157.

Vranova, V., Zahradnickova, H., Janous, D., Skene, K.R., Matharu, A.S., Rejsek, K. and Formanek, P. (2012) The significance of D-amino acids in soil, fate and utilization by microbes and plants: review and identification of knowledge gaps. *Plant and soil*, **354**, 21-39.

Wu, Y.M., Silverstone, A.L., Liu, Y. and Yang, S.F. (1995) Partial-Purification and Characterization of Tryptophan N-Malonyltransferase from Tomato Leaves. *Phytochemistry*, **40**, 699-704.

Figure Legends

Figure 1: AtDAT1 as a candidate protein for D-AA metabolization in Arabidopsis (a) Scheme of the genomic structure of AtDAT1 (exons and introns in black and grey, respectively) with the positions of T-DNA insertions in *dat1-1* and *dat1-2* as well as the mutations found in Ler and M7323S. Arrows indicate primers used for genotyping the T-DNA insertions and RT-PCR (for primer sequences see Table S1) (b) RT-PCR analysis of AtDAT1 expression in Col-0, Ler, *dat1-1*, *dat1-2* and M7323S (top: *AtDAT1*; bottom: *AtACT2*) (c) Contents of D-Ala (white), D-Glu (grey) and L-Met (black) in seedlings of Col-0, Ler, *dat1-1* and *dat1-2* without (water) and with D-Met treatment for 16 h (D-Met); For each measurement four seedlings were pooled and further processed. Error bars represent the standard deviation from three independent measurements.

Figure 2: D-Met metabolization in lines overexpressing AtDAT1 and in F1 seedlings from crosses of Col-0, Ler and *dat1-2*

Contents of D-Ala, D-Glu and L-Met after overnight exposure to D-Met (a) in Ler and *dat1-2* overexpressing AtDAT1 (AtDAT1 Ox) and their corresponding background

SUBMITTED MANUSCRIPT

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

753 lines and (b) in F1 progeny seedlings of crosses of Col-0, Ler and *dat1-2* and their
754 corresponding parental lines; for further information see Fig. 1c

755 **Figure 3:** Relative D-Ala producing activity of AtDAT1 with different D-AAs as amino
756 group donor and pyruvate as acceptor.
757 Activity of reaction with D-Met was set to 100% and all other reactions were
758 calculated in relation to it. Each bar represents the mean of measurement of three
759 independent assays. Error bars (± SD).

760 **Figure 4:** Activities of AtDAT1 variants
761 Transaminase activities of $AtDAT1_{(Col-0)}$, $AtDAT1_{(Ler)}$, $AtDAT1_{(A77T)}$, and $AtDAT1_{(T303S)}$
762 with D-Met as amino group donor and (a) 2-oxoglutarate or (b) pyruvate as acceptor
763 molecule are displayed. Each bar represents the mean of measurement of three
764 independent assays. Error bars (± SD).

765 **Figure 5**: Seedling growth is differentially suppressed by D-Met in *AtDAT1* knock out-
766 lines
767 Seeds of Col-0, *dat1-1*, *dat1-2*, and Ler were germinated either (a) in permanent light
768 for 10 d or (b) in permanent darkness for 6 d on different solid growth media (without
769 supplementation, with 500µM D-Met or with 500µM L-Met supplemented).
770 Afterwards, hypocotyl growth of the dark grown plants was measured (c). Bars (Col-
771 0: black, *dat1-1*: checked, *dat1-2*: striped; n=30) represent the average of relative
772 difference compared to the control (without D-Met). The difference between Col-0
773 and the *dat1* mutants for each tested D-Met concentration was highly significant
774 ($p<0.001$). Error bars (± SD).

775 **Figure 6**: D-Met leads to increase of ethylene in AtDAT1 knock out-lines
776 Ethylene contents in seedlings of Col-0, *dat1-1*, *dat1-2* were measured after growth
777 (a) in permanent light or (b) in permanent darkness in vials with solid growth media
778 supplemented with 200µM and 500µM D-Met, and additionally without
779 supplementation (control). The bars (Col-0: black, *dat1-1*: checked, *dat1-2*: striped)
780 represent averages of three biological replicates. The asterisks indicate the
781 significance level of difference between *dat1* mutants and wild type at given D-Met
782 concentrations (* $p<0.05$; ** $p<0.01$; *** $p<0.001$). Error bars (± SD).

783 **Figure 7**: D-Met affects formation of malonyl-methionine and malonyl-ACC stronger
784 in *dat1* mutants
785 Malonyl-methionine contents in seedlings of Col-0 (black), *dat1-1* (checked), *dat1-2*

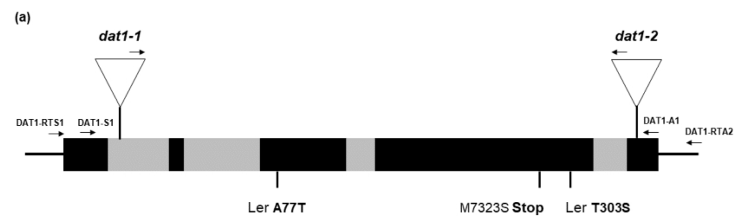
CONFIDENTIAL

(striped) were measured after growth (a) in permanent light or (b) in permanent darkness grown on agar plates supplemented with 200µM and 500µM D-Met, and additionally without supplementation (control). The relative values are given in percent with the values of Col-0 at 500µM D-Met set to 100%. Furthermore, the relative malonyl-ACC contents in Col-0 and *dat1-1* seedlings were measured after growth on the given D-Met concentrations plus 10 µM ACC (c). Seedlings were either grown in permanent light (Col-0: white, *dat1-1*: white-dotted) or darkness (Col-0: grey, *dat1-1*: grey-dotted). For further information, see Figure 6.

CONFIDENTIAL

SUBMITTED MANUSCRIPT

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24



25
26
27
28
29
30
31
32

Figure 1: AtDAT1 as a candidate protein for D-AA metabolization in Arabidopsis
(a) Scheme of the genomic structure of AtDAT1 (exons and introns in black and grey, respectively) with the positions of T-DNA insertions in dat1-1 and dat1-2 as well as the mutations found in Ler and M7323S. Arrows indicate primers used for genotyping the T-DNA insertions and RT-PCR (for primer sequences see Table S1) (b) RT-PCR analysis of AtDAT1 expression in Col-0, Ler, dat1-1, dat1-2 and M7323S (top: AtDAT1; bottom: AtACT2) (c) Contents of D-Ala (white), D-Glu (grey) and L-Met (black) in seedlings of Col-0, Ler, dat1-1 and dat1-2 without (water) and with D-Met treatment for 16 h (D-Met); For each measurement four seedlings were pooled and further processed. Error bars represent the standard deviation from three independent measurements.

33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

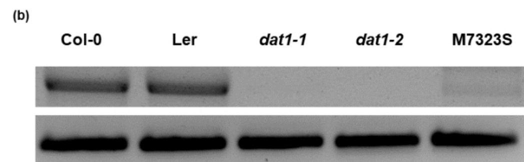
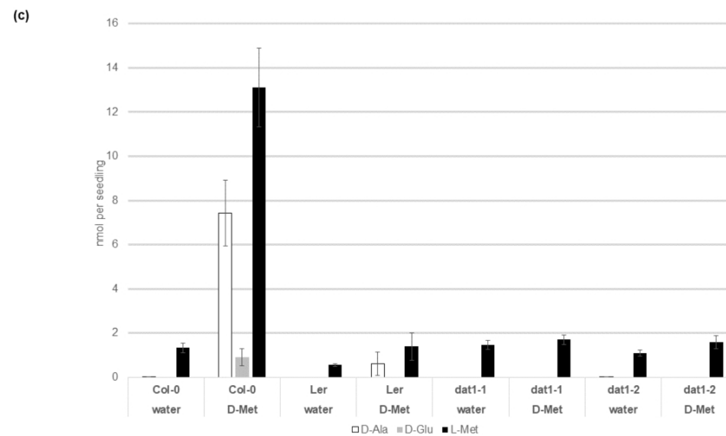


Figure 1: AtDAT1 as a candidate protein for D-AA metabolization in Arabidopsis
(a) Scheme of the genomic structure of AtDAT1 (exons and introns in black and grey, respectively) with the positions of T-DNA insertions in dat1-1 and dat1-2 as well as the mutations found in Ler and M7323S. Arrows indicate primers used for genotyping the T-DNA insertions and RT-PCR (for primer sequences see Table S1) (b) RT-PCR analysis of AtDAT1 expression in Col-0, Ler, dat1-1, dat1-2 and M7323S (top: AtDAT1; bottom: AtACT2) (c) Contents of D-Ala (white), D-Glu (grey) and L-Met (black) in seedlings of Col-0, Ler, dat1-1 and dat1-2 without (water) and with D-Met treatment for 16 h (D-Met); For each measurement four seedlings were pooled and further processed. Error bars represent the standard deviation from three independent measurements.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24



25 Figure 1: AtDAT1 as a candidate protein for D-AA metabolization in Arabidopsis
26 (a) Scheme of the genomic structure of AtDAT1 (exons and introns in black and grey, respectively) with the
27 positions of T-DNA insertions in dat1-1 and dat1-2 as well as the mutations found in Ler and M7323S.
28 Arrows indicate primers used for genotyping the T-DNA insertions and RT-PCR (for primer sequences see
29 Table S1) (b) RT-PCR analysis of AtDAT1 expression in Col-0, Ler, dat1-1, dat1-2 and M7323S (top:
30 AtDAT1; bottom: AtACT2) (c) Contents of D-Ala (white), D-Glu (grey) and L-Met (black) in seedlings of Col-
31 0, Ler, dat1-1 and dat1-2 without (water) and with D-Met treatment for 16 h (D-Met); For each
32 measurement four seedlings were pooled and further processed. Error bars represent the standard deviation
from three independent measurements.

33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

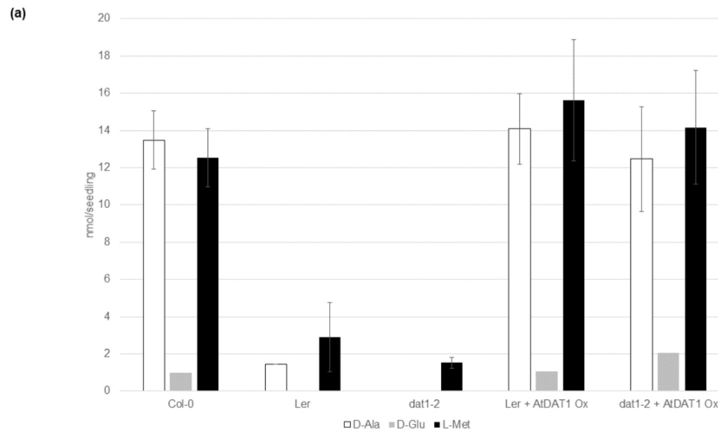
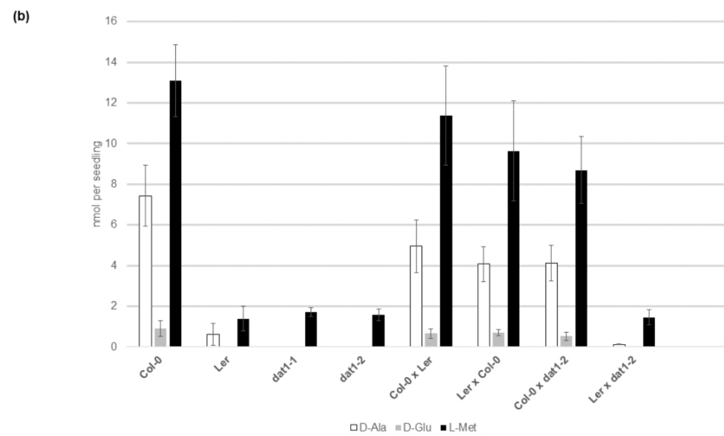


Figure 2: D-Met metabolization in lines overexpressing AtDAT1 and in F1 seedlings from crosses of Col-0, Ler and dat1-2
Contents of D-Ala, D-Glu and L-Met after overnight exposure to D-Met (a) in Ler and dat1-2 overexpressing AtDAT1 (AtDAT1 Ox) and their corresponding background lines and (b) in F1 progeny seedlings of crosses of Col-0, Ler and dat1-2 and their corresponding parental lines; for further information see Fig. 1c.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24



25 Figure 2: D-Met metabolization in lines overexpressing AtDAT1 and in F1 seedlings from crosses of Col-0,
26 Ler and dat1-2
27 Contents of D-Ala, D-Glu and L-Met after overnight exposure to D-Met (a) in Ler and dat1-2 overexpressing
28 AtDAT1 (AtDAT1 Ox) and their corresponding background lines and (b) in F1 progeny seedlings of crosses of
29 Col-0, Ler and dat1-2 and their corresponding parental lines; for further information see Fig. 1c.

30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

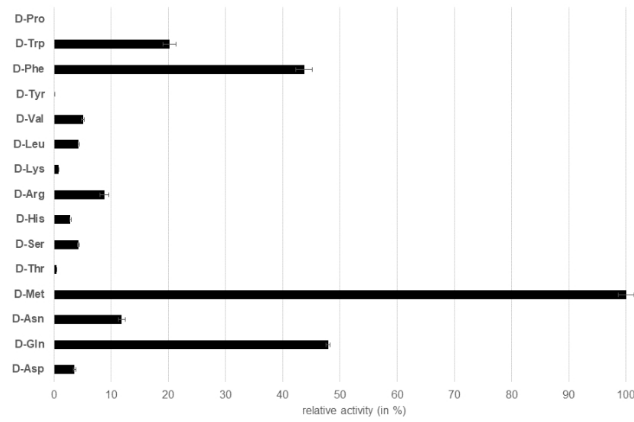


Figure 3: Relative D-Ala producing activity of AtDAT1 with different D-AAs as amino group donor and pyruvate as acceptor.
Activity of reaction with D-Met was set to 100% and all other reactions were calculated in relation to it. Each bar represents the mean of measurement of three independent assays. Error bars (± SD).

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Fig 4a Activities of AtDAT1 variants
Transaminase activities of AtDAT1(Col-0), AtDAT1(Ler), AtDAT1(A77T), and AtDAT1(T303S) with D-Met as amino group donor and (a) 2-oxoglutarate or (b) pyruvate as acceptor molecule are displayed. Each bar represents the mean of measurement of three independent assays. Error bars (± SD).

SUBMITTED MANUSCRIPT

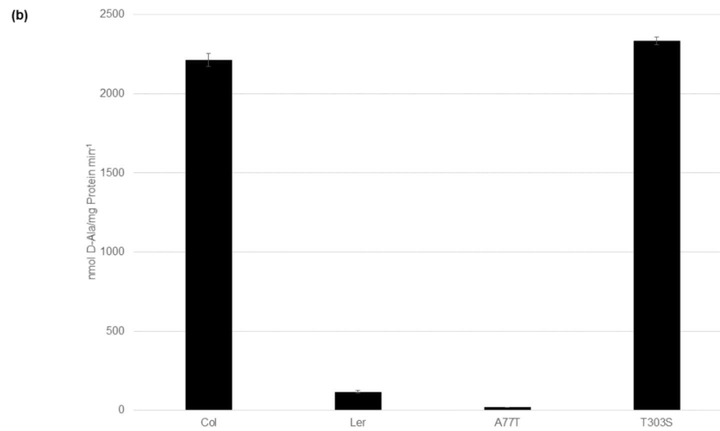
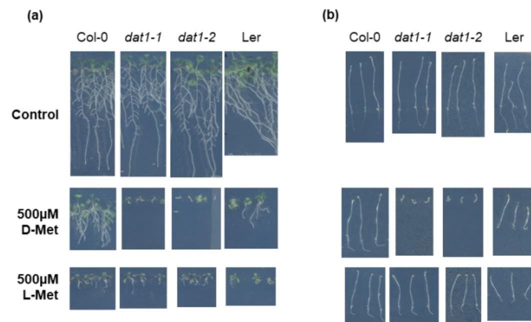


Fig 4b Activities of AtDAT1 variants
Transaminase activities of AtDAT1(Col-0), AtDAT1(Ler), AtDAT1(A77T), and AtDAT1(T303S) with D-Met as amino group donor and (a) 2-oxoglutarate or (b) pyruvate as acceptor molecule are displayed. Each bar represents the mean of measurement of three independent assays. Error bars (± SD).

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24



25 Figure 5: Seedling growth is differentially suppressed by D-Met in AtDAT1 knock out-lines
26 Seeds of Col-0, dat1-1, dat1-2, and Ler were germinated either (a) in permanent light for 10 d or (b) in
27 permanent darkness for 6 d on different solid growth media (without supplementation, with 500µM D-Met or
28 with 500µM L-Met supplemented). Afterwards, hypocotyl growth of the dark grown plants was measured (c).
29 Bars (Col-0: black, dat1-1: checked, dat1-2: striped; n=30) represent the average of relative difference
30 compared to the control (without D-Met). The difference between Col-0 and the dat1 mutants for each
tested D-Met concentration was highly significant ($p<0.001$). Error bars (± SD).

31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

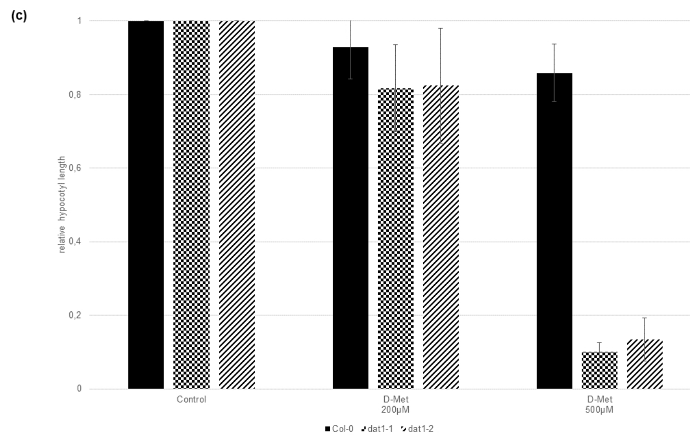


Figure 5: Seedling growth is differentially suppressed by D-Met in AtDAT1 knock out-lines
Seeds of Col-0, dat1-1, dat1-2, and Ler were germinated either (a) in permanent light for 10 d or (b) in permanent darkness for 6 d on different solid growth media (without supplementation, with 500µM D-Met or with 500µM L-Met supplemented). Afterwards, hypocotyl growth of the dark grown plants was measured (c). Bars (Col-0: black, dat1-1: checked, dat1-2: striped; n=30) represent the average of relative difference compared to the control (without D-Met). The difference between Col-0 and the dat1 mutants for each tested D-Met concentration was highly significant ($p<0.001$). Error bars (± SD).

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

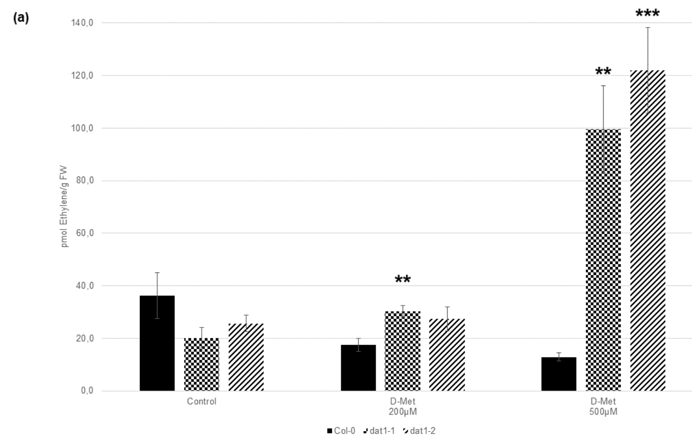
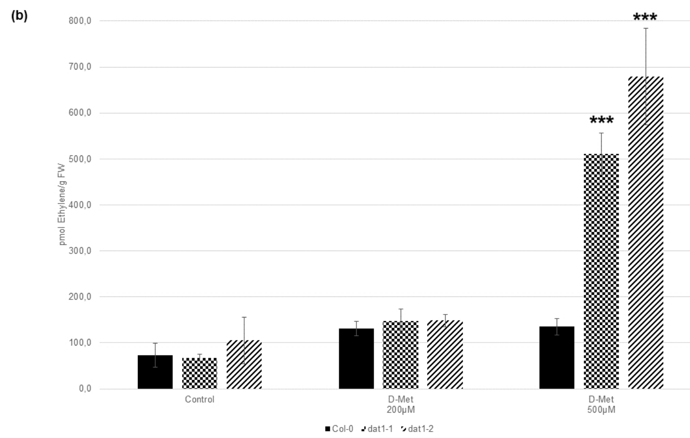


Figure 6: D-Met leads to increase of ethylene in AtDAT1 knock out-lines
Ethylene contents in seedlings of Col-0, dat1-1, dat1-2 were measured after growth (a) in permanent light or (b) in permanent darkness in vials with solid growth media supplemented with 200µM and 500µM D-Met, and additionally without supplementation (control). The bars (Col-0: black, dat1-1: checked, dat1-2: striped) represent averages of three biological replicates. The asterisks indicate the significance level of difference between dat1 mutants and wild type at given D-Met concentrations (* $p<0.05$; ** $p<0.01$; *** $p<0.001$). Error bars (± SD).

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24



25 Figure 6: D-Met leads to increase of ethylene in AtDAT1 knock out-lines
26 Ethylene contents in seedlings of Col-0, dat1-1, dat1-2 were measured after growth (a) in permanent light
27 or (b) in permanent darkness in vials with solid growth media supplemented with 200µM and 500µM D-Met,
28 and additionally without supplementation (control). The bars (Col-0: black, dat1-1: checked, dat1-2:
29 striped) represent averages of three biological replicates. The asterisks indicate the significance level of
30 difference between dat1 mutants and wild type at given D-Met concentrations (* $p<0.05$; ** $p<0.01$; *** $p<0.001$). Error bars (± SD).
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

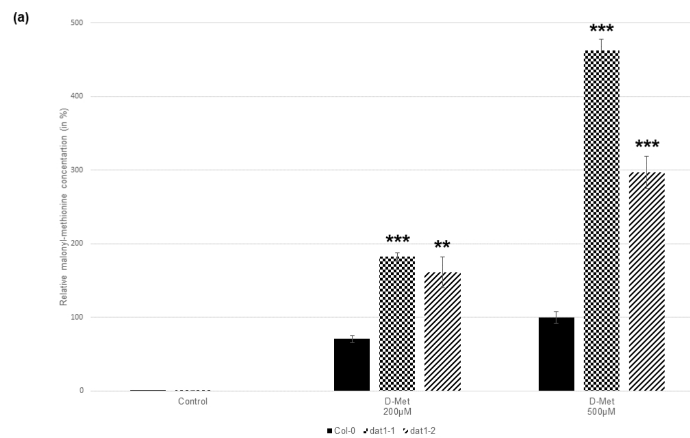


Figure 7: D-Met affects formation of malonyl-methionine and malonyl-ACC stronger in dat1 mutants
Malonyl-methionine contents in seedlings of Col-0 (black), dat1-1 (checked), dat1-2 (striped) were measured after growth (a) in permanent light or (b) in permanent darkness grown on agar plates supplemented with 200µM and 500µM D-Met, and additionally without supplementation (control). The relative values are given in percent with the values of Col-0 at 500µM D-Met set to 100%. Furthermore, the relative malonyl-ACC contents in Col-0 and dat1-1 seedlings were measured after growth on the given D-Met concentrations plus 10 µM ACC (c). Seedlings were either grown in permanent light (Col-0: white, dat1-1: white-dotted) or darkness (Col-0: grey, dat1-1: grey-dotted). For further information, see Figure 6.

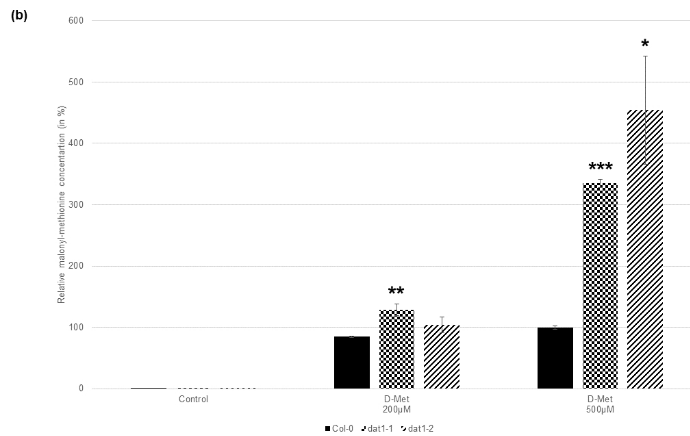
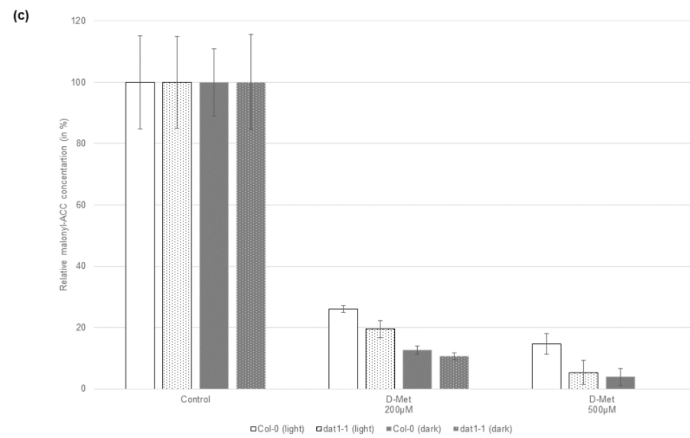


Figure 7: D-Met affects formation of malonyl-methionine and malonyl-ACC stronger in dat1 mutants Malonyl-methionine contents in seedlings of Col-0 (black), dat1-1 (checked), dat1-2 (striped) were measured after growth (a) in permanent light or (b) in permanent darkness grown on agar plates supplemented with 200µM and 500µM D-Met, and additionally without supplementation (control). The relative values are given in percent with the values of Col-0 at 500µM D-Met set to 100%. Furthermore, the relative malonyl-ACC contents in Col-0 and dat1-1 seedlings were measured after growth on the given D-Met concentrations plus 10 µM ACC (c). Seedlings were either grown in permanent light (Col-0: white, dat1-1: white-dotted) or darkness (Col-0: grey, dat1-1: grey-dotted). For further information, see Figure 6.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24



25 Figure 7: D-Met affects formation of malonyl-methionine and malonyl-ACC stronger in dat1 mutants
26 Malonyl-methionine contents in seedlings of Col-0 (black), dat1-1 (checked), dat1-2 (striped) were
27 measured after growth (a) in permanent light or (b) in permanent darkness grown on agar plates
28 supplemented with 200µM and 500µM D-Met, and additionally without supplementation (control). The
29 relative values are given in percent with the values of Col-0 at 500µM D-Met set to 100%. Furthermore, the
30 relative malonyl-ACC contents in Col-0 and dat1-1 seedlings were measured after growth on the given D-
31 Met concentrations plus 10 µM ACC (c). Seedlings were either grown in permanent light (Col-0: white, dat1-
1: white-dotted) or darkness (Col-0: grey, dat1-1: grey-dotted). For further information, see Figure 6.

32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

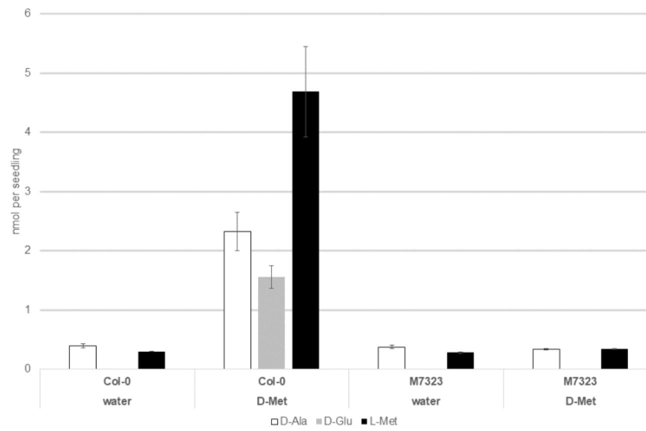


Figure S1: D-Met metabolization in M7323S and Col-0
Contents of D-Ala (black), D-Glu (grey) and L-Met in seedlings of Col-0 and M7323S without (water) and with D-Met treatment (D-Met) for 16 h; for further information see Figure 1c.

5.2 Supplementary Materials:AtDAT1 Plays a Regulatory Role in D-Amino Acid Stimulated Ethylene Production in *Arabidopsis thaliana*

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

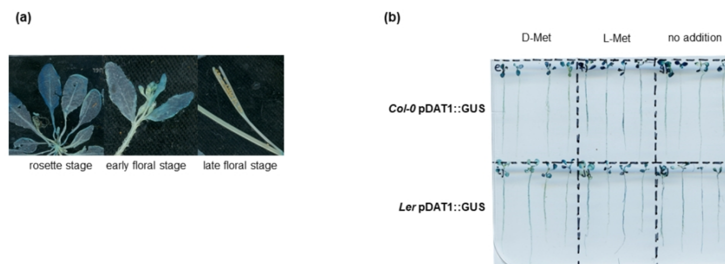


Figure S2: Expression patterns of AtDAT1
(a) GUS staining of rosette leaves (left), early (middle) and late flower stages (right) expressing the ß-Glucuronidase under the control of the AtDAT1 promotor from Col-0
(b) GUS staining of seedlings expressing the ß-Glucuronidase either under the control of the AtDAT1 promotor from Col-0 (top) and Ler (bottom) after addition of 1 mM D-or L-Met and without (no addition)

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

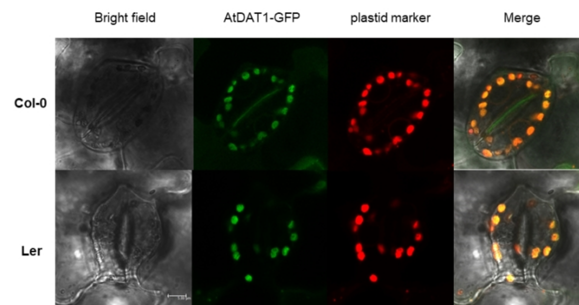
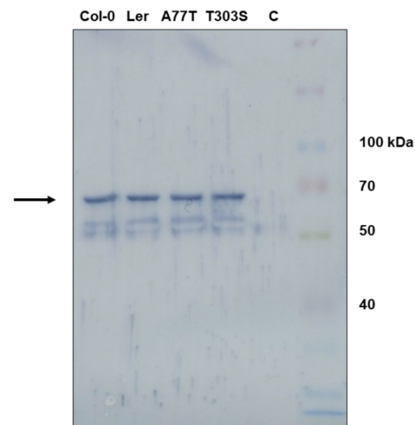


Figure S3: AtDAT1 from Col-0 and Ler localize both in plastids
Localization of AtDAT1 from Col-0 (top) and Ler (bottom) in stomata of N. benthamiana leaves. Left: AtDAT1-GFP; middle: pt-rk-CD3-999 (plastid marker); right: image merge

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24



25 Figure S4: Western blot of the purified AtDAT1 variants
26 In this figure a western blot is shown of purified His tagged protein from BL21 with pGEX-2TM(Col-0) (lane
27 1), pGEX-2TM(Ler) (lane 2), pGEX-2TM(A77T) (lane 3), pGEX-2TM(T303S) (lane 4), empty vector (C, lane
28 5) and the protein size standard (Spectra Multicolor Broad Range Protein Ladder; Thermo Fisher Scientific,
29 Karlsruhe, Germany). The arrow points at the purified protein at the calculated size. The molecular mass of
30 selected bands of the protein standard are given at the right side.

31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

```
AtDAT1  -------------------------------------MAGLSLEFT-VNTWNLRSLSQVPCP-LRHGFRFPRRLTRRRTI
BnDAT1  ----------------MTSRFVSFPG----------PIAGFLPELSSVNTCNLHFLSRAPCSSGRHGFRLPR--TRGRT-
BnDAT2  ----------------MTSRFVSFPE----------PIVGFLPELSSFNTWNLHFVSRAPCSSGRHGFRLPR--TRGRT-
OsDAT1  ----------------MMASLSTPPA---TAG--VSPSPR--PSLLAYKKAAGLTPSPWCG------WRRAAVATAATSS
HvDAT1  -----------------MAHLSAPPA---AAGHRVLPSHR--RRLALKNIAPSSGGAVATGG---LPFWRAAVSKAAASS
ZmDAT1  MRVDHRSFPLRPPSQNPMAHLPSSPAAAVPTGHRVSPSPHSCRSLPQLKTVLLS-IGAAAGP-PRRSWRGMESVAAAPTG
ZmDAT2  MRVDHRSFPLRPPSQNPMAHLPSSPAAAVPTGHRVSPSPHSCRSLPQLKTVLLS-IGAAAGP-PRRSWRGMESVAAAPTG
SbDAT1  -----------------MAHLPASPAAAVSTGHRVSPSPRSCPSLPQLKTTVLSSTGAAAGPRPSRPWRGLAATGSDKAG
PsDAT1  ---------------MRSTAVSPQ---------------------------------------------SVQTTETAQVN
SmDAT1  --------------------------------------------------------------------------------
OsDAT2  --------------------------------------------------------------------------------
SbDAT2  --------------------------------------------------------------------------------
ZmDAT3  --------------------------------------------------------------------------------
ZmDAT4  --------------------------------------------------------------------------------
ZmDAT5  --------------------------------------------------------------------------------
HvDAT2  --------------------------------------------------------------------------------
TaDAT1  --------------------------------------------------------------------------------
OsDAT3  --------------------------------------------------------------------------------
PpDAT1  -------MAMAINLHHTHARGLAELQRSSSPDLTRVLQQRATPGCSPHSLRKISTSIPSKNFSPRFRDLQIVPLCHALTT
PpDAT2  -------------------------------------------------MALSSSSPTATPPPPPPHDGHSISLPPRPDG
PpDAT3  -------------------------------------------------MAFLDAT-----------QCDAVDFPPGLNR
VcDAT1  ---------------------------MEAASTSSRCQGNGPVSRVGQRGWRMPPARVLRLRSQA--LVEVGKLNHEPVP
CrDAT1  ---------------------------MEAS-CSSHCQG-AATSRLGQRGWRMPPARVLRLRMQASMPVEIGKLEKQPIP
        1.......10........20........30........40........50........60........70........80

AtDAT1  ----LMCSDSSSQSWNVPVLSS-YEVGERLKLARG------GQQFLAMYSSVVDGITTDPAAMVLPLDDHMVHRGHGVFD
BnDAT1  ----LMCSGSSSHSWNVPVLSS-NEVVERLKLVGK------GKQFLAMYSSVVGGITTDPAAMVLPLDDHMVHRGHGVFD
BnDAT2  ----LMCSGSSSHSWNVPVLSS-NEVVERLKLVGE------GKQFLAMYSSVVGGITTDPAAMVLPLDDHMVHRGHGVFD
OsDAT1  -NRTAAPAETIVTGNDVPLLSF-AEVAERLDEFHAS--GTRNQNYMAMYSSIFGGITTNPSAMVIPIDDHMVHRGHGVFD
HvDAT1  -SR-AALAGTIVNPIDVPILSF-SEVAERLDAFQES--GARSQSYVAMYSSIFGGITTNPSAMVIPIDDHMVHRGHGVFD
ZmDAT1  -SDNAAATGTIAHANEVPVLSF-SEVAERLDTFHAS--GARNQNYMAMYSSIFGGITTNPSAMVIPIDDHMVHRGHGVFD
ZmDAT2  -SDN-AATGTIAHANEVPVLSF-SEVAERLDTFHAS--GARNQNYMAMYSSIFGGITTNPSAMVIPIDDHMVHRGHGVFD
SbDAT1  YKEGESSTGTIANANEVPVLSF-SEVAERLDTFHAS--GARNQNYMAMYSSIFGGITTGPSAMVIPIDDHMVHRGHGVFD
PsDAT1  EFRSQELTMQKRDEADVPVLSV-SEVIDKLRERFG---GCSKQPYPAMYSSIFGGITTDPTMMVIPIDDHMVHRGHGVFD
SmDAT1  -----------------------------------------------MYSSLIDGITTDPAAMVIPMDDHMVHRGHGVFD
OsDAT2  --------MQGEHHDHVPVYESGTEVFQKLQEKWNS---TKHKRYRAMYSSVVGGIILDPSMMVIPIDDHMVHRGHGVFD
SbDAT2  --------MQGEDR-VVPVYASGTQVLQKLQEKWES---TKQG-YPAMYSSVVGGIILDPSMMVIPIDDHMVHRGHGVFD
ZmDAT3  ----MGSYIQGDDE--VPVYESGAEVLAKVQEKWAAAAASRPPPYPAMYSSVFGGIILDPAMMVLPIDDHMVHRGHGVFD
ZmDAT4  -----------------------------------------------MYSSVFGGIILDPAMMVLPIDDHMVHRGHGVFD
ZmDAT5  ---------------------------------MGGGGGIAASSLPGHVLERVRRHHPGPGHDVLPIDDHMVHRGHGVFD
HvDAT2  ----MGSYVQGEDQ--VPVYESGADALQKLQEKWKS----TAAPYPAMYSSFLGGIVLDPAMMALPIDDHMVHRGHGVFD
TaDAT1  ----MGSYVQGEDE--VPVYESGAHALQKLQEKWKS----TAAPYPAMYSSFLGGIILDPAMMALPIDDHMVHRGHGVFD
OsDAT3  ----MGSYVQGEDE--VPVYESGAEIVQKLQEKWKS----TAAPFPAMYSSVLGGIILDPAMMSVPLDDHMVHRGHGVFD
PpDAT1  DVGTVEISNDHITDDSIPVLTF-LEVTERMQSLATT----RPTTFRAMYSSVIGGITTDTAAMVIPIDDHMVHRGHGVFD
PpDAT2  VADEAGGLAIGSAKVTIPVLGL-TEIISRLQKEASA---AKFKNFRSMYSSIVGAITTDVAAMVIPLDDHMVHRGHSVFD
PpDAT3  ACEEAEVPINGSSNVNIPVLGL-AEIILRLQVEASQ---AKYKNFRSMYSSVVGAITTDVAAMVVPLDDHMVHRGHSVFD
VcDAT1  PPKGFRPAVVPGSRTKPTPLLTPDVMVQRLRSSMHDY---GQENFGAFYSSIMGGIVVDPALMMLPVDDQFVCKGYGVSE
CrDAT1  VPKDYKPSVVPGSRTRPTPRLDAVQMIDRLQRTLPVY---VREQFGSFYSSHMGGIVTDPALMMLPPDDQLVCKGHGVSE
        ........90.......100.......110.......120.......130.......140.......150.......160

AtDAT1  TALIINGYLYELDQHLDRILRSASMAKIPL-PFDRETIKRILIQTVSVSGCRDGSLRYWLSAGPGDFLLSPSQCLKPTLY
BnDAT1  TAMVIKGYLYELDQHLDRILRSASMAKIPL-PFDRETIRSILIQTVSVSGCRYGSLRYWLSAGPGDFSLSPSQCPKPSLY
BnDAT2  TAMIVNGHLYELDQHLDRIIRSASMAKIPL-PFDRETIRRILIQTVSVSGCRYGSLRYWLSAGPGDFSLSPSQCPKPSLY
OsDAT1  TAAIMNGHLYELEQHLDRFLKSASMAKITL-PFDRSTIRSILIQTVSASKCTQGSLRYWLSVGPGDFQLSSAGCANSALY
HvDAT1  TAAIMDGHLYELEQHIDRFLNSAQMAKIPL-PFDRSKIRSVLIQTVCASKCSQGSLRYWLSVGPGDFQLSSSGCRNPALY
ZmDAT1  TAAIMDGHLYELEQHLDRFLKSASMAKIPL-PFNRSTIRSILIQTVSASNCTQGSLRYWLSAGPGDFQLSSSGCTNPALY
ZmDAT2  TAAIMDGHLYELEQHLDRFLKSASMAKIPL-PFNRSTIRSILIQTVSASNCTQGSLRYWLSAGPGDFQLSSSGCTNPALY
SbDAT1  TAAIMDGHLYELEQHLDRFLRSALMAKIPL-PFDRSTIRSILIQTVSASNCTQGSLRYWLSVGPGDFQLSSSGCANPALY
PsDAT1  TAMIIDGYLYELDSHLDRFLRSASKAKVIP-PFDRSSVRSILLQTVAASQCRKGSLRYWLSAGPGDFLLSPAGCPNSAFY
SmDAT1  TATIADGYLYELDAHLDRFHKSAAAAKIQP-PFDRATMREILIQTVASSGCKLGSLRYWLSAGPGGFGLSSSECTMSTLY
OsDAT2  TAMLSDGYLYELDSHLDRLLLSASKAKISS-PFSRETLRAILVQMTAASKCRNGSIKYWLSAGPGDFLLSPKGCTAPAFY
SbDAT2  TATISDGYLYELDSHLDRLLVSASKAKIDP-PFPRETLRNILLQMTAASGCKNGSIKYWLSAGPGDFLVSPKGCTGSAFY
ZmDAT3  TAMILDGALYELDAHLDRFLRSAAAARVGTAPFPREALRRILIQMTAASGCRMGSIRYWLSSGPGDFLLSSRGCPSPAFY
ZmDAT4  TAMILDGALYELDAHLDRFLRSAAAARVGTAPFPREALRRILIQMTAASGCRMGSIRYWLSSGPGDFLLSSRGCPSPAFY
ZmDAT5  TAMILDGALYELDAHLDRFLRSAAAARVGTAPFPREALRRILIQMTAASGCRMGSIRYWLSSGPGDFLLSSRGCPSPAFY
HvDAT2  TAMLLDGHLYELDAHLDRFLRSAAQAKVGT-PFPRDTLRSILVQMTAASGCRKGSIRYWLSSGPGDFLLSSSGCPGPAFY
TaDAT1  TAMLLDGHLYELDAHLDRFLRSAAQAKVGT-PFPRDTLRSILVQMTAASGCRKGSIRYWLSSGPGDFLLSSSGCPGPAFY
OsDAT3  TAMILDGHLYELDPHLDRFLRSAAKARIGT-PFPRDTLRSILVQMTAASNCRRGSIRYWLSAGGGDFLLSSAGCAGPAFY
PpDAT1  TAIIYNGYLYELDDHLDRILRSAEKAKIQS-PFDRATLRDILVQTVAASGCRRGALRYWLSAGLGGFALSSKECFKSTFY
PpDAT2  TSILVNGYLYELDAHLDRFLSSATKAKITP-PFDRATIREILLQTVSAGKCQHGILRFWMSVGRGNFELSAKNCLESSLF
PpDAT3  TTTLVNGNLYELDTHLDRFLDSAAKAKILP-PFNRAMIREILMQTVAAGSCKDGTLRFWLSAGRGNFELSTKNC-EASLY
VcDAT1  TVVLRDGHLYMLDEHIARLTAACAQVGLSL-PFSVPAVKRIVLDTAAASGKLNGQLRFWVTPGRGGFSPVELGGSEPALY
CrDAT1  VVVLREGHIYLLDRHIQRLKESCEQVGIAL-PFEEASLKRILLDVAAASGRVNGVVRFWATPGRGGFSTVETGGAEPAFY
        .......170.......180.......190.......200.......210.......220.......230.......240
```

Figure S5: Alignment of DAT1 protein sequences from different plants and algae Protein sequences were taken from the Phytozome 12 database (https://phytozome.jgi.doe.gov/pz/portal.html#) with AtDAT1 as search sequence.

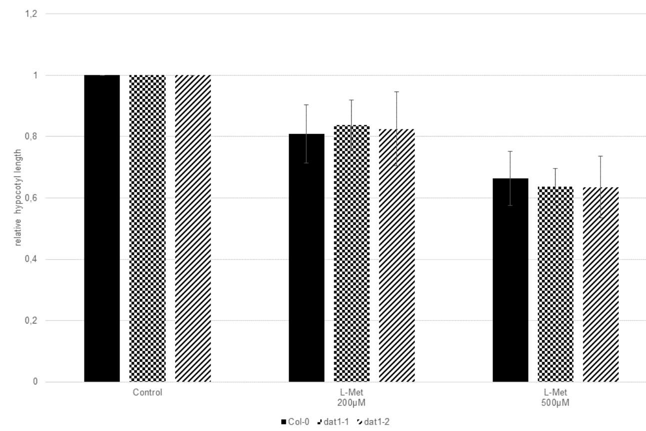
The alignment was constructed with ClustalX 2.1 (Larkin et al. 2007). AtDAT1 in the first line of the alignment is marked by a blue box. The chloroplastic transit peptide cleavage site (arrow) was determined according to the respective entry in the Plant Proteome Database (PPDB; http://ppdb.tc.cornell.edu/). Red triangles mark the sites of amino acid exchanges A77T and T303S between AtDAT1 from Col-0 and Ler. The blue star denotes the site of nonsense mutation in M7323S.

CONFIDENTIAL

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Figure S6: Loss of AtDAT1 does not lead to apparent growth defects in adult plants
Plants of dat1-1 (left), dat1-2 (middle) and Col-0 (right) were grown for 28 d under greenhouse conditions.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24



25 Fig s7 Seedling growth is not differentially suppressed by L-Met in AtDAT1 knock out-lines
26 For this experiment seedlings were grown as given in Figure 5 with the difference that instead of D-Met
27 growth media was supplemented with L-Met. None of the measurements revealed a significant difference
28 between dat1 mutants and Col-0 at all tested L-Met concentrations. For further information see Figure 5.

29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Table S1 Primers used in this study

Primer name	Gene	Primer sequence	Purpose
DAT1-S1	At5g57850	5´-AGGTCTCTCTCTCAAGTTCCATGTC-3´	genotyping
DAT1-Start		5´-caccATGGCAGGTTTGTCGCTGGAGTTTACAG-3´	cloning cDNA
DAT1-A1		5´-GTAAGGAACAAGAACACGAACGGAAG-3´	genotyping, cloning cDNA
DAT1-RTS1		5´-CTCTATTCTTTTGAGTCCCAAACC-3´	RT-PCR
DAT1-RTA2		5´-AGACAGATACCTAAAAACCCCATG-3´	RT-PCR
ProDAT1-SGW		5´-CACCCTCTTCTTCCGCTGCCGATTCACAAGAC-3´	cloning DAT1-Promoter
ProDAT1-AGW		5´-CAAACCTGCCATGGGGTTTGGGACT-3´	cloning DAT1-Promoter
ACT2-F		5´-TCCAAGCTGTTCTCTCCTTG-3´	RT-PCR
ACT2-R		5´-GAGGGCTGGAACAAGACTTC-3´	RT-PCR
SALK-LB1		5´-AATCAGCTGTTGCCCGTCTCACTGGTGAA-3´	genotyping

CONFIDENTIAL

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

		Amino donor		
		D-Met	D-Trp	D-Ala
Amino acceptor	Pyruvate	2686.4 (±36.7)	543.0 (±29.2)	Not determined
	2-OG	16.3 (±0.8)	5.1 (±0.7)	17.1 (±2.7)

Table S2: Comparison of the transaminase activity of AtDAT1 with different amino donors and acceptors

The transaminase activity (V_{max}) of the *Col-0* variant of AtDAT1 was compared with D-Met, D-Trp and D-Ala as amino donor and 2-OG and pyruvate as acceptor molecules (in nmol product mg^{-1} Protein min^{-1}); Each value represents the average of three independent assays (standard deviations are given in parentheses).

CONFIDENTIAL

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Table S3: Relative malonyl-methionine contents (in %) in seedlings grown with or without addition of 10µM ACC
For these measurements seedlings of Col-0 and *dat1-1* were grown and analyzed as given in Figure 7.

	-ACC	*±SD*	*+ACC*	*±SD*
Col-0 (light), 0 mM D-Met	0,0	0,0	0,0	0,0
Col-0 (light), 200 mM D-Met	70,5	5,0	54,6	5,9
Col-0 (light), 500 mM D-Met	100,0	8,0	100,0	7,9
dat1-1 (light), 0 mM D-Met	0,1	0,0	0,0	0,0
dat1-1 (light), 200 mM D-Met	182,2	5,2	129,1	13,5
dat1-1 (light), 500 mM D-Met	463,1	15,3	392,0	15,0
Col-0 (dark), 0 mM D-Met	0,0	0,0	0,0	0,0
Col-0 (dark), 200 mM D-Met	84,5	0,7	83,2	5,8
Col-0 (dark), 500 mM D-Met	100,0	2,8	100,0	8,7
dat1-1 (dark), 0 mM D-Met	0,0	0,0	0,0	0,0
dat1-1 (dark), 200 mM D-Met	128,5	9,8	95,6	2,7
dat1-1 (dark), 500 mM D-Met	335,0	6,5	229,8	4,7

CONFIDENTIAL

Chapter 6

Results and Discussion

In this chapter I want to summarize and discuss the results of the investigations presented in Hener et al. (2018); Kolukisaoglu and Suarez (2017) and in (Suarez et al., submitted). For doing so, I will split this chapter in three main sections, each of them devoted to discuss one of the main findings of my work.

6.1 Transport of D-Amino Acids in *Arabidopsis thaliana*

The investigation of D-AA transport in plants is one of the prerequisites to understand their uptake, distribution and release to the plant environment. With this in mind, I presented investigations that covered three main aspects of this topic: First, I found indications that D-AAs are transported by known transporter proteins Kolukisaoglu and Suarez (2017). Second, I confirmed that D-AAs are exuded into the rhizosphere, but that this may not be the only way plants avoid accumulation of D-AAs to toxic levels Hener et al. (2018). Third, I developed the first protocols for the usage of fluorescent D-Ala in higher plants as a tool for intracellular D-AA transport analysis Kolukisaoglu and Suarez (2017). In the present section, I will present an extended discussion of these findings.

6.1.1 AAPs, LHT1, and MRPs are Putative D-Amino Acids Transporters

To elucidate whether L-AAs transporters may also be involved in the transport of D-AAs, *lht1* and *aap1* knock-out mutants were analysed. These mutants were treated with different D-AAs and seedling growth was compared to the corresponding wild type. In these experiments, *lht1* mutants were found to be more tolerant against D-Ala Kolukisaoglu and Suarez (2017).

This finding confirmed previous reports Svennerstam et al. (2007), where the resistance to D-Ala was used to identify *lht1* mutants. Recently, it was shown that LHT1 is responsible for the uptake of ACC and that *lht1* seedlings displayed reduced triple response when treated with exogenous ACC Shin et al. (2014). This is another indication that LHT1 is not confined

to the transport of neutral and acidic proteinogenic AAs Tegeder and Ward (2012).

Similar observations were made for the amino acid permease (AAP) transporter family. Initially, AAP1 was characterised as a broad range AA transporter Lee et al. (2007). Previous experiments of Fischer et al. (2002) demonstrated that AAP1 to AAP6 transporters are able to transport D-Ala. In that work, six transporter proteins showed different affinities towards D-Ala with AAP6 having the highest. In my experiments with *aap1* and *aap5*, the enhanced tolerance of *aap1* mutants against D-Met and D-Phe implies that this transporter is also involved in the uptake of other D-AAs different from D-Ala Kolukisaoglu and Suarez (2017). The reasons for the enhanced tolerance of *aap1* mutants against D-Met and D-Phe, as well as the enhanced tolerance of *lht1* towards D-Ala can be due to reasons other than the absence of AAP1 and LHT1. It is possible that secondary mutations that lead to the observed phenotype are harboured in this mutant line. Therefore, the usage of more alleles of T-DNA insertion mutants is necessary to confirm this findings. Additionally, the expression of this proteins in yeast and oocytes followed by D-AA feeding experiments can shed more light to the D-AA transport capacities of these proteins.

The interpretation of the decreased tolerance of *mrp5* mutants against D-Phe is more complicated to explain than in the cases described above. MRP5 belongs to the Multi-Drug Resistance-related proteins (MRP), part of the ATP Binding Cassette (ABC) transporter family Verrier et al. (2008). To my knowledge, no AA transport function has been reported for this protein family to date. Instead, it has been reported that in yeast and animals, MRPs are involved in the secretion and excretion of compounds such as organic ions, anions, heavy metals; alkaloids, and other drugs. In contrast, in plants they are involved in hypocotyl elongation Sidler et al. (1998), in the transport of glutathione conjugates, chlorophyll catabolites, glucuronidates, and inositol hexakisphosphate Gaedeke et al. (2001); Nagy et al. (2009).

As discussed in Hener et al. (2018), there are reports of aberrant L-AA exudation in MRP mutants, as *mrp2* showed higher concentrations of L-Pro, L-Tyr, L-Phe, and L-Ala in root exudate Badri and Vivanco (2009). An opposite case was shown for *mrp5* mutants, as they displayed decreased exudation of L-AAs Zhou et al. (2016). However, this role is different to the ones described before for LHT1 and AAP1. In those cases, the transporter mutants revealed a decreased sensitivity against D-AAs, most probably caused by disturbed D-AA export. To explain the enhanced sensibility against D-Phe displayed by *mrp5*, accumulation of this compound due to the lack of MRP5 must be postulated. Increased sensitivity of *mrp5* mutants to D-Phe compared to Col-0 can be taken as an indication of MRP5 playing a role in the export of D-Phe and probably of other D/L-AAs in *Arabidopsis thaliana*, but further confirmation of this hypothesis is needed.

Nevertheless, these examples show that MRPs are further candidates for D-AA transport in plants. The toxicity test experiments in Kolukisaoglu and Suarez (2017), provide a good

indication of D-AAs transport by the tested mutant lines, but by no means it is an ultimate prove of it. Finally, just as discussed previously for AAP1 and LHT1, further experiments in yeast and oocytes as well as the usage of more alleles of *mrp5* in different experiments is needed to confirm whether this protein is involved in D-AA transport. Moreover, it has been shown that another ABC transporter belonging to the pleiotropic drug resistance protein (PDR) is involved in root exudation of phytochemicals that provoke an overhaul of natural soil microbiotaBadri et al. (2009), opening the possibility that other members of the ABC transporters such as the MRPs could be involved in root exudation of AAs.

6.1.2 Passive Exudation and Active Uptake of D-Amino Acids

In initial experiments analysing the D-AA profile of D-Met treated seedlings over time, it was noticed that the subsequently produced D-Ala and D-Glu were not degraded continuously. In contrast, the levels of these D-AAs increased transiently during their decline (data not shown). This observation gave rise to the assumption that this effect could be caused by root exudation and re-uptake of D-AAs. This was confirmed by the works reported in Hener et al. (2018). In this report, we were able to show that accumulation of D-Ala and D-Glu during the D-AA treatment is followed by a drastic decrease of their concentrations inside the plant during the first two hours. Followed by a slow but constant decrease until concentrations similar to those of control plants just after 72 hours. Interestingly, accumulation of L-Ala and L-Glu in D-AA treated seedling was also detected, implying the involvement of racemases in the reduction of D-Ala and D-Glu levels Hener et al. (2018).

Exudation of D-AAs was not restricted to D-Ala and D-Glu, but occurred with all other tested D-AAs (D-Asp, D-Leu, D-Lys, D-Phe, and D-Pro)Hener et al. (2018). The exudation of D-Ala from the root into the rhizosphere was proven to be passive, and to take place in comparable amounts to the L-AA secretion in roots Hener et al. (2018). Altogether, our works revealed that D-AAs are exuded by plants to the rhizosphere like L-AAs, which has been shown different times before Badri and Vivanco (2009); Farrar et al. (2003); Frenzel (1957, 1960); Juma and McGill (1986).

Additionally, we were able to show that this process is at least partially actively energised. As secretion of D-/L-Ala by Col-0 was increased when seedlings were treated with inhibitors of active transporters such as orthovanadate (OV) or CCCP. Simultaneously, the D-Ala content within treated and untreated plants decreased comparably over time Hener et al. (2018). This indicates a passive exudation, as the ATP-dependent transporters are blocked. If uptake and exudation of D-AAs are both active processes, OV and CCCP treatment should block or at least suppress the exudation of D-AAs. Nonetheless, the opposite took place. A possible explanation for this scenario is that a portion of D-AA exudation happens by passive efflux, whereas a significant part of the uptake, especially the re-uptake of AAs, is ATP-dependent

or coupled to H^+gradient. This scenario is supported by the fact that D-Ala uptake has been shown to be mediated by LHT1, which is an active transporter Hirner et al. (2006). It is important to highlight that despite the fact that levels of D-Ala and D-Glu within the seedlings were significantly high, the D-AA levels in the medium did not increase significantly over time Hener et al. (2018).

Although exudation of D-AAs was shown to take place, it did not appear to play a major role in the reduction of D-AA content in *Arabidopsis thaliana*. In spite of that, over time concentration of D-Ala *in planta* decreased considerably. This argues against our initial hypothesis that exudation plays a relevant role in the D-Ala detoxification process, but supports our ideas about enzymes metabolising D-AAs.

With the discovery of D-AA root exudation several questions arise: Is it possible that the little amount of D-Ala (and other D-AAs) exuded by the plants into the rhizosphere can actually be enough to influence the AA composition of the rhizosphere, and is it used by the plant as a plant-microbe interaction strategy? D-Ala and D-Glu are AAs that can be utilised by bacteria for direct incorporation into PG biosynthesis. Some bacteria are known to use L-Ala to racemise it into D-Ala for pyruvate productionBardaweel et al. (2011). Additionally, assuming that AAs are strong osmolytes like for instance L-Pro Yoshiba et al. (1997), D-AAs may be also used to regulate the osmotic balance in the rhizosphere.

It is also tempting to speculate whether there are more transporter proteins in plants besides LHT1, AAP1, and MRP5 that can specifically transport D-AAs. The question whether there is long distance transport of D-AAs, as in the case of L-AAs Dinkeloo et al. (2018), or if it is limited to the root-rhizosphere interface. Now, knowing that D-AAs are not mainly disposed into the rhizosphere, the mobility of D-AAs from the roots to overground organs becomes a far more interesting question to address. In this regard it must be noticed that almost all yet published experiments were made with seedlings and no data are available about whole plant distribution of D-AAs. Therefore, independent analysis of D-AA concentration in different organs of the plant must be done in future experiments. The usage of transporter mutants for this experiment, would reveal if the D-AA content in different organs of these mutants is different to that of the corresponding wild type.

6.1.3 Fluorescent D-Ala "HADA", a tool for D-Amino Acid detection in *Arabidopsis thaliana*

Based on experiments with 7-hydroxycoumarin-3-amino-D-alanine (HADA), a D-Ala fluorescent analog, it was possible to observe D-Ala accumulation in the chloroplast of treated seedlings Kolukisaoglu and Suarez (2017). The usage of this tool for tracking D-Ala was already described and stablished in bacteria Kuru et al. (2012); Zhang et al. (2018). To my knowledge, the usage of HADA in plants and the record of its accumulation in chloroplast is

mentioned for the first time in Kolukisaoglu and Suarez (2017). According to Kolukisaoglu and Suarez (2017), the even distribution of HADA in *A. thaliana* contrasts with the ring-like structure found in the chloroplasts envelope of mosses Hirano et al. (2016). This observations suggests a different function of D-Ala in higher plants compared to mosses, where PG-like structures in the chloroplast envelope were described.

Nevertheless, refining this method for D-Ala detection in higher plants in future experiments, can result in a fine detection of small residues of D-Ala in the chloroplast. Which eventually could disclose the accumulation of D-Ala in the envelope or division plane of the this organelle. The efforts in the course of this thesis to localise D-Ala using HADA as well as click chemistry are a solid starting point for more experiments to resolve the intracellular transport and localisation of D-Ala.

But the reason of D-Ala accumulation in this organelle remains an open question. One possible explanation is that AtDAT1 locates in this organelle as well, and seems to be the main producer of D-Ala in the cell. Nevertheless, this does not explain why exogenous applied D-Ala or HADA somehow accumulates in the chloroplast. Experiments with knock out mutants of chloroplast transporter proteins could reveal if accumulation of D-Ala in the chloroplast is disturbed or impeded. Additionally, the feeding of stable isotope labeled D-Ala, and its consecutive tracking, would reveal if this amino group is being use or incorporated into other molecules in the cell. It is worth mentioning that in most of our HADA experiments, accumulation of it happened in the chloroplast of the guard cells of the stomata (Data not shown).

6.2 Metabolism of D-Amino Acids in *Arabidopsis thaliana*

As already discussed in the previous section, plants have the capacity to import D-AAs. Hence, once D-AAs are inside the plant, the questions about their fate needs to be answered. The intracellular concentrations of them have to be kept below toxic levels, and as mentioned above, exudation of D-AAs into the rhizosphere is limited. Therefore, specific metabolic routes must be available for the plant to achieve its regulation. This raises the question of how plants process D-AAs, and why D-Ala is the preferred product of D-AA metabolism as it has been show in Gördes et al. (2011) and (Suarez et al., submitted). Along this work, I was able to summarise an inventory of putative D-AA metabolising enzymes encoded in the *Arabidopsis* genome, and to characterise the D-AA specific transaminase AtDAT1. Consequently, I will discuss in more detail these two achievements in the following subsections.

6.2.1 Putative D-Amino Acid Metabolising Enzymes

The list of putative D-AA metabolising enzymes in Kolukisaoglu and Suarez (2017), reveals that numerous enzymes are encoded in the genome of *Arabidopsis thaliana* with the potential of metabolising D-AAs. There is no information on the expression levels of most of them, or under which circumstance the expression takes place. Few of these enzymes have been characterised and described to date Kolukisaoglu and Suarez (2017). Therefore, it needs to be proven that indeed all of them are involved in D-AA metabolism. However, this list provides a framework for future investigations on the metabolism and physiological functions of D-AAs in plants. In this subsection I will focus on those enzymes potentially involved in the metabolisation of D-AAs and the production of D-Ala.

D-AA specific transaminases are one of the candidate enzymes for the production of D-Ala from other D-AAs. There are four D-AA specific transaminases listed, from which just one has been shown to produce D-Ala and D-Glu using several D-AAs as substrates. On the other hand, the other three remaining transaminases are predicted to possess specificities for other AAs, but, they have not been characterised to date Funakoshi et al. (2008).

Another class of enzymes that might be related to D-AA metabolism in *Arabidopsis* are amino acid racemases like AtDAAR1 and AtDAAR2, which are specific for isoleucineStrauch et al. (2015). Furthermore, a Ser racemase (AtSR1) has been described. It catalyses the racemisation of Ser, and to a lower extent, Ala, Arg, and Gln. Besides its racemase activity, this enzyme also acts as a dehydratase on D- and L-Ser Ono et al. (2006). Additionally, a putative Asp-Glu racemase was also found, however, there are no conclusive reports about it. Examples for other known D-AA specific enzymes in *Arabidopsis thaliana* are the D-Cys desulfhydrase, which is able to catabolise D-Cys to pyruvate, NH_3 and H_2S Riemenschneider et al. (2005). And a D-Ala-D-Ala ligase, which was described by Hirano et al. (2016). In both cases, the physiological function of these enzymes in *Arabidopsis thaliana* still remains unclear.

Apart from the enzymes identified due to their homology, several D-AA specific enzymatic activities have been reported in plants for which the encoding genes are still unknown, as in the case of the Ala Racemase and the ACC-Malonyltransferase Kolukisaoglu and Suarez (2017). Another example was shown in Kawasaki et al. (1982), where σ-glutamylation is highly specific to D-Ala. Altogether, there are several examples of enzymatic activities metabolising D-AAs in plants, and there are even more putative ones. This implies that plants have adapted to a D-AA containing environment.

It is remarkable that for six of the enzymes listed in Table 1 of Kolukisaoglu and Suarez (2017) , the localisation is unknown. While for another six, they are predicted to be localised in the chloroplast and two more in the cytosol. It is interesting that a representative group of enzymes related to D-AA metabolism are predicted to localise in the same place,

the chloroplast. In case of confirmation, this would be an indication that major metabolic processes regarding D-AAs take place in this organelle. This would not be too surprising due to the cyanobacterial ancestry of chloroplasts. In these bacteria, specially D-Ala and D-Glu are utilised for the cell wall production Zhang et al. (2018). The questions are now, which functions D-AAs fulfil in higher plants, and how do D-AAs get specifically into the chloroplast. Efforts to elucidate how this happens must be part of future research.

6.2.2 DAT1 is a major determinant of D-Amino Acid Metabolisation in *Arabidopsis thaliana*

As it has been shown in Gördes et al. (2013) and investigated in (Suarez et al., submitted), after treatment with any D-AA, led to high concentrations of D-Ala, D-Glu, and of the corresponding L-enantiomer in Col-0. This did not hold true for the accession Landsberg erecta (Ler). One possible explanation was given by Vranova et al. (2012), suggesting that a D-AA specific aminotransferase causes these effects.

The results of our experiments in this regard, provided conclusive evidence of a single D-AA specific aminotransferase, named D-amino acid transferase 1 (AtDAT1), to be responsible for this effect. Moreover, our genetic and biochemical experiments showed that Ler possesses a AtDAT1 enzyme with reduced enzymatic activity, due to a point mutation in the active site of AtDAT1. This explains the observations of Gördes et al. (2013) regarding the D-AA concentration profile in D-AA treated Ler plants. Our biochemical experiments showed that AtDAT1 is a broad range D-AA specific aminotransferase with highest affinity towards D-Met as substrate and D-Ala as product (Suarez et al., submitted). This fits to the observation that D-Met leads to the highest conversion rates in most *Arabidopsis* accessions Gördes et al. (2013),

Considering the findings described above, AtDAT1 appears to be a major regulator of D-AA catabolism and D-Ala biosynthesis in *Arabidopsis thaliana*. Furthermore, it confirms the idea formulated in Vranova et al. (2012) suggesting a single transamination event to be responsible for most of the D-AA metabolisation observed in plants. The increase of L-Met after D-Met treatment, is explained most probably, due to a secondary transamination of D-Met.

The predominant increase in D-Ala concentration after D-AA treatment can be explained by the abundance of pyruvate in the cell, as it is used as amino group acceptor by AtDAT1 producing D-Ala. It is critical to evaluate if there are more reasons or explanations that support the general production of D-Ala as a step in D-AA metabolism in *Arabidopsis*. For now, the fate of the D-Ala produced by AtDAT1 remains to be an open question. Therefore, as mentioned previously, future efforts should be made to elucidate the fate of D-Ala in *Arabidopsis thaliana* as different metabolic routes are possible, like oxidation, malonylation,

ligation, or translocation to other organs Kolukisaoglu and Suarez (2017) and (Suarez et al., submitted).

Additionally, the great variety of *Arabidopsis thaliana* accessions with and without active AtDAT1, reveals that DAT1 is not indispensable for growth, development, and evolution of plants. But, it points to the idea that only under particular conditions or environments, this enzyme offers an adaptive advantage as suggested by the preservation of this enzyme in the genome of many different plants (Suarez et al., submitted). One of the starting points to define such a condition, was the germination of *dat1* in D-Met containing media (see below).

6.3 Physiological functions of D-Amino Acids in *Arabidopsis thaliana*

Although plants have the capacity to transport and metabolise D-AAs, the question about the physiological role of these compounds remained unanswered until now. But more than 30 years ago, it was reported that feeding D-Met to seedlings of different plants led to increased ethylene production Kionka and Amrhein (1984); Liu et al. (1983); Satoh and Esashi (1980). This phenomenon was referred as "D-Amino Acid Stimulated Ethylene Production"Satoh and Esashi (1980). There were attempts for explaining this by means of competitive malonylation of D-Met and 1-aminocyclopropane (ACC), the precursor of ethylene. But this hypothesis could not be verified because the corresponding malonyl transferase had not been identified to date. This phenomenon offered a starting point for the phenotypic analysis of AtDAT1 mutants. In this thesis it is reported that an *in planta* rise of D-Met concentration leads to an increase in ethylene biosynthesis.

We could show that *Arabidopsis thaliana* with DAT1 loss of function or affected alleles, did not have any apparent alterations in growth and development or in response to stress, but were less resistant against the exogenous application of D-Met (Suarez et al., submitted). DAT1 impaired seedlings were not able to metabolise this D-Met, leading to the accumulation of it inside the plant. As mentioned above, biochemical assays revealed that the affinity of DAT1 for D-Met as a substrate is also the highest compared to all other D-AAs. When grown in media with the addition of D-Met (500μM), *dat1* mutants and Ler displayed a triple response phenotype when grown in the dark and a strong reduced growth under permanent light conditions. Additionally, increasing ethylene concentrations were measured in *dat1* mutants and Ler samples compared to Col-0 in etiolated as well as in light grown seedlings (Suarez et al., submitted).

This was an important finding, as it was the first time ever reported, that a physiological phenotype is directly linked to the loss of function of an enzyme specific to D-AA. The fact that the triple response phenotype is triggered by the lack of DAT1 after D-Met treatment,

and consequent excess of D-Met inside the plant, is in accordance with our previous finding that D-Met is the main substrate for this enzyme. Nevertheless, investigations on the way to confirm if the production of ethylene can also be induced by other D-AAs in DAT1 defective lines are currently under way. It may also answer the question whether all toxic effects of D-AAs are due to ethylene overproduction and its consequences.

Under normal conditions, ACC is oxidised by the ACC Oxidase (ACCO) to produce ethylene. Alternatively, ACC conjugation with Malonyl-coenzyme A by the N-Malonylase to produce N-Malonyl-ACC inhibits ethylene over production Taiz and Zeiger (2010). The fact that D-AAs are involved in ACC Malonylation and ethylene production was found in the late seventies and eighties. It was already known that N-Malonylation of mainly non-polar D-AAs such as D-Phe, D-Ala, and D-Met happens in plants Kawasaki et al. (1982); Liu et al. (1983). Additionally, it was proven that ethylene synthesis inhibition by D-Phe and D-Ala was reverted by addition of high concentrations of ACC. ACC inhibited the formation of N-malonyl-D-Met, after D-Met treatment, which was proven to be exclusive for D-AAs Yang and Hoffman (1984).

It was postulated by Yang and Hoffman (1984) that the synthesis of N-Malonyl-ACC and N-Malonyl-D-Met may be catalysed by the same enzyme or by separate enzymes that exhibit cross-specificity for both D-Met and ACC. In addition, they described that D-AAs stimulate an increase in ACC concentration followed by an increase in ethylene production and proposed that the physiological significance of this phenomenon was to inactivate external and potentially toxic D-AAs Yang and Hoffman (1984). Nevertheless, it was not taken into account that D-AAs might be important for the ethylene regulation *in planta*.

In this thesis, I provided the biochemical and physiological characterisation of an active DAT1 in *Arabidopsis thaliana* that is involved in the regulation of ethylene biosynthesis mediated by D-Met. In our experiments with *Arabidopsis thaliana*, detection of higher amounts of ethylene and Malonyl-Met in *dat1* seedlings treated with exogenous D-Met compared to controls, support the idea that this D-Met competes with ACC as substrate of the N-Malonylase. This is reflected in the increase of Malonyl-D-Met, and ACC that is mainly oxidised by the ACC oxidase leading to increased ethylene production. It is intriguing that the molecular machinery for ethylene production and regulation to which DAT1 is linked to (Suarez et al., submitted), is not located in the chloroplast but in the cytosol Taiz and Zeiger (2010).

As shown in (Suarez et al., submitted) and discussed previously , the activity of DAT1 with D-Met in the growth media results in a production of D-Ala inside the plant. Interestingly, D-Ala has been shown to inhibit ACC oxidase Brunhuber et al. (2000). This could be an explanation for the reduced ethylene production in Col-0 after D-Met treatment. The D-Ala produced by DAT1 with D-Met as a substrate, would be adding to the repression of ethylene biosynthesis by inhibiting the ACC oxidase, resulting in a wild type phenotype. In mutants

lacking DAT1, the addition of D-Ala and D-Met to the growth medium should revert the effects of D-Met alone.

The fact that DAT1 is directly involved in ethylene regulation becomes very interesting particularly in ripening fruits as they are known to contain D-AAs Brückner and Westhauser (2003); Liu et al. (1985). It is pertinent to discuss if the physiological substrate for the induction of ethylene production and in particular of the triple response phenotype in *dat1* mutants is indeed D-Met or another chemical compound with similar biochemical characteristics. But, it would be interesting to investigate if DAT1 homologs from other plants display the same affinity to D-Met or if it shifts to other D-AAs. Furthermore, silique and seed development of *dat1* mutants as well as their AA profile should be analysed in the future.

Another interesting question is the origin of D-Met, as no studies can account for its *de novo* synthesis in plants. Furthermore, soils contains a vast number of D-AAs, but D-Met is rarely found Amelung and Zhang (2001); Vranova et al. (2012). Nevertheless, several reports point out that D-Met is produced and released by bacteria. For instance, non canonical D-AAs, such as D-Met, are incorporated within *V. cholerae* as well as in in *E. coli* and *P. aeruginosa* peptidoglycan subunits and may aid bacteria in adapting to, and protecting themselves against a variety of environmental challenges Cava et al. (2011). D-Met, among other D-Amino acids, has been associated to the dissolution of biofilms Kolodkin-Gal et al. (2010). In *V cholerae*, a dedicated racemase produces D-Met, and accumulation of D-AAs coincides with the transition into stationary phase, appearing to down-regulate peptidoglycan synthesis. Therefore, D-amino acids may enable to coordinate metabolic slowing in bacterial synthesis of cell wall and cytoplasmic compartments when resources become scarce Lam et al. (2009). In many cases, bacterial D-AAs have been shown to influence important physiological aspects of eukaryotic organisms Aliashkevich et al. (2018); Ollivaux et al. (2014).

Taken all together, these findings lead to the conclusion that, relevant concentrations of D-Met available for the plant do not originate from the bulk soil, but directly from the rhizosphere where bacteria can deposit it as product of its biofilms. Even if the presence and correct function of DAT1 is not essential for *Arabidopsis thaliana*, we hypothesise that DAT1 can be beneficial, under particular scenarios for the interaction with microorganisms in the rhizosphere. As ethylene is also produced in response to bacterial pathogens Hase et al. (2003); Lund et al. (1998); van Loon et al. (2006), sensing and reacting to D-Met accumulation may be a microbe response mechanism worth to be investigated in the future.

Chapter 7

Outlook

As many questions remained open, further research about D-AA transport, metabolism, and physiological function in higher plants is needed. To confirm whether D-AAs are transported by MRP5, AAP1 and LHT1, more experiments are required. One possibility would be expressing these proteins in plant protoplast or frog oocytes, followed by exposing the cells to D-AAs such as D-Met, D-Ala and D-Phe and by determining the concentration of D-AAs inside the cells. This would be a direct proof for the D-AA transport capacities of these transporters. In addition, toxicity test with other *lht* and *aap* mutants would be a first step to find more candidate transporters.

It was speculated in this thesis whether D-AAs in plant exudates could influence the rhizosphere biomass and composition. To address this issue, experiments characterising and comparing the bacterial and fungi microbiome within the rhizosphere of different ecotypes and mutants (e.g. *dat1, dao, ddt, lht1, aaps*, Ler, Col-0, M7323S) of *Arabidopsis thaliana* after D-AA treatment, should be performed. This would reveal whether D-AAs are indeed used as intermediates in plant-microbe interaction. To evaluate whether D-AAs that have been exuded into the rhizosphere by plants are directly utilised by bacteria, experiments tracking labeled D-AAs from inside the plant into structural features such as peptidoglycans or metabolites of rhizosphere bacteria are required. An alternative would be to feed *Arabidopsis thaliana* with HADA. After the accumulation of the D-AAs inside the plant, it must be transferred into media containing rhizosphere bacteria, and after incubation, to aim to detect HADA in these bacteria microscopically.

The fate of D-Ala remains an open question. Nevertheless, it appears to be the major product of D-AA metabolism in *Arabidopsis thaliana*, and at the same time it is one of the most toxic D-AAs. The analysis of the translocation of D-Ala within the plant from roots to other organs using HADA and click chemistry is required. Putative enzymes able to metabolize D-Ala should be identified in *Arabidopsis thaliana* and characterized biochemically. Some candidate proteins like DAO and DDL are already in our focus. It would be interesting

to study the localisation of other D-AA metabolism related enzymes, as some putative enzymes might be located in the cytosol, where all enzymes related to ethylene production are located. Interestingly, as shown in this thesis, DAT1 and D-Ala are localised in the chloroplast. Nonetheless, a linking mechanism and intermediates between, on one hand side DAT1 and D-Ala, in the chloroplast, and on the other side, ethylene singling and metabolism in the cytosol, are awaiting to be unraveled.

It is important to investigate whether LHT1 plays another role, besides its recently shown capacity to facilitate ACC transport, in the regulation of ethylene biosynthesis, especially, whether it is somehow related to DAT1 and D-Met. Profiling D-Ala in *lht1* mutants and Col-0 after D-AAs treatment, could reveal characteristic D-Ala concentration patterns in the plants, that could be assigned to the lack of LHT1 transporter. Recently, it was proposed that D-Ala could interact with the ACC oxidase, competing with ACC and blocking its oxidation. As a consequence, down-regulation of the ethylene production would be expected. Experiments to prove this hypothesis are currently running.

Another experiment that would be interesting is to analyse the DAT1 function in climacteric plants. It would address the question whether this enzyme is involved in the autoinduction of ethylene production during fruit ripening. Additionally, it would be interesting to evaluate if plants respond to treatment with combinations of two, three, and four D-AAs in a differential manner compared to their respond to D-Met treatment. This would reveal if the D-AAs have a synergetic effect on plants, as it has been proven to be in bacterial biofilm disassembly.

Finally, due to the scarcity of D-Met in plants, its relevance in the modulation of ethylene synthesis is still under debate. To confirm that D-Met is indeed the trigger of the observed ethylene phenotype in *Arabidopsis thaliana*, it is important to repeat the experiments performed with D-Met and AtDAT1 with different molecules that resemble D-Met. This molecules should also be more abundant in the plant, or be more available to them. This experiments would support with experimental data that D-Met or an unknown natural similar compound is indeed the genuine substrate for DAT1.

After confirmation that DAT1 is a crucial enzyme in D-AA metabolism in *Arabidopsis thaliana*, and that D-AAs homeostasis influences *Arabidopsis* hormone physiology, it is easy to postulate that more enzymes related to the D-AAs metabolism should be involved and yet to be found and characterise. Accordingly, more questions arise, as well as possibilities about D-AA metabolic routes that can take place to maintain the D-AA homeostasis in plants.

Bibliography

Agranat, I., Caner, H., and Caldwell, J. (2002). Putting chirality to work: the strategy of chiral switches. *Nature Reviews Drug Discovery*, 1(10):753.

Aldag, R. and Young, J. (1970). D-amino acids in soils. i. uptake and metabolism by seedling maize and ryegrass. *Agronomy Journal*, 62(2):184–189.

Aldag, R., Young, J., Kickuth, R., and Vardakis, F. (1972). D-valine metabolism in sunflower seedlings1) 2). *Biochemie und Physiologie der Pflanzen*, 163(4):363–374.

Aliashkevich, A., Alvarez, L., and Cava, F. (2018). New insights into the mechanisms and biological roles of d-amino acids in complex eco-systems. *Frontiers in microbiology*, 9:683.

Amelung, W. and Zhang, X. (2001). Determination of amino acid enantiomers in soils. *Soil Biology and Biochemistry*, 33(4):553–562.

Amelung, W., Zhang, X., and Flach, K. (2006). Amino acids in grassland soils: climatic effects on concentrations and chirality. *Geoderma*, 130(3):207–217.

Badri, D. V., Quintana, N., El Kassis, E. G., Kim, H. K., Choi, Y. H., Sugiyama, A., Verpoorte, R., Martinoia, E., Manter, D. K., and Vivanco, J. M. (2009). An abc transporter mutation alters root exudation of phytochemicals that provoke an overhaul of natural soil microbiota. *Plant Physiology*, 151(4):2006–2017.

Badri, D. V. and Vivanco, J. M. (2009). Regulation and function of root exudates. *Plant, Cell & Environment*, 32(6):666–681.

Balu, D. T. and Coyle, J. T. (2015). The nmda receptor ?glycine modulatory site? in schizophrenia: D-serine, glycine, and beyond. *Current opinion in pharmacology*, 20:109–115.

Bardaweel, S., Ghosh, B., Chou, T.-F., Sadowsky, M. J., and Wagner, C. R. (2011). E. coli histidine triad nucleotide binding protein 1 (echint) is a catalytic regulator of d-alanine dehydrogenase (dada) activity in vivo. *PloS one*, 6(7):e20897.

Bari, R. and Jones, J. D. (2009). Role of plant hormones in plant defence responses. *Plant molecular biology*, 69(4):473–488.

Benichou, M., Martinez-Reina, G., Romojaro, F., Pech, J.-C., and Latché, A. (1995). Partial purification and properties of a 36-kda 1-aminocyclopropane-1-carboxylate n-malonyltransferase from mung bean. *Physiologia Plantarum*, 94(4):629–634.

Brodowski, S., Amelung, W., Lobe, I., and Du Preez, C. C. (2005). Losses and biogeochemical cycling of soil organic nitrogen with prolonged arable cropping in the south african highveld?evidence from d-and l-amino acids. *Biogeochemistry*, 71(1):17–42.

Brückner, H. and Westhauser, T. (2003). Chromatographic determination of l-and d-amino acids in plants. *Amino acids*, 24(1):43–55.

Brunhuber, N. M., Mort, J. L., Christoffersen, R. E., and Reich, N. O. (2000). Steady-state kinetic mechanism of recombinant avocado acc oxidase: initial velocity and inhibitor studies. *Biochemistry*, 39(35):10730–10738.

Buchanan, B. B., Gruissem, W., and Jones, R. L. (2015). Biochemistry and molecular biology of plants.

Cava, F., Lam, H., De Pedro, M. A., and Waldor, M. K. (2011). Emerging knowledge of regulatory roles of d-amino acids in bacteria. *Cellular and molecular life sciences*, 68(5):817–831.

Chen, L. and Bush, D. R. (1997). Lht1, a lysine-and histidine-specific amino acid transporter in arabidopsis. *Plant Physiology*, 115(3):1127–1134.

Di Fiore, M. M., Santillo, A., and Chieffi Baccari, G. (2014). Current knowledge of d-aspartate in glandular tissues. *Amino Acids*, 46(8):1805–1818.

Dinkeloo, K., Boyd, S., and Pilot, G. (2018). Update on amino acid transporter functions and on possible amino acid sensing mechanisms in plants. In *Seminars in cell & developmental biology*, volume 74, pages 105–113. Elsevier.

Dubois, M., Van den Broeck, L., and Inzé, D. (2018). The pivotal role of ethylene in plant growth. *Trends in plant science*.

Dunlop, D. S., Neidle, A., McHale, D., Dunlop, D. M., and Lajtha, A. (1986). The presence of free d-aspartic acid in rodents and man. *Biochemical and biophysical research communications*, 141(1):27–32.

Dworkin, J. (2014). The medium is the message: interspecies and interkingdom signaling by peptidoglycan and related bacterial glycans. *Annual review of microbiology*, 68:137–154.

Erikson, O., Hertzberg, M., and Näsholm, T. (2004). A conditional marker gene allowing both positive and negative selection in plants. *Nature biotechnology*, 22(4):455–458.

Eriksson, T., Björkman, S., and Höglund, P. (2001). Clinical pharmacology of thalidomide. *European Journal of Clinical Pharmacology*, 57(5):365–376.

Eschrich, W. and Hartmann, T. (1969). Translokation und biochemisches verhalten von d-und l-phenylalanin bei vicia fabatranslocation and biochemical behaviour of d-and l-phenylalanine in vicia faba. *Planta*, 85(3):213–227.

Espaillat, A., Carrasco-López, C., Bernardo-Garcia, N., Pietrosemoli, N., Otero, L. H., Alvarez, L., De Pedro, M. A., Pazos, F., Davis, B. M., Waldor, M. K., et al. (2014). Structural basis for the broad specificity of a new family of amino-acid racemases. *Acta Crystallographica Section D: Biological Crystallography*, 70(1):79–90.

Farrar, J., Hawes, M., Jones, D., and Lindow, S. (2003). How roots control the flux of carbon to the rhizosphere. *Ecology*, 84(4):827–837.

Farrell, M., Hill, P. W., Farrar, J., Bardgett, R. D., and Jones, D. L. (2011). Seasonal variation in soluble soil carbon and nitrogen across a grassland productivity gradient. *Soil Biology and Biochemistry*, 43(4):835–844.

Farrell, M., Prendergast-Miller, M., Jones, D. L., Hill, P. W., and Condron, L. M. (2014). Soil microbial organic nitrogen uptake is regulated by carbon availability. *Soil Biology and Biochemistry*, 77:261–267.

Fischer, W.-N., Loo, D. D., Koch, W., Ludewig, U., Boorer, K. J., Tegeder, M., Rentsch, D., Wright, E. M., and Frommer, W. B. (2002). Low and high affinity amino acid h+-cotransporters for cellular import of neutral and charged amino acids. *The Plant Journal*, 29(6):717–731.

Forde, B. G. and Roberts, M. R. (2014). Glutamate receptor-like channels in plants: a role as amino acid sensors in plant defence? *F1000prime reports*, 6.

Forsum, O., Svennerstam, H., Ganeteg, U., and Näsholm, T. (2008). Capacities and constraints of amino acid utilization in arabidopsis. *New Phytologist*, 179(4):1058–1069.

Frenzel, B. (1957). Zur abgabe von aminosäuren und amiden an das nährmedium durch die wurzeln von helianthus annuus l. *Planta*, 49(2):210–234.

Frenzel, B. (1960). Zur ätiologie der anreicherung von aminosäuren und amiden im wurzelraum vonhelianthus annuus l. *Planta*, 55(2):169–207.

Friedman, M. (1999). Chemistry, nutrition, and microbiology of d-amino acids. *Journal of Agricultural and Food Chemistry*, 47(9):3457–3479.

Fuchs, S. A., Berger, R., Klomp, L. W., and de Koning, T. J. (2005). D-amino acids in the central nervous system in health and disease. *Molecular genetics and metabolism*, 85(3):168–180.

Fujii, N. (2002). D-amino acids in living higher organisms. *Origins of Life and Evolution of Biospheres*, 32(2):103–127.

Fujii, N., Kaji, Y., and Fujii, N. (2011). D-amino acids in aged proteins: analysis and biological relevance. *Journal of Chromatography B*, 879(29):3141–3147.

Fujitani, Y., Horiuchi, T., Ito, K., and Sugimoto, M. (2007). Serine racemases from barley, hordeum vulgare l., and other plant species represent a distinct eukaryotic group: gene cloning and recombinant protein characterization. *Phytochemistry*, 68(11):1530–1536.

Fujitani, Y., Nakajima, N., Ishihara, K., Oikawa, T., Ito, K., and Sugimoto, M. (2006). Molecular and biochemical characterization of a serine racemase from arabidopsis thaliana. *Phytochemistry*, 67(7):668–674.

Funakoshi, M., Sekine, M., Katane, M., Furuchi, T., Yohda, M., Yoshikawa, T., and Homma, H. (2008). Cloning and functional characterization of arabidopsis thaliana d-amino acid aminotransferase–d-aspartate behavior during germination. *The FEBS journal*, 275(6):1188–1200.

Gaedeke, N., Klein, M., Kolukisaoglu, U., Forestier, C., Müller, A., Ansorge, M., Becker, D., Mamnun, Y., Kuchler, K., Schulz, B., et al. (2001). The arabidopsis thaliana abc transporter atmrp5 controls root development and stomata movement. *The EMBO journal*, 20(8):1875–1887.

Good, N. E. and Andreae, W. (1957). Malonyltryptophan in higher plants. *Plant physiology*, 32(6):561.

Gördes, D., Koch, G., Thurow, K., and Kolukisaoglu, Ü. (2013). Analyses of arabidopsis ecotypes reveal metabolic diversity to convert d-amino acids. *SpringerPlus*, 2(1):559.

Gördes, D., Kolukisaoglu, Ü., and Thurow, K. (2011). Uptake and conversion of d-amino acids in arabidopsis thaliana. *Amino Acids*, 40(2):553–563.

Hamase, K. (2007). Sensitive two-dimensional determination of small amounts of d-amino acids in mammals and the study on their functions. *Chemical and pharmaceutical bulletin*, 55(4):503–510.

Hamase, K., Morikawa, A., and Zaitsu, K. (2002). D-amino acids in mammals and their diagnostic value. *Journal of chromatography B*, 781(1):73–91.

Hase, S., Van Pelt, J. A., Van Loon, L., and Pieterse, C. M. (2003). Colonization of arabidopsis roots by pseudomonas fluorescens primes the plant to produce higher levels of ethylene upon pathogen infection. *Physiological and molecular plant pathology*, 62(4):219–226.

Hashimoto, A., Nishikawa, T., Oka, T., and Takahashi, K. (1993). Endogenous d-serine in rat brain: N-methyl-d-aspartate receptor-related distribution and aging. *Journal of neurochemistry*, 60(2):783–786.

Hener, C., Hummel, S., Suarez, J., Stahl, M., and Kolukisaoglu, Ü. (2018). D-amino acids are exuded by arabidopsis thaliana roots to the rhizosphere. *International journal of molecular sciences*, 19(4):1109.

Hill, P. W., Farrell, M., and Jones, D. L. (2012). Bigger may be better in soil n cycling: does rapid acquisition of small l-peptides by soil microbes dominate fluxes of protein-derived n in soil? *Soil Biology and Biochemistry*, 48:106–112.

Hill, P. W., Quilliam, R. S., DeLuca, T. H., Farrar, J., Farrell, M., Roberts, P., Newsham, K. K., Hopkins, D. W., Bardgett, R. D., and Jones, D. L. (2011). Acquisition and assimilation of nitrogen as peptide-bound and d-enantiomers of amino acids by wheat. *PLoS One*, 6(4):e19220.

Hirano, T., Tanidokoro, K., Shimizu, Y., Kawarabayasi, Y., Ohshima, T., Sato, M., Tadano, S., Ishikawa, H., Takio, S., Takechi, K., et al. (2016). Moss chloroplasts are surrounded by a peptidoglycan wall containing d-amino acids. *The Plant Cell*, 28(7):1521–1532.

Hirner, A., Ladwig, F., Stransky, H., Okumoto, S., Keinath, M., Harms, A., Frommer, W. B., and Koch, W. (2006). Arabidopsis lht1 is a high-affinity transporter for cellular amino acid uptake in both root epidermis and leaf mesophyll. *The Plant Cell*, 18(8):1931–1946.

Hockachka, P., Hartline, P., and Fields, J. (1977). Octopine as an end product of anaerobic glycolysis in the chambered nautilus. *Science*, 195(4273):72–74.

Huang, Y., Zhang, W., Shi, Q., Toyo?oka, T., and Min, J. Z. (2018). Determination of d, l-amino acids in collagen from pig and cod skins by uplc using pre-column fluorescent derivatization. *Food Analytical Methods*, pages 1–8.

Jilek, A., Mollay, C., Lohner, K., and Kreil, G. (2012). Substrate specificity of a peptidyl-aminoacyl-l/d-isomerase from frog skin. *Amino acids*, 42(5):1757–1764.

Jones, D. L., Owen, A. G., and Farrar, J. F. (2002). Simple method to enable the high resolution determination of total free amino acids in soil solutions and soil extracts. *Soil Biology and Biochemistry*, 34(12):1893–1902.

Jones, D. L., Shannon, D., Junvee-Fortune, T., and Farrar, J. F. (2005). Plant capture of free amino acids is maximized under high soil amino acid concentrations. *Soil Biology and Biochemistry*, 37(1):179–181.

Juma, N. G. and McGill, W. B. (1986). Decomposition and nutrient cycling in agro-ecosystems. In *Microfloral and faunal interactions in natural and agro-ecosystems*, pages 74–136. Springer.

Kamatani, Y., Minakata, H., Kenny, P. T., Iwashita, T., Watanabe, K., Funase, K., Ping, S. X., Yongsiri, A., Kim, K. H., Novales-Li, P., et al. (1989). Achatin-i, an endogenous neuroexcitatory tetrapeptide from achatina fulica ferussac containing a d-amino acid residue. *Biochemical and biophysical research communications*, 160(3):1015–1020.

Kaneko, I., Yamada, N., Sakuraba, Y., Kamenosono, M., and Tutumi, S. (1995). Suppression of mitochondrial succinate dehydrogenase, a primary target of β-amyloid, and its derivative racemized at ser residue. *Journal of neurochemistry*, 65(6):2585–2593.

Katane, M. and Homma, H. (2011). d-aspartate?an important bioactive substance in mammals: a review from an analytical and biological point of view. *Journal of Chromatography B*, 879(29):3108–3121.

Kawasaki, Y., Ogawa, T., and Sasaoka, K. (1982). Two pathways for formation of d-amino acid conjugates in pea seedlings. *Agricultural and Biological Chemistry*, 46(1):1–5.

Keglević, D., Ladešić, B., and Pokorny, M. (1968). Biochemical studies in tobacco plants: Iv. n-malonylmethionine, metabolite of d-methionine in nicotiana rustica. *Archives of biochemistry and biophysics*, 124:443–449.

Kionka, C. and Amrhein, N. (1984). The enzymatic malonylation of 1-aminocyclopropane-1-carboxylic acid in homogenates of mung-bean hypocotyls. *Planta*, 162(3):226–235.

Kolodkin-Gal, I., Romero, D., Cao, S., Clardy, J., Kolter, R., and Losick, R. (2010). D-amino acids trigger biofilm disassembly. *Science*, 328(5978):627–629.

Kolukisaoglu, Ü. and Suarez, J. (2017). D-amino acids in plants: New insights and aspects, but also more open questions. In *Amino Acid-New Insights and Roles in Plant and Animal*. InTech.

Kuru, E., Hughes, H. V., Brown, P. J., Hall, E., Tekkam, S., Cava, F., de Pedro, M. A., Brun, Y. V., and VanNieuwenhze, M. S. (2012). In situ probing of newly synthesized peptidoglycan in live bacteria with fluorescent d-amino acids. *Angewandte Chemie*, 124(50):12687–12691.

Ladešić, B., Pokorny, M., and Keglević, D. (1971). Metabolic patterns of l-and d-serine in higher and lower plants. *Phytochemistry*, 10(12):3085–3091.

Lam, H., Oh, D.-C., Cava, F., Takacs, C. N., Clardy, J., de Pedro, M. A., and Waldor, M. K. (2009). D-amino acids govern stationary phase cell wall remodeling in bacteria. *Science*, 325(5947):1552–1555.

Lee, Y.-H., Foster, J., Chen, J., Voll, L. M., Weber, A. P., and Tegeder, M. (2007). Aap1 transports uncharged amino acids into roots of arabidopsis. *The Plant Journal*, 50(2):305–319.

Lehmann, S., Gumy, C., Blatter, E., Boeffel, S., Fricke, W., and Rentsch, D. (2010). In planta function of compatible solute transporters of the atprot family. *Journal of experimental botany*, 62(2):787–796.

Li, J. and Wang, N. (2014). Foliar application of biofilm formation–inhibiting compounds enhances control of citrus canker caused by xanthomonas citri subsp. citri. *Phytopathology*, 104(2):134–142.

Lippincott, J. A. and Lippincott, B. B. (1970). Lysopine and octopine promote crown-gall tumor growth in vivo. *Science*, 170(3954):176–177.

Lipson, D. and Näsholm, T. (2001). The unexpected versatility of plants: organic nitrogen use and availability in terrestrial ecosystems. *Oecologia*, 128(3):305–316.

Liu, Y., Hoffman, N. E., and Yang, S. F. (1983). Relationship between the malonylation of 1-aminocyclopropane-1-carboxylic acid and d-amino acids in mung-bean hypocotyls. *Planta*, 158(5):437–441.

Liu, Y., Su, L.-Y., and Yang, S. F. (1985). Ethylene promotes the capability to malonylate 1-aminocyclopropane-1-carboxylic acid and d-amino acids in preclimacteric tomato fruits. *Plant Physiology*, 77(4):891–895.

Lund, S. T., Stall, R. E., and Klee, H. J. (1998). Ethylene regulates the susceptible response to pathogen infection in tomato. *The Plant Cell*, 10(3):371–382.

Martínez-Rodríguez, S., Martínez-Gómez, A. I., Rodríguez-Vico, F., Clemente-Jiménez, J. M., Heras-Vázquez, L., and Javier, F. (2010). Natural occurrence and industrial applications of d-amino acids: an overview. *Chemistry & biodiversity*, 7(6):1531–1548.

Michard, E., Lima, P. T., Borges, F., Silva, A. C., Portes, M. T., Carvalho, J. E., Gilliham, M., Liu, L.-H., Obermeyer, G., and Feijó, J. A. (2011). Glutamate receptor–like genes form ca2+ channels in pollen tubes and are regulated by pistil d-serine. *Science*, 332(6028):434–437.

Monselise, E.-I., Levkovitz, A., and Kost, D. (2015). Ultraviolet radiation induces stress in etiolated landoltia punctata, as evidenced by the presence of alanine, a universal stress signal: a 15n nmr study. *Plant Biology*, 17(s1):101–107.

Montecucchi, P. C., DE CASTIGLIONE, R., PIANI, S., GOZZINI, L., and ERSPAMER, V. (1981). Amino acid composition and sequence of dermorphin, a novel opiate-like peptide from the skin of phyllomedusa sauvagei. *International journal of peptide and protein research*, 17(3):275–283.

Nagy, R., Grob, H., Weder, B., Green, P., Klein, M., Frelet-Barrand, A., Schjoerring, J. K., Brearley, C., and Martinoia, E. (2009). The arabidopsis atp-binding cassette protein atmrp5/atabcc5 is a high affinity inositol hexakisphosphate transporter involved in guard cell signaling and phytate storage. *Journal of Biological Chemistry*, 284(48):33614–33622.

Nishikawa, T. (2005). Metabolism and functional roles of endogenous d-serine in mammalian brains. *Biological and Pharmaceutical Bulletin*, 28(9):1561–1565.

Ogawa, T., Fukuda, M., and Sasaoka, K. (1973). Occurrence of n-malonyl-d-alanine in pea seedlings. *Biochimica et Biophysica Acta (BBA)-General Subjects*, 297(1):60–69.

Ohta, N., Kubota, I., Takao, T., Shimonishi, Y., Yasuda-Kamatani, Y., Minakata, H., Nomoto, K., Muneoka, Y., and Kobayashi, M. (1991). Fulicin, a novel neuropeptide containing a d-amino acid residue isolated from the ganglia of achatina fulica. *Biochemical and biophysical research communications*, 178(2):486–493.

Ollivaux, C., Soyez, D., and Toullec, J.-Y. (2014). Biogenesis of d-amino acid containing peptides/proteins: where, when and how? *Journal of Peptide Science*, 20(8):595–612.

Ono, K., Yanagida, K., Oikawa, T., Ogawa, T., and Soda, K. (2006). Alanine racemase of alfalfa seedlings (medicago sativa l.): first evidence for the presence of an amino acid racemase in plants. *Phytochemistry*, 67(9):856–860.

Pollock, G. E., Cheng, C., and Cronin, S. (1977). Determination of the d and l isomers of some protein amino acids present in soils. *Analytical chemistry*, 49(1):2–7.

Prendergast-Miller, M. T., de Menezes, A. B., Farrell, M., Macdonald, L. M., Richardson, A. E., Bissett, A., Toscas, P., Baker, G., Wark, T., and Thrall, P. H. (2015). Soil nitro-

gen pools and turnover in native woodland and managed pasture soils. *Soil Biology and Biochemistry*, 85:63–71.

Raab, T. K., Lipson, D. A., and Monson, R. K. (1999). Soil amino acid utilization among species of the cyperaceae: plant and soil processes. *Ecology*, 80(7):2408–2419.

Radkov, A. D. and Moe, L. A. (2014). Bacterial synthesis of d-amino acids. *Applied Microbiology and Biotechnology*, 98(12):5363–5374.

Raven, P. H., Evert, R. F., Eichhorn, S. E., et al. (2005). *Biology of plants*. Macmillan.

Rentsch, D., Schmidt, S., and Tegeder, M. (2007). Transporters for uptake and allocation of organic nitrogen compounds in plants. *FEBS letters*, 581(12):2281–2289.

Riemenschneider, A., Wegele, R., Schmidt, A., and Papenbrock, J. (2005). Isolation and characterization of a d-cysteine desulfhydrase protein from arabidopsis thaliana. *The FEBS journal*, 272(5):1291–1304.

Rosa, N. and Neish, A. (1968). Formation and occurrence of n-malonylphenylalanine and related compounds in plants. *Canadian journal of biochemistry*, 46(8):797–806.

Sanders, A., Collier, R., Trethewy, A., Gould, G., Sieker, R., and Tegeder, M. (2009). Aap1 regulates import of amino acids into developing arabidopsis embryos. *The Plant Journal*, 59(4):540–552.

Sasabe, J., Miyoshi, Y., Rakoff-Nahoum, S., Zhang, T., Mita, M., Davis, B. M., Hamase, K., and Waldor, M. K. (2016). Interplay between microbial d-amino acids and host d-amino acid oxidase modifies murine mucosal defence and gut microbiota. *Nature microbiology*, 1(10):16125.

Satoh, S. and Esashi, Y. (1980). D-amino-acid-stimulated ethylene production in seed tissues. *Planta*, 149(1):64–68.

Shin, K., Lee, S., Song, W.-Y., Lee, R.-A., Lee, I., Ha, K., Koo, J.-C., Park, S.-K., Nam, H.-G., Lee, Y., et al. (2014). Genetic identification of acc-resistant2 reveals involvement of lysine histidine transporter1 in the uptake of 1-aminocyclopropane-1-carboxylic acid in arabidopsis thaliana. *Plant and Cell Physiology*, 56(3):572–582.

Sidler, M., Hassa, P., Hasan, S., Ringli, C., and Dudler, R. (1998). Involvement of an abc transporter in a developmental pathway regulating hypocotyl cell elongation in the light. *The Plant Cell*, 10(10):1623–1636.

Snyder, S. H. and Kim, P. M. (2000). D-amino acids as putative neurotransmitters: Focus on d-serine. *Neurochemical Research*, 25(5):553–560.

Soper, F. M., Paungfoo-Lonhienne, C., Brackin, R., Rentsch, D., Schmidt, S., and Robinson, N. (2011). Arabidopsis and lobelia anceps access small peptides as a nitrogen source for growth. *Functional Plant Biology*, 38(10):788–796.

Strauch, R. C., Svedin, E., Dilkes, B., Chapple, C., and Li, X. (2015). Discovery of a novel amino acid racemase through exploration of natural variation in arabidopsis thaliana. *Proceedings of the National Academy of Sciences*, 112(37):11726–11731.

Svennerstam, H., Ganeteg, U., Bellini, C., and Näsholm, T. (2007). Comprehensive screening of arabidopsis mutants suggests the lysine histidine transporter 1 to be involved in plant uptake of amino acids. *Plant Physiology*, 143(4):1853–1860.

Svennerstam, H., Jämtgård, S., Ahmad, I., Huss-Danell, K., Näsholm, T., and Ganeteg, U. (2011). Transporters in arabidopsis roots mediating uptake of amino acids at naturally occurring concentrations. *New Phytologist*, 191(2):459–467.

Taiz, L. and Zeiger, E. (2010). Plant physiology 5th ed. *Sunderland, MA: Sinauer Associates*.

Tegeder, M. and Ward, J. M. (2012). Molecular evolution of plant aap and lht amino acid transporters. *Frontiers in plant science*, 3.

Typas, A., Banzhaf, M., Gross, C. A., and Vollmer, W. (2012). From the regulation of peptidoglycan synthesis to bacterial growth and morphology. *Nature Reviews Microbiology*, 10(2):123–136.

Van de Poel, B. and Van Der Straeten, D. (2014). 1-aminocyclopropane-1-carboxylic acid (acc) in plants: more than just the precursor of ethylene! *Frontiers in plant science*, 5:640.

van Loon, L. C., Geraats, B. P., and Linthorst, H. J. (2006). Ethylene as a modulator of disease resistance in plants. *Trends in plant science*, 11(4):184–191.

Verrier, P. J., Bird, D., Burla, B., Dassa, E., Forestier, C., Geisler, M., Klein, M., Kolukisaoglu, Ü., Lee, Y., Martinoia, E., et al. (2008). Plant abc proteins–a unified nomenclature and updated inventory. *Trends in plant science*, 13(4):151–159.

Vollhardt, K. P. C. and Schore, N. E. (2007). *Química orgánica: estructura y función*. Number 547. Omega,.

Vranova, V., Zahradnickova, H., Janous, D., Skene, K. R., Matharu, A. S., Rejsek, K., and Formanek, P. (2012). The significance of d-amino acids in soil, fate and utilization by microbes and plants: review and identification of knowledge gaps. *Plant and soil*, 354(1-2):21–39.

Wade, D., Boman, A., Wåhlin, B., Drain, C., Andreu, D., Boman, H. G., and Merrifield, R. B. (1990). All-d amino acid-containing channel-forming antibiotic peptides. *Proceedings of the National Academy of Sciences*, 87(12):4761–4765.

Wedyan, M. A. and Preston, M. R. (2008). The coupling of surface seawater organic nitrogen and the marine aerosol as inferred from enantiomer-specific amino acid analysis. *Atmospheric Environment*, 42(37):8698–8705.

Yang, S. F. and Hoffman, N. E. (1984). Ethylene biosynthesis and its regulation in higher plants. *Annual review of plant physiology*, 35(1):155–189.

Yoshiba, Y., Kiyosue, T., Nakashima, K., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1997). Regulation of levels of proline as an osmolyte in plants under water stress. *Plant and Cell Physiology*, 38(10):1095–1102.

Zenk, M. and Scherf, H. (1963). D-tryptophan in höheren pflanzen. *Biochimica et Biophysica Acta*, 71:737–738.

Zhang, J.-Y., Lin, G.-M., Xing, W.-Y., and Zhang, C.-C. (2018). Diversity of growth patterns probed in live cyanobacterial cells using a fluorescent analog of a peptidoglycan precursor. *Frontiers in microbiology*, 9:791.

Zhang, Z., Yuan, Y., Zhao, W., He, H., Li, D., He, W., , Q., and Yin, H. (2017). Seasonal variations in the soil amino acid pool and flux following the conversion of a natural forest to a pine plantation on the eastern tibetan plateau, china. *Soil Biology and Biochemistry*, 105:1–11.

Zhou, D., Huang, X.-F., Chaparro, J. M., Badri, D. V., Manter, D. K., Vivanco, J. M., and Guo, J. (2016). Root and bacterial secretions regulate the interaction between plants and pgpr leading to distinct plant growth promotion effects. *Plant and soil*, 401(1-2):259–272.